STUDIES ON BACTERIAL EXOTOXINS WITH SPECIAL REFERENCE TO THOSE OF THE STAPHYLOCOCCI AND THE GROUP OF SPORING ANAEROBES.

BY

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General Introduction.

At an early stage in the development of bacteriological science it was clearly shown that certain micro-organisms were pathogenic by virtue of their diffusible poisons, now designated exotoxins.

The exact definition of exotoxins in the light of recent knowledge has become a matter of great difficulty. Formerly they were characterised according to certain properties, their ready diffusibility in bacterial culture and filtrability from the living organism, thermolability at 65°C, high toxicity in the animal body after an incubation period following their injection, particularly their selective action on certain tissues and tissue elements and their antitoxinogenic effect. Two toxins, those of B. diptheriae and B. tetani exhibit all these properties. Certain bacterial toxins which may reasonably be classified among the exotoxins differ from these of B. diptheriae and B. tetani in certain important features and the characterisation stated above is therefore too rigid. The most important character in the determination of an exotoxin is now considered to be the ability to stimulate the production of a specific antitoxin in the animal body. It is suggested that this character may in some measure be shared with the endotoxins. In the

absence of any well defined characterisation of exotoxins in general the toxin of each organism must be characterised individually.

This also applies to the elaboration of exotoxins. There is no proof they are invariably a product of bacterial metabolism or are freed on autolysis of the bacterial cell and there is no constant correlation between bacterial growth and toxin production, some toxins, such as that of the classical B. welchii, being obtained from young actively growing cultures and others, for example that of B. diphtheriae, only from older cultures. It seems probable that the mechanism of toxin production varies with each type of bacterium.

Exotoxins also play parts of widely different importance in the mechanism of infection. Some organisms, such as B. diphtheriae and B. tetani, have low invasive powers and are pathogenic only by virtue of their toxins while in the case of others, for example the Staphylococci, the exotoxin is a less dominant feature in the pathogenic mechanism of the organism.

In this thesis special study is made of the exotoxins of Staphylococcal and B. welchii strains. In addition the absorption of these and V. septique toxins from the alimentary tract is considered.

Part 1.

The Toxic Properties of Staphylococcal Filtrates.

REVIEW OF LITERATURE.

Staphylococci were described by Ogston as early as 1881, but their pathogenic potentialities were disregarded for many years. Van de Velde and Denys (1894), Neisser and Wechsberg (1901) and others, demonstrated the presence of filtrable haemolysin and leuococidin but their work was neglected and has only comparatively recently been considered when later workers, Burnet (1929), Kellaway, Burnet and Williams (1930) and Parker Weld and Gunther (1931) were stimulated to investigate staphylococcal toxin as a result of the Bundaberg disaster of 1928 in which a number of children were inoculated with diptheria toxin-antitoxin mixture contaminated with pathogenic Staphylococci. Many of these children succumbed, particularly the younger ones, and those with resistance lowered by other disease. A Royal Commission appointed to inquire into the cause of the deaths came to the conslusion that the Staphylococci were responsible. They assumed that the organisms multiplied rapidly in the body and liberated toxin sufficient to cause a severe, and in some cases a fatal toxaemia. The virulence of the Bundaberg Staphylococcus was demonstrated by the death in 24 hours of rabbits following intravenous injection. The effects produced being similar to

those exhibited by the children.

Van de Velde (1894) produced two varieties of Staphylococcus aureus from a strain originally feebly pathogenic. One variety exhibited a great attenuation of virulence due to prolonged culture on artificial media, whereas, the other had its virulence exalted by passage through rabbits. Apart from the difference in virulence the two varieties exhibited other peculiarities. Rabbits inoculated intraperitoneally with either variety were killed at stated intervals and it was found that the attenuated forms had in a few seconds undergone characteristic alterations in that they became swollen and lost their staining properties whereas the virulent variety rarely showed any signs of degeneration but remained small and could still be stained readily. Further, in the exudates produced several hours after the intraperitoneal injections the white blood corpuscles were numerous and actively phagocytic when the attenuated forms had been used but they lost their vitality and became markedly degenerated if the inoculum had contained the organisms of exalted virulence.

Van de Velde (1894) found that if fresh active leucocytes were added "in vitro" to the exudate formed by the virulent Staphylococci, they altered rapidly and in a similar manner, but if, the exudate had previously been heated for 10 minutes at

58°C. the leucocytes retained their vitality. It thus became evident that a thermolabile substance, highly deleterious for leucocytes, was formed in the exudate provoked by the injection of the virulent Staphylococci. Van de Velde named this "Substance leucocid" or "Leucocidin".

It was found possible to obtain leucocidin "in vitro" as well as "in vivo". In culture media such as broth and pabula enriched with blood and blood serum, leucocidin was produced in equal quantities by the virulent and avirulent strains, but on injection into the rabbit the attenuated forms were destroyed before the leucocidin was able to develop.

In studying the defensive mechanism of the body Van de Velde considered that the blood serum and serous exudates constitute the chief defence and that leucocytes act on organisms which escape humoral destruction. Here again, the attenuated forms were more easily attacked and destroyed than the virulent, though both secreted "Lysin" a principle capable of neutralising the bactericidal power of the serum.

Virulent Staphylococci injected into dogs were destroyed by the blood and serum to the same extent as the attenuated variety. As a result Van de Velde found it impossible to raise the virulence of Staphylococci by passage through dogs.

Rabbits inoculated with filtrates, cultures killed by heat, or weakly virulent cultures produced antileucocidin although deaths from cachexia and

abscesses were numerous (Denys and Van de Velde, 1894).

Leucocidin had "in vitro" the power of immobilising motile leucocytes and rendering them vacuolated. This can be observed in 2 minutes with warm stage preparations. The addition of the serum of a rabbit immunised with Staphylococci inhibited the action of the luecocidin on the leucocytes. Thus a rabbit on immunisation developed in its serum an antileucocidin principle capable of preserving leucocytes against the action of leucocidin.

These immunised rabbits were able to withstand doses of virulent Staphylococci several times greater than the average lethal dose for normal rabbits. As in the dog, however, intoxication in immunised rabbits is very marked with the result that while they withstood small doses of pathogenic cultures they succumbed to large doses on account of the toxaemia produced. Their leucocytes, protected by the antileucocidin, remained intact till the death of the animal.

Denys and Van de Velde (1899) considered the mode of action of anti-leucocidin on leucocidin and devised experiments to determine whether the former attacked the latter directly, or whether it impaired the toxic action by uniting with the leucocytes.

They first demonstrated in a series of experiments that Leucocidin is thermolabile and anti-

leucocidin thermostable on exposure to 58°C for 10 minutes. This difference enabled Denys and Van de Velde to study the effect of neutralisation on these agents. A mixture of toxin and an amount of antitoxin which exactly neutralised it had no effect on leucocytes. Heating for 10 minutes at 58°C should have by destroying the toxin liberated antitoxin, if the two substances had preserved their individuality. Denys and Van de Velde were, however, unable to demonstrate freed antitoxin and concluded that this substance was destroyed in neutralisation.

Neisser and Wechsberg (1901) separated most of the haemolysin from a haemolysin leucocidin mixture by absorbing the leucocidin and part of the haemolysin with excess of leucocytes. As estimated by its action on rabbit red blood corpuscles the resultant toxin was strongly haemolytic in spite of the absorption of some of the haemolysin by the red blood corpuscles ever present in the leucocyte extracts.

Neisser and Wechsberg (1901) found that staphylococcal haemotoxin always exhibited similar properties no matter from what source it was obtained. Thus the toxin of any staphylococcal strain could be neutralised by any artificially produced staphylococcal antiserum. It is, therefore, assumed that all pyogenic Staphylococci, both aureus and albus,

form the same haemolysin. Non pyogenic types of Staphylococcus aureus and Staphylococcus albus differ from the pyogenic in being apparently entirely unable to form haemolysin.

Under anaerobic conditions active leucocytes reduce methylene blue to leuco-methylene blue, the change being evident by a visible loss of colour. Exposure of normal white blood corpuscles to leucocidin inactivates them and renders them unable to reduce methylene blue. The leucocytes, however, of an immunised rabbit similarly treated retain their activity and their power of reduction of methylene blue. This reaction can be carried out quantitatively by using differing amounts of toxin, with constant amounts of the other reagents.

Leucocidin is produced by pyogenic but not by non-pyogenic strains of Staphylococcus aureus and Staphylococcus albus. Like the haemolysin, the leucocidin of different strains is believed to be identical, as whatever strain its origin may be it can always be neutralised by one artificially produced antileucocidin.

The production of a specific exotoxin by Staphylococci was confirmed by Kraus and Pribram (1906). Rabbits died on intravenous injection of strongly haemolytic filtrates, the symptoms exhibited agreeing with those produced by a true toxin. They concluded the staphylococcal toxin was specific since it was neutralised by staphylococcal antisera, but not

by Vibrio antisera.

In regard to the neutralisation of haemolysin and leucocidin results similar to those of Neisser and Wechsberg (1901) were obtained by Nicolle and Césari (1914) who working with strains from botryomycosis and human lesions found in both groups great variation in toxicity. Toxins from either source on subcutaneous injection in guinea-pigs and intravenous injection in mice and rabbits exhibited acute lethal actions.

Pharmacological Action of Staphylococcal Toxin:

That staphylococcal toxin entered the circulation was shown by Kraus and Pribram (1906) who considered it was primarly a cardiac poison.

In a pharmacological study Russ (1916) observed the effect of the toxin on the blood pressure, respiratory system and heart of rabbits. The blood pressure fell twice (1) a primary fall with subsequent recovery followed by (2) a terminal fall; respiration diminished as a result of failure of the pulmonary circulation and the heart stopped with right auricle and ventricle dilated.

Kellaway, Burnet and Williams (1930) confirmed and considerably extended Russ's work. They first showed that the lethal effect of intravenous injection of staphylococcal filtrate in young cats was due to an exotoxin since the effect could be annulled if the toxin injected was first treated with

antitoxic serum. The effect of the filtrate on blood pressure had three phases of which the last one only was directly due to toxic action. Firstly a short transient fall occured, attributed to pharmacologically active constituents of the media; this was followed by a rise above normal, probably due to the stimulation of the suprarenals with increased secretion of adrenalin; finally a rapid terminal fall, indicative of the acute killing toxin, was observed. In agreement with the findings of Russ (1916) these effects were shown on Starling's heart lung preparations to be principally due to obstruction of the pulmonary circulation (Kellaway, Burnet and Williams, 1930).

A direct action on the heart was demonstrated on isolated heart preparations (Kellaway, Burnet and Williams 1930) the contractions being invariably arrested on introduction of toxin.

Nélis (1934) attributed the rapid lethal effect produced on intravenous injection of toxin in dogs and rabbits to cardiac deficiency and death occuring after longer intervals to secondry causes. Lesions were produced in a number of organs and were manifested by destruction of the cellular protoplasm. In certain areas of the body, however, the toxin was rendered atoxic; notably where strong body acids are present.

Production of Haemolysin:

In an investigation of the conditions

required for haemolysin production Walbum (1922) found that amounts of potassium and magnesium were necessary but calcium and salts which increased the osmotic pressure of the medium were inhibitory. Ordinary meat extract, therefore, with peptone and traces of potassium dihydrogenphosphate and magnesium sulphate was found to favour haemolysin production and has been used by recent workers (Burnet, 1929; Parker-Weld, 1931).

Burnet (1930) solidified this medium with agar and prepared the haemolysin by emulsifying the growth in saline. He showed, however by mincing the agar and extracting it with saline that most of the haemolysin diffused into the medium. He demonstrated in addition that most cultures of Staphylococcus aureus possess two colonial types, the typical aureus and an albus variant. The aureus type did not produce appreciable amounts of haemolysin unless a proportion of carbon dioxide, 10-20%, was present, whereas the albus variant was capable of producing haemolysin in a normal atmosphere. The haemolysin produced by the two types was identical. Bigger, Boland and O'Meara (1927) prepared staphylococcal haemolysin by the emulsification in saline of an agar growth of staphylococci and the subsequent centrifugalisation to deposit the organisms. The lysin so prepared was more rapidly produced, was in a purer form and was more active than toxins previously investigated. Its chief effect took place not at 37°C

but immediately on cooling after removal from the incubator and the phenomenon was termed "hot-cold" lysis.

Bigger (1933) considered the reaction of the medium was the first essential for the production of an active lysin. Hence the presence of phosphate buffer, carbohydrates or carbon dioxide in the atmosphere assists the production of toxin by neutralising alkalinity developed by the growth of the staphylococci and stabilising the hydrogenion concentration at the optimum reaction (pH7.2 - pH7.4). Strains varied in their requirements. Some produced lysin in quantity in the presence of buffer salts and glycerol without carbon dioxide in the atmosphere; some were dependant for lysin production on carbon dioxide in the atmosphere alone while a few strains produced little lysin unless carbon dioxide was absent.

Nélis (1934) found Ramon's medium for diptheria toxin, with the addition of glucose, was favourable for the production of staphylococcal toxins.

Properties of Staphylococcal Toxins:

Forssman (1933) showed that the activity of the haemolysin varied in direct proportion to the numbers of red cells with which it was allowed to act. He found the optimum temperature for lytic action was in general 37°C with subsequent storage at a lower temperature but with some kinds of corpuscles, for example those of the rabbit, the whole experiment could be equally satisfactorily carried out at 0°C.

Forssman showed that red cells and lysin became bound together before lysis occurred. This binding could also occur independently of haemolysis and could be demonstrated at 0°C. Sometimes a subsequent reverse action, which might occur with great rapidity, was detected. Forssman considered that these phenomena indicated a ferment like nature of the haemolysin.

Gengou (1930 a and b) demonstrated that staphylococcal lysin exerts a lytic power not only on red blood corpuscles but also on other cells such as haematoblasts and cells of the suprarenal capsules.

Burnet (1929) observed that rabbits immunised, passively or actively, against the necrotoxin (skin toxin) were immune from the killing effect of staphyococcal filtrates. He considered that the necrotoxic, lethal and haemolytic toxins were a single toxic entity since he obtained a fairly constant quantitative relationship between the lethal and haemolytic effects in the three toxic filtrates that he studied. He believed that any fluctuation which occurred in the proportional relationships might be accounted for either by (1) environmental factors such as cultural conditions or by (2) a partial conversion of the toxin to toxoid in which state it retained its full combining capacity and some of its haemolytic power but lost its necrotoxic and lethal activities.

Five antitoxic sera produced by Burnet (1929) by different methods all showed a constant relation-

ship in neutralising the three toxic activities. This was additional proof of the staphylococcal toxin being a single entity capable of action on red blood corpuscles and skin and of causing death.

Gross (1931) believed that the characteristic features of staphylococcal toxin were, haemolysin, necrotoxin, leucocidin and lethal toxin all of which were the outcome of a single antigenic substance. As proof of this he showed that: (1) a potent toxin possessed all the qualities already mentioned; (2) a single antitoxic serum neutralised all these properties at once; (3) all the properties were destroyed by the same degree of heat. He discovered one principle, plasma coagulable substance, present only in certain haemolysing filtrates which could not be identified with any of the other toxic properties. It was produced before the other toxic elements, being followed first by haemolysin production and then by necrotoxin and lethal toxin development. This plasma coagulable substance degenerated rapidly and was usually entirely absent by the time necrotoxin and lethal toxin were abundant. It also differed in being thermostable at 70°- 80°C whereas the other toxic elements were all destroyed in 60 minutes at 56°C. It was found impossible to produce any antibody to plasma coagulable substance. Hence Gross did not regard it as a true toxic property although it undoubtably has a damaging effect on

introduction into the body.

Gengou (1930 a and b) noted that the haemolytic, leucocidal and lethal activities of staphylococcal filtrates were not always present in equal proportions. He considered, however, that proof as to the unity or plurality of the toxin could only be provided by the separation of the different toxic properties. He, therefore, performed absorption tests and found that one property alone could not be removed; for example, a filtrate on exposure to erythrocytes lost not only its haemolytic power but also its leucocidal and lethal effects. This, he concluded, showed the different activities of staphylococcal filtrates are all due to the effects of one substance.

