THE MODE OF ACTION OF MUCIN IN PROMOTING BACTERIAL INFECTION.

A.F. MACCABE, M.B., Ch.B.; D.P.H.

BACTERIOLOGY DEPARTMENT,

UNIVERSITY OF EDINBURGH.

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INTRODUCTION.

As an introduction to this subject it will be appropriate first to discuss certain aspects of microbic infection, and the factors that determine the results of infection.

In the production of disease several factors must be considered, these are concerned with (1) the subject of infection, and (2) the infecting agent, both of which possess considerable powers of adaptation.

Micro-organisms have been divided into two main classes, (1) parasites (2) saprophytes. A true division of organisms cannot however be made in this way, for it is known that some parasitic organisms can lead a saprophytic existence outside the body under special conditions, namely on artificial culture media, e.g. Neisseria gonorrhoeae, Treponema pallidum and Mycobacterium tuberculosis.

A third class, the commensals, are those which can live and flourish on the skin and mucous membranes of the body without producing any harmful effects. Again, certain of these commensal organisms can, under certain conditions, invade the body and produce disease, the term 'potential pathogen' is applied to these.

The term 'pathogenic' in relation to bacteria has been defined as the power an organism has of

producing disease, either under natural conditions, or in conditions specially prepared.

Virulence has been described as an important property of micro-organisms in relation to their pathogenic action. In early times the word virulence referred solely to the micro-organism, and the possession of virulence by an organism was regarded as a well defined and intrinsic quality of the bacterium in question. However, with our ever increasing knowledge of the relationship of bacteria to disease, this problem is really a complex one.

One factor which must be considered is the path of entrance of the bacteria. The swallowing of pyogenic cocci etc., would not produce any harmful effects, whereas the ingestion of Vibrio comma or Shigella dysenteriae would, in the majority of cases, produce manifestations of disease. It was shown by Webster and Clow (1933) that certain strains of pneumococci were highly virulent on intraperitoneal injection into mice, but were much less virulent when injected intranasally, whilst other strains were of low virulence both by the intraperitoneal and intranasal route.

The power possessed by an organism to overcome the initial defence of the host on first contact will be an important factor in determining its pathogenicity. Arnold, Gustafson and others (1930)

found that such organisms as Serratia marcescens (B. prodigosus), Salm. typhosa, Esch. coli etc., were rapidly killed on normal human skin, whereas staphylococci and diphtheroid bacilli were not so easily killed; the low pH of the skin 5.2-5.8 was considered an important factor. In the mouth, the saliva removes bacteria and debris in a mechanical manner, and in the intestinal tract bacteria are taken up by the mucus and carried forward by peristalsis. Florey (1933) believed that one of the functions of the mucous secretion was to cleanse the villi of the intestine from small adherent particles, but from experiments on the colon of the cat he did observe that in spite of a secretion of mucus, bacteria may lodge and thrive in the crypts. In some cases bacteria could thrive in the colon, the normal flow of mucus being insufficient to dislodge them, but on stimulating the mucus, e.g. by mustard oil, the bacteria were completely removed from the depths of the crypts.

There are certain secretions in the body which exert an antagonistic action on bacteria such as the H Cl of the gastric juice, and lysozyme which is present in tears. Apart from the mechanical action of the tears in washing away any bacteria which settle on the conjunctiva, lysozyme, as Fleming (1929) pointed out, has the power of killing bacteria, although its action is most marked against

non-pathogenic types. He regarded lysozyme as a ferment which is widely distributed throughout the body, in spinal fluid, ascitic fluid, pleural effusion (clear), ovarian cyst fluid, semen, liver, tonsil, kidney and skin etc.etc., with the strongest concentration in cartilage, and a high concentration in leucocytes. Intestinal streptococci are the most susceptible, while Esch.coli are least susceptible. Webb (1948) demonstrated the action of lysozyme on rendering killed gram positive organisms gram negative.

The anatomical structure of the anterior nares partially prevents the access of organisms to the body via this route; lysozyme also plays its part here by killing off bacteria, more especially the saprophytic types. Organisms which do penetrate more deeply will be propelled backwards by the ciliated epithelium, and eventually swallowed on meeting the secretions from the mouth. The reverse flow of mucus in the trachea and bronchi set up by the ciliated epithelium will prevent organisms penetrating deeply into the respiratory tract. It is interesting to note that Armstrong (1935) rendered white mice relatively resistant to the intranasal administration of the St.Louis encephalitis virus by a preliminary instillation of 3% sodium alum, while Armstrong and Harrison (1935) rendered monkeys resistant to the fatal effects of the virus of

poliomyelitis by the instillation of sodium aluminium sulphate into the nostrils, the action being due to an alteration which decreased the permeability of the mucous membrane of the nose, rather than to an antiseptic one. Salim, Olitsky and Cox (1936) observed the same effect using sodium alum and tannic acid, although Rake (1937) did not find that tannic acid influenced the passage of pneumococci through the olfactory mucosa in mice.

Most bacteria select certain avenues of entrance into the body, e.g. the organisms of enteric fever and dysentery select the intestinal tract, the gonococcus - the urogenital system, while the respiratory tract is in most cases the avenue of infection of the tubercle bacillus and the pneumococcus. Most organisms have an affinity for particular tissues, the name given to this phenomenon is 'Tropism'.

There are of course accidental ways by which bacteria can gain entrance to the body, e.g. trauma, where the tissues are opened up and the blood vessels injured, and where tissues are killed, organisms like staphylococci and streptococci are able to flourish. For the multiplication of such organisms as Cl.tetani and other anaerobic organisms, deep penetrating wounds with dead tissue are necessary, as it is in these conditions that such organisms find the reduced oxygen tension so

necessary for their growth. The importance of the reduced oxygen tension in relation to tetanus was stressed by Campbell and Fildes (1931). It was pointed out by Fildes (1927) that the cause of absence of germination of tetanus spores was not due to phagocytosis, but to a positive tension of oxygen in the tissues, but in necrotic tissue the tension of oxygen may fall to zero and any spores therein will germinate. Again, a pre-existing infection often favours the development of a second infection, e.g. severe pneumococcal or influenzal infection following on whooping cough, measles etc., and it is often observed that in virus diseases of the central nervous system a preceding debilitating condition such as measles, mumps etc., has been present. In these cases the resistance of the individual has been broken down by the initial infection and the path opened up for the attack of the more virulent organism, to which the individual might have had, in many cases, a strong resistance. The experimental work of Shope (1931) indicated that swine influenza was caused by both the filterable virus and Haemophilus suis acting in concert, and that neither by itself was capable of inducing the disease.

In the human subject we can also see this apparent symbiotic virulence enhancement in mixed infections e.g. the presence of Strept.pyogenes complicating a case of diphtheria, where the disease is much more severe than when the normal naso-pharyngeal flora is present. The reason for this may

be as Holman (1928) suggested to be due to such factors as pH, production of H_2O_2, CO_2, and various amino acids.

Certain species of animal are more susceptible to infection with particular organisms, e.g. mice are very highly susceptible to the pneumococcus, but guinea pigs are resistant. Again, the inoculation of large numbers of meningococci into mice produces no harmful effects. In some human beings Strept. pyogenes produces scarlet fever, in others, tonsillitis is the only manifestation of its effects, whilst in a number of people these organisms are carried in the throat for long periods without producing disease.

In determining the outcome of an infection, it is essential therefore to consider both the parasite and the host. The part played by the parasite will now be discussed.

The ability to produce disease varies not only between species but also between strains, and it also varies within the same strain. It depends upon the state of dissociation of the culture and also with its history both in vivo and in vitro.

There appears to be a certain periodicity regarding virulence. It is seen, that for a period, a certain organism produces a rather mild type of infection, then suddenly a more potent strain is developed when the disease takes on an epidemic form.

There is a definite relationship between

virulence and capsule formation. Such organisms as the pneumococcus, streptococcus and anthrax bacillus can exist in the capsulated form. Virulent pneumococci isolated from pathological conditions are always in the encapsulated form, and immunisation with one of the purified polysaccharides produces anti-bodies which will protect against infection with a pneumococcus of the homologous type. It is believed that the capsule protects the pneumococcus against phagocytosis both in vivo and in vitro. In experiments with pneumococci and rabbits, Enders, Shaffer and Wu (1936) found that phagocytosis by leucocytes of the normal animal in vitro and in vivo was observed only when the capsule was impaired. Non-encapsulated forms undergo phagocytosis much more readily than capsulated forms. In 1896 Migula maintained that all bacteria possessed capsules but only a few could be stained. It is only comparatively recently that capsules have been demonstrated on a large number of organisms e.g. Cooper in 1925 demonstrated capsules on cells of Salm.typhosa, Salm.Schottmülleri, Esch.coli, Shig.dysenteriae, Salm.enteritidis, Alkaligenes faecalis, Proteus and A.cloacae, and that those organisms which were normally motile were found to be non-motile during the period when capsules could be demonstrated. An encapsulated strain of M.aureus, highly virulent for guinea pigs was described by Gilbert in 1931.

He found this strain could dissociate into an unencapsulated relatively avirulent strain, which on "passage" through guinea pigs could be made to revert to the capsulated form. In the same year Pittman described two strains of Haem.influenzae - a smooth type which was capsulated, and a rough type non-capsulated. Lyons (1937) demonstrated how the capsule of Micrococcus (Staph.) was lost in old cultures and that encapsulated strains were resistant to phagocytosis. The possession of capsules by haemolytic streptococci groups A-F while in the mucoid phase was demonstrated by Hobby and Dawson in 1937, and they were of the opinion that when group A streptococci are isolated from disease in the human subject they are usually encapsulated and in the mucoid phase.

There is a definite relationship between encapsulation and virulence, in that encapsulated organisms are more virulent than non-encapsulated ones. It was pointed out by Danysz (1900) that encapsulated organisms were able to withstand the lytic action of sera, whereas non-encapsulated organisms could not. It would be quite reasonable to assume that most bacteria have the potential power of capsule formation but it is only developed to a marked degree when the organism takes on a pathogenic role, as may happen in the case of those organisms designated 'potential pathogens'. It is possible that only under certain environmental

conditions can an organism assimilate material around its cell and form a capsule. The power to encapsulate may, therefore, be one of the conditions necessary for rendering an organism virulent and this power to encapsulate would only be possessed by living organisms. It was pointed out by Duguid (1948) that in artificial culture media, the proportion of utilisable carbohydrate was the main factor in determining the size of the capsule. Felix and Pitt (1934) observed that the inagglutinability of certain strains of Salm. typhosa was only possessed by living organisms and that these inagglutinable strains were virulent ones. Perhaps the possession of a capsule rendered these strains virulent, as shown in one way by their inagglutinability. Even in those bacteria which do not show a demonstrable capsule, there may be as Eisenberg (1908) suggests, changes in the peripheral portion of the bacterial cell analagous to true encapsulation, as he maintained that non-encapsulated bacteria show swelling from time to time under conditions in which their offensive activities are called into play. Sia (1926) believed that the presence of a capsule was indicative of great protoplasmic activity resulting in the elaboration of substances capable of neutralising the defensive forces of the body. Even in those micro-organisms which do not show capsule form, there have been observed evidences of ectoplasmic change such as enlargement or swelling

during periods when the bacterium was actively engaged in its struggle against the body's defences. Some workers believe the capsule is not a true morphological structure but corresponds to the accumulation of viscous material around the cell.