Nélis (1934) carried out absorption tests with erythrocyte stroma and found haemolysin and necrotoxin to be inseparable and concluded that the action of these substances was due to one toxin.

Parker-Weld and Gunther (1931) attempted to prove that staphylococcal toxin was made up of two or more distinct components and was not, as Burnet (1929) suggested, a single entity. They considered that Burnet had not given an adequate explanation of the fact that the different toxic activities did not always occur in constant proportions and that his theory was not in accordance with Neisser and Wechsberg's (1901) experiments demonstrating the separation of haemotoxin from leucocidin.

Parker-Weld and Gunther (1931) found that there was a constant quantitative relationship between haemolysin, necrotoxin and killing toxin in strongly haemolytic filtrates. This was not however evident in weaker heamotoxic filtrates (cf. Gross 1931) for the leucocidin never appeared in definite relationship to the haemolysin and necrotoxin. They came to the conclusion that there was little evidence to be gained from these experiments as to the unity or diversity of the toxic principle and, therefore, attempted to separate haemotoxin and leucocidin by absorption with leucocyte suspensions and erythrcyte stroma respectively. In both cases it was found that the absorbing agent frequently removed haemotoxin and leucocidin so that the results were not conclusive. One definite fact was, however, brought to light by these experiments, namely, that although all the other toxic elements might be removed by absorption the necrotoxic action was left unaltered, thus proving that necrotoxin was a separate toxic entity.

Panton and Valentine (1932) carried out a quantitative and qualitative investigation of toxins produced by strains obtained from diverse human staphylococcal infections. They determined the relationship of the haemolytic, leucocidal, necrotoxic and lethal properties and came to the conclusion that the haemolytic, necrotoxic and lethal activities were present in constant proportions whereas no association could be traced between the leucocidal action and any

of the other effects. They divided their strains into four groups on the grounds of their haemolysin and leucocidin content and the source of the strains. Toxins derived from strains isolated from different types of lesions corresponded with certain groups. For example, Group 1 contained toxins with a high leucocidin and low haemolysin content and all the strains producing these toxins were isolated from severe lesions. Group 11 consisted of toxins possessing a high leucocidin and haemolysin content and all the strains were obtained from carbuncles and boils. Group 111 contained toxins with a low leucocidin and high haemolysin content which were produced by strains isolated from infections of deffering severity; all sycosis cases were included in this group. Group 1V toxins had low leucocidin and haemolysin content and all the strains were of saprophytic origin. Panton and Valentine emphasised the importance of this classification in relation to treatment of the various types of staphylococcal infections with specific antitoxins.

Burky (1933 a) found that pigmentation afforded no indication as to whether the Staphylococci normally present on the conjunctiva could set up ocular infection. He, therefore, investigated the cultural and toxinogenic properties of three representative strains in an attempt to correlate these properties with pathogenicity. One strain was highly virulent and produced a potent toxin but had

low invasive powers while another was highly invasive but produced little toxin. These types could be correlated with certain sources, the former being isolated from chronic lesions of the skin and mucous membrane and the latter from purulent lesions. These characteristics were independant of haemolysin or pigment production. For example, aureus and albus strains were isolated from abscesses and neither was invariably haemolytic or non-haemolytic, also when Staphylococci, which under favourably conditions produced active toxin, were grown anaerobically they formed little pigment or haemolysin though the lethal activity was unimpaired.

Nelis (1934) demonstrated a similar lack of parallelism between virulence and toxicity by showing the virulence of strains increased after animal passage whereas there was no corresponding increase in the toxic activity.

Panton, Valentine and Dix (1931) treated thirteen cases of staphylococcal infection with antisera. The cases so treated fell into three groups: 1-5 carbuncle, 6-10 septicaemia and pyaemia, 11-13 those of a fulminating character in which there was little hope that treatment could be successful. They owned that the difficulty of estimating the efficacy of the treatment when factors such as the resistance of the patient and the virulence of the organism cannot be controlled. They are, however, of the opinion that serum therapy prevented death and

shortened the illness in a large proportion of the cases, particularly those of septicaemia and pyaemia, and is a valuable method of treatment. A similar view is held by Gross (1931).

Gross (1931) showed that staphylococcal antitoxin might be standardised. Constant amounts of the different toxic constituents (haemolysin, leucocidin, necrotoxin and lethal toxin) were added to varying amounts of the antitoxin and the inhibitory effect noted.

Parish and Clark (1932) tested the lethal, necrotoxic and haemolytic powers of various toxins. The conversion of active toxin to toxoid by the addition of 0.3 per centage formalin caused a definite and rapid fall in toxicity and a slight loss in combining power with antitoxin in horse serum. Lethal tests in mice gave irregular results and were therefore unsuitable for its titration but haemolytic and intradermal tests in guinea-pigs were satisfactory. The results obtained showed that the antisera possess neutralising powers similar for haemolytic and necrotoxins. The protective antibody was contained in the pseudoglobulin and it could be concentrated three or four times by precipitation with ammonium sulphate. The intravenous injection of antisera in rabbits prior to an otherwise certainly fatal dose of virulent culture prolonged life and on one occasion afforded complete protection. This finding suggests that the action of the antisera is

mainly antitoxic and that it does not inhibit the invasion and multiplication of the living organisms.

Parish and Clark (1932) have only used antisera clinically in a few cases but they consider that it is of a definite therapeutic value causing alleviation of symptoms and sometimes rapid recovery.

Panton and Valentine (1932) advocated for immunisation the use of toxin in preference to vaccine owing to the ease with which the toxicity of the inoculum may be measured.

Bryce and Burnet (1932) found that natural immunity to staphylococcal toxin corresponded with the presence of antitoxin. It was acquired as a result of antigenic stimuli from the environment and was not an inborn characteristic. Infants possessed antitoxin as a result of the passive transfer of maternal antitoxin across the placenta but the antitoxic titre fell rapidly during the first few weeks of life and unless a gross staphylococcal infection occurred was only gradually regained during childhood.

Burky (1933 c) reported similar findings in young rabbits which until they were four months old were not sensitive either to the lethal or necrotoxic effects of staphylococcal filtrates. He considered that these animals are born without a reacting mechanism which is only acquired after the first four months of life. Immunity could be produced in rabbits by intracutaneous inoculations of filtrate and was manifested by a change in the skin reactions

and a decrease in the lethal effect of filtrates injected (Burky 1933, b).

Recently two antigenically distinct toxins, "a" and "b", have been demonstrated in staphylococcal filtrates (Glenny and Stevens, 1935). The "a" toxin was lethal for mice, caused necrosis on intracutaneous inoculation in guinea-pigs and lysed rabbit and sheep red blood corpuscles. The "b" toxin, on the other hand, was non-lethal for mice, produced an erythema but no necrosis on intradermal injection in guinea-pigs and haemolysed sheep but not rabbit cells. The "hot-cold" type of lysis was exhibited and the toxin corresponded to that described by Bigger, Boland and O'Meara (1927).

"b" was present only in some filtrates and was invariably associated with "a". Among the thirty eight filtrates examined by Glenny and Stevens thirty two possessed "a" toxin without "b", six "a" and "b" mixed and none "b" alone. In order to obtain a "b" "a"-free filtrate for toxin-antitoxin neutralisation experiments Glenny and Stevens flocculated the "a" fraction out by exposure of the mixed filtrate to an antiserum with a low "b" and high "a" content of antibodies.

By means of toxin-antitoxin neutralisation tests "a" and "b" were shown to be antigenically distinct.

Food Poisoning with Staphylococcal Toxin:

During recent years cases of food-poisoning

have been reported which, on investigation have apparently been due to toxin formed by staphylococci (aureus, albus, or citreus types) growing in the incriminated food material, e.g. chicken gravy, layered cream cake, cheese and milk. The symptoms described have been those of an acute gastroenteritis developing a few hours after consumption of the contaminated food (Jordan, 1930; Jordan and Hall, 1931; Jordan, 1931).

Jordan and Hall (1931) were unable to produce symptoms of poisoning in laboratory animals by feeding with staphylococcal filtrates but found that a severe and characteristic gastro-enteritis could be set up in human volunteers after consumption of 2 to 10 c.c. of culture filtrates of staphylococci isolated from incriminated foods, and other sources. Ramsay and Tracy (1931) fed kittens on milk cultures of strains of Staphylococcus aureus known to have been responsible for gastro-enteritis in man. After several days these animals developed a diarrhoea which persisted until the milk culture was withdrawn from the food; in some cases blood and mucus appeared in the dejecta.

Jordan and Burrows (1933) demonstrated various physical properties of the gastro-intestinal poison. Symptoms of food poisoning can be produced on feeding monkeys with staphylococcal gastro-intestinal toxin. Therefore, using these mammals for its detection it was shown that the poison will not distil

nor readily dialyse, is destroyed by exposure to an acid solution in the presence of heat or to an alkaline solution and may be extracted from either solution with ethyl ether or chloroform though removal from the alkaline solution is difficult owing to its destructive effect. Further, the food poisoning toxin differs from the haemolysin and necrotoxin.

In the same year Woolpert and Dack (1933) using a technique similar to that of Jordan and Burrows (1933) for the detection of the gastro-intestinal poison investigated the relationship between it and the haemolytic, necrotoxic and lethal activities. Four filtrates were examined; of these two contained all the properties mentioned, the gastro-intestinal toxin was absent from one and the fourth was entirely inactive. From this Woolpert and Dack (1933) deduced that the food poisoning toxin occurred always in association with active toxin though not invariably present. They also showed this food-poisoning toxin was thermostable to a greater extent than the other toxic properties and was not neutralised by antitoxin effective against these properties.

Experimental Work carried out in the following Investigations:

(1) Filtrates produced by strains, obtained from as many and varied sources as possible, have been tested for their haemolytic, leucocidal, necrotising and lethal abilities. A comparison of the results

obtained was made to discover what relationship, if any, existed between the various toxic properties and if there was any correlation between the source of a strain and the properties possessed by its filtrate. (2) It was considered of importance to determine whether strains, grown under constant cultural conditions, produce filtrates of uniform activity. (3) Since fresh filtrates were not always available the effect on the activity of keeping at various temperatures was tested. (4) Monovalent antitoxins were prepared for several filtrates. Their neutralising abilities were tested against their particular toxic filtrates and also against other toxins with a view to determining the antitoxinogenic relationship between (a) the different properties of a filtrate (b) different filtrates. (5) In addition staphylococcal food poisoning was studied by expxrimental methods.

METHODS.

Strains tested for toxin production and their source and date of isolation.

1. Staphylococcus aureus Wood - A type culture obtained from the Lister Institute, London.

2. Staphylococcus aureus Glover - Isolated from a case of Osteomyelitis at the Sick Childrens Hospital, Edinburgh. 2/12/32.

3. Staphylococcus aureus Mackay - Isolated post mortem from a case of meningitis at the Royal Infirmary, Edinburgh. 25/1/32.

4. Staphylococcus aureus Philip - Isolated from a chronic staphylococcal infection of the subcutaneous tissues following a carbuncle and septicaemia. 12/1/32.

5. Staphylococcus aureus Laboratory - Stock laboratory culture at least 9 years old.

6. Staphylococcus aureus Tweedlie - Isolated from a fatal case of cellulitis at the Royal Infirmary, Edinburgh. 8/2/32.

7. Staphylococcus albus - A type culture obtained from the Lister Institute.

8. Staphylococcus aureus Rae - Isolated from an acute and fatal case of Osteo-myelitis at the Royal Infirmary, Edinburgh. 29/2/32.

9. Staphylococcus aureus Millar - Isolated from empyema of the thorax in a child at the Sick Childrens Hospital, Edinburgh. 22/3/32.

10. Staphylococcus aureus McMillan - Isolated from an inflamed gall bladder. 26/3/32.

11. Staphylococcus aureus 1 - Isolated from a case of sycosis. 1/4/32.

12. Staphylococcus aureus 11 - Isolated from a case of sycosis. 1/4/32.

13. Staphylococcus aureus Watson - Isolated post mortem from a case of pyaemia following boils. 8/4/32.

14. Staphylococcus aureus Farmer - Isolated from a case of recurrent boils. 14/4/32.

15. Staphylococcus aureus Hogg - Isolated from infected tissues at the site of a compound fracture at the Astley Ainslie Institute. 22/4/32.

16. Staphylococcus aureus Ovens - Isolated from a hip abscess at the Astley Ainslie Institute. 22/4/32.

17. Staphylococcus aureus Thomson - Isolated from infected tissues at the site of a compound dislocation at the Astley Ainslie Institute. 22/4/32.

18. Staphylococcus aureus Spence - Isolated from a case of osteo-myelitis at the Sick Childrens Hospital. 23/4/32.

19. Staphylococcus aureus Fraser - Isolated from a case of osteo-myelitis at the Astley Ainslie Institute. 22/4/32.

20. Staphylococcus aureus McNally - Isolated from a case of osteo-myelitis subsequent to staphylococcal pericar-ditis in the previous year. 22/4/32.

21. Staphylococcus aureus Burnie - Isolated from one a case of recurrant boils. 9/9/32.

22. Staphylococcus aureus 58 - Isolated from a cystic gland near the gall bladder at the Royal Infirmary, Edinburgh. 8/10/32.

23. Staphylococcus aureus Spence (2) - Isolated from a boil. 14/10/32.

24. Staphylococcus aureus Hartley - Isolated from a

thigh abscess at the Royal Infirmary, Edinburgh. 14/10/32.

25. Staphyloccus aureus Hostley - Isolated from an abscess of the axilla at the Royal Infirmary, Edinburgh. 14/10/32.

26. Staphylococcal aureus Stanley - Isolated from a throat swab. 24/10/32.

27. Staphylococcus aureus Henrie - Isolated from a case of suppurative sinusitis at the Royal Infirmary, Edinburgh. 24/10/32.

28. Staphylococcus aureus Campbell - Isolated from a case of osteo-myelitis at the Sick Childrens Hospital. 19/10/32.

29. Staphylococcus aureus Crawford - Isolated from a boil. 26/10/32.

30. Staphylococcus aureus Duncan - Isolated from a boil. 26/10/32.

31. Staphylococcus aureus Elliot - Isolated from a boil. 26/10/32.

32. Staphylococcus aureus Hamilton - Isolated from a case of osteo-myelitis at the Astley Ainslie Institute. 5/11/32.

33. Staphylococcus aureus Allen - Isolated from a stye. 14/11/32.

34. Staphylococcus aureus Bentley - Isolated from a carbuncle. 14/11/32.

35. Staphylococcus aureus Todd - Isolated from a throat swab. 15/11/32.

36. Staphylococcus aureus Gon[illegible] - Isolated from a carbuncle. 21/11/32.

37. Staphylococcus aureus Ramsay - Isolated from a whitlow 21/11/32.

38. Staphylococcus aureus Thornburn - Isolated from a hair follicle infection. 21/11/32.

39. Staphylococcus aureus Bowden - Isolated from a whitlow. 23/11/32.

40. Staphylococcus aureus Robertson - Isolated from a running ear. 23/11/32.

41. Staphylococcus aureus Hendry - Isolated from a boil. 29/11/32.

42. Staphylococcus aureus Menzies - Isolated from a boil. 29/11/32.

43. Staphylococcus aureus Black - Isolated from a mouth abscess. 30/11/32.

44. Staphylococcus aureus McGregor - Isolated from a suppurating burn. 2/12/32.

45. Staphylococcus aureus Kennedy - Isolated from a case of recurrent boils. 7/12/32.

Preparation of Toxin.

The filtrates employed were prepared from cultures grown in Walbum's (1922) liquid medium as recommended by Burnet (1930) which was prepared in the following way. Ox heart extract was made by adding 1 litre of tap water to 500 grms of beef heart, which was then diluted to half strength and

0.5 per cent Witte's peptone,

0.2 per cent KH_2PO_4,

and 0.03 per cent $MgSO_4$ were added

without the usual addition of NaCl as Walbum (1922)

considered the presence of salts which increased the osmotic pressure of the medium was definitely inhibitory to haemolysin production. The reaction of the medium was adjusted to pH 7-0.

In some cases 1 per cent agar was added and a semi-solid medium was obtained.

In one experiment ordinary bouillon was used.

The liquid medium was distributed in 50 c.c. to 100 c.c. amounts in centrifuge tubes which were then inoculated with the Staphylococci and incubated at 37°C in jars containing an atmosphere with 20 to 30 per centage of carbon dioxide. After 10 to 12 days the cultures were centrifuged and the clear supernatant fluid was passed through a British Berkfeld filter. The resulting filtrate was distributed, in amounts of about 6 c.c., in sterile test tubes and preserved under vaseline seals.

The semi-solid medium was placed in Petri dishes and, after inoculation, incubated at 37°C in an atmosphere of 20 to 30 per cent carbon dioxide. After 48 hours 10 c.c. of normal saline was added to the surface browth on the agar which was then scraped loose as far as possible. After standing half an hour the saline emulsion of the growth was removed and centrifuged. The clear supernatant fluid was passed through a Seitz filter and the filtrate was preserved in tubes under vaseline seals.

The agar left after the removal of the growth was chopped into small pieces and passed

through a tissue mincer. 5 c.c. of normal saline was added to the mince and left to extract half an hour before the fluid was drained off and centrifuged. As in the previous case the supernatant fluid was passed through a Seitz filter and the resulting filtrate similarly preserved.

Tests for Estimation of the Toxic Properties of Filtrates.

Haemolysin Tests.

Serial dilutions of the filtrates ranging from 1 in 15 to 1 in 3840 were made up in 0.3 c.c. amounts in tubes, and 0.3 c.c. of a 1 per cent. suspension of washed rabbit red cells was added. The tubes were incubated at 37°C. for one hour; readings were then made, and also after the tubes had remained at room temperature overnight.

Leucocidin Tests.

The methylene-blue reduction test as originally employed by Neisser and Wechsburg (1901) was adopted. The white cells employed for the leucocidin tests were obtained from the leucocyte cream separated by centrifugalization from citrated ox blood (see Mackie, Finkelstein and Van Rooyen, 1932). By using a large amount of blood a preparation almost entirely free from red cells was obtained. 0.5 c.c. of a 5 per cent. leucocyte suspension was added to 0.25 c.c. of a series of dilutions of toxin in small test-tubes and also to two control tubes, one containing toxin heated at 60°C. for half an hour, and the other normal

saline. The tubes were incubated at 37°C. for one hour; 0.05 c.c. of a 1 in 2000 dilution of methylene-blue was then added to each tube and the contents were transferred to agglutination tubes, and after the superimposition of paraffin seals they were reincubated for one hour, when readings were made.

Skin Tests.

0.4 c.c. amounts of dilutions of toxic filtrate, and also 0.4 c.c. toxin heated at 55°C for one hour as a control, were injected intracutaneously in the depilated skin on the abdomen of guinea-pigs. Readings were made after 18, 24 and 48 hours, necrosis at the site of inoculation being the criterion of a positive result.

Lethal Tests.

The lethal effect of varying amounts of filtrate and also of Walbum's liquid medium and filtrate heated at 55°C for one hour as controls was observed on intravenous injection in mice. The time elapsing before death was noted.

Intra-gastric Administration of Toxin to Guinea-pigs and Rabbits.

Filtrates prepared from Staphylococcus aureus "Gon" were administered in the majority of experiments. In a few instances, however, filtrate from Staphylococcus aureus "Wood" was substituted.

The toxin preparations were introduced directly into the stomach by means of a syringe

attached to a No.3 rubber catheter. In experiments in which the hydrogen-ion concentration of the stomach contents was adjusted, a sample of the contents was aspirated, after introduction of the organisms or toxin and its reaction tested by means of a B.D.H. field capillator and a suitable indicator. The normal reaction varied from pH 3 to pH 6. Frequently, feeding caused a rapid secretion of acid, so that a 5 per cent. solution of sodium bicarbonate was introduced into the stomach in order to adjust the reaction to the range desired, pH 7.2 to pH 7.4. After a short interval a further specimen was aspirated, and the hydrogen-ion concentration was again tested and adjusted if necessary. This procedure was repeated until samples gave the required reaction.

Selection of Walbum's Liquid Medium for Use.

Walbum's liquid medium was found to be more practicable for toxin production than the 1 per cent. agar medium as considerably greater amounts of filtrate could be prepared from the liquid than from the semi-solid medium. In addition, filtrate prepared from the liquid medium contained as much haemolysin as that from the minced agar. Filtrate obtained from the growth emulsion exhibited considerably weaker haemolytic properties (see Table 1). That most of the haemolysin produced in agar diffuses into the medium was demonstrated by Burnet (1930).

Table 1.

Toxin.	Preparation of Toxin.	Haemolytic Titre.
Glover.	From Walbum's liquid medium.	1 in 960 dilution.
"	Filtrate from surface growth on 1 per cent agar	1 in 120 dilution.
"	Filtrate from agar extract	1 in 960 dilution.
Wood.	Filtrate from Walbum's liquid medium.	1 in 2,840 dilution.
"	Filtrate from surface growth on 1 per cent agar	1 in 480 dilution.
	Filtrate from agar extract	1 in 2,840 dilution.
Albus.	Filtrate from Walbum's liquid medium.	1 in 240 dilution.
	Filtrate from surface growth on 1 per cent agar	1 in 60 dilution.
	Filtrate from agar extract	1 in 240 dilution.

Comparison of the Efficacy of Walbum's Liquid Medium and Bouillon on Toxin Production.

The strain Staphylococcus aureus "Wood" was grown in Walbum's liquid medium and in ordinary bouillon. Filtrates were prepared and the toxic properties compared (see Table 2).

Table 2.

Test.	Bouillon Filtrate.	Filtrate from Walbum's Medium.
Haemolytic Test - highest dilution which produced complete haemolysis when 0.3 c.c. of filtrate was added to 0.3 c.c. of 1 per cent suspension of rabbit red cells.	1 in 960 dilution.	1 in 2,840 dilution.
Leucocidin Test - highest dilution of filtrate which showed a leucocidal effect.	1 in 30 dilution.	1 in 640 dilution.
Skin Test - highest dilution of filtrate which gave a necrotic lesion when 0.4 c.c. was injected intracutaneously in guinea-pigs.	1 in 30 dilution.	1 in 30 dilution.
Lethal Test - smallest amount of filtrate lethal to mice, within 24 hours, when injected intravenously.	0.4 c.c.	0.4 c.c.

The results show the strain "Wood" produces toxin in either medium but that Walbum's medium allows a freer formation of toxin.

Haemolysin Content of Toxin before and after Preparation.

Haemolytic tests were carried out before and after filtration of the toxin and it was found that there was no loss in the haemolytic titre due to preparation of the toxin.

Action of Filtrate on Red Cells of Various Mammals.

Rabbit red cells were more sensitive than ox or sheep red cells to haemolytic filtrate prepared from the strain "Wood" (See Table 3).

Table 3.

Toxin.	Haemolytic Titre		
	1 per cent ox red cells.	1 per cent sheep red cells.	1 per cent rabbit red cells.
Wood.	Undiluted no effect.	1 in 30 dilution.	1 in 2,840 dilution.

Rate of Toxic Action.

Considerable difference in the rate of lytic action on rabbit red blood corpuscles was observed. The first reading of the haemolytic test with "Wood" filtrate on removal from the incubator gave a titre of 1 in 60 but after standing at room temperature all night the titre attained a dilution of 1 in 2,840. Tweedlie, on the contrary, showed rapid haemolysis and little subsequent rise in titre. With the majority of filtrates the titre of the haemolytic tests rose to about four times its original dilution after standing overnight at room temperature. Some toxins, however, ("Laboratory", "Mackay" and "Roe") showed no increase after the first reading. Table 4 shows the haemolytic titres of toxins after 1 hour of incubation at 37 ^{o}C and after a subsequent 24 hours at room temperature.

Table 4.

Toxin.	Haemolytic Titre.	
	Titre after 60 minutes incubation at 37 C.	Titre after a subsequent 24 hours at room temp.
Wood.	1 in 60 dilution.	1 in 2,840 dilution
Glover.	1 in 240 "	1 in 960 "
Mackay.	1 in 60 "	1 in 60 "
Philip.	1 in 30 "	1 in 240 "
Laboratory.	1 in 30 "	1 in 30 "
Tweedlie.	1 in 960 "	1 in 1,920 "
Albus.	1 in 60 "	1 in 240 "
Roe.	1 in 60 "	1 in 60 "
Millar.	1 in 60 "	1 in 1,920 "
McMillan.	1 in 2 "	1 in 240 "
Sycosis 1.	1 in 480 "	1 in 5,680 "
Sycosis 11.	1 in 240 "	1 in 480 "
Farmer.	1 in 480 "	1 in 2,840 "

According to these results "Wood" produces the slowest acting haemolysin.

These differential rates of action are apparent in the skin tests. Necrotic areas appeared within 24 hours after intracutaneous injection in guinea-pigs of "Tweedie" filtrate but not till after 48 hours with "Wood" toxin.

Comparison of the Toxic Properties of Filtrates produced by various Strains.

Filtrates prepared from different strains

were investigated for four toxic properties, namely haemolytic, leucocidal, necrotoxic and lethal. The results of these tests are given in Table 5.

The lethal dose of the filtrates must be accepted with some reserve since the number of mice inoculated was insufficient to overcome fallacies resulting from the individual factor.

TABLE 5.

Comparison of the Toxic Properties of Filtrates obtained from various strains.

Toxin.	Source and Date of Isolation.	Haemolytic Test. Highest dilution which produced complete haemolysis when 0.3 cc of filtrate was added to 0.3 cc of 1% suspension of rabbit red cells.	Leucocidin Test. Highest dilution of filtrate which showed a leucocidal effect.	Skin Test. Highest dilution of filtrate which gave a necrotic lesion when 0.4cc was injected intradermally in guinea-pigs.	Lethal Test. Smallest amount of filtrate lethal, within 24 hours, to mice when injected intravenously.
1 Wood.	Type culture. Lister Institute, London. (Originally isolated from septic burns).	1 in 3,840 dilution.	1 in 512 dilution.	1 in 30 dilution.	0.4 c.c.
11 Glover. (treated successfully).	Isolated from a case of Osteo-myelitis at the Sick Children's Hospital, Edinburgh. 2/12/31.	1 in 960 dilution.	1 in 64 dilution.	1 in 120 dilution.	1 c.c.
111 Mackay.	Isolated from a post mortem case of Meningitis at the Royal Infirmary, Edinburgh. 25/1/32.	1 in 60 dilution.	No effect undiluted.	Undiluted.	1 c.c. non-lethal.
1V Philip.	Chronic Staphylococcal infection of the subcutaneous tissues following carbuncle septicaemia. 12/1/32.	1 in 240 dilution.	Undiluted.	Undiluted No effect.	1 c.c. non-lethal.

TABLE 5 continued.

V Laboratory	Stock laboratory culture at least 9 years old.	1 in 30 dilution.	No effect undiluted.	No effect undiluted.	1 c.c. non-lethal.
V1 Tweedlie (treated, died).	Isolated from a case of Staphylococcal Cellulitis. R.K.E. 8/2/32.	1 in 1,920 dilution.	1 in 128 dilution.	1 in 30 dilution.	0.2 c.c.
VII Albus.	Type culture, Lister Institute, London.	1 in 120 dilution.	1 in 8 dilution.	No effect undiluted.	1 c.c.
VIII Roe (treated, died).	Isolated from a case of acute Osteo-myelitis. R.I.E. 29/2/32.	1 in 60 dilution.	No effect undiluted.	No effect undiluted.	1 c.c. non-lethal.
IX Millar.	Isolated from empyema of the thorax in a child at the Sick Children's Hospital, Edinburgh. 22/3/32.	1 in 1,920 dilution.	1 in 64 dilution.	1 in 15 dilution	1 c.c.
X McMillan.	Isolated from the mucosa in a case of cholecystis. 26/3/32.	1 in 240 dilution.	1 in 32 dilution.	Undiluted.	1 c.c. non-lethal.
XI S.I.	Isolated from a Sycosis case. 1/4/32.	1 in 5,680 dilution.	1 in 512 dilution.	1 in 30 dilution.	0.6 c.c.
XII S.II.	Isolated from a Sycosis case. 1/4/32.	1 in 480 dilution.	1 in 16 dilution.	Undiluted.	1 c.c. non-lethal.
XIII Watson.	Isolated post-mortem from a case of pyemia following boils. 8/4/32.	No effect undiluted.	No effect undiluted.	No effect undiluted.	1 c.c. non-lethal.

TABLE 5 continued.

XIV Farmer.	Isolated from a boil. 14/4/32.	1 in 3,840 dilution.	1 in 128 dilution.	1 in 30 dilution.	0.6 c.c.
XV Hogg.	Isolated from a compound fracture at the Astley Ainslie Institute. 22/4/32.	No effect undiluted.	No effect undiluted.	No effect undiluted.	1 c.c. non-lethal.
XVI Ovens.	Isolated from a hip abscess at the Astley Ainslie	Undiluted.	No effect undiluted.	No effect undiluted.	1 c.c. non-lethal.
XVII Thomson.	Isolated from a compound dislocation at the Astley Ainslie Institution. 22/4/32.	No effect undiluted.	No effect undiluted.	No effect undiluted.	1 c.c. non-lethal.
XVIII Spence.	Isolated from a case of Osteomyelitis at the Sick Children's Hospital. 23/4/32.	1 in 240 dilution.	1 in 8 dilution.	Undiluted.	0.6 c.c.
XIX Fraser.	Isolated from a case of Osteomyelitis (multiple abscesses) at the Astley Ainslie Institute. 22/4/32.	1 in 60 dilution.	No effect undiluted.	No effect undiluted.	1 c.c.
XX McNally. (treated with some success).	Isolated from a case of Osteomyelitis subsequent to a Staphylococcal pericarditis in the previous year. 22/4/32.	1 in 60 dilution.	Undiluted.	Undiluted.	1 c.c.
XXI Burnie.	Isolated from a case of recurrent boils. 9/9/32.	1 in 60 dilution.	No effect undiluted.	No effect undiluted.	1 c.c.

TABLE 5 continued.

XXII 58.	Isolated from a cystic gland near the gall bladder at the Royal Infirmary. 8/10/32.	1 in 120 dilution.	No effect undiluted.	Undiluted.	1 c.c.
XXIII Spence (2)	Isolated from a boil. 14/10/32.	1 in 960 dilution.	1 in 4 dilution.	Undiluted.	0.8 c.c.
XXIV Hartley.	Isolated from a thigh abscess at the Royal Infirmary. 14/10/32.	1 in 480 dilution.	1 in 32 dilution.	Undiluted.	0.8 c.c.
XXV Hostley.	Isolated from an axilla abscess at the Royal Infirmary. 14/10/32.	No effect undiluted.	No effect undiluted.	No effect undiluted.	1 c.c. non-lethal.
XXVI Stanley.	Isolated from a throat swab. 24/10/32.	1 in 15 dilution.	No effect undiluted.	No effect undiluted.	1 c.c. non-lethal.
XXVII Henrie.	Isolated from a case of suppurative sinusitis at the Royal Infirmary. 24/10/32.	1 in 960 dilution.	1 in 32 dilution.	1 in 15 dilution.	0.6 c.c.
XXVIII Campbell.	Isolated from a case of osteomyelitis at the Sick Children's Hospital. 19/10/32.	No effect undiluted.	No effect undiluted.	No effect undiluted.	No effect undiluted.
XXIX Crawford.	Isolated from a boil. 26/10/32.	1 in 120 dilution.	1 in 4 dilution.	Undiluted.	1 c.c.
XXX Duncan	Isolated from a boil. 26/10/32.	1 in 960 dilution.	1 in 16 dilution.	1 in 15 dilution.	0.6 c.c.
XXXI Elliot.	Isolated from a boil 26/10/32.	1 in 1,920 dilution.	1 in 16 dilution.	1 in 15 dilution.	0.6 c.c.
XXXII Hamilton.	Isolated from a case of osteomyelitis at the Astley Ainslie Institute. 5/11/32.	1 in 30 dilution.	1 in 8 dilution.	Undiluted.	1 c.c.