The virulence of an organism can be increased or decreased. Increase in virulence can be brought about by the method of 'passage' (Pasteur). This is also seen at the beginning of an epidemic when the organism is allowed to invade the tissues.

Virulence can be lost or decreased by (1) cultivation on artificial culture media, (2) growing the organism in the presence of an anti-serum, (3) growing the organism in the presence of antiseptics etc. Gaseous environment etc., also plays a part in virulence, for example, the optimum for a strain of tubercle bacillus was found to be 2.5% CO_2 at pH 7.4. In artificial pneumothorax the CO_2 tension in the lung is increased, and it is believed by some that it is this increased CO_2 tension which helps to kill off the tubercle bacilli.

It is interesting to note that Seastone (1939) demonstrated how bile accelerated the loss of capsular material from young group C streptococci.

The relationship between virulence and adaptation to body temperature was exemplified by Pasteur (1878) who demonstrated that the chicken which is usually resistant to anthrax could be made susceptible by immersion in cold water. It has

been said that one of the reasons for the immunity shown by birds to both the human and bovine type of the tubercle bacillus is due to their high body temperature, and again, Carpenter et al (1933) noted the disappearance of gonococci from the urethra on raising the body temperature.

Virulence is characterised by various organisms in different ways, e.g. Cl. tetani does not invade the tissues to any extent, its virulence is characterised by the powerful diffusible exotoxin it produces. On the other hand, B. anthracis invades and multiplies rapidly in the tissues, invasiveness is therefore the characteristic of its virulence. However, invasiveness and toxigenicity are not always successful in producing disease, e.g. Trypanosoma lewisi in the rat, and the spirochaete of relapsing fever in man, also group A. strept. or pneumococcus cause a fulminating septicaemia in mice or rabbits on intravenous or intraperitoneal injection, but they do not cause spontaneous infection in these animals. A third attribute is therefore apparently necessary, to which Dubos (1945) gave the name "communicability", which is the power an organism possesses of establishing itself in the host under natural conditions. The power of an organism to adapt itself to body conditions leads us on to the question of latent infections, where although the infectious agent does not manifest itself by producing signs and symptoms of disease, it can live in the body. This

is due to a balance set up between the tissues and the organisms. Nevertheless, disease is manifest from time to time, as is often seen in such conditions as syphilis, tuberculosis and malaria. Zinsser et al (1939) record a case where a patient had recovered from a haemolytic streptococcal infection of the hand, - a second operation to improve function was undertaken when haemolytic streptococci were found.

In such a disease as chronic malaria one can appreciate the importance of this latency, where the spleen harbours parasites for long periods, perhaps years. Also, in chronic diseases like rheumatism, latency probably plays a great part, where here, the allergic nature of the disease must be considered. Zinsser and Yu (1928) isolated non-haem.streptococci from the spleen in cases of chronic endocarditis, while in another case they isolated non-haem.strept. from the myocardium. From his experimental work with streptococci in guinea pigs, Bordet (1897) was of the opinion that organisms may remain quiescent but viable in phagocytic cells, while Rous and Jones (1916) pointed out that living phagocytes can actually protect ingested organisms (e.g.Salm.typhosa) from the action of destructive substances in the surrounding fluid and even from the action of a strong homologous antiserum. These facts, they believed, should be taken into consideration in the study of

diseases caused by infectious agents capable of living within tissue cells.

As mentioned previously there may be a periodicity re virulence, e.g. a new disease on first introduction into a virgin population is usually very severe, e.g. syphilis in Europe in the 15th century, also tuberculosis and measles on first introduction among aboriginal tribes caused high mortality. As time proceeds, an adaptation between organism and host is eventually set up when manifestations of the disease are not so severe. According to Yu (1930), smooth, virulent, and toxic diphtheria bacilli are transformed into non-virulent 'R' forms in the throats of patients during convalescence, and Shibley and Rogers (1932) recovered 'R' pneumococci from the lungs of patients with lobar pneumonia by lung puncture.

It has long been observed that on artificial culture the colony characteristics of a strain show change. One of the main changes is the 'S'$\longrightarrow$'R'; the smooth variant becoming rough. This change can also be brought about by growing the organism in the presence of an antiserum. This dissociation causes loss of the type specific polysaccharide antigen designated 'O'. The 'O' antigen of the typhoid bacillus exerts a repellent action against leucocytes and the injection of these substances into animals

causes an intense leucopenia. The 'O' antigens also inhibit the bactericidal power of the serum against the homologous organism. These 'O' antigens which are characteristic of smooth variants of Gram negative bacilli protect the organism against the defence mechanism of the host. 'Vi' is said to be similar in chemical composition to 'O'. All virulent strains of group 'A' streptococci possess what is termed the 'M' substance which confers type specificity, and the 'M' substance and the capsular polysaccharide are the requirements for rendering the organism capable of establishing a pathological state. Pittman (1931) found two strains of H. influenzae, one smooth and capsulated, the other rough and non-capsulated, while Wright and Ward (1932) showed how the virulent 'S' form of the influenza bacillus was completely resistant to the bactericidal action of diluted normal unheated serum, whilst the 'R' form was easily killed by the action of the serum. The 'S' culture filtrate contained a substance with a strong antibactericidal effect which was not found in the 'R' culture.

The experiments of Griffith (1928) appear to have answered the question as to whether a rough and therefore relevantly avirulent organism can revert to the 'S' encapsulated form when introduced into the body under certain conditions. Mice were injected intracutaneously with a mixture of rough avirulent pneumococci and heat killed smooth

pneumococci of the same or other type, and smooth virulent pneumococci were frequently recovered from the mice.

There may, of course, be loss or partial loss of virulence without loss of the specific antigenic constituent that characterises the 'S' forms, these avirulent 'S' forms, must, according to Wilson and Miles (1946), be regarded as true variants as judged by their failure to revert easily to the virulent parent type.

Virulence has a relation to the animal employed- it may be increased for one species yet diminished for another, e.g. streptococci 'passed' through mice have their virulence increased for mice, but diminished for rabbits, and E.rhusiopathiae inoculated from rabbit to rabbit loses its virulence for pigs.

Another factor which must be considered is the number of organisms introduced. Most animals and human beings can withstand a certain number of organisms, and there is for each animal a minimum lethal dose. The healthy peritoneum of a rabbit can withstand a large number of pyogenic cocci, but when the dose is considerably increased, a fatal result follows.

The path of entrance must also be taken into consideration. In the human subject for example, pyogenic cocci in the healthy mouth or throat would produce a far less severe infection than would the

introduction of these organisms into an open wound. The organisms may be destroyed at the point of entry or they may be dealt with in the lymphatic glands which may be regarded as the second line of defence.

The biological and physiological nature of the host as well as the organism must be considered in determining the result of infection.

Susceptibility to disease varies among healthy individuals. Factors influencing susceptibility are (1) age, (2) race, (3) inherent individual peculiarities, (4) environmental conditions, etc. It is well known that young children are more susceptible to diphtheria than adults, although very young infants are usually resistant to diphtheria, due to a passive immunity received from the mother. Young people are also more susceptible to fungus infections, particularly of the hair. In early life the bones and joints are more susceptible to tuberculosis. Certain races are more prone to tuberculosis than others. This may be due to the individuals concerned having the proper 'soil' for the development of the organisms, to damp and unhygienic conditions of living, or to lack of opportunity of infection, thereby not allowing an immunity to be developed. This is exemplified in the case of country dwellers taking up residence in a heavily

industrialised town, when many develop a severe tuberculous infection. A disease on first introduction into a community usually takes heavy toll, e.g. syphilis in this country in the 16th century was more severe than at the present day. Certain diseases are peculiar to certain races, e.g. syphilis is peculiar to the human subject, only in a very few of the lower animals can the disease be produced.

Even among members of the same race there are individual differences in regard to susceptibility to infection, e.g. tuberculosis, streptococcal infections, the virus of the common cold etc. Environmental conditions also play an important part in diminishing natural resistance to infection. It has long been recognised that physical or mental fatigue, exposure to cold, starvation etc., decrease the body's resistance. The importance of vitamin deficiency, especially vitamin A, in lowering general resistance to infection is well established. Alcoholic intoxication is also a recognised method of lowering resistance. In this connection Koch (1884) observed higher fatality rates during cholera epidemics among excessive users of alcohol, while Lushbaugh (1943) showed how rabbits subjected to alcoholic intoxication, died of a septicaemia after dermal infection with pneumococcus type 1, in spite of their being actively or passively immunised with the same strain. The effect of alcoholic intoxication, ether, and avertin anaesthesia, was found by

Pickrell (1938) to destroy the resistance to pneumococcal infection in rabbits. There is a great tendency for tuberculosis to develop in lungs exhibiting silicosis. Kettle (1924, 1927, 1934) pointed out the relationship between silicosis and tuberculosis. He thought that silica appeared to have some specific action on the growth of the tubercle bacillus in the tissues, while Gye and Kettle (1922) believed silica to be a cell poison; they observed degeneration of leucocytes around silicotic lesions produced by the subcutaneous injection of silica alone in mice. Endocrine disturbances may lower the body's resistance as Asher (1924), Furruya (1924), Masuno (1924) and Abe (1925) found the removal of certain organs of internal secretion from the rabbit resulted in decreased phagocytosis. The effect was most marked in the removal of thyroid, then spleen, ovary and testicle in that order. Bullock and Cramer (1919) emphasised the effect of ionisable calcium salts in promoting infection with Cl.welchii and other anaerobes. They showed that when Cl.welchii, Cl.septicum, Cl.novyi (oedematiens) and Cl.tetani were completely freed from their toxins, they did not produce the specific disease when injected into a mouse or guinea pig, but if a small dose of soluble ionisable calcium salt was injected together with the bacterium or their spores, the specific disease was elicited in a very resistant form. The effect of the calcium was

to bring about a local breaking down of the defensive mechanism. The name 'kataphylaxis' or 'defence rupture' was suggested. It may be that many more substances exist which can cause a tissue breakdown and which may provide an explanation for the occurrence of infections for which no obvious sign is apparent. Camp and Baumgartner (1915) showed that in rabbits with severe leucopenia there were apparently no antibacterial bodies present.

Finlay (1928) pointed out the relationship between injury and localisation of organisms in injured tissue as being due to the liberation by the injured tissue of histamine like substances. Such was his conclusion from his experiments with the virus of fowl pox, vaccina and Rous sarcoma, M.aureus, streptococcus and pneumococcus. The findings of Duran-Reynals (1928, 1929) re the effect of testicular extract on increasing tissue permeability may be of a similar nature.

The possible part played by the secretions of the endocrine glands in relation to infection has been receiving importance in recent years.

In 1936 Fox and Whitehead studied the effect on the haemolysin titres of the serum by injecting albino rats with cortico adrenal extract. A daily dose of 1 cc cortico adrenal extract was administered for two days prior and for three days subsequent to

the injection of 0.5cc 10% sheep cells intraperitoneally. On examination of the serum they found an increase of 60-70% in the haemolysin titres of the test animals over those of the controls.