TABLE 5 continued.

XXXIII Allen.	Isolated from a stye. 14/11/32.	1 in 480 dilution.	1 in 128 dilution.	1 in 30 dilution.	0.4 c.c.
XXXIV Bentley.	Isolated from a carbuncle. 14/11/32.	1 in 480 dilution.	1 in 128 dilution.	1 in 15 dilution.	0.4 c.c.
XXXV Todd.	Isolated from a throat swab. 15/11/32.	No effect undiluted.	No effect undiluted.	No effect undiluted.	1 c.c. non-lethal.
XXXVI Gon.	Isolated from a carbuncle. 21/11/32.	1 in 3,840 dilution.	1 in 256 dilution.	1 in 30 dilution.	0.2 c.c.
XXXVII Ramsay.	Isolated from a whitlow. 21/11/32.	1 in 15 dilution.	No effect undiluted.	No effect undiluted.	1 c.c. non-lethal.
XXXVIII Thorburn.	Isolated from a hair follicle infection. 21/11/32.	1 in 15 dilution.	No effect undiluted.	No effect undiluted.	1 c.c. non-lethal.
XXXIX Bowden.	Isolated from a whitlow. 23/11/32	1 in 120 dilution.	1 in 2 dilution.	Undiluted.	1 c.c.
XL Robertson.	Isolated from a running ear. 23/11/32.	1 in 60 dilution.	No effect undiluted.	Undiluted.	1 c.c. non-lethal.
IXL Hendry.	Isolated from a boil. 29/11/32.	1 in 60 dilution.	1 in 16 dilution.	No effect undiluted.	1 c.c.
VIIIL Menzies.	Isolated from a boil. 29/11/32.	1 in 960 dilution.	1 in 16 dilution.	1 in 15 dilution.	0.6 c.c.
VIIL Black.	Isolated from a mouth abscess. 30/11/32.	1 in 1,920 dilution.	1 in 32 dilution.	1 in 15 dilution.	0.4 c.c.
VIL McGregor.	Isolated from a suppurating burn. 2/12/32.	1 in 1,920 dilution.	1 in 64 dilution.	1 in 15 dilution.	0.4 c.c.
VL Kennedy.	Isolated from a case of recurrent boils. 7/12/32.	1 in 960 dilution.	1 in 128 dilution.	1 in 30 dilution.	0.2 c.c.

Irregularity of Toxin Production.

The ability of toxin production of a number of strains, ("Farmer", "Wood", "Tweedlie" and "Gon"), was tested on several occasions over a period of 8 to 12 months. Every test was carried out under similar conditions and the results given in Table 6 show that toxin production fluctuated. The most stable toxin was obtained from "Wood", the only strain which had been cultured for some years. The other strains produced potent filtrates fairly regularly when freshly isolated but frequently their activity, with the exception of the lethal effect, later decreased.

Preservation of Toxin.

Filtrate of tested activity from three strains ("Farmer", "Wood" and "Gon") was kept in tubes under vaseline seals, some at room temperature and some in the ice chest, for one year. During this period haemolytic and lethal tests were carried out after 1, 3, 6 and 12 months preservation. The results given in Table 7 show that, both in filtrates kept at room temperature (about 15°C), and in the ice chest, (0°C) after a slight deterioration in the first month there is, in general, little loss of either haemolytic or lethal properties.

TABLE 6.

The Toxic Activities of Batches of Filtrate prepared from the same Strain.

Toxin.	Batch of Filtrate.	Haemolytic Test. Highest dilution which produced complete haemolysis when 0.3 ccm of filtrate was added to a 1% suspension of rabbit red cells.	Leucocidin Titre. Highest dilution of filtrate which showed a leucocidal effect.	Skin Test. Highest dilution of filtrate which gave a necrotic lesion when 0.4 ccm was injected intracutaneously in guinea-pigs.	Lethal Test. Smallest amount of filtrate lethal, within 24 hours, to mice when injected intravenously.
Farmer.	I	1 in 3,840 diln.	1 in 128 diln.	1 in 30 diln.	0.6 ccm.
	II	1 in 3,840 diln.	1 in 128 diln.	1 in 30 diln.	0.2 ccm.
	III	1 in 60 diln.	1 in 8 diln.	No effect undiluted.	0.6 ccm.
	IV	1 in 120 diln.	1 in 16 diln.	No effect undiluted.	0.4 ccm.
	V	1 in 1,920 diln.	1 in 128 diln.	1 in 15 diln.	0.1 ccm.
Wood.	I	1 in 3,840 diln.	1 in 640 diln.	1 in 30 diln.	0.4 ccm.
	II	1 in 1,920 diln.	1 in 640 diln.	1 in 15 diln.	0.2 ccm.
	III	1 in 960 diln.	1 in 32 diln.	1 in 15 diln.	0.2 ccm.
	IV	1 in 120 diln.	1 in 32 diln.	1 in 15 diln.	0.2 ccm.
Tweedlie.	I	1 in 1,920 diln.	1 in 128 diln.	1 in 30 diln.	0.2 ccm.
	II	1 in 1,920 diln.	1 in 256 diln.	1 in 30 diln.	0.2 ccm.
	III	1 in 3,840 diln.	1 in 256 diln.	1 in 30 diln.	0.1 ccm.
	IV	No effect undiluted.	No effect undiluted.	No effect undiluted.	1 ccm.non-letha
	V	1 in 960 diln.	1 in 128 diln.	1 in 15 diln.	0.2 ccm.
Gon.	I	1 in 3,840 diln.	1 in 256 diln.	1 in 30 diln.	0.2 ccm.
	II	1 in 3,840 diln.	1 in 512 diln.	1 in 30 diln.	0.2 ccm.
	III	No effect undiluted.	No effect undiluted.	No effect undiluted.	1 ccm.non-lethal
	IV	1 in 1,920 diln.	1 in 256 diln.	1 in 15 diln.	0.4 ccm.
	V	No effect undiluted.	No effect undiluted.	No effect undiluted.	1 ccm.non-lethal
	VI	1 in 1,920 diln.	1 in 128 diln.	1 in 15 diln.	0.4 ccm.

TABLE 7.

Filtrate.	Haemolytic Test: Highest dilution which produced complete haemolysis when 0.3 c.c. of filtrate was added to a 1% suspension of rabbit red cells.			Lethal Test: Smallest amount of filtrate lethal within 24 hours to mice when injectected intravenously.		
	Farmer	Wood	Gon	Farmer	Wood	Gon
Fresh.	1 in 1,920 diln.	1 in 960 diln.	1 in 960 diln.	0.05 c.c.	0.4 c.c.	0.4 c.c.
After keeping at room temperature for:						
1 Month	1 in 960 diln.	1 in 480 diln.	1 in 960 diln.	0.05 c.c.	0.4 c.c.	0.5 c.c.
3 Months	1 in 480 diln.	1 in 480 diln.	1 in 960 diln.	0.05 c.c.	0.4 c.c.	0.5 c.c.
6 Months	1 in 960 diln.	1 in 480 diln.	1 in 960 diln.	0.1 c.c.	0.4 c.c.	0.6 c.c.
12 Months	1 in 960 diln.	1 in 480 diln.	1 in 960 diln.	0.05 c.c.	0.4 c.c.	0.5 c.c.
After keeping in the Ice Chest for:						
1 Month	1 in 960 diln.	1 in 480 diln.	1 in 480 diln.	0.05 c.c.	0.5 c.c.	0.4 c.c.
3 Months	1 in 960 diln.	1 in 240 diln.	1 in 480 diln.	0.01 c.c.	0.4 c.c.	0.5 c.c.
6 Months	1 in 480 diln.	1 in 240 diln.	1 in 480 diln.	0.01 c.c.	0.3 c.c.	0.5 c.c.
12 Months	1 in 480 diln.	1 in 240 diln.	1 in 240 diln.	0.05 c.c.	0.4 c.c.	0.5 c.c.

Production of Antitoxin.

Monovalent antitoxins were prepared in rabbits with the toxins of "Wood", "Farmer" and "Tweedlie". Weekly injections, first intracutaneously then intravenously, with increasing doses of toxin were made over a period of 8 to 9 weeks. A protocol indicating the dosage and routes of inoculation is given as an example of the method adopted.

0.2 c.c.	filtrate	injected	intracutaneously.
0.4 c.c.	"	"	"
0.2 c.c.	"	"	intravenously.
0.4 c.c.	"	"	"
0.6 c.c.	"	"	"
0.8 c.c.	"	"	"
1 c.c.	"	"	"
1 c.c.	"	"	"

Three to four days later the rabbits were bled by heart puncture and the serum separated.

Estimation of Potency of Antitoxin.

The antihaemolytic, antileucocidal, antinecrotoxic and protective powers of the antitoxin were tested against Wood filtrate and also against other filtrates. In all tests a constant amount of antiserum was added to varying dilutions of the filtrate and after incubation for 1 hour at 37°C the tests were carried out and read in the usual way. Control tests were carried out at the same time by substituting sterile saline and normal serum for antiserum.

Antihaemolytic and Antileucocidin Tests.

The additon of 0.01 c.c. of antitoxin to the filtrate was found to give well defined results in the case of the haemolytic and leucocidin tests (see Table 8). It will be noted that antitoxins from the defferent strains neutralise all haemolysins and all leucocidins to the same extent.

TABLE 8.

Filtrate.	(1) Sterile Saline, (2) Normal serum, or (3a,b,c) Antiserum added.	Haemolytic Titre*	Leucocidin Titre*
Wood.	(1) 0.01 c.cm. sterile saline.	1 in 3,840 diln.	1 in 640 diln.
	(2) 0.01 c.cm. normal serum I.	1 in 1,920 diln.	1 in 128 diln.
	" " " " II.	1 in 240 diln.	1 in 32 diln.
	(3a) 0.01 c.cm. Wood Antiserum.	1 in 15 diln.	1 in 4 diln.
	(3b) 0.01 c.cm. Farmer antiserum.	1 in 15 diln.	1 in 4 diln.
	(3c) 0.01 c.cm. Tweedlie antiserum.	1 in 15 diln.	1 in 2 diln.
Farmer.	(1) 0.01 c.cm. sterile saline.	1 in 3,840 diln.	1 in 128 diln.
	(2) 0.01 c.cm. normal serum I.	1 in 960 diln.	1 in 64 diln.
	" " " " II.	1 in 480 diln.	1 in 32 diln.
	(3a) 0.01 c.cm. Wood antiserum.	1 in 15 diln.	1 in 4 diln.
	(3b) 0.01 c.cm. Farmer antiserum.	1 in 60 diln.	1 in 16 diln.
	(3c) 0.01 c.cm. Tweedlie antiserum.	1 in 30 diln.	1 in 8 diln.
Tweedlie.	(1) 0.01 c.cm. sterile saline.	1 in 1,920 diln.	1 in 128 diln.
	(2) 0.01 c.cm. normal serum I.	1 in 960 diln.	1 in 64 diln.
	" " " " II.	1 in 480 diln.	1 in 32 diln.
	(3a) 0.01 c.cm. Wood antiserum.	1 in 2 diln.	Undiluted.
	(3b) 0.01 c.cm. Farmer antiserum.	1 in 2 diln.	Undiluted.
	(3c) 0.01 c.cm. Tweedlie antiserum.	1 in 15 diln.	1 in 2 diln.

* The haemolytic and leucocidal tests were carried out by methods which have been described on pages 28 and 29.

Antinecrotoxic Tests.

In the necrotoxic tests 0.06 c.c. of antiserum was added to 0.5 c.c. of the various filtrate dilutions and after incubation for 1 hour at 37°C 0.4 c.c. of the mixtures were injected intradermally in guinea-pigs. Controls in which normal serum or sterile saline were substituted for antiserum were always inoculated at the same time.

It was found that larger amounts of antiserum were required to produce neutralisation of the necrotoxin than was necessary for the inactivation of the haemolytic and leucocidal activities. Even with the greater amount of antitoxin used neutralisation occurred only in relatively low dilutions (see Table 9) and the different antitoxins protected equally against the necrotic activity of all the toxins.

The protective powers of the antitoxin were not investigated since it was considered that the lethal dose of the filtrates, due to its estimation by insufficient numbers of mice, did not form an accurate enough basis to justify the development of a series of tests depending on the neutralisation of such uncertain doses.

TABLE 9.

(1) Sterile Saline, (2) Normal serum, (3a,b,c) Antiserum added.	Filtrates. Wood. Undiluted.	Wood. 1:10 diln.	Wood. 1:20 diln.	Wood. 1:40 diln.	Farmer. Undiluted.	Farmer. 1:10 diln.	Farmer. 1:20 diln.	Farmer. 1:40 diln.	Tweedlie. Undiluted.	Tweedlie. 1:10 diln.	Tweedlie. 1:20 diln.	Tweedlie. 1:40 diln.
0.043 c.cm sterile saline in 0.4 c.cm. of mixture inoculated.	++	++	++	+	++	++	+	-	++	++	++	-
0.043 c.cm normal serum in 0.4 c.cm. of mixture inoculated.	++	++	++	-	++	++	+	-	++	++	+	-
0.043 c.cm. Wood antiserum in 0.4 c.cm of mixture inoculated.	++	++	-	-	++	++	-	-	++	+	-	-
0.043 c.cm Farmer antiserum in 0.4 c.cm of mixture inoculated.	++	++	+	-	++	++	-	-	++	++	-	-
0.043 c.cm Tweedlie antiserum in 0.4 c.cm of mixture inoculated.	++	++	-	-	++	+	-	-	++	+	-	-

\- No necrotoxic reaction at side of injection.
\+ Necrotoxic reation at site of injection.
++ Considerable necrotoxic reaction at site of injection.

Estimation of the Effect of Gastric Contents on Toxins by in vitro Tests.

As it seemed possible that staphylococcal toxin, when introduced into the stomach, might be inactivated readily by the gastric secretion, especially by a secretion of acid reaction, the effect on the toxin of gastric contents of varying reactions was examined, the haemolysin and leucocidin being used to estimate toxic activity. The technique already described for these tests was employed (see pages 29 and 30), the only modification being that an equal volume of gastric contents, or saline as a control, was added to the dilutions of filtrate and incubated for half an hour at 37°C.

It was observed that even slightly acid (pH 6.8) or slightly alkaline (pH 7.8) contents considerably impaired the activity of the filtrate, whereas a specimen with a reaction of pH 7.2 to pH 7.4 had no appreciable effect on the toxin. The hydrogen ion concentration of the toxic filtrate itself was pH 7.3. Typical results are given in Table 10.

TABLE 10.

	End-titre of haemolytic test.	End-titre of leucocidin test.
Toxin + equal volume of gastric contents with a reaction of pH 6.8.	Only undiluted preparation active	Only undiluted preparation active
Toxin + equal volume of gastric contents with a reaction of pH 7.8.	Ditto	Ditto
Toxin + equal volume of gastric contents with a reaction of pH 7.3.	1 in 120	1 in 64
Toxin + equal volume of normal saline.	1 in 120	1 in 64

Animal Experiments.

Introduction of Toxin into Stomach.

The animals used in the experiments were rabbits and guinea-pigs. These animals were dieted for two days on green food and water, and on the day of the experiment they received no food till some hours after the introduction of the toxin.

In the preliminary work the required amount of toxin was introduced into the stomach without adjustment of the hydrogen ion concentration of the gastric contents.