Dougherty, Chase and White (1945) demonstrated that the rate of release of the immune globulin from lymphocytes was under pituitary-adrenal cortical control. Working on this observation Chase, White and Dougherty (1946) thought that the administration of adrenal cortical steroids might influence antibody levels. Rabbits were used as the test animals and it was found that an increase in the antibody content of the serum was observed in those animals receiving injections of adrenal cortical extract, the maximum increase was observed six hours after injection. It was also found that agglutinin titres to sheep erythrocytes were enhanced in rabbits as a result of prolonged or single injections of adrenal cortical extracts or pituitary adrenotrophin. Von Hamm and Rosenfeld (1942) found that the administration of oestrone and pneumococcus type 1 to castrated and non-castrated rabbits produced a remarkable increase in the specific agglutins and protective antibodies. Further evidence of the protective effect of estrogenic hormone was demonstrated by Sprunt, McDearman and Raper (1938) in that the resistance of the rabbit to vaccinia was increased if the rabbit had been castrated and then given estrogenic hormone for a period of three weeks before being vaccinated.

They also observed that after a course of estrogenic hormone, the spread of india ink in the skin was slowed up.

The favourable influence of certain hormones in the course of tuberculosis was demonstrated by Steinbach and Klein (1937) who showed how experimental tuberculosis in rabbits and guinea pigs was favourably influenced by the administration of antuitrin S., pregnant mare serum, and to a lesser extent by folluetin. No retardation of disease was obtained by the use of either anterior pituitary extract or emmenin. These results suggested that the gonodotropic hormone may be a factor in the temporary amelioration of symptoms observed in tuberculous women during pregnancy.

Lewis and Adler (1936) observed the beneficial effect of treating gonococcal vaginitis in children with estrogenic substance in ethylene glycol while Aycock (1936) found that in castrated female monkeys oestrin prevented the development of experimental poliomyelitis.

It does seem quite probable that an unbalanced state in the endocrine system may play a part in the question of susceptibility to infection. Such an upset even though temporary may throw some light on the causation of those infections termed endogenous.

One must therefore conclude that in any infectious process it is essential to study the

reaction of the host to the micro-organism. As Kettle (1927) remarked, it is extremely difficult to explain why the meningococcus which is often a normal inhabitant of the perfectly healthy person's naso-pharynx should be able to cause meningitis.

One of the first lines of defence possessed by the body against micro-organisms is phagocytosis. In relation to bacteria this is meant ingestion by the white cells of the blood and certain tissue cells. Metchnikoff (1883) called the white blood cells 'motile' phagocytes; the 'fixed' phagocytes being certain connective tissue cells, cells of the splenic pulp, endothelial cells, and certain cells present in lymph nodes. Large mononuclear cells of the blood, wandering tissue cells and fixed phagocytes are classed as macrophages.

Two stages can be observed in phagocytosis, (1) contact of particle and cell, (2) the particle is engulfed by the cell. It may be that a form of chemical attraction is concerned as Fenn (1921) observed re the ingestion of carbon and quartz by leucocytes. He also advanced the theory that phagocytosis was proportional to the chance of collision between cell and particle and this depended on the size and density of the particles in relation to the size and density of the cells.

It also depended on the relative velocities of cell and particle in the rotating tube. Carbon was found to be ingested four times as easily as quartz. Menkin (1938) gave the name leucotaxine to the substance in inflammatory exudates that induces in cutaneous tissue a rapid immigration of polymorphonuclear cells through the endothelial wall of small vessels, and this substance also causes increased capillary permeability. He believed this substance to be an intermediate product of protein catabolism. Leber (1888, 1891) found leucocytes were actually attracted by powdered copper and mercury compounds but not by powdered gold or iron. Dead bacteria also exerted a powerful chemotactic influence.

Extensive work has been carried out on the question of the mechanism of phagocytosis. According to Abramson (1927) the migration of leucocytes to a point of injury is in part dependent on the electromotive forces at play in the tissues, while Buchner (1890) extracted from Klebsiella pneumoniae a protein which exhibited strong attraction for leucocytes. He also noted that glycine and leucine were definitely chemotactic, while tryosine and trimethylamine were inert in this respect. Intravenous calcium gluconate, calcium chloride, sodium salicylate, sodium iodide, dextrose and

neosalvarsan, in rabbits, were shown by Tunnicliff (1931) to cause a marked increase in phagocytosis by the white blood cells.

It is the polymorphonuclear cells of the blood that are the main agents concerned in phagocytosis in most infections, although Gay and Morrison (1923) maintained that in experimental infection in the rabbit the 'clasmatocytes' or 'tissue macrophages' were the cells that were mainly concerned. Further evidence of the importance of these cells was produced by Gay and Clark (1925) in their observations on the mechanism of protection in rabbits against streptococcal empyema by intrapleural injection of normal and immune sera. In the case of the immune serum sterilisation of the infected cavity was complete in three hours or less with a rise in clasmatocytes and polymorphs. With the normal serum, there was an initial drop in the clasmatocytes and polymorphs with the clasmatocytes rising slowly to a maximum in twelve hours, while the infected cavity was not sterilised until the second or third day.

During the course of an acute infection the polymorphs in the blood usually show a steady increase, which is taken as a favourable sign, as indicating that one of the main defence forces is being mobilised to combat the attack of the invading

organisms. It was however shown by Wright (1927) in experiments with pneumococci in rabbits, that leucocytes may be considerably reduced by the injection of benzole without interfering with the capacity of the animal to dispose of organisms introduced into the blood. However, Schnitzer and Goddard (1943) found that rabbits suffering from a leucopenia as the result of repeated injections of benzene, failed to develop a typical local lesion when injected intravenously with strept.pyogenes, but died of streptococcal septicaemia. In normal rabbits a progressive local inflammatory reaction was observed but no generalisation.

The fact that organisms are ingested by leucocytes does not necessarily mean they are destroyed as Fothergill, Chandler and Dingle (1937) have pointed out that in some cases of "influenzal" meningitis the organisms may become ingested by the phagocytes without being destroyed by them, and may be actually protected by the phagocytes from the action of the specific antibody, which fact may explain the failure of some of these cases to respond to specific therapy. The phenomenon of leucocytes protecting ingested organisms was also stressed by Rous and Jones (1916) who pointed out that such organisms as Myco.leprae, the tubercle

bacillus, the gonococcus and Leishmania live habitually within tissue cells.

In relation to phagocytosis, the part played by the opsonin of the serum must be considered. The opsonin in some way sensitises the bacterium rendering it susceptible to phagocytosis, although the viability of the organism is not affected.

Robertson and Sia (1927) observed that after virulent pneumococci had been in contact with the serum of pneumococcus resistant animals they were actively phagocytosed, not only by the homologous leucocytes but also by leucocytes of other resistant and susceptible animals; but pneumococci exposed to the action of the serum of pneumococcus susceptible animals were not phagocytosed by leucocytes of either resistant or susceptible species. Rosenow (1907) found that avirulent pneumococci absorb opsonin and become susceptible to phagocytosis whereas virulent pneumococci do not absorb opsonin and are insusceptible to phagocytosis.

The presence of two enzymes occurring in leucocytes was demonstrated by Opie (1906). These enzymes have the power of digesting protein. One occurring in polymorphs called leucoprotease which is active in the presence of alkali or neutral solution and practically incapable of proteolysis in acid medium, the other occurring in large mononuclear

cells and called lymphoprotease is active in weak acid but not in alkali.
The serum, in virtue of its anti-leucoprotease activity protects the tissues from the action of the proteolytic enzymes.

According to Ward and Enders (1933) two separate antibodies are concerned in normal serum which act in conjunction with complement, one, the type specific carbohydrate antibody reacting with the carbohydrate fraction of the pneumococcus, the other also probably type specific, but reacting with a different antigenic constituent of the bacterium. They did not think that complement was really essential in the phagocytic process, but merely increased the speed at which the reaction takes place, and may be similar to a catalyst. In an immune serum the only antibody that can induce phagocytosis is the carbohydrate antibody. An enzyme present in polymorphs which destroyed the basophilic character of heat killed pneumococci ('R' and 'S' variants) and inactivated the type specific polysaccharide antigen of encapsulated cells was demonstrated by Dubos and Macleod (1938).

It is generally agreed that leucocytosis and phagocytosis run hand in hand, although intraleucocytic destruction of the organisms may not occur simultaneously. Rosenow (1910) concluded from his work on pneumococcal endocarditis that intraleucocytic destruction was dependent on a substance or property

always present in normal serum and in normal leucocytes after treatment with normal serum, but was frequently absent in the serum and leucocytes in chronic cases of endocarditis. This observation may throw some light on the reason for so many diseases which do not become completely arrested but tend to pass into a subacute or chronic state. In diseases like tuberculosis and leprosy, ingestion of organisms takes place without apparent destruction; these are diseases which tend very often to run a chronic course.

The opsonic power of the serum and the leucocytic power of destroying organisms plays such an important part in checking invading organisms, that anything interfering with either of these functions will greatly impair the body's power of dealing successfully with an infection. Micro-organisms of course, show various antagonistic actions against the defence mechanism of the host. Ward (1930) maintained that the specific carbohydrate of the pneumococcus had a specific antiphagocytic action which was not neutralised by either weak or too strong serum. Phagocytosis was only effective when the serum was present in optimum concentration. Rosenow (1907) believed that a substance or group of substances could be extracted from virulent pneumococci which inhibited the action of pneumococcal opsonin. After extraction of the substance, virulent pneumococci

acquired the power to absorb pneumococcal opsonin.

The peculiar susceptibility exhibited by some individuals to particular infections might be attributed to a lack of sufficient opsonin or to the opsonin not being present in optimum concentration. Lyons and Ward (1935) thought that the serum of infants was deficient in opsonin for virulent haemolytic streptococci. They also believed that the haemolytic streptococci could be typed by means of specific opsonins.

The effect of temperature on phagocytosis has been studied by many workers. It would be rational to assume that phagocytosis would be maximal at or around 37°C. Madsen and Wulff(1919) maintained that human leucocytes displayed maximum phagocytosis at 37°C. Ledingham (1908) found a proportionately steady increase in the number of bacteria phagocytosed by leucocytes at temperatures 18°C - 37°C and at 43°C it was four to five times greater than at 18°C, although Kämmerer (1928) failed to find much difference in phagocytosis of staphylococci at 0°C and 37°C, he did nevertheless find that tubercle bacilli were more rapidly ingested at 37°C than at zero.

The effect of the exposure of rabbits to benzene in causing leucopenia resulting in susceptibility to streptococcal infection was shown by Schnitzer (1943). Hamburger (1912) found that iodide ions, and anions of fluoride, sulphide, and citrate of sodium, hyper and hypo-tonic sodium chloride,

depressed phagocytosis. There was practically no difference between the chloride of sodium and potassium. In experiments with streptococcus viridans in the rabbit, Evans (1922) found that phagocytosis declined with the pH. Maximal phagocytosis occurred near neutrality, and organic acids were found to be more toxic than mineral acids. There are substances which can increase phagocytosis as was for example observed by Tunnicliff (1931) with calcium salts, and Loos (1931) with histamine in concentration of about 1 in 40,000. The phagocytosis promoting property of phage was discovered by d'Herelle (1926).

The effect of the removal of certain organs of internal secretion from the rabbit by Asher (1924), Furruya (1924), Masuno (1924) and Abe (1925) was found to cause a decrease in phagocytosis.

The importance of viscosity in relation to phagocytosis was demonstrated by Lewis (1923) who showed the effects of acids and alkalis on the viscosity of mammalian cells in that the nuclear substance was more readily coagulable than the cytoplasm by acids, while the cytoplasm was more rapidly dissolved by an alkali than the nucleus. At temperatures below 30°C Fenn (1922) noted that the cells were too rigid for phagocytosis. Ponder (1927) also stressed his belief that great cytoplasmic viscosity or turbulence of flow in the surrounding

fluid tended to prevent phagocytosis.

The most important substances which can promote phagocytosis are, as Mudd et al (1934) state, components of normal and immune sera, known respectively as opsonins and tropins. The increase in phagocytosis is due to the deposition on the particle surface of serum protein and this makes conditions in the interface between particle and phagocyte, and particle and suspending medium, favourable for the spreading of the phagocyte over the particle.

It appears from the extensive work on this subject that the mechanism of phagocytosis is extremely complex, and that the action of the phagocyte is conditioned both by internal and external influences, of which viscosity and interfacial tension will play a major part.

The main types of cells concerned in phagocytosis are the polymorphs and macrophages. In most acute infections it is the polymorphs which are the most active in this role. They appear much earlier than the macrophages in the inflammatory reaction. The part played by the macrophages is seen more in such conditions as leprosy and tuberculosis. Opinions vary as to whether lymphocytes play a part in phagocytosis; some maintain they do, others say not. Mudd, McCutcheon and Lucké (1934) believed there was increasing evidence to show that the cells of the macrophage system were as at least as important as polymorphs in removal of bacteria from the blood and

tissues, and they were of the opinion that polymorphs play an important role in such chronic infections as tuberculosis and in the removal of animal cells or their debris.

In studying the defence of the host against infection it is important to realise the part played by leucocytes as the first line of defence against the organisms. Any rupture of this defence will, in most cases, allow an infection to become established and lead to symptoms and signs of disease with pathological changes in the tissues. In investigating results of experimental infection in animals, a method of determining any damage to leucocytes is essential. According to Neisser and Wechsberg (1900), Ehrlich was the first to recognise the fact that certain colours were reduced by living cells and organisms. From the time of Neisser to the present day the methylene blue reduction test has been universally used as an indicator of the presence of living cells. No explanation except that of Neisser has been forthcoming as to the exact rationale of the test, the test has merely been accepted without much question. It may be, of course, that a more complex mechanism than that described by Neisser is involved. Neisser and Wechsberg (1901) were apparently the first workers to make use of the test to determine the viability of leucocytes. Their explanation of the phenomenon was that live leucocytes require oxygen, and they take the oxygen out

of their surroundings, and, therefore, if we introduce a colouring matter which is easily reducible in the liquid in which the leucocytes are suspended, it would be possible to conclude by the reduction of the colour, the presence of living leucocytes. They proved conclusively that the leucocytes were responsible for the reduction of the dye, for by separating the leucocytes and using the diluent or leucocyte free liquid, no reduction took place. There was no reduction if leucocytes were inactivated or killed by heating, or by the addition of quinine or alcohol. Proof was therefore presented of the reduction being dependent on the presence of the leucocytes, and, moreover, living leucocytes. They pointed out that the amount of reduction was proportional to the number of living leucocytes. In some cases, they suggested, it might be conceived that constituents of cells might reduce methylene blue whether the cell was alive or not, but with other suitable controlled experiments false conclusions could be overcome.

The work of Quastel (1924) on the reduction of methylene blue by organisms is worthy of consideration. He demonstrated how the rate of decolourising of methylene blue by resting Esch. coli and B. pyocyaneus was considerably accelerated by the presence of sodium succinate. According to Quastel and Whetham (1924) the following reaction takes place in the presence of the above organisms:-

succinic acid + methylene blue ⇌ fumaric acid + leuco methylene blue, and that resting bacteria (B.coli and B.pyocyaneus) produce an enzyme similar to that in muscle, which is capable of bringing about the reduction of methylene blue in the presence of sodium succinate; Quastel, Stephenson and Whetham (1925) believed that methylene blue and leuco methylene blue were substances which are easily capable of accepting and donating hydrogen respectively in vitro, that is without activation. Succinic acid cannot donate hydrogen in vitro, hence its power of reducing methylene blue in vivo must be ascribed to an oxidation of the acid by the cell.

The following substances have, according to Quastel and Whetham (1925), an inhibiting action on the reducing action of hydrogen donators:- monohydric alcohols and gelohexanol. Benzene, toluene, phenol and acetone have definite but relatively slight inhibiting effects.

AGGRESSINS.

Bacteria produce their effects on the body by the production of toxins. There are two types of toxin, (1) exotoxins which diffuse from the body of the organism and are carried throughout the circulation and which can be fairly readily separated from the organism in vitro. (2) Endotoxins which remain incorporated in the substance of the bacterial cell. They can only be extracted by a process which results in disintegration of the cell. Organisms produce

different types of toxin, for example streptococcus pyogenes possesses toxins which destroy the red and white cells of the blood, known as leucocidins and haemolysins respectively; the erythrogenic toxin possessed by streptococcus pyogenes causes the characteristic erythematous rash of scarlatina. There are other bacterial products, which, although not toxic in themselves, can play a part in influencing the course of a bacterial infection. The coagulase produced by the more virulent staphylococci has the property of coagulating human and rabbit plasma. Fibrinolysin found in streptococcus pyogenes can dissolve human fibrin and is a product of the same category. This substance is produced by most strains of group A, C and G streptococci. A third and very important so-called non-toxic bacterial product is the spreading or diffusing factor. Duran-Reynals (1928, 1929) demonstrated how the vaccinal injection of the rabbit was considerably enhanced when the virus was injected in the skin along with aqueous extracts of rabbit, guinea pig, and rat testicle.

The 'spreading' or 'diffusing' factors have been identified with hyaluronidase (Chain and Duthie 1940), and the action is apparently a hydrolysis of the hyaluronic acid by the enzyme.
According to Duran-Reynals (1942) 'spreading' factors are present in the tissues, especially the testes of many animal species including man and in many

bacteria, reaching high concentration in certain intrinsic species.

O'Meara (1940) put forward the hypothesis that the diffusion factor was an integral part of diphtheria toxins and that exotoxins in general consist of at least two factors, one of which actively promotes the diffusion of the other through and into the tissues by increasing their permeability. In further studies on diphtheria toxin (1941) he believed that the effect of the substance B component of the toxin was to promote the penetration of the tissues by substance A, thus enabling it to cause its specific damage.

McLean (1941) however, found that only a small proportion of strains of C.diph. produced detectable amounts of diffusing factor. He did not find any evidence of any association between diffusing factor or hyaluronidase and substance B postulated by O'Meara to be present in toxic B.diph. filtrates.

McLean (1941) stressed the importance of the elaboration of diffusing substance or hyaluronidase in influencing the behaviour of such organisms as Strept.pyogenes, Staph. and Cl.welchii.

It is interesting to note how in 1947 Hecter, Deepkeen and Mundell showed how minute amounts of hyaluronidase (obtained from bovine testes) could remove the barrier to rapid diffusion in the subcutaneous tissues of the guinea pig, thus facilitating the administration of fluid.

There are other bacterial products, which, although in themselves are not very toxic to the host, can influence the course of an infection by interfering with the defence mechanism. Bail (1900,02,03, 04,05) was the first to study these products to which he gave the name 'Aggressin'. There is evidence that these toxins are of a distinct nature by the fact that the exudates produced in experimental animals with such bacteria as Salm.typhosa, Vibrio comma, etc., had the effect of rendering a non-lethal dose of the bacterium into one with fatal effects. When aggressins are added to a lethal dose of bacteria, a much more acute and more rapidly fatal type of disease is produced. Bail (1905) pointed out that cholera aggressin neutralises the bactericidal action of an anti-cholera serum. It is generally agreed that the action of the aggressins is partly due to their antiphagocytic action on the leucocytes. It has also been stated that the immunity produced by aggressins is far more effective than that produced by the inoculation of bacteria.

Since the hypothesis of Bail (1905) increasing evidence has been produced in favour of the 'aggressive' action of these specific bacterial products. Cole (1917) demonstrated the presence in empyema fluids resulting from pneumococcal infection of large amounts of soluble substances which could neutralise pneumococcus antibodies. He observed that

when immune serum was injected into infected rabbits, the immune substances disappeared very quickly and therefore prevented from activity in overcoming the infection. These results are, in certain respects, in agreement with those of Felton and Bailey (1926) who proved that the specific soluble substance of type II pneumococcus when injected into mice in extremely small doses along with type II pneumococcus, produced an antagonistic effect on the defence of the animals, and an increase in the virulence of the organisms from ten to one hundredfold. The specific soluble substances in themselves even in greater doses was non-toxic. Sia (1926) claimed that the specific soluble substance of pneumococcus (type II and type III) could confer on avirulent pneumococci of the same type, the growth capacity of a virulent organism.

From the work of Rosenow (1907) it would seem likely that the specific soluble substance neutralises the opsonin, thereby allowing the organisms to resist phagocytosis. Similar findings were obtained by Enders, Schaffer and Wu (1936) in noting that phagocytosis of the leucocytes either in vitro or in vivo only occurred when the capsule had become impaired. The observations of Seastone (1934) who showed that the loss of resistance of haemolytic streptococci to phagocytosis was evident with the loss or partial degeneration of the capsule; and the

findings of Lyons (1937) of the resistance of capsulated staphylococci to phagocytosis confirms the anti-phagocytic action of the capsular substances.

ARTIFICIAL AGGRESSINS.

Many substances and methods have been used to promote experimental infection in animals.

It was shown by Blake and Cecil (1920) that normal monkeys were resistant to the inoculation of a large dose of pneumococcal culture into the nose and throat. The same phenomenon was observed by Stillman (1923) in his experiments with mice, but Stillman and Branch (1924) often produced a fatal septicaemia in mice by spraying the animals with pneumococci after they had been intoxicated with alcohol. Further evidence of the effect of alcohol on the defence of the host is found in the observations of Koch (1884) on the high fatality rates among excessive users of alcohol during cholera epidemics, while the work of Lushbaugh (1943) and Pickrell (1938) amply demonstrated how alcohol destroyed the resistance of rabbits to pneumococcal infection. The effect of excessive alcohol is that of depressing leucocytosis, and any factor which interferes with this important line of defence of the body to infection will greatly impair the host's chances of recovery.

Evidence of the effects of cold, alcohol, and

irritating gases, in predisposing rabbits to pneumococcal infection was given by Kline and Winternitz (1915) while Terrell and Robertson (1929) and Terrell, Robertson and Coggeshall (1933) produced lobar pneumonia in the dog by intrabronchial injections of the pneumococcus suspended in a starch broth medium. Gum tragacanth was used by Benians (1921) who found that on injecting bacteria along with gum tragacanth into animals, organisms of the coli-typhoid group in the subcutaneous tissues of a rabbit or guinea pig were protected from the defence mechanism of the host, and could survive for some weeks. When he injected the organism intravenously and the gum subcutaneously Esch. coli and Salm. typhosa could be recovered from the gum. The same results were obtained with commercial gastric mucin. Starch, egg albumen and mutton fat were also employed but with negative results. The results with agar were inconclusive. It is easy to appreciate from the experiment that the organisms might have been protected from the defences of the body, but it is also possible that the injected substance may have had some aggressive action, by, for example, actively depressing the defences of the host. The same investigator carried out a series of experiments on rabbits using gum tragacanth, bile and Esch. coli. He found that Esch. coli and gum intraperitoneally produced a fatal

result with acute toxic symptoms. Esch. coli and bile intravenously, and gum intraperitoneally, also caused a fatal result. He suggested that the passage of organisms from the blood stream into the gum in the peritoneum was greatly facilitated by the presence of the bile in the blood stream. He thought that the penetration of the organisms from the blood stream into the peritoneum and their protection there by the gum may throw some light on the problems of blood borne peritonitis. He believed that it was highly improbable that a few organisms arriving in the blood stream would gain access to the peritoneum without some preliminary irritation or injury of the membrane, or that they would survive peritoneal destruction without some protection in the peritoneum, so that a preliminary extravasation of mucus from some source would most easily explain a peritoneal infection arising apart from a perforation. The effect of gum tragacanth on promoting infection in dogs was observed by Steinberg and Goldblatt (1927) who produced a fatal effect by intraperitoneal injection of Esch. coli and gum.