The majority of animals, apparently, were not affected by the toxin, but certain of them succumbed rapidly. Guinea-pigs were more easily affected than rabbits. Nine guinea-pigs and five rabbits were all given 8 to 10 c.c. of a toxic staphylococcal filtrate and symptoms of poisoning were produced in two guinea-pigs. One died within an hour after the inoculation of 8 c.c. of toxin. The second showed signs of illness and intense muscular weakness within an hour or two, and was killed after six hours and a post-mortem examination carried out. Both showed the pathological condition described later, though in the former it was more typical and pronounced than in the latter.

In subsequent work the hydrogen ion concentration of the gastric contents was adjusted, as described in the methods (see pages 29 and 30), to

as nearly pH 7.3 as possible.

Seven guinea-pigs and one rabbit were all inoculated in this manner with 8 to 10 c.c. of toxin, and all died after intervals ranging from two minutes up to five days. General post-mortem findings in the different animals varied only in so far as the patholoical condition was more pronounced in animals dyeing after several days than in those succumbing after a shorter interval. The prominent post-mortem features were intense congestion of the mucosa of the stomach and duodenum, associated with small haemorrhages in the tissue and effusion of blood into the lumen, and congestion of various internal organs with haemorrhages, e.g. kidney, liver and lung. The autopsy on one guinea-pig which died five days after inoculation revealed also haemorrhage into the abdominal cavity.

Histological examination was made of the stomach-wall, liver and kidney, and in certain instances the lung, intestine and spleen. Sections of the stomach-wall showed intense congestion with areas of haemorrhage, and in several cases, extreme degeneration of glandular tissue. A similar condition was found in the intestinal wall, except that less haemorrhage was present, and in addition to the preceding features severe catarrhal inflammation was observed. In sections of the liver, congestion, cloudy swelling and, in animals which survived

several days, fatty degeneration were noted. The kidney showed intense congestion and some capillary haemorrhages and, also, general cloudy swelling of the tubular cells. Sections of lung examined revealed marked congestion of the alveolar capillaries with slight haemorrhages into the alveoli.

Two control experiments were performed on guinea-pigs. In one animal the gastric contents were made alkaline (pH 7.8), in the other acid (pH 6.8), previous to the addition of 8 c.c. of toxin. The health of the animals was apparently not impaired.

Intrarectal Inoculation of Toxin.

Guinea-pigs and rabbits were used for these experiments, and a number 3 rubber catheter was passed a short distance into the rectum. In the first experiments the filtrate was introduced directly into the rectum, and the catheter withdrawn. The results of experiments performed by this method were uniformly negative.

In later experiments the rectum was irrigated with saline and, after a suitable interval, its reaction, usually slightly acid, was adjusted as nearly as possible to pH 7.3, by addition of a solution of 5 per cent. sodium bicarbonate and N/10 hydrochloric acid in a manner similar to that already described for adjustment of the reaction of the stomach contents (see pages 29 and 30).

Two experiments were carried out by this method, and symptoms of poisoning resulted in both instances. One guinea-pig died two days after inoculation of 10 c.c. of toxic filtrate, the general post-mortem features being similar to those described in the preceding experiments, with the exception that the large intestine was deeply congested and contained mucus and blood. In addition, pronounced haemorrhagic lesions were observed in the lungs. A second guinea-pig was inoculated with 10 c.c. toxic filtrate and killed after two days. Intense congestion of the stomach, liver, spleen and kidneys was the prominent post-mortem feature along with haemorrhages in the kidneys. The lungs in this case were apparently normal.

Discussion.

Staphylococcal filtrates have aroused much controversy as to the unity or plurality of their toxic principle.

A constant quantitative relationship between the haemolytic and necrotoxic properties was established by Burnet (1929) who deduced from this that the toxin was a single entity. Gross (1931) came to a similar conclusion observing that potent staphylococcal filtrates invariably contained haemolysin, leucocidin, necrotoxic and lethal toxins. As additional proof he showed that one antitoxin could neutralise all these properties and also that they were all thermolabile to the same degree. Likewise Gengou(1930) and Nélis (1934) produced strong evidence in favour of the unity of the toxin by failing to separate any single property.

Other investigators have favoured the theory of the diversity of staphylococcal toxin. Parker-Weld and Gunther (1931) and Panton and Valentine (1932) demonstrated a definite correlation between the haemolytic, necrotoxic and lethal activities but not with the leucocidal activity. According to Burky (1933) repeated subculture of a strain resulted in a decrease of haemolysin production and a simultaneous increase in lethal effect. Similarly both anaerobic culture and the use of synthetic media inhibited the production of haemolysin but not the lethal factor. Burky suggested that the increase of lethal activity in the absence of haemolysin might be due to an

inhibitory effect on it of the haemolysin when present.

The finding reported in this thesis that strains vary from time to time in their ability to produce toxin under apparently constant cultural conditions is in agreement with that of Burky (1933). Bigger (1933) attributed fluctuation in the production ~~in~~ of haemolysin to the presence of active and inactive strains in the culture, the relative overgrowth of one or the other influencing the lysogenic power of the culture as a whole.

The amount of toxin produced has been shown to be markedly influenced by cultural conditions (Walbum, 1922; Bigger, Boland and O'Meara, 1927; Burky, 1933 and Nelis, 1934). This influence is probably due to their effect on the production of single toxic factors (Burky, 1933) and may account to some extent for the discrepancies between the results of different investigators.

Absorption tests have been used in attempts to remove from staphylococcal toxins certain properties and leave others intact. The evidence obtained thereby has been conflicting as to the unity or plurality of the constituents. As before mentioned the results of Gengou (1930) and of Nélis (1934) favour the idea of unity but Neisser and Wechsberg (1901) by exposure to an excess of leucocytes absorbed leucocidin and left the haemolysin intact. Parker-Weld and Gunther (1931) too by absorption with leucocyte and erythrocyte stroma attempted to separate haemolysin and leucocidin respectively but their results were not conclusive. They found, however, that all

the other toxic elements except that producing the necrotoxic effect might be removed by absorption. This, they considered, showed that the necrotoxin at least was a separate entity.

The experimental work detailed in this thesis has demonstrated divergences in the ratio of the toxic properties. All four properties were found in potent filtrates (cf.Gross 1931) and the various toxic activities, including leucocidal action, appeared to correspond on broad lines. For instance, the toxins showing the highest activity were selected and it was found that, with a few exceptions, the groups contained the same filtrates.

Filtrate.	Haemolytic Test.	Leucocidin Test.	Skin Test.	Lethal Test.
Sl.	1:5680	1:512	1:30	-
Wood.	1:3840	"	"	0.4 c.c.
Farmer	"	1:128	"	-
Gon.	"	1:256	"	0.2 c.c.
Tweedlie.	1:1920	1:128	"	"
Millar.	"	-	-	-
Elliott.	"	-	-	-
Black.	"	-	-	-
McGregor.	"	-	-	-
Kennedy.	-	1:128	1.30	0.2 c.c.
Allen.	-	"	"	0.4 c.c.
Bentely.	-	"	-	0.4 c.c.
Glover.	-	-	1.120	-

- = not outstandingly active.

Panton and Valentine (1932) elaborated a classification of Staphylococci on the apparently inadequate

basis of the differences in the haemolysin and leucocidin content of filtrates. The four groups thus demonstrated were each correlated with the types of lesion for which the strains were responsible. For instance, of the twenty two strains classified the seven placed in Group 1 produced much leucocidin and little haemolysin. These seven were subsequently found to have been isolated from severe lesions. In the present work several strains from severe lesions have been studied but neither their filtrates or those of any other strains have been found to be actively leucocidal without being also actively haemolytic. The three strains placed by Panton and Valentine in their Group 11 had both a high leucocidin and high haemolysin content. They were derived from boils or carbuncles. In the present study numerous strains were obtained from similar sources and they too had a high leucocidin and high haemolysin content. The strains of Panton and Valentine's Group 111 were isolated from lesions of differing severity and had a low leucocidin and high haemolysin content. None of the strains examined here corresponded. Many of the strains examined would fall into Panton and Valentine's saprophytic Group 1v on account of their poor leucocidal and poor haemolytic powers. They were not, however, with the possible exceptions of one from a throat swab and one in a laboratory culture saprophytic as they were derived from cases of osteomyelytis ("Fraser", "Roe", "Spence" and "Hamilton"), from a case of recurrant boils ("Burnie") and from a case of cholecystis ("McMillan").

Panton and Valentine found among the twenty two examined one strain isolated from a case of osteomyelytis, which produced neither leucocidin nor haemolysin. In this work seven such non-toxic strains were found. "Watson" was isolated from a fatal case of pyemia; "Ovens" and "Hartley" from abscesses of the hip and axilla respectively; "Todd" from a throat swab; "Thomson" from an infected compound dislocation and "Hogg" from an infected compound fracture.

Nélis (1934) drew attention to the lack of parallesism between invasiveness and toxin production. Burky (1933) attempted to correlate both properties with the type of lesion in which the strains were found. Those with well developed invasive and weak toxic powers were associated with acute staphylococcal conditions; a statement diverging from that of Panton and Valentine (1932) regarding the saprophytic origin of such strains. Highly toxic strains with little aggressiveness were associated with chronic lesions of the skin and mucous membranes. The general trend of the findings reported in this thesis follow the lines suggested by Burky (1933). Examples of highly invasive strains with low grade toxicity have already been given in this discussion and the most active toxin producers have been isolated from boils and carbuncles, ("Kennedy", "Elliot", "Bentley", "Gon" and "Farmer"): from cellulitis, ("Tweedlie"); from sycosis, ("Sl"); from an abscess, ("Black") and from suppurative conditions, ("McGregor" and "Wood"). No definite statement can be made as to the invasiveness of these toxic strains. It will, however, be noted that in agreement with the

findings of Burky (1933) most of these strains were isolated from infections of the skin or mucosa. The few exceptions were isolated from adjacent tissues. Fluctuation in the production of toxin (cf. Burky 1933 Bigger 1933 and this thesis) would, however, have to be taken into consideration and, in addition, the difficulty of defining many staphylococcal infections as acute or chronic decreases the value of a classification on such grounds.

The findings of the toxin-antitoxin neutralisation experiments reported in the experimental work are in accordance with those of other investigators (Neisser and Wechsberg, 1901; Burnet, 1929; Gross, 1931; Parish and Clark, 1932). They afford proof of the antigenic similarity of all haemolysins, all leucocidins and all necrotoxins.

Attention has recently been drawn to the presence of additional toxic factors in staphylococcal filtrates. Glenny and Stevens (1935) demonstrated the presence of two antigenically distinct toxins ("a" and "b"). The "B" toxin was identical with the "Hot-cold" lysis of Bigger, Boland and O'Meara (1927) and its occurance was comparitively rare.

Jordan and Burrows (1933) separated a gastro-intestinal toxin which proved non-haemolytic and incapable of producing a skin reaction. They, thus, considered this property distinct from the haemolysin and necrotoxin. Woolpert and Dack (1933) came to a similar conclusion on finding that the food poisoning toxin was not neutralised by staphylococcal antitoxin and that it possessed greater thermostability than

the other toxic properties.

The experiments with staphylococcal gastro-intestinal toxin recorded in the experimental work in this thesis illustrate how laboratory animals such as rabbits and guinea-pigs may naturally be protected against staphylococcal toxin when it is introduced directly into the alimentary tract. The gastro-intestinal poison is, however, readily destroyed by exposure to an alkaline solution or by acid in the presence of heat (Jordan and Burrows, 1933). The results in the present work suggest that the inactivating effect of the acid contents of the stomach is an essential factor in this protection, and explain the failure of previous workers to reproduce experimentally in these animals the condition of "food-poisoning" by staphylococcal toxin as observed in the human subject. On the other hand, the local and general toxic effects produced by staphylococcal filtrates introduced into the stomach of guinea-pigs after neutralization of the contents, afford an interesting demonstration that the toxins of the staphylococci growing in certain articles of food may produce serious poisoning. It seems likely also that the hydrogen ion concentration of the stomach contents is a factor which determines the occurence of such poisoning in the human subject, and this may explain the variable results observed by Jordan and Hall when volunteers were fed with milk containing staphylococcal toxin. Further the experiments indicate how small laboratory animals can, under certain conditions, be utilized for further inquiry

into this form of food-poisoning, and may have a more general application in the study of the effects of other bacterial toxins on the alimentary tract.

Summary and Conclusions.

1) Walbum's liquid medium encourages the production of staphylococcal toxin and is more practible than a solid medium.

2) No haemolysin is lost in the process of filtration.

3) Rabbit red cells are more sensitive than those of ox or sheep to "Wood" lysin.

4) The rate of action of different haemolysins and necrotoxins varies.

5) The origin of strains could not be correlated to their toxic properties. Parallelism in the haemolytic, leucocidal, necrotoxic and lethal properties of each filtrate was observed. This affords no proof as to the unity of the toxic principle.

6) Strains fluctuate in their ability to produce toxin.

7) Toxin kept anaerobically at room temperature (15°C) or in the ice chest (0°C), after a slight deterioration in the first month, loses little activity.

8) More staphylococcal antitoxin is required to neutralise the necrotoxic than the haemolytic and leucocidal effects. Haemolysins are all neutralised to the same extent by antitoxins as are also leucocidins and necrotoxins. This shows the antigenic identity of these specific properties.

9) When staphylococcus toxin is added to gastric contents in vitro a slightly acid (pH 6·8) or a slightly alkaline (pH 7·8) reaction impairs its activity whereas no inactivation occurs at pH 7·3.

10) Symptons of poisoning can only occasionally be produced in guinea-pigs and rabbits when toxin is introduced directly into the stomach. Uniformly positive results are obtained in experiments in which the reaction of the stomach is adjusted to pH 7·3 at the time of introduction of the toxin. The animals die within five days, and post-mortem show signs of acute gastroenteritis and marked congestion of internal organs, associated with haemorrhage in the stomach and kidneys.

11) Symptons of poisoning can be produced by intrarectal inoculation of toxin when the rectum has been irrigated with saline and the reaction adjusted to pH 7·3. Post-mortem features are similar to those obtained after introduction of toxin into the stomach. This method is not so effective as innoculation into the stomach.

PART II

The Antigenic Relationship of the Toxins of the B. welchii Group, and an Account of Factors predisposing to Infection from the Alimentary Tract by (1)Vibrion septique, and (2) B. ovitoxicus.

Introduction.

Though numerous bacteria may be involved in gas gangrene infections, four, namely B. welchii, Vibrion septique, B. oedematiens and B. histolyticus are of particular importance (Weinberg and Sequin, 1918; Zeissler, 1930). They can be distinguished by cultural reactions (Zeissler, 1917 and 1930) and by the antigenic structure of their toxins (Klose, 1916; Ficker, 1917; Bull and Pritchett, 1917; Weinberg and Sequin, 1918). Bull and Pritchett (1917) were the first to demonstrate the exotoxin produced by B. welchii and the significance of specific toxins in the classification of the sporing anaerobes. Their findings have since been confirmed (Medical Research Committee, 1919). More recent work has demonstrated the production of multiple toxins by certain members of the group.

Vibrion septique.

Gaiger (1922,a) established Vibrion septique as the causal organism of braxy or bradsot. It is generally agreed that the initial lesion is in the mucosa or submucosa of the abomasum but that the clinical features are due to absorption of the exotoxin(Nielson, 1897; Gaiger, 1922, b). The fact that certain strains though capable of immunising sheep in some areas of Britain are not effective in other localities suggests serological differences in the toxins of different strains of the organism (Glenny, Barr, Llewellyn-Jones, Dalling and Ross, 1933).

B. welchii Group.

Organisms from various sources have been

included in the B. welchii group. Gaiger and Dalling (1923) isolated a bacillus from lambs suffering from lamb dysentery which is an enteritis varying from mild congestion of the intestinal mucosa to necrosis and ulceration in both the small and large intestines. Practically pure cultures of the bacillus were obtained from the lesions and the organism was held to be responsible for the toxaemia. The inflammation of the intestinal mucosa, however, they regarded as caused primarly by B. coli but the rôle played by that organism was shown by Dalling (1926) to be negligible for he reproduced the disease by feeding healthy young lambs with cultures of the lamb dysentery bacillus.