The apparent virulence enhancement of glucose was observed by Pillsbury and Kulcher (1935) who noted that the administration of glucose caused a very marked increase in the extent of skin infections when using a haemolytic Micrococcus pyogenes aureus (Staph. aureus) as the test organism.

Parfentyev and Collins (1940) also demonstrated the enhancement effect of glucose in that mice injected with live staphylococci, mucin, then glucose, succumbed to a smaller number of organisms than did those injected with only live staphylococci and mucin, while Miller and Castles (1934) found the same phenomenon using a suspension of 5% mucin, 1% glucose, and meningococci. Hoyt, Holden and Rawson (1939) observed that mice in the symptomatic stage of various neurotropic virus infections were more susceptible than normal mice to the lethal effect of the intraperitoneal injection of glucose. Apparently the dehydration produced by intraperitoneal glucose was thought to be a major factor in this differential susceptibility.

The susceptibility of diabetics to infections, particularly those due to M.aureus and Myco.tuberculosis is, in view of the above work, probably associated with the high glucose content of the blood, and any disturbances of endocrine function leading to depressed bactericidal activity of the blood may be connected with carbohydrate metabolism.

By means of daily intravenous injections of Scillaren B.-a squill glucoside, Wallace, Wallace and Robertson (1933) produced the typical clinical picture of Vincent's angina in the dog. Smears from the lesions in the mouth showed the characteristic fusiform bacilli and spirochaetes found in human

beings suffering from Vincent's angina. Pure cultures of the fusiform bacilli were grown from material from the dog's mouth, but attempted cultivation of the spirochaetes was not successful. The mode of action of Scillaren B was suggested in the work of Tunnicliff and Klein (1934) who found that the subcutaneous injection of small doses of Scillaren B appeared to produce a leucopenia and to dilate the blood vessels in the skin of guinea pigs, so that abscesses were produced in the prepared area by the injection of non-virulent cultures of fusiform bacilli. Fusiform bacilli and spirilla-like organisms were demonstrated in smears and sections of the abscess, and were recovered in pure culture. In 1937 Tunnicliff and Hammond produced abscesses 1 - 2 cmm in diameter with subcutaneous injections of fusiform bacilli after preparing the skin by injection of 0.1 cc Scillaren B, whereas the bacilli alone produced abscesses of only 0.5 cmm in diameter. They also produced peritonitis in mice by the intraperitoneal inoculation of Scillaren B and fusiform bacilli.

The first mention of the use of gastric mucin in experimental animals was found in the work of Benians (1921) who demonstrated that when organisms of the coli-typhoid group were injected into animals along with commercial gastric mucin they were protected from the defence mechanism of the host.

It was in the same year that Ivy and Oyamma observed that the secretion of the mucous membrane of the pyloric antrum was mucoid, viscous, tenacious, transparent, odourless and slightly salty in taste. The secretion was alkaline (pH 7-7.5). It did not contain enzymes or an anti-peptic ferment and did not form antibodies when injected parenterally, nor did it contain any substance that was of specific digestive importance.

Kim and Ivy (1931) in their experimental work on duodenal ulcer in dogs, concluded that the administration of mucin prevented ulcer formation in dogs with biliary fistula, while in the same year, Fogelson used hogs' gastric mucin in treating patients suffering from duodenal ulcer. He maintained that complete relief was afforded from symptoms for varying periods from two to five months. These observations, and those of Heidenhein (1879), Pavlov (1910) Lim (1924) and Ivy (1921) suggested gastric mucin as an ideal antacid in that (1) it combined readily with the free acid (2) it was a natural substance which plays normally, a protective, soothing and lubricating role in the functioning of mucous membranes, and (3) it's secretion or ingestion causes no chemical disturbance in the body and no unfavourable effect on gastro-intestinal secretory or motor activity.

These findings are very interesting in view of

the work carried out by many workers on the apparent virulence enhancement effect possessed by mucin towards various species of bacteria.

The earliest account of experimental work on these lines was carried out by Nungester, Wolf and Jourdonnais in 1932 who found that mice receiving intraperitoneal inoculation of pneumococci suspended in mucin died sooner than the control animals receiving the same dose in saline. With inoculations in the tail vein the effect of mucin was not apparent.

Miller (1933) was apparently the first to use gastric mucin to promote infection in animals with such organisms as the gonococcus and meningococcus; while gastric mucin was found to be more satisfactory than other vehicles for the inoculation of gonococci, the value of mucin in promoting experimental infection with meningococci was even more striking. He prepared his mucin suspension by washing the crude mucin for several days in many changes of 70% alcohol in order to remove unnecessary ingredients, and to kill the spores of contaminating bacteria. It was then dried between blotting paper and a 6% solution in saline was made and buffered to pH 7.4. The solution was sterilised in an Arnold steriliser at 100°C for one hour every other day for three days, and incubated on the intervening days. The precipitate was discarded and only the clear supernatant used. 2 ccs mucin were used for the inoculum, the injections being given intraperitoneally. Death

usually occurred in 6-24 hours with positive cultures from heart blood, if cultures were made soon after death. The peritoneal exudate was found to contain large numbers of organisms and relatively few leucocytes. Maximum virulence was obtained by using as the inoculum peritoneal exudate diluted with mucin. By this method one strain of meningococcus was found to have a minimum lethal dose of less than one hundred organisms. Parallel titrations of peritoneal exudate made with saline and mucin showed the minimum lethal dose with saline as the diluent to be approximately a million times as great as with mucin as the diluent.

Following on this work Miller and Castles (1934) produced a lethal effect with meningococci in mice by using a 5% suspension of mucin and 1% glucose. Rake (1935) increased the pathogenicity of typhoid cultures for mice by the use of mucin. His attempt to enhance the virulence of strains by growth in media containing mucin was not successful. Miller and Castles (1936) found that organisms recovered from the exudates or blood of moribund mice proved no more virulent when titrated in saline than cultures grown on artifical media. They were also of the opinion that the assumption that mucin might function as a kind of aggressin was rendered untenable by the observation that antimucin serum

failed to protect mice against infection, and by the fact that mucin injected subcutaneously failed to immunise against infection. It was stated that their experimental data (so far) failed to support the notion that mucin enhanced the virulence of the meningococcus unless one takes liberties with the conventional concept of virulence. This therefore suggests that the action of mucin was not so much on the organism as on the host. This point was actually confirmed by Nungester, Jourdonnais and Wolf (1936) from their work with Klebsiella pneumoniae in mice, where there was no marked reversion of the avirulent form (non-mucoid) in vivo in the presence of mucin, to the colony type (mucoid) associated with virulence. Further confirmation of this fact was obtained when they observed that a saline suspension of M.pyogenes aureus recently isolated from a human lesion failed to kill constantly when suspended in mucin, which fact indicated the inability of mucin to lend virulence to avirulent cultures. This was substantiated when mice inoculated intraperitoneally with old laboratory strains of M.pyogenes aureus, M.pyogenes albus, Esch.coli, B.subtilis and N.catarrhalis failed to cause death with either test (with mucin) or controls (with saline). These workers further showed that rats inoculated intraperitoneally with B.anthracis and mucin died on an average in 1.1 days, the bacilli

being recovered from the heart blood at autopsy, while only one of the thirty control rats died, after six days. They found evidence of a change in virulence of bacteria after growth in vivo in the presence of mucin.

It would have been interesting to have discovered whether the change in virulence after growth in mucin was due to the development of a form of capsule. Their experimental work went on to show how mucin enabled bacteria to survive the defences of the peritoneal cavity and lungs. They believed that the viscosity and cohesive properties of the mucin appeared to be important indices as to the effectiveness of mucin in bacterial infections.

The effect of mucin in enhancing the power of H.influenzae to infect mice was observed by Chandler, Fothergill and Dingle (1937), and in vivo multiplication of the organisms occurred with subsequent death of the animal. In the same year Tunnicliff and Hammond produced peritonitis in mice by intraperitoneal inoculation of fusiform bacilli and mucin, while intracutaneous injection of fusiform bacilli and mucin produced abscesses. Experimental pneumonia in white rats was studied by Nungester and Klepser (1938). They produced a pneumonia in white rats by injecting intrabronchially 0.1 cc sterile mucin solution, and twenty four hours later spraying with a

culture of pneumococci or placing a suspension of pneumococci in the nose. While none of the control rats developed pneumonia, eighty per cent of those receiving mucin did. They found that certain factors such as exposure to cold, prolonged deep ether anaesthesia or alcoholic intoxication increased the aspiration of mucous material placed in the nose of white rats. Cold or alcoholic intoxication were found to interfere with closing of the glottis, thereby permitting entry of mucous and pneumococci.

A new mode of action of mucin was suggested by Keefer and Spink (1938) in that it depressed the bacteriolytic power of whole blood, and of anti-gonococcal serum, while Pacheco and Peres (1940) reported that mucin opposed the bactericidal action of cholera antiserum in vitro and in 1942 Griffitts noted the virulence enhancing effect of mucin towards the cholera vibrio in that relatively small numbers of organisms with mucin intraperitoneally were capable of proliferating and killing mice. According to Anderson and Oag (1939) marked variation occurred among samples of mucin, both in regard to solubility, and power to raise the pathogenicity of various organisms. The more soluble the mucin, the greater the virulence raising properties. Mucin was found to increase the virulence of the meningococcus, M.pyogenes aureus, and some strains of Strept.pyogenes,

on intraperitoneal inoculation in mice, but not of Streptococcus viridans, Salm. typhosa, Esch. coli, B. anthrocoides etc., they did not think the action of mucin was of a physical nature. They also showed that agar in various concentrations 2-10% solution was active, but such substances as gum acacia (10% solution), olive oil, liquid paraffin, soluble starch (10%), charcoal, kaolin, egg albumin (5%) gelatin (10%), glucose (5%), and milk were inactive.

In 1942 Wheeler and Nungester produced gross lesions in the lungs of hamsters by intratracheal injections of mucin and mouse lung infected with influenza 'A' virus.