McEwen (1930) believed that B. paludis, a B. welchii like organism, was the aetiological agent of struck, a disease of sheep on Romney Marsh. The infection originated in the alimentary tract, and frequently no further bacterial invasion of the tissues could be demonstrated. An exotoxin elaborated by the organism was, therefore, held responsible for the injury of the intestinal wall and subsequent death of the animal. He reproduced the disease by feeding sheep with culture but not with even large amounts of sterile toxic filtrates.

Bennetts (1932) identified B. ovitoxicus as the causal organism of infectious-enterotoxaemia of sheep, and was able to reproduce the disease in healthy sheep by feeding with the living organism.

Mason, Ross and Dalling (1931) defining the

relationship of B. paludis (McEwen) to the B. welchii group of organismsshowed that the antisera for B. paludis and the lamb dysentery bacillus protect against the toxins of the classical B. welchii of the lamb dysentery bacillus and of B. paludis. Antisera for the classical B. welchii, however, neutralise B. welchii toxin only and not the lamb dysentery or B. paludis toxins. These findings are not in accordance with those previously made by Dalling (1926) and Weinberg and Ginsbourg (1927) that B. welchii antitoxin neutralises lamb dysentery toxin, and by McEwen (1930) that B. paludis antitoxin does not neutralise B. welchii toxin. Wilsdon (1931) found that the toxins produced by a large number of B. welchii strains isolated from different sources could be arranged according to their antigenic structure in four sub-groups. He distinguished three toxic constituents W, X and Z. Factor W was produced to a certain extent by types B, C and D and was the only factor produced by type A. Factor X was found in types B and D, and factor Z was present and predominant in types B and C. Each of these sub-groups contained organisms isolated from a particular source: type A were the classical B. welchii types as described originally in cases of gas gangrene; type B the lamb dysentery organism; type C B. paludis and type D consisted of various strains of animal origin and (Wilsdon, 1933) B. ovitoxicus (Bennetts).

Glenny, Barr, Llewellyn-Jones, Dalling and Ross (1933) demonstrated by means of titrations with antisera the presence of four toxins in the culture filtrates of B. welchii, B. paludis and the lamb dysentery bacillus. They indicated that still further constituents might be present. Wilsdon's type D certainly produced a fifth element.

Recent experiments have demonstrated in the alimentary tracts of sheep and lambs the presence of the specific toxins of various anaerobes responsible for infections such as pulpy kidney (due to B. ovitoxicus), lamb dysentery and struck. Sterile filtrates of the intestinal contents of such animals killed mice on intravenous inoculation. Intravenous inoculation of similarly prepared material from cases of pulpy kidney was found to be toxic for healthy lambs and produced symptoms and post mortem appearances identical with those of the natural disease(Montgomerie and Dalling, 1933; Montgomerie and Rowlands, 1933) Antisera prepared for Wilsdon's type D and B. ovitoxicus neutralised filtrates of the intestinal contents from cases of pulpy kidney (Montgomerie and Dalling, 1933; Montgomerie and Rowlands, 1933). The same sera according to Montgomerie and Rowlands,(1933) neutralised intestinal filtrates from animals suffering from struck but this is not confirmed by the work of Wilsdon (1931 and 1933). A close relationship between B. welchii type D and B. ovitoxicus was, therefore demonstrated. The serum prepared from B.paludis

neutralised lamb dysentery toxin and that from lamb dysentery occasionally neutralised intestinal filtrates from cases of pulpy kidney (Montgomerie and Rowlands, 1933). Montgomerie and Dalling (1933) showed that certain lamb dysentery sera which had been prepared before 1930 neutralised filtrates of the intestinal contents from cases of pulpy kidney and struck but later batches of sera similarly prepared did not give the same results.

Experimental Work.

There appears to be general agreement that the members of the B. welchii group are antigenically dissimilar (Dalling, 1926; Weinbourg and Ginsbourg, 1927; Mason, Ross and Dalling, 1931; Wilsdon, 1931 and 1933; Glenny and his co-workers, 1933;Montgomerie and Rowlands, 1933). The exact inter-relationship has not, however, been clearly defined. Further investigations, therefore, on the relationship of the toxins of the members of the B. welchii group both to each other and to other members of the gas gangrene group have been carried out. The neutralising ability of various B. welchii commercial antisera against the different type toxins was studied. The protective power of the B. welchii type and commercial antisera against experimental infection by the different B. welchii strains was tested. The production of infection and intoxication from the alimentary tract by members of the group of sporing anaerobes has been studied.

The results are reported in the following experimental work.

Methods.

Strains.

The B. welchii strains used in the experimental work were Wilsdon's type strains, A, B, C and D, and also a strain of B. ovitoxicus (Bennetts). These strains were all obtained from the Institute of animal pathology at the University of Cambridge.

Two strains of Vibrion Septique were employed. One, "AɳM", had been isolated from a case of braxy and was obtained from the Moredun Institute of the Animal Diseases Research Association. The second, "Bedford" was obtained from the National Collection of Type Cultures, and had originally been isolated from an amoebic abscess of the brain in the human subject.

Purity of B. welchii Cultures.

Typical colonies were produced on Zeissler's (1930) dextrose blood agar by all the B. welchii strains and cultures were made from single colonies in liver bouillon.

Concentration of Cultures.

In order to reduce the volume introduced into the stomach when Vibrion septique culture was fed to guinea-pigs, the fluid portion of an 18 to 24 hours' culture in cooked-meat medium was decanted and centrifuged, after which the supernatant fluid, except for 2 to 3 c. cm. was discarded. The deposit of organisms, mixed

with the remaining fluid, was employed in the feeding experiments.

Preparation of Toxin

The medium used for the preparation of toxin from the B. welchii strains was a peptic digest broth known as "Bouillon V.F.". This medium was recommended by Wilsdon (1931) and was prepared according to the method used at the Pasteur Institute in Paris. 500 grams of pig's stomach were minced and added to 5 litres of water warmed to 50°C and 50 c.c. of normal HCL in a glass vessel. After standing for 24 hours at 48° C to 52° C it was heated to 80° C for 5 to 10 minutes to arrest digestion and then filtered. 0.1% of potassium nitrate and 0.2% of glucose were added to the filtrate and the reaction was adjusted with 10.N.NaOH to pH 7.6. The medium was then boiled to precipitate the phosphates, filtered, distributed in about 500 c.c. amounts in litre flasks and autoclaved for half an hour.

About 10 c.c. of 24 hour bouillon cultures of the different strains were inoculated into the flasks. To ensure rapid growth the inoculation was made while the medium was at a temperature of 37° C to 40° C. The flasks were then incubated anaerobically at 37° C. The period of incubation for optimum toxin production varied with the different B. welchii types: types A,

B and C required 15 to 24 hours and type D, 3 to 5 days.

Filtration was carried out first through fine filter paper and then through a Seitz filter. The toxins were then concentrated by precipitation with ammonium sulphate, dried and ground to powder.

Potency was estimated by two methods, (1) intravenous inoculation in mice of different doses of toxin dissolved in a constant amount (0.5 c.cm) of saline (2) intracutaneous inoculation in the shaved abdomen of light skinned guinea-pigs of different doses of toxin dissolved in a constant amount (0.2 c.cm.) of saline.

Vibrion septique toxin was prepared according to Robertson's (1920) method, and its potency estimated by intracutaneous inoculation into the depilated skin on the abdomen of guinea-pigs, 0.3. c.c. of the varying dilutions of the toxic filtrate being injected. The hydrogen-ion concentrations of the filtrate and the saline diluent were adjusted to pH 5 and the mixtures kept for two hours at room temperature before injection. This hydrogen-ion concentration is later shown to be favourable to the activity of the toxin. Control inoculations of saline at pH 5 and the filtrate, heated at 60° C for half an hour, were also made. Necrosis at the site of inoculation, which was observed most readily after 48 hours, was the criterion of a positive result.

Preparation of B. welchii Antitoxin.

Monovalent sera were prepared from rabbits

against all the different type toxins. Weekly intravenous injections of toxoid and later toxin were given over a period of eight to ten weeks. The toxoid was prepared by incubating the toxin with formol at 37° C. The period of incubation and the amount of formalin used varied with the different types: types A and B were treated with 0.1 formalin for three days, type C with 0.3 formalin for seven days and type D with 0.2 formalin for four days. The first dose injected contained two average lethal doses of toxin for mice which doses were converted to toxoid. Subsequent doses of toxoid contained increasingly greater amounts of toxin till eventually a series of small doses of toxin, one to five average lethal doses for mice, were injected.

Testing of Antitoxin.

The antitoxic value of the sera was determined by its protective and antinecrotoxic properties. Doses of toxin of known lethal and necrotoxic activity, dissolved in 0.25 c.cm. and 0.1. c.cm. of saline respectively, along with the same volume of different dilutions of sera in saline were injected intravenously in mice and intracutaneously in guinea-pigs as described previously. The toxin-antitoxin mixtures were allowed to stand one hour at room temperature before inoculation. The criteria of the results, in the mice intravenous tests was death, and, in the guinea-pig intracutaneous tests, necrosis. Survival of mice for three days after injection of a toxin-antitoxin mixture was

accepted as proof of the protective powers of the antisera and the absence of necrosis in guinea-pig skins two to three days after inoculation as evidence of its antinecrotic powers.

The Protective Powers of Sera against Infection.

The average lethal dose of organisms from twenty four hour liver bouillon cultures was determined by intramuscular injection in guinea-pigs. Two to three average lethal doses of organisms and, at the same time, the sera to be tested were then injected intramuscularly in guinea-pigs. The injections were made separately and as nearly as possible in the same site. The protective power of the serum was judged by the survival or death of the guinea-pigs within twenty four hours of inoculation.

Experimental.

The Inter-relationship between the B.welchii Type Toxins.

It seemed advisable to study the inter-relationship of the different types of B. welchii toxins by the investigation of more than one toxic property. Cross neutralisation tests between the toxins and the monovalent sera for the B. welchii types was therefore carried out by two methods (1) intravenous tests in mice as described under methods; this being a repitition of Wilsdon's (1931) work. (2) intracutaneous tests in guinea-pigs also as described under methods. These experiments and their results are recorded in tables (1) and (2). Two and a half to five average lethal doses and about two average doses of necrotoxin were found to be suitable amounts of toxin for the preliminary experiments. Subsequent lethal tests with less toxin were carried out where it was considered necessary, as when the preliminary findings did not agree with the results of the skin tests or with Wilsdon's (1931) findings.

There is a general agreement of the results of the lethal and skin tests, with two exceptions; C antitoxin affords mice no protection against the lethal effects of toxin A, and yet actively neutralises A necrotoxin, and also, D. and B. ovitoxicus sera appear to neutralise C lethal toxin slightly but have no action on C necrotoxin. It will be noted from tables (1) and (2) that the results obtained with D and B. ovitoxicus toxins and antitoxins invariably correspond showing these toxins are antigenically identical. The

TABLE I

CROSS NEUTRALISATION TESTS BETWEEN B. WELCHII TYPE TOXINS AND THEIR MONOVALENT ANTITOXINS AS DETERMINED BY INTRAVENOUS INJECTION IN MICE.

Toxins. (mgrs. in 0.25 ccm.)	Neutralising Doses of Monovalent Antitoxins in 0.25 ccm.														
	Antitoxin A.			Antitoxin B.			Antitoxin C.			Antitoxin D.			Ovitoxicus Antitoxin.		
	Number of mice inoculated.	Dose of Antitoxin.	Number of mice surviving	Number of mice inoculated.	Dose of Antitoxin.	Number of mice surviving.	Number of mice inoculated.	Dose of Antitoxin.	Number of mice surviving.	Number of mice inoculated.	Dose of Antitoxin.	Number of mice surviving.	Number of mice inoculated.	Dose of Antitoxin.	Number of mice surviving.
Toxin A. (average lethal dose = 0.02 mgrs.)															
0.1 mgrs. in 0.25 ccm.	2	1:80	2	2	1:5	0	2	1:5	0	2	1:5	0	2	1:5	2
		1:160	0										2	1:10	0
0.05 mgrs. in 0.25 ccm.	2			2	1:5	2	2	1:5	0	2	1:5	0			
				2	1:10	0									
0.025 mgrs. in 0.25 ccm.							2	1:5	0	2	1:5	2			
							2	undiluted	0	2	1:10	0			
Toxin B. (average lethal dose = 0.01 mgrs.)															
0.025 mgrs. in 0.25 ccm.	2	1:5	0	4	1:80	3	2	1:80	2	2	1:5	0	2	1:5	0
				4	1:160	0	2	1:160	0						
Toxin C. (average lethal dose = 0.0025 mgrs.)															
0.00625 mgrs. in 0.25 ccm.	2	1:5	0	2	1:20	2	2	1:80	2	2	1:5	Prolongation of life	2	1:5	1
				2	1:40	0	2	1:160	0				2	1:10	0
Toxin D. (average lethal dose = 0.005 mgrs.)															
0.025 mgrs. in 0.25 ccm.	2	1:5	0	2	1:5	2	2	1:5	0	4	1:10	3	4	1:10	4
				2	1:10	0				5	1:20	0	4	1:20	2
													2	1:40	0
Ovitoxicus Toxin (average lethal dose =0.02 mgrs.)															
0.1 mgrs. in 0.25 ccm.	2	1:5	0	2	1:5	0	2	1:5	0	4	1:20	4	4	1:80	3
										4	1:40	0	4	1:160	0
0.05 mgrs. in 0.25 ccm.				2	1:5	2									
				2	1:10	0									

TABLE 2.

CROSS NEUTRALISATION TESTS BETWEEN B. WELCHII TYPE TOXINS AND THEIR MONOVALENT ANTITOXINS AS DETERMINED BY INTRACUTANEOUS INJECTION IN GUINEA-PIGS.

Toxins. (mgrs. in 0.1 ccm.)	Neutralising Doses of Monovalent Antitoxins in 0.1 ccm.									
	Antitoxin A.		Antitoxin B.		Antitoxin C.		Antitoxin D.		Ovitoxicus Antitoxin	
	Dose of Antitoxin.	Result.	Dose of Antitoxin.	Result.	Dose of Antitoxin.	Result.	Dose of Antitoxin.	Result.	Dose of Antitoxin.	Result.
Toxin A. (average necrotoxic dose = 0.3 mgrs.) 0.6 mgrs. in 0.1 ccm.	1:40	-	1:10	-	1:10	-	1:5	-	1:40	-
	1:80	++	1:20	+	1:20	+	1:10	++	1:80	++
			1:40	++	1:40	++				
Toxin B. (average necrotoxic dose = 0.005 mgrs.) 0.01 mgrs. in 0.1 ccm.	1:5	+++	1:5	-	1:10	-	1:5	++	1:5	++
			1:10	+	1:20	+				
			1:20	+++						
Toxin C. (average necrotoxic dose = 0.007 mgrs.) 0.015 mgrs. in 0.1 ccm.	1:5	++	1:20	-	1:20	-	undiluted	++	undiluted	++
			1:40	++	1:40	++				
Toxin D. (average necrotoxic dose = 0.02 mgrs.) 0.04 mgrs. in 0.1 ccm.	1:5	++	1:5	-	1:5	++	1:5	-	1:10	-
			1:10	+			1:10	+	1:20	+
			1:20	++						
Ovitoxicus Toxin. (average necrotoxic dose = 0.04 mgrs.) 0.08 mgrs. in 0.1 ccm.	1:5	++	1:10	-	1:5	++	1:10	-	1:40	-
			1:20	+			1:20	*	1:80	++
							1:40	++		

\- * = no reaction at site of injection.
\+ = a slight necrotic reaction at site of injection.
++ = a considerable necrotic reaction at site of injection.

greater amount of both D and B.ovitoxicus anti-toxins required to neutralise B.ovitoxicus toxin is explained by the fact that the sample of B.ovitoxicus toxin contained more toxoid than that of type D.