The most recent study of the pathogenising effect of various substances was carried out by Olitski, Shelbubsky and Hestrin (1946). They classified carbohydrates into three classes as regards their pathogenising effect viz:-

a. Powerful pathogenisers - levan, dextran, mucin, and agar-agar.

b. Moderate pathogenisers - gum acacia, glycogen, mannan, and possibly inulin.

c. Non-pathogenising carbohydrates in concentrations 0.2 - 10% arabinose, xylose, glucose, fructose, galactose, maltose, trehalose, lactose, cellobiose, sucrose, and raffinose, galactomannan (0.2%), soluble starch (5%) rice starch (5%) and pectin (5%). Kaolin, they found exerted a marked effect on the pathogenising activity of carbohydrates of class (b) but

little or no effect in class (a) and (c). Among the carbohydrates examined no consistent relationship between viscosity and pathogenising activity was observed. They suggested that the activity of a polysaccharide in this respect was a function of patterns specific to the colloidal state.

It was in view of the apparent diversity of opinion as to the apparent virulence-enhancing action of gastric mucin that tempted one to pursue a study on the mode of action of this complex substance, and to attempt to obtain a purified fraction, for it was appreciated that crude gastric mucin might contain several impurities, one or more of which might be responsible for the observed effects of the product in enhancing virulence or depressing natural resistance to various bacteria.

THE CHEMISTRY OF MUCIN.

The mucins belong to the class of chemical compounds known as mucoproteins. These are complex proteins and are composed of a protein and a carbohydrate radical. After prolonged boiling with dilute mineral acid or the action of alkalis, they are split into their two constituents - protein radical and carbohydrate radical. The mucoproteins possess characters common to all proteins but differ in (1) being precipitated by acetic acid in the cold and (2) by their solutions being very viscous. According

to Levene (1925), the chief variable component is the protein group and this protein part differs widely from tissue to tissue and from species to species. The carbohydrate group of all mucoproteins is conjugated with sulphuric acid and is built up of four components in equimolecular proportions viz:- sulphuric acid, acetic acid, hexosamine, and glucuronic acid. The carbohydrate radical is not a simple monosaccharide but a complex polysaccharide. Mucins have been defined as complex compounds of amino sugars, glucuronic acid and sulphuric acid. Some mucins yield chondroitin sulphuric acid, others mucoitin sulphuric acid. Tissues such as cartilage, tendons, aorta and sclera contain chondroitin sulphuric acid, which is a tetra-saccharide composed of two amino galactose and two glucuronic acid units, the amino groups being acetylated and the primary alcohol groups esterified with sulphuric acid. Mucoitin sulphuric acid is a disaccharide containing glucosamine and glucuronic acid. Mucoitin sulphuric acid according to Levene (1925) differs from chondroitin sulphuric acid principally in the nature of the hexosamine, whereas the nitrogenous sugar of chondroitin sulphuric acid is chondrosamine (2 - galactosamine or 2 talosamine) that of mucoitin sulphuric acid is chitosamine (2 - glucosamine or 2 mannoseamine).

Mucoitin sulphuric acid has been isolated from the mucoproteins of the following tissues:-

1. From the mucin of the gastric mucosa.
2. " serum mucoid
3. " ovomucoid
4. " funis mucin
5. " humor vitreous
6. " cornea

According to Levene (1925) gastric mucin is composed of the following constituents:-

Mucoitin sulphuric acid (N 3.47%, S 1.48%, glucuronic acid 13% acetyl 7.8%);

Glucuronic acid derivatives,

Mucosin (amino N 2.12%) and

Glucosamine.

Mucins form extremely viscous solutions with water, are soluble in dilute alkalis, and insoluble in acetic acid. Several unclassified substances of the same type as chondroitin and mucoitin sulphuric acid have been isolated from various tissues. Van Lier (1909) obtained a substance from skin which he regarded as chondroitin sulphuric acid, while Mörner (1889) isolated a conjugated sulphuric acid from bones. In 1901 Levene isolated a sulphuric ester of a complex carbohydrate from submaxillary mucin, while from the spleen he isolated a conjugated sulphuric acid which was either a chondroitin or mucoitin sulphuric acid. Alzona (1914) obtained from the mucous membrane of the intestinal wall, urinary bladder and human prostate a substance having the character of a chondroitin sulphuric acid. From mammary gland, kidneys, pancreas, liver, leucocytes, and from amyloid tissues of various organs, Mandel

and Levene (1905 and 1907) isolated a sulphuric ester of a complex carbohydrate.

The work of Blix, Oldeft and Karlberg (1935) throws additional light on the chemical analysis of the mucins. They found that submaxillary mucin contained two carbohydrate complexes - of which one, the smaller amount (about 5%) was identical with dimannose glycosamine, whereas the other, the major part (about 25%) represented a new carbohydrate containing the following constituents:- glycosamine, two acetyl groups, and probably a polyoxyacid which is, according to these workers, certainly not hexuronic acid, the chemical formula being $C_{14}H_{25}O_{11}$, N. The substance has acid characteristics and gives on heating without treatment with alkali as well in the free state as when combined with protein, a stable red colour with Ehrlich's reagent. This colour reaction gives the submaxillary mucin a special place apart from the hitherto well known mucoids. The strong acid character of submaxillary mucin is most probably due to the acid carbohydrate group. From the results of their analysis they believed that in these substances one has probably also to reckon with the existence of two carbohydrate groups of which one belongs to the widespread type dimannose-glycosamine, whereas the second represents a different glycosamine compound. They maintained that all mucins and mucoids were rich in carbohydrates (10-30%) but contain only insignificant

amounts of sulphuric acid split off through acid hydrolysis corresponding to 0.04% up to at the most 0.3% sulphur. Accordingly, mucoitin sulphuric acid at anyrate is not to be considered as a predominant carbohydrate group in this class of compounds. In 1943 Morgan and King isolated from crude hog gastric mucin a polysaccharide-amino-acid complex possessing the serological properties of a hapten corresponding to the human A blood-group. This material (1) appeared to account for both serological properties of the crude commercial mucin and the extreme viscosity of its solutions. Chemically this material showed unusual structure. It was composed (80%) of a polysaccharide apparently consisting of galactose and N-acetylglucosamine and (20%) an amino-acid complex. The latter appeared to be bound to the polysaccharide by primary valency bonds, since it was not possible to separate the two save by drastic chemical treatments which destroyed both. The material was not antigenic, but the amino-acids tyrosine and tryptophane were absent. These workers also isolated the human group A factor from human ovarian cysts, and found its chemical and serological properties to correspond very closely with those of the hog gastric mucin group A factor.

(1) which appeared to be the main component of the crude commercial mucin.

Bray, Henry and Stacey (1946) also isolated from human gastric contents a highly complex muco-polysaccharide.

It is of interest to note that Kendall, Heidelberger and Dawson (1937) isolated a serologically inactive polysaccharide from cultures of group A haemolytic streptococci in the mucoid phase; this polysaccharide was composed of N-acetyl-glucosamine and glucuronic acid units, which appears to be very similar to that occurring in gastric mucin. The mucoid streptococcal carbohydrate according to Kendall, Heidelberger and Dawson resembles the specific polysaccharides of the pneumococcus in being associated with the encapsulated phase of the micro-organism, and resembles types 1 and 111 pneumococci in containing a high content of uronic acid.

Dawson, Hobby and Olstead (1938) examined the nature of the mucoid substance of group A haemolytic streptococci and their results were in agreement with Kendall, Heidelberger and Dawson (1937) in that the soluble polysaccharide was serologically inactive. They showed that rabbits and other animals immunised with encapsulated organisms prepared in a variety of ways developed precipitins for filtered washings of the mucoid growth from neopeptone blood agar plates but the reactions were not type specific. The results of immunising with the filtrate indicated that the mucoid material was either completely inactive or only feebly antigenic. There does, therefore, appear

to be a similarity in chemical composition between gastric mucin and the mucoid substance of group A haemolytic streptococci. It may be mentioned that in experiments carried out by the writer, crude commercial gastric mucin was not antigenic in that it failed to give rise to a specific antiserum on immunising rabbits. This was also true of the mucoid substance of the group A streptococci as shown by Dawson, Hobby and Olstead (1938). This fact again brings out the similarity of these two substances. It is possible that mucin may take on the rôle of a hapten.

PART I.

EXPERIMENTAL STUDIES ON THE VIRULENCE ENHANCING EFFECT OF MUCIN.

EXPERIMENTAL STUDIES ON THE VIRULENCE ENHANCING EFFECT OF MUCIN.

Part 1.

BASIC EXPERIMENTS.

In the writer's experimental work the meningococcus has been mainly used as the infecting organism, on the lines of the earlier work of Benians (1921), Miller (1933), Rake (1935), Miller and Castles (1936), Nungester, Jourdonnais and Wolf (1936), Chandler, Fothergill and Dingle (1937), and others. The mouse was the experimental animal, and the mode of inoculation was by intraperitoneal injection.

Throughout the various studies to be recorded in this thesis, several hundred experiments have been carried out in which mice have been inoculated with mixtures of mucin and doses of meningococcal culture which were non-lethal *per se*, these being in all cases carefully controlled by inoculation of animals with the test doses of the organism in saline.

The results abundantly demonstrate the remarkable effect of mucin in promoting meningococcal infection in these animals. Thus, when the test dose of the meningococcal culture has proved innocuous, the same dose along with mucin has brought about the death of the animal within 24-48 hours. The organisms not only flourish in large numbers in the peritoneal cavity, but rapidly invade the blood,

and produce death by septicaemia.

In addition to experiments with the meningococcus, various other organisms were used, including certain which are not naturally pathogenic, viz. B.mycoides, B.anthracoides, B.subtilis, Esch.coli, Salm.typhosa, Strept.viridans, Staph.pyogenes albus, C.diphtheriae, and C.hofmanni.

It must be emphasised that though most of this experimental work concerning the virulence enhancement action of mucin has been done with the meningococcus, the effect is a fairly general one, and applies to a wide variety of bacteria.

The following protocols of certain of the experiments will serve to illustrate the general results.

1. MUCIN AND MENINGOCOCCAL CULTURE INJECTED INTRAPERITONEALLY IN MICE.

MUCIN. Granular mucin, type 1701-W, Wilson Laboratories, Chicago. 5% suspension made in distilled water, sterilised by heating at 100°C for twenty minutes and adjusted to pH 7.2 with phosphate buffer.
Amount injected - 0.5 c.c.

MENINGOCOCCUS. A fresh strain isolated from the cerebro-spinal fluid, from a case of cerebro-spinal meningitis. Twenty four hours cultures on heated blood agar slopes were washed off with

sterile normal saline and made up to a strength of 500 million organisms per c.c. (estimated by Brown's opacity standards). Amount injected 0.5 c.c. (250 million organisms).

The approximate lethal dose of this strain per se for mice was 1,250 million organisms.

MICE. Healthy animals of 25-30 grams in weight. Three mice were used for the test and three for each control.

Mucin and meningococcal culture.	Normal saline and meningococcal culture.	Normal saline and mucin.
All three animals died in 24-48 hours with signs of blood infection. Cultures from heart blood, spleen and peritoneal exudates yielded a growth of the meningococcus on blood agar in twenty four hours.	Alive and well after five days.	Alive and well after five days.

The above experiment was repeated on nine other occasions with identical results.

GENERAL SUMMARY OF RESULTS AFTER INTRAPERITONEAL INOCULATION OF MUCIN (VARYING PERCENTAGES) AND MENINGOCOCCAL CULTURE (VARYING CONCENTRATIONS).

Mucin 0.5 c.c. at	Meningococcal culture 0.5 c.c.	Mice	Deaths within 48 hours	Survivals	Percentage death rate.
5%	250 million	30	30	-	100%
3%	350 "	60	54	6	90%
3%	10 "	35	30	5	86%
2%	350 "	135	80	55	59%
1%	350 "	35	12	23	34%
0%	350 "	60	-	60	0%
5%	no organisms	30	-	30	0%

All dead animals exhibited signs of blood infection, cultures from heart blood and spleen yielding growths of meningococcus on blood agar in 24 hours.

2. As these results were obtained with a commercially prepared mucin, it was considered desirable to confirm them with mucin fractions extracted in the laboratory from pigs' stomachs. In collaboration with Dr. H.K.King seven preparations were produced and tested for virulence enhancement of the meningococcus in mice, on the lines of the previous experiments. These preparations will be referred to as 3 E1, 3 E2, EG 4, EG 5, EG 6, EG 7, and 3 E10.

PREPARATION OF MUCIN FROM PIG STOMACHS.

The mucosa was stripped from six fresh pigs' stomachs and well minced with an ordinary domestic mincer.

1200 c.c. water and sufficient 2N hydrochloric acid (70 c.c.) were added to adjust the pH to pH 3-4. Accurate pH determination and even adequate mixing of the gelatinous mixture, was very difficult. 200 c.c. toluene were added (sufficient to saturate the digest) and the mixture left to autolyse for seven days at 37°C. The pH rose to pH 5-6 after the first twenty four hours. Each day the mixture was thoroughly stirred and more toluene added to replace that lost by evaporation.

After four days autolysis, 2000 c.c. water were added and the mixture thoroughly stirred and centrifuged (RCF 2000 xg). The partly digested residue was re-extracted with two lots of water - 2 litres and 1.5 litres respectively - at 40°C. The

insoluble residue was discarded.

Two dry preparations were made from this crude extract, 3 E1 and 3 E2.

PREPARATION OF 3 E1.

1000 c.c. were evaporated carefully *in vacuo* at a temperature below 30°C till the volume was 200 c.c. approximately, and then freeze-dried. 10.4 g. of hygroscopic material was obtained.

EXPERIMENTAL.

Mucin 0.5 c.c.	Meningococcal culture 0.5 c.c.	Mice	Deaths	Survivals
4%	350 million	4	3	1
3%	" "	8	7	1
2%	" "	4	-	4
1%	" "	4	-	4
5%	no organisms	5	-	5
0%	350 million	8	-	8

PREPARATION OF 3 E2.

In order to obtain a less hygroscopic preparation, 250 c.c. of the crude extract were run slowly and with thorough mechanical stirring into 1000 c.c. of ice-cold alcohol. The precipitate was washed twice with alcohol, and once with ether, then dried *in vacuo* yielding a fine white powder (1.95g).

EXPERIMENTAL.

Mucin 0.5 c.c.	Meningococcal culture 0.5 c.c.	Mice	Deaths	Survivals
4%	350 million	4	-	4
2%	" "	4	1	3

It will be seen that precipitation with alcohol seriously impaired the activity of the material.

PREPARATION OF EG5.

In view of the above, a preparation was made using acetone in place of alcohol.

25 c.c. 3 E1 were poured slowly into 100 c.c. acetone. The precipitate was ground up with fresh acetone, washed in ether and dried in vacuo.

EXPERIMENTAL.

Mucin 0.5 c.c.	Meningococcal culture 0.5 c.c.	Mice	Deaths	Survivals
4%	350 million	6	3	3

No significant loss of activity was found.

PREPARATION OF EG4.

This preparation was made in order to determine whether the material was dialysable or not. It was obtained by dialysis of 3 E1.

EXPERIMENTAL.

Mucin 0.5 c.c.	Meningococcal culture 0.5 c.c.	Mice	Deaths	Survivals
4%	350 million	2	2	-

It was concluded that the material was not diffusible.

PREPARATION OF EG6 AND EG7.

To determine whether the active material was protein in character a solution of crude mucin was deproteinised by shaking with amyl alcohol and chloroform, Sevag (1934), the denatured material was suspended in water and freeze-dried (EG7). The supernatant was also freeze-dried (EG6).

EXPERIMENTAL EG6 - (90% of original material)

Mucin 0.5 c.c.	Meningococcal culture 0.5 c.c.	Mice	Deaths	Survivals
4%	350 million	6	1	5
4%	" "	6	2	4

EXPERIMENTAL EG7 - (10% of original material)

Mucin 0.5 c.c.	Meningococcal culture 0.5 c.c.	Mice	Deaths	Survivals
4%	350 million	2	2	-

It appears there is a considerable loss of activity with this method.

PREPARATION OF 3 E10. (Repeat of EG5).

25 c.c. 3E1 were poured slowly into 100 c.c. acetone, precipitate ground up with fresh acetone, washed in ether, and dried in vacuo.

EXPERIMENTAL.

Mucin 0.5 c.c.	Meningococcal culture 0.5 c.c.	Mice	Deaths	Survivals
4%	350 million	2	2	-
3%	" "	2	-	2
2%	" "	2	-	2

SUMMARY.

With fraction 3 E1 and preparations made from 3 E1, (viz. EG4, EG5, EG7 and 3 E10) a virulence enhancement effect was observed. (See table below).

Mucin	Meningococcal culture	Mice	Deaths	Survivals	Percentage death rate.
4% (3E1 EG4, EG5, EG7, 3 E10)	350 million	16	12	4	75%

THE EFFECT OF MUCIN ON THE VIRULENCE OF VARIOUS OTHER ORGANISMS.

B. mycoides - approximate lethal dose for mice - 750 million organisms.

Mucin	Organism		Mice	Deaths	Survivals
3%	B. mycoides	375 million	3	3	-
"	"	190 "	3	3	-
"	"	95 "	21	20	1
"	"	47 "	12	10	2
"	"	12 "	3	2	1
"	"	6 "	3	2	1
2%	"	95 "	9	5	4
"	"	47 "	3	2	1
0%	"	375 "	24	5	19
"	"	95 "	18	-	18
3% crude	B. anthracoides	900 "	6	4	2
Saline	"	900 "	6	-	6
3% crude	B. mesentericus	900 "	3	-	3
Saline	"	900 "	3	1 (-)	2
3% crude	B. subtilis	5 "	3	3	-
Saline	"	5 "	3	-	3

Mucin	Organism		Mice	Deaths	Survivals
5% crude	Salm. typhosa (Cole 'R' form)	450 million	2	2	-
3% "	"	" "	3	3	-
1% "	"	" "	2	-	2
Saline	"	" "	7	-	7
3% crude	Esch. coli	375 "	3	-	3
Saline	"	" "	3	-	3
3% crude	Staph. pyogenes albus (Coag. Positive)	375 "	3	1 (-)	2
Saline		" "	3	-	3
5%	Strept. viridans	600 "	3	-	3
water	"	" "	3	-	3
3%	"	" "	3	-	3
Saline	"	" "	3	-	3
3%	C. diph. mitis	900 "	3	-	3
2%	"	" "	5	3	2
2%	"	550 "	2	-	2
2%	"	175 "	2	-	2
1%	"	900 "	2	-	2
Saline	"	" "	5	-	5

Mucin			Mice	Deaths	Survivals
3%	C. diph. gravis	900 million	3	-	3
Saline	"	900 "	5	-	5
3%	C. hofmanni	900 "	3	-	3
Saline	"	900 "	3	-	3

Unless otherwise stated mice died of a blood infection.

(-) = No blood infection.

THE HEAT STABILITY OF MUCIN SUSPENSIONS.

It will be noted from the details of the typical experiments recorded, that the mucin was sterilised by heating at 100°C for twenty minutes and adjusted to pH 7.2 with phosphate buffer.

The stability of the active principle at 100°C is of particular interest; heating at 100°C for twenty minutes seemed to suffice in general for purposes of sterilisation in these experiments and this was made the standard procedure. It may be noted that the active principle can withstand longer than twenty minutes at 100°C, and even higher temperatures.

Mucin	Sterilisation	Meningococcal culture	Mice	Deaths	Survivals
	autoclaved				
3%	15 lbs. press. 15 mins.	350 million	5	5	-
3%	60 mins. 100°C	350 "	2	2	-
2%	60 mins. 100°C	350 "	2	2	-
2%	3 hours 100°C	350 "	2	1	1

pH STABILITY OF MUCIN AT 100°C.

A 2% solution of the commercial mucin was prepared and the pH adjusted by N H_2SO_4 or N Na_2CO_3 to 20 c.c. of the solution. The pH achieved is indicated. The samples were then placed in a boiling water bath for twenty minutes, and the pH adjusted to 7.2. 0.5 c.c. of each sample was injected into two mice with meningococcal culture (350 million). A second experiment was carried out using 3% mucin.

pH	Mucin	Meningococcal culture	Mice	Deaths	Survivals
1.80	2%	350 million	2	1	1
3.95	2%	350 "	2	2	-
5.25	2%	350 "	2	2 (±)	-
6.20	2%	350 "	2	2	-
7.15	2%	350 "	2	2	-
9.60	2%	350 "	2	2	-
2	3%	350 "	3	-	3
2.9	3%	350 "	3	-	3
3.75	3%	350 "	3	3	-
9.6	3%	350 "	3	3	-

(±) = culture from spleen positive
" " heart blood negative.

STABILITY TO HEAT AND EXTREME pH.

15 c.c. 2% commercial mucin adjusted to pH 2.1 and heated at 100°C for twenty minutes when the pH was readjusted pH 6.5. There was no change in pH during heating.

EXPERIMENTAL.

The activity of the preparation was tested with meningococcal culture in mice.

Meningococcal culture	Mice	Deaths	Survivals
350 million	2	-	2

A second sample was adjusted to pH 11 sterilised at 100°C for twenty minutes and pH readjusted to pH 5.5.

Meningococcal culture	Mice	Deaths	Survivals
350 million	2	-	2

EXTREME HEAT AND EXTREME pH STABILITY.

A 2% suspension of mucin was adjusted to pH 2. It was heated at 100°C for one hour, cooled and pH readjusted pH 7 (approx.).

Meningococcal culture	Mice	Deaths	Survivals
350 million	2	-	2

A further sample was adjusted to pH 11.8. It was heated at 100°C for one hour, cooled and pH readjusted to pH 7 (approx.).

Meningococcal culture	Mice	Deaths	Survivals
350 million	2	1	1

Owing to the irregular supply of animals during this period, a satisfactory number of experiments could not be performed and a clear cut result was not obtained.

There did not however, appear to be any marked inactivation over the range pH 3.75 - pH 9.6. Extreme heat and extreme pH did appear to cause some degree of inactivation.

Some authors have stated that there is an appreciable fall in activity on heating at pH 7: at this (or higher values) there is a marked change on heating, a small amount of solid material settling out leaving a relatively clear supernatant. This latter is the 'clear mucin' referred to by McLeod (1940) and said to have a slightly lower activity than mucin heated at pH 5. One was unable either to confirm definitely or disprove this: there is however no striking difference. But as a matter of routine, the mucin was suspended in distilled water, the pH of this suspension was pH 5. This was sterilised by heating for 20 minutes at 100°C, and the pH was then adjusted to pH 7.2 by addition of sterile phosphate buffer, whose strength was such that the final mixture was isotonic with normal saline.

In the earlier experiments the pH of the mucin suspension was adjusted before sterilisation. It was however found to be more accurate to determine the pH of the mucin suspension after sterilisation.