The neutralising Ability of Commercial B.welchii Sera against the Type B.welchii Toxins.

The neutralising ability of commercial B.welchii antisera from London, Paris and Marburg was tested against the different types of B.welchii toxins by the mouse intravenous and the guinea-pig intracutaneous methods. These experiments are recorded in tables (3) and (4) and from the results it may be seen that the sera neutralise both the lethal and necrotoxins of A but have no action on the toxins of the other types.

The Toxins of the B.welchii Types and Their Antigenic Relationship to other Members of the Gas Gangrene Group.

The neutralising ability of B.histolyticus, V.septique, and B.oedematiens antisera against the different B.welchii type toxins was investigated by guinea-pig intracutaneous tests as described under methods. The experiments are recorded in table(5) and from the results it will be seen that the antisera have no neutralising action on any of the toxins, with perhaps the exception against C toxin. It was decided to investigate thoroughly the question of a possible antigenic relationship between B.welchii type C and B.oedematiens. Neutralisation experiments were therefore carried out between C toxin and B.oedematiens antitoxin and B.oedematiens toxin and

TABLE 3.

NEUTRALISATION EXPERIMENTS BETWEEN B. WELCHII TYPE TOXINS AND COMMERCIAL B. WELCHII ANTISERA AS DETERMINED BY INTRAVENOUS INJECTION IN MICE.

Toxins. (mgrs. in 0.25 ccm.)	Neutralising Doses of Standard Antiserums in 0.25 ccm.								
	London Antitoxin.			Paris Antitoxin.			Marburg Antitoxin.		
	Number of mice inoculated.	Dose of Anti-toxin.	Number of mice surviving.	Number of mice inoculated.	Dose of Anti-toxin.	Number of mice surviving.	Number of mice inoculated.	Dose of Anti-toxin.	Number of mice Surviving.
Toxin A. (average lethal dose = 0.02 mgrs.) 0.1 mgrs. in 0.25 ccm.	2	1:320	2	2	1:80	2	2	1:160	2
	2	1:640	0	2	1:460	0	2	1:320	0
Toxin B. (average lethal dose = 0.01 mgrs.) 0.025 mgrs. in 0.25 ccm.	2	1:5	0	2	1:5	1	2	1:5	0
					1:10	0			
Toxin C. (average lethal dose = 0.00625 mgrs. in 0.25 ccm.	4	1:5	0	2	1:15	0	2	1:5	0
Toxin D. (average lethal dose = 0.005 mgrs.) 0.025 mgrs. in 0.25 ccm.	4	1:5	1	2	1:5	0	2	1:5	0
Ovitoxicus Toxin. (average lethal dose = 0.02 mgrs.) 0.1 mgrs. in 0.25 ccm.	2	1:5	0	2	1:5	0	2	1:5	0

TABLE 4.

NEUTRALISATION EXPERIMENTS BETWEEN B. WELCHII TYPE TOXINS AND COMMERCIAL B WELCHII ANTISERA AS DETERMINED BY INTRACUTANEOUS INOCULATION IN GUINEA-PIGS.

Toxins. (mgrs. in 0.1 ccm.)	Neutralising Doses of Monovalent Antitoxins in 0.1 ccm. London Antitoxin. Doses of Antitoxin.	Result.	Paris Antitoxin. Doses of Antitoxin.	Result.	Marburg Antitoxin. Doses of Antitoxin.	Result.
Toxin A. (average necrotoxic dose = 0.3 mgrs.) 0.6 mgrs. in 0.1 ccm.	1:80	-	1:160	-	1:640	-
	1:160	+	1:320	+	1:1280	+
Toxin B. (average necrotoxic dose = -.--5mgrs.) 0.01 mgrs. in 0.1 ccm.	1:5	++	1:5	++	1:5	++
Toxin C. (average necrotoxic dose = 0.007 mgrs.) 0.015 mgrs. in 0.1 ccm.	1:5	++	1:5	++	1:5	++
Toxin D. (average necrotoxic dose = 0.04 mgrs.) 0.04 mgrs. in 0.1 ccm.	1:5	++	1:5	++	1:5	++
Ovitoxicus Toxin. (average necrotoxic dose = 0.04 mgrs.) 0.08 mgrs in 0.1 ccm.	1:5	++	1:5	++	1:5	++

- = no reaction at site of injection.
+ = a slight necrotic reaction at site of injection.
++ = a considerable necrotic reaction at site of injection.

TABLE 5.

NEUTRALISATION EXPERIMENTS BETWEEN B WELCHII TYPE TOXINS AND ANTISERA OF OTHER MEMBERS OF THE GAS GANGRENE GROUP AS DETERMINED BY INTRACUTANEOUS INOCULATION IN GUINEA-PIGS.

Toxins. (mgrs. in 0.1 ccm.)	Neutralising Doses of Antitoxins in 0.1 ccm.					
	Histolyticus Antitoxin.		V. septique Antitoxin.		B. oedematiens Antitoxin.	
	Dose of Antitoxin.	Result.	Dose of Antitoxin.	Result.	Dose of Antitoxin.	Result.
Toxin A. (average necrotoxic dose = 0.3 mgrs.) 0.6 mgrs. in 0.1 ccm.	1:5	++	1:5	++	1:5	++
Toxin B. (average necrotoxic dose = 0.005 mgrs.) 0.01 mgrs. in 0.1 ccm.	1:5	++	1:5	++	1:5	++
Toxin C. (average necrotoxic dose = 0.007 mgrs.) 0.015 mgrs. in 0.1 ccm.	1:5	++	1:5	++	1:5	+ (reaction delayed)
Toxin D. (average necrotoxic dose = 0.007 mgrs.) 0.015 mgrs. in 0.1 ccm.	1:5	++	1:5	++	1:5	++
Ovitoxicus Toxin. (average necrotoxic dose = 0.04 mgrs.) 0.08 mgrs. in 0.1 ccm.	1:5	++	1:5	++	1:5	++

± = no reaction at site of injection.
\+ = a slight necrotic reaction at site of injection.
++ = a considerable necrotic reaction at site of injection.

C antitoxin by both tests in mice and in guinea-pigs as previously described. The results are given in table (6) and show that neither type C or B.oedematiens antisera afford any protection against B.oedematiens and C toxins respectively, but C sera neutralises B.oedematiens necrotoxin markedly and B.oedematiens C necrotoxin slightly.

The Protection afforded by B.welchii Sera against Infection.

The technique for estimating the protective power of sera described under methods was used in studying the effect of the B.welchii type and Marburg commercial sera on infection by the different B.welchii types in guinea-pigs. The results of the experiments are given in table 7. Two animals were used for each test and the results for each animal is given as they did not always correspond exactly.

Production of Infection and Intoxication from the Alimentary Tract.

For the experimental study of infection and intoxication from the alimentary tract, organisms known to be responsible for anaerobic intestinal toxaemias in animals were chosen viz Vibrion septique which causes braxy and B. ovitoxicus which is responsible for infectious-enterotoxaemia. In braxy the factors determining the occurance of the abomasal infection are still obscure, though external cold and the ingestion of frozen grass have been regarded as predisposing (Nielson, 1897) (Hamilton, 1901: Gaiger, 1922 b). In infectious-enterotoxaemia inhibition of

TABLE 6.

THE ANTIGENIC RELATIONSHIP OF B. WELCHII TYPE C AND B. OEDEMATIENS AS DETERMINED BY NEUTRALISATION EXPERIMENTS BETWEEN C TOXIN AND B OEDEMATIENS ANTITOXIN AND B. OEDEMATIENS TOXIN AND C ANTITOXIN BY THE MOUSE INTRAVENOUS AND GUINEA-PIG INTRACUTANEOUS METHODS.

	LETHAL TESTS.					NECROTOXIC TESTS.			
	Neutralising Doses of Sera in 0.25 ccm.					Neutralising Doses of Sera in 0.1 ccm.			
	B. welchii Type C Antitoxin.			B. oedematiens Antitoxin.		B. welchii Type C Antitoxin.		B. oedematiens. Antitoxin.	
	Number of mice inoculated.	Dose of Antitoxin.	Number of mice surviving.	Number of mice inoculated.	Dose of Antitoxin.	Number of mice surviging.	Dose of Antitoxin.	Result.	Dose of Antitoxin.	Result.
Toxin B.welchii Type C.										
2.5 average lethal doses in 0.25 ccm.	---	---		2	1:5	0				
2 average nectotoxic doses in 0.1 ccm.							---	---	1:5	+(reaction delayed)
B. oedematiens Toxin.										
2 average lethal doses in 0.25 ccm.	2	1:5	0	---	---	---				
2 average necrotoxic doses in 0.1 ccm.							1:20 1:40	- ++	---	---

- = no reaction at site of injection.
+ = a slight necrotic reaction at site of injection.
++ = a considerable necrotic reaction at site of injection.

TABLE 7.

THE PROTECTIVE POWERS OF B. WELCHII SERA AGAINST INFECTION BY THE DIFFERENT B. WELCHII TYPE STRAINS IN GUINEA - PIGS.

	Strain A (dose 0.5 ccm of culture).		Strain B (dose 0.25 ccm of culture)		Strain C ccm of cluture.)		1 Strain D (dose 0.25 ccm of culture)	
	Result in Guinea-pigs 1	and 2	Result in Guinea-pigs. 1	and 2	Result in Guinea-pigs. 1 and	2.	Result in Guinea-pigs. 1	and 2.
Monovalent serum A Dose 1 ccm. undiluted.	+	+	-	-	-	-	+	+
Monovalent serum B dose 1 ccm. undiluted.	+	±	+	+	+	±	±	-
Monovalent serum C dose 1 ccm undiluted.	-	-	+	±	+	±	-	-
Monovalent serum D dose 1 ccm undiluted.	-	-	-	-	-	-	*	+
Marburg Commercial serum dose 0.5 ccm. undiluted.	+	+	-	-	-	-	+	+
Controls - No serum	+		+		+		+	

\- * Death within 24 hours of inoculation.
± = III 24 hours after inoculation and died within three days.
\+ = Lived.

secretion and of peristalsis with an alkaline reaction of the gastric contents facilitate the onset of the disease (Bennetts, 1932).

Guinea-pigs were used for the experiments with Vibrion septique and sheep for the experiments with B.ovitoxicus.

Vibrion septique cultures and toxin very rarely set up an infection or intoxication when fed to normal guinea-pigs. Observations were therefore carried out to ascertain the conditions which predispose to infection or toxic action.

The following conditions were investigated: (1) The optimal hydrogenion concentration for toxic activity. In culture medium pH 7.6 to pH 7.8 has been accepted as the optimal concentration for toxin production (Robertson, 1920; McEwan, 1926). (2) The inhibition of peristalsis. Since narcotine has been shown to exert a depressant action on the movements of the whole gastro-intestinal system in cats (Chopra, Mukhjee and Dikshit, 1930; Dikshit, 1932), it was thought of interest to determine whether this drug had a similar effect in guinea-pigs and, if so, to study the effect of adminstering Vibrion septique and its toxin when the alimentary tract was in a state of decreased activity. (3) The influence of cold, which has been acknowledged to predispose was also investigated.

The Influence of pH on the Activity of the Toxin.

Intracutaneous inoculations, such as those described for determining the potency of the toxin, were employed to estimate the influence of pH on its

TABLE 8. Results obtained on Intracutaneous Inoculation in Guinea-pigs of 0.3 c.c. Amounts of Dilutions of Toxin Adjusted to Varying H-ion Concentrations.

pH.	Toxin Dilutions. 1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	Controls Saline	Toxin heated to 60°C. for ½ hour.
4.	+	+	-	-	-	-	-	-
5.	++	++	++	++	++	-	-	-
6.	++	++	++	++	+	-	-	-
7.	++	++	++	++	-	-	-	-
8.	++	++	++	-	-	-	-	-
9.	-	-	-	-	-	-	-	-

- No reaction at site of injection. + A slight necrotic reaction at site of injection. ++ A considerable necrotic reaction at site of injection.

activity. Toxin and saline diluent were adjusted to various H-ion concentrations ranging from pH to pH 9. Dilutions from 1/2 to 1/64 were made at each pH and kept at room termperature for 2 hours before being injected. Control inoculations of toxin heated to 60°C. for half an hour and of saline at each pH were made. These tests were repeated five times, and on each occasion controls failed to show any reaction. In all cases the most favourable H-ion concentration for the activity of the toxin was found to lie between pH and pH 6. A typical example of the results obtained is given in Table 8.

The Rate of Passage of Meals in the Alimentary Tract of the Guinea-pig.

In preliminary experiments methylene-blue thickened with gum acacia was fed to (a) normal guinea-pigs, (b) guinea-pigs which had received intramuscular injections of narcotine chloride 1 hour previously (3 mgm. of nar-

cotine per kg. of body-weight). The animals were killed 15 minutes later, and it was observed that in normal guinea-pigs the dye had passed as far as the junction of the small and the large intestine, whereas in the treated animals it had not passed out of the stomach.

More detailed investigations were made by observing the rate of passage of barium meals by X-ray screening in (a) normal guinea-pigs, (b) guinea-pigs treated with narcotine, and (c) guinea-pigs chilled by exposure to a temperature of approximately 5°C. for about an hour in the open or, when the weather was too warm by exposure to a temperature of 0°C. in the refrigerator for a short time.

Four normal guinea-pigs were given barium meals and examined by X-rays immediately after feeding, and at frequent intervals thereafter till all the meal had become diffused in the small intestine. The same animals were then treated with narcotine, fed with barium meals and examined by X-rays as before. The observations showed that narcotine in a dose of 3 mgm. per kg. of body-weight approximately doubled the time of passage.

Three guinea-pigs were chilled as described above. The normal rectal temperature varies from about 102° to 103°F. Chilling in the majority of animals produced only a slight fall in temperature (approximately 0.1°F.). A comparison of results obtained from normal and chilled animals showed no significant difference in the times required for a barium meal to pass through the stomach.

The results of these experiments suggest that narcotine is capable of arresting the movements of the gastro-intestinal

system, and thus increasing the time of passage of meals, in guinea-pigs, whereas cold causes little difference in the rate of food passage.

Experimental Infection of Guinea-Pigs.

The preparation of Vibrion septique culture and toxin has been described. The method of its administration to guinea-pigs was similar to that used for the introduction of staphylococcal toxin into the stomach of laboratory animals and has been described on pages 29 and 30. Part I. The reaction of the gastric contents was, however, adjusted to the range pH 5-6 or pH 7.6-7.8. In view of the observations recorded it was decided to feed the following groups of guinea-pigs with concentrated culture:

Group 1. Gastric contents adjusted to pH 7.6, this being the accepted H-ion concentration for the growth of Vibrion septique and the production of its toxin.

Group 2. Gastric contents adjusted to pH 5, in view of the finding that this H-ion concentration is optimal for the maintenance of the activity of the toxin.

Group 3. Treated with narcotine to decrease the movements of the alimentary canal. Two doses of narcotine were given; 3 mgm. per kg. of body-weight followed by half this quantity 3-4 hours later.

Group 4. Treated with narcotine, and gastric contents adjusted to pH 7.6.

Group 5. Exposed to a low temperature.

Group 6. Exposed to a low temperature, and the gastric contents adjusted to pH 7.6.

Group 7. Concentrated cultures cooled to approximately 0°C. before administration. Concentrated culture was placed in a freezing mixture until it had just frozen solid. It was then removed, and as each 2 to 3 c.c.

became sufficiently liquid it was administered to the guinea-pigs.

Group 8. Controls.

Further control groups of guinea-pigs in which the culture was not administered, were included: (1) Treated with narcotine, as in Groups 3 and 4 ; (2) exposed to cold, as in Groups 5 and 6.

TABLE 9. Results obtained from Experimental Feeding of Guinea-pigs with Vibrion septique

Group.	Number of animals	Animals with defence evidence of infection & toxic effects. Died.	Killed.	per cent. infected.	Survival times in days.
Gastric contents adjusted to pH 7.6	21	1	1	9.5	11, 8
Gastric contents adjusted to pH 5	21	3	..	14	1,3,6
Treated with narcotine	21	15	2	81	2 to 19, except one which lived 42 days.
Treated with narcotine and the gastric contents adjusted to pH 7.6	20	15	..	75	1 to 22
Exposed to a low temperature	20	9	1	50	3 to 19
Exposed to a low temperature and the gastric contents adjusted to pH 7.6	20	16	..	80	1 to 31
Culture administered at o. C.	22	4	..	18	1 to 21
Controls	30	..	1	3.3	14

ditional Controls.

6 Guinea-pigs treated with narcotine remained in good health.
6 guinea-pigs exposed to a low temperature remained in good health.

The guinea-pigs which were killed had, apparently, begun to recover from infection.

Resistance to cold varied among the guinea-pigs; some animals appeared chilled and sat huddled with raised hair, while others appeared to be unaffected. The experiments with Groups 5 and 6 were therefore repeated on the same animals on more than one occasion, so that

the results might not be invalidated by feeding guinea-pigs in which cold had produced no effect.

One feature of interest observed was that neither the drug nor exposure to cold caused any noticeable alteration in the H-ion concentration of the stomach contents.

The summarized results of the experiments are given in Table 9.

Post Mortem Findings.

These showed only slight variations. Care was taken to examine animals immediately after death, and when this was impracticable the possiblility of post-mortem changes was taken into consideration in the histological examination.

Marked congestion and, usually, small haemorrhages were present in the wall of the stomach; the small intestine also showed acute congestion, which was occasionally associated with haemorrhages. Histological examination showed that the haemorrhages were caused mainly by rupture of capillaries in the submuccosa. In addition, a considerable amount of atrophied epithelium was, generally, desquamated into the lumen.

The lungs were invariable intensely congested, and showed patchy or, occasionally, complete consolidation by infiltration with mononuclear cells. In some cases areas of both consolidation and emphysema were present.

The kidneys were intensely congested and showed irregularly distributed areas in which the tubular cells had undergone degeneration. The stages of degeneration ranged from cloudy swelling to necrosis and, frequently,

both, the early and late stages were present in the same kidney. Infiltration with mononuclear cells and commencing hyalinization of the glomeruli were regularly found.

The suprarenals were usually enlarged and discoloured. Histological examination revealed a condition of acute congestion and the presence of diffuse degenerative changes in the cells of the medulla, some of which were necrotic.

Routine histological examination of the liver was not carried out. This organ was, however, occasionally investigated, when its mottled appearance and great friability indicated that it had undergone pathological alterations. The changes found in such cases were striking. Intense congestion and dilation of the veins were usual. In some instances lobulation had entirely disappeared, and the liver-cells showed fatty degeneration and, in some areas, necrosis. Where liver-cells could be distinguished the peripheral cells were found to be more affected than the central.

It is of particular significance that the histological findings indicated that all the pathological changes were of toxic origin.

Vibrion septique occurred constantly in the alimentary lesions and frequently in the lung lesions. On rare occasions it was isolated from the heart blood of animals which died within a few days of feeding.

The 5 guinea-pigs recorded as killed in Table II, had been in poor condition and losing weight; they then apparently began to recover and were killed and examined at this stage. In all five typical lesions

were present.

The death of one guinea-pig from Group 5, not included in Table 9, was attributed to a mediastinal abscess from which Gram-positive cocci were isolated. In addition to this infection, however, a lesion was present in the stomach mucosa from which Vibrion septique was isolated.

Administration of Toxin.

Toxin was administered in 8 to 10 c.c. amounts to guinea-pigs in the following groups, each of which contained 6 animals.

Group 1. Gastric contents adjusted to pH 7.6

Group 2. Gastric contents adjusted to pH 5

Group 3. Treated with narcotine as described for the animals fed with culture, and gastric contents adjusted to pH 5

Group 4. Controls.

The animals in all these groups remained in good health and showed no signs of toxic effects.

B. ovitoxicus

No infection or toxaemia was set up on feeding four sheep with B. ovitoxicus toxic filtrate or culture. Observations were therefore carried out to ascertain the factors favourable to infection or intoxication experiments, after those of Bennetts (1932) being made under conditions of reduced peristalsis and inhibited gastric secretion on animals treated with drugs. Four sheep were fed from a bottle, two with

about 40 c.cm. of B. ovitoxicus toxin and two with about 50 c.cm. of a twenty four hour liver bouillon culture. Two of these sheep, one from the first two and one from the second two, received just prior to feeding 20c.cm. tincture of opium diluted in water by mouth and a subcutaneous injection, of atropine, diluted 1/100. These experiments were without result. Thirty c.cm. of a solution of sodium bicarbonate was, therefore, added to the toxin and culture administered to the sheep and twenty four hours later the treated sheep fed with culture died. At post mortem examination there was a blood stained mucous effusion from the nose and mouth and in the throat subcutaneous oedema and a purplish discolouration of the muscles. A patch of haemorrhage, the size of a pigeon's egg and with a sharply defined edge, was found in the mucosa and sub-mucosa of the colon and many small haemorrhagic areas were present in the intestinal mucosa. Except that the liver was soft and pale in colour the other internal organs were normal. Organisms resembling B. welchii morphologically and producing typical B.welchii colonies were recovered from the throat oedema, the intestinal contents, liver and heart blood. The conclusion was reached death was due to a toxaemia set up by toxins produced by the B. welchii like organism, probably B.ovitoxicus and absorbed from the colon and intestine a bacterial invasion into the blood stream occurring only at or near the time of death. These findings agree with Bennetts (1932) description of infectious-enterotoxaemia.

An attempt made to reproduce the condition by feeding a young sheep with a sample of the intestinal contents met with no success.

Discussion.

The neutralisation tests detailed in the experimental work of this thesis show that the toxins produced by Wilsdon's four types of *B. welchii* though differing considerably exhibit a degree of interrelationship between themselves sufficiently close to separate them from the toxins of the other members of the gas gangrene group. Of the others *B. oedematiens* appears to have a distant relationship on account of its necrotoxin. The lethal factors of all the *B. welchii* types differ from that of *B. oedematiens*. The neutralisation of the necrotoxin of type C by *B. oedematiens* antisera and of the necrotoxin of *B. oedematiens* by type C antitoxin are somewhat difficult to explain since Glenny and his co-workers (1933) regarded the necrotoxins of the lamb dysentery bacillus (Wilsdon's type B) and *B. paludis* (Wilsdon's type C) as identical. Some slight difference, however, must exist between the necrotoxins of type C and of types A, B and D since *B. oedematiens* necrotoxin has relationship only with the toxin of type C and not with those of the other types.

The intravenous injection of mice for testing the neutralisation of *B. welchii* type toxins with antitoxins gave results identical with those of Wilsdon (1931) except that in this research C antitoxin did not neutralise A toxin and that D and *B. ovitoxicus* sera exerted a slight protective action against C toxin. Intracutaneous injection of toxin-antitoxin mixtures in guinea-pigs, however, gave

results agreeing exactly with the findings of Wilsdon (see table 10).

Table 10

The Inter-relationship of the Toxins of the B. welchii Group

Antitoxin.	Necrotoxins.				Lethal Toxins.			
	A	B	C	D	A	B	C	D
A.	+	-	-	-	+	-	-	-
B.	+	+	+	+	+	+	+	+
C.	+	+	+	-	-	+	+	-
D.	+	-	-	+	+	-	+	+

\+ indicates neutralisation.
\- indicates no neutralisation.

Glenny and his co-workers (1933) stated that neutralisation of lethal toxins and necrotoxins did not always correspond within the gas gangrene group. Certain sera did not protect against their "gamma" toxin of lamb dysentery and B. paludis filtrates but neutralised the necrotoxic factor. They advanced as an explanation of these discrepancies the different antitoxins present in normal horse sera. These influence the rate and the quantity of antitoxin produced on immunisation so that the use of one filtrate in different animals may result in the production of antitoxic sera with different contents.

The identity of the toxins of type D and B. ovitoxicus as established by Wilsdon (1933), Montgomerie and Dalling (1933) and Montgomerie and Rowlands (1933) has been confirmed. The experiments with the commercial sera are particularly significant as these sera,

though to a more pronounced extent, possess the neutralising properties of type A serum only.

The protective power of the sera against experimental infection by the different B. welchii types coincides with its ability to neutralise the toxin. When the neutralisation of the toxin with the antisera was slight, protection was not established, for example as when type A and D toxins were not neutralised to any marked extent with type D and B antisera respectively. The one exception to this rule is that type A sera and the commercial antisera, which both neutralise only A toxin, protect against infection by either types A or D. According to Wilsdon (1931) type A toxin contains only one toxic factor while type D toxin contains factor X along with a small amount of factor W. The results suggest that in guinea-pigs type D organisms do not produce factor X but only factor W. The latter is, therefore, neutralised by type A serum and the commercial sera. The toxins of types B and C contain principally factor Z. The factor Z is evidently produced in guinea-pigs and is not neutralised by type A serum or the commercial antisera.

Type A is recognised as causing infection in man. There appears, however, to be no reason why types B, C and D, all of which contain some of the A factor W, should not also infect man. In such cases, if all the possible toxic constituents were produced, the commercial sera would be of little value in treatment. It is sometimes reported that B. welchii sera is ineffective in the treatment of gas gangrene and it may be that in

these cases the infection is caused by organisms other than those of type A and the antisera administered has therefore not contained the appropriate antibodies. Further investigation is, therefore, required as to the types of B. welchii actually occurring in human infections.

It may be that multiple toxins depend for their production on environmental conditions (Glenny and his co-workers, 1933). These environmental conditions may act directly by effecting metabolism and consequently toxin production or indirectly by influencing the production of variants. The determination of the types of B. welchii antitoxin present in B. welchii sera is therefore of importance - a finding supported by the experimental work detailed in this thesis.

The conditions influencing the infection of guinea-pigs by the enteral introduction of cultures of Vibrion septique has been studied. The hydrogen-ion concentration of the gastric contents is not, either by itself or associated with other factors, of special importance. When, however, administration of drugs has caused diminished activity of the gastro-intestinal tract or when animals have been exposed to a low temperature, infection with Vibrion septique can be set up by intra-gastric administration of culture. The apparent factor influencing the onset of infection in the drug-treated animals is the opportunity to penetrate the mucosa of the stomach and small intestine afforded to the organisms by the increased time they

are retained in this situation.

Cultures at room temperature, introduced into the stomach of guinea-pigs which have been exposed to a low temperature, set up an infection more readily than cultures at approximately 0° C. administered to normal animals. This suggests that the influence of external cold, rather than the ingestion of frozen food, is an important factor in Vibrion septique infection.

The findings reported here, therefore, suggest that two factors may be at work in producing alimentary infection in guinea-pigs: (1) Stasis of the stomach and intestine; (2) low temperature, which is more effective when applied to the animal as a whole than when applied locally to the mucous membrane of the alimentary tract. The experiments on the production of infectious-enterotoxaemia in sheep by the administration of B. ovitoxicus culture agree with those of Bennetts (1932); decreased peristalsis and inhibited gastric secretion facilitating infection and intoxication particularly when B. ovitoxicus is fed along with an alkaline solution.

Bennetts suggested that constipation is an important factor in determining the onset of enterotoxaemia. If this is so, stasis would have a similar influence in the production of infection by Vibrion septique from the alimentary tract.

There is one difference in the factors favouring the onset of the two infections. The hydrogen-ion

concentration of the alimentary contents of guinea-pigs was not found to influence infection and intoxication with Vibrion septique whereas an alkaline reaction was favourable to the onset of infectious-enterotoxaemia in sheep. This finding, however, loses its significance since different species of animals were used in the two sets of experiments.

Guinea-pigs which died from Vibrion septique infection and sheep from infectious-enterotoxaemia showed extensive toxic changes in the internal organs. The toxic filtrates, however, when introduced into the stomach of guinea-pigs or fed to sheep did not produce a similar pathological condition. It, therefore, seems probable that the toxins are not readily absorbed from the intact mucosa of the gastro-intestinal tract.

Summary and Conclusions.

(1) Wilsdon's four types of B. welchii and B. ovitoxicus all produce typical B. welchii colonies on Zeissler's dextrose blood agar.

(2) Cross neutralisation tests between the toxins and antitoxins of the B. welchii types show that the toxins, though each is antigenically distinct, are all inter-related. The monovalent antisera do not possess equal antilethal and antinecrotoxic properties. The toxins of B. welchii type D and B ovitoxicus are antigenically identical.

(3) Commercial B. welchii antisera neutralise type A toxin only and have no action on the toxins of types

B, C and D.

(4) The B. welchii type toxins are antigenically distinct from those of other members of the gas ganrene group. The necrotoxins of type C and B. oedematiens however, bear some slight relationship to each other.

(5) Hydrogen-ion concentrations within the range pH 5 to pH 6 are most favourable for the maintenance of activity of Vibrion septique toxin.

(6) Narcotine has a depressant influence on the movements of the gastro-intestinal system of guinea-pigs.

(7) Exposure of the guinea-pig to a low temperature (0° C.-5° C.) produces no alteration in the movements of the alimentary canal.

(8) The Hydrogen-ion concentration of the gastric contents is of little importance in determining the production of a Vibrion septique infection from the alimentary canal.

(9) Animals in which alimentary activity has been reduced by the drug narcotine frequently show evidence of infection by Vibrion septique after intra-gastric administration of culture.

(10) Exposure of guinea-pigs to a low temperature before administration of culture, predisposes to infection by Vibrion septique. Culture at approximately 0° C., introduced into the stomach of normal animals, does not readily cause infection.

(11) The general character of the changes found in the internal organs on post-mortem examination indicates

the presence of a toxaemic condition in animals infected by Vibrion septique.

(12) Intra-gastric administration of toxin alone to guinea-pigs, whether normal, with the gastric contents adjusted to pH 5 or pH 7.6, or treated with narcotine, does not produce intoxication. The toxin is apparently not absorbed from the lumen of the stomach.

(13) B. ovitoxicus toxin does not produce toxaemia when fed to normal sheep or to sheep with their alimentary activity reduced by drugs.

(14) Infectious-enterotoxaemia is produced on feeding an alkaline B. ovitoxicus culture to sheep which have had the gastric secretion inhibited and peristalsis reduced with opium and atropine. The alkalinity of the culture is important as without it infection does not result either in normal sheep or in sheep to whom opium and atropine have been given.

General Discussion.

The toxins of the Staphylococci and the B.Welchii group show differences as great as those between the organisms which produce them.

Grouping of the Staphylococci by means of the origin and toxin production of the strains (see Review of Literature on Staphylococcal Toxins) has not been convincing. The members of the group may still be regarded as having similar antitoxinogenic structure. The composition of the toxin has been the subject of prolonged investigations. Doubt still remains as to its component factors and opinion is divided as to its unity or plurality.

Members of the B. welchii group are, on the other hand, capable of being classified as they are antigenically distinct (see Part 11 of the thesis). This has been clearly demonstrated by neutralisation tests in mice and guinea-pigs.

Both Staphylococci and members of the gas gangrene group have been found to be responsible for toxaemias originating in the alimentary tract. In both naturally occurring cases of food poisoning as well as in the experimental disease in human volunteers and later in animals the ingestion of the staphylococcal toxin, if not inactivated by an acid or alkaline reaction of the intestinal contencts, can produce a profound and sometimes fatal toxaemia. On the other hand for production of alimentary toxaemia by members of the gas gangrene group. (viz Vibrion septique and B. ovitoxicus), the presence in the alimentary

tract of the organisms themselves is required in addition to certain rigid controlled conditions such as reduced alimentary movement and secretion.

From the practical aspect the work has brought to light two outstanding facts. Firstly, the Staphylococci responsible for the toxaemic type of food poisoning can be incriminated by tests on laboratory animals.

Secondly, it is probable that the commercial antisera at present used for infections with members of the B. welchii group do not contain antitoxins specific for all the toxins which may possibly be produced in such infections.

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