Mucin	Meningococcal culture	Mice	Deaths	Survivals
3% (pH before sterilisation)	350 million	6	4	2
3% (pH after sterilisation)	350 "	5	5	-
2% (pH before sterilisation)	350 "	4	-	4
2% (pH after sterilisation)	350 "	4	4	-

STABILITY OF MUCIN SUSPENSIONS AT DIFFERENT TEMPERATURES.

It was found that mucin suspensions retained their activity for ten days if kept at a temperature below 4°C.

Mucin	Age of suspension	Meningococcal culture	Mice	Deaths	Survivals
2%	3 days-room temp.	350 million	2	1	1 -
2%	4 " " "	350 "	2	-	2
2%	5 " below 4°C	350 "	2	2	-
2%	6 " " 4°C	350 "	2	2	-
2%	7 " " 4°C	350 "	2	2	-
2%	8 " " 4°C	350 "	2	2	-
2%	10 " " 4°C	350 "	2	2	-
2%	11 " " 4°C	350 "	2	1	1
2%	8 weeks " 4°C	350 "	2	1	1

SUMMARY OF EXPERIMENTAL STUDIES - PART 1.

1. Mucin enhances the virulence of the meningococcus, B.mycoides, and Salm.typhosa for mice.
   An apparent virulence enhancement was noted with B.anthracoides and B.subtilis.
   No apparent virulence enhancement effect was observed with Esch.coli, Staph.pyogenes albus, Strept.viridans, or C.hofmanni.
   The results with C.diphtheriae were too doubtful for definite conclusions to be drawn.
2. Mucin suspensions withstand autoclaving at 15 lbs. pressure for fifteen minutes without loss of activity.
3. Experimental work indicated that it was more accurate to estimate the pH of the mucin after sterilisation than before.
4. Mucin suspensions retain their activity for ten days if kept at temperatures below 4°C.

PART II.

OBSERVATIONS ON THE INFLAMMATORY EXUDATES AND GROWTH OF THE ORGANISMS IN PERITONEUM AND BLOOD.

PART II.

OBSERVATIONS ON THE INFLAMMATORY EXUDATES AND GROWTH OF THE ORGANISMS IN PERITONEUM AND BLOOD.

As the results so far amply demonstrated the effect of mucin in promoting meningococcal infection in mice, it was necessary to study the mechanism by which this remarkable effect was produced. From the examination of large numbers of peritoneal exudates withdrawn at various times after inoculation, some very interesting observations were made, the most remarkable and consistent being the degenerate condition of the polymorphonuclear cells of the inflammatory exudates in those animals inoculated with meningococcal culture and mucin as compared with those inoculated with culture alone. In making differential counts 200 cells were counted.

EXAMINATION OF PERITONEAL EXUDATES OF MICE INOCULATED WITH MENINGOCOCCAL CULTURE AND MUCIN (5%- pH 7.2) AND MENINGOCOCCAL CULTURE SUSPENDED IN SALINE.

Three mice were used for the test and three for the control.

Exudates examined at 6, 10 and 24 hours after inoculation.

Test- Meningococcal culture and mucin.	Control-Meningococcal culture suspended in saline.
EXPERIMENT I. Majority of meningococci extra-cellular. Irregular and distorted outline of the cells of the exudate. At death the cells of the exudate were so indistinguishable that it was only with great difficulty the different types of cell could be identified. The polymorph leucocytes did not appear in any great numbers until ten hours after injection. At death most of the cells were of the mononuclear type. All animals died within 24 - 48 hours as a result of a blood infection.	All meningococci seen at six hours were intra-cellular. Polymorphs appeared in great numbers six hours after injection. Most of the polymorphs were young forms. At ten hours the exudate contained very few cells, and no growth of organisms was obtained on culture. At twenty-four hours no exudate was obtained. All mice alive and well after five days.
Cultures from heart blood, spleen and peritoneal exudate yielded a growth of meningococcus on blood agar in twenty-four hours.	

EXPERIMENT 2.

A fresh sample of commercial gastric mucin was tested at the following strengths; 5%, 4%, 3%, 2%, 1%. The bacterial suspension was standardised to 700 million.

TEST SERIES. 10 mice were used. Mucin 0.5 c.c. and meningococcal culture 0.5 c.c. (350 million). The mucin suspension was not accurately adjusted to pH 7.2.

CONTROL SERIES. 10 mice were used. Sterile normal saline 0.5 c.c. and meningococcal culture 0.5 c.c. (350 million).

Examination of peritoneal exudates was carried out at 3, 5 and 9 hours, and immediately after death.

TEST SERIES (MUCIN AND ORGANISMS).

	Polymorphs	Large lymphocytes	Small lymphocytes	Monocytes
3 hours	30% (25% damaged)	11% (3% damaged)	18% (1% damaged)	41% (16% damaged)

Arneth Count

Lobes					
1.	2.	3.	4.	5.	6.
15%	32%	44%	8%	1%	-

78% contained organisms.

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5 hours	19% (88% damaged)	14% (12% damaged)	33% (0.5% damaged)	34% (30% damaged)

Arneth Count

Lobes					
1.	2.	3.	4.	5.	6.
10%	20%	50%	15%	5%	-

82% contained organisms.

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9 hours	41%	15%	24%	20% (40% damaged)

80% damaged
16% contained organisms.

POST MORTEM- Most cells of large mononuclear type; majority of meningococci extra-cellular.
All mice died 24 - 48 hours.

CONTROL SERIES (ORGANISMS AND SALINE).

3 hours No exudate obtained

	Polymorphs	Large lymphocytes	Small lymphocytes	Monocytes
5 hours little exudate obtained	82% (less than 3% damaged)	5%	4%	9%

Arneth Count

Lobes

1.	2.	3.	4.	5.	6.
23%	34%	30%	9%	2.75%	1.25%

60% contained organisms.

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9 hours	55%	18%	5%	22%

Arneth Count

Lobes

1.	2.	3.	4.	5.	6.
42%	29%	20.5%	7.5%	-	1%

60% contained organisms.

---

24 hours No exudate obtained

All control mice alive and well after five days.

EXPERIMENT 3. Examination of peritoneal exudates at 1, 3½, 6½, 10 and 24 hours after inoculation of meningococcal culture (250 million) and mucin 5%, and meningococcal culture and saline. (Two mice were used for test and two for control).

Test (Mucin and meningococcal culture).	Control (Saline and meningococcal culture).
1 hour. Very much granular material, more than in control, cells mainly lymphocytes (69%), monocytes (19%), polymorphs (12%). The polymorphs were irregular in outline. Monocytes showed irregular cell outline, fenestrated margins and pseudopodial projections.	Much granular material. Cells very scanty, and mostly lymphocytic type.
3½ hours. Many more cells than at one hour. Majority polymorphs with irregular outlines, and nucleus in many cases fragmented; cells shrunken and cytoplasm poorly staining, cell margin difficult to define. Lymphocytes much more regular. Very few meningococci.	Very poor cellular reaction compared with test. Cells mainly polymorph, some a little shrunken. Very few meningococci, all intracellular.
6½ hours. Not so much granular material as at 3½ hours. 75% cells polymorphs which appeared swollen, irregular in outline and poorly staining. Many nucleii fragmented. Lobes of polymorphs difficult to distinguish. No organisms seen.	Cells much more regular than in test series. Majority polymorphs. Majority of meningococci intracellular.

10 hours. Granular material as at 6½ hours, majority of cells polymorph- still irregular in outline, but not so swollen as at 6½ hours. Nucleus fragmented, lobes indistinct. Meningococci fairly numerous - majority extra-cellular.	Very little exudate obtained. Cells regular.
24 hours. Most cells polymorph. Moderate number of meningococci, majority extra-cellular.	Minimal amount of exudate. Majority of cells normal. No meningococci seen.
48 hours. Both animals died of a blood infection.	Both mice alive and well after five days.

EXPERIMENT 4. Examination of peritoneal exudates from mice at 3, 6 and 24 hours after inoculation with meningococcal culture (350 million), and mucin 3% (buffered pH 7.2); and meningococcal culture (350 million), and saline.
Two mice were used for the test and two for control.

TEST SERIES (MENINGOCOCCAL CULTURE AND MUCIN).

3 hours

Polymorphs.	Large lymphocytes.	Small lymphocytes.	Monocytes.
60% (60% damaged)	10% (3% damaged)	19% (1% damaged)	11% (5% damaged)

60% contained organisms.

The damaged polymorphs showed irregular and distorted outlines with pseudopodial projections and vacuolation of the cytoplasm, the nucleus stained poorly, and was fragmented.

6 hours

Polymorphs.	Large lymphocytes.	Small lymphocytes.	Monocytes.
58% (85% damaged)	16% (5% damaged)	18% (1% damaged)	8% (30% damaged)

70% contained organisms.
Many more meningococci than at 3 hours.

24 hours Both mice died of a blood infection.
Excessive numbers of meningococci in all fields examined. Most of the cells appeared to be of the mononuclear type, but owing to the excessive numbers of organisms, it was difficult to identify the exact type of cell.

CONTROL SERIES (MENINGOCOCCAL CULTURE AND SALINE).

	Polymorphs.	Large lymphocytes.	Small lymphocytes.	Monocytes.
3 hours	68% 4% damaged. 55% contained organisms. Few organisms seen in film.	10%	18%	4%
6 hours	90% 2% damaged 70% contained organisms.	2%.	8%	

24 hours Very little exudate obtained.
All cells normal.
Very few organisms seen.
Both mice alive and well after five days.

EXPERIMENT 5. Examination of peritoneal exudates from mice at 6 and 24 hours after inoculation with meningococcal culture (350 million), and mucin 1% (refined fraction G 321 buffered pH 7.2), and meningococcal culture (350 million) and saline. Five mice were used for the test, and five for the control.

TEST SERIES (MENINGOCOCCAL CULTURE AND MUCIN).

	Polymorphs.	Large lymphocytes.	Small lymphocytes.	Monocytes.
6 hours	62%	13%	19%	6%
	(88% damaged)	(2% damaged)	(1% damaged)	(25% damaged)

58% contained organisms.

The damaged polymorphs stained poorly. In many cases differentiation between the cytoplasm and the nucleus was impossible. The cytoplasm showed pseudopodia and vacuolations, and in many cases had disappeared, while the nucleii exhibited fragmentation and in some cases had totally disappeared. Meningococci were fairly numerous.

	Polymorphs.	Large lymphocytes.	Small lymphocytes.	Monocytes.
24 hours	49%	18%	19%	14%
	(90% damaged)	(2% damaged)	(2% damaged)	(10% damaged)

75% contained organisms.

Excessive numbers of meningococci present.

24-48 hours — All mice died of a blood infection.

CONTROL SERIES (MENINGOCOCCAL CULTURE AND SALINE).

	Polymorphs.	Large lymphocytes.	Small lymphocytes.	Monocytes.
6 hours	90%	3%	5%	2%

3% damaged.
85% contained organisms.

24 hours — All cells normal. Very few organisms seen.
All five mice alive and well after five days.

EXPERIMENT 6. Examination of peritoneal exudates from mice at 3, 6, 9 and 24 hours after inoculation with meningococcal culture (350 million), and mucin 0.5% (refined fraction G 327 buffered pH 7.2) and meningococcal culture and saline.
Five mice were used for the test, and five for the control.

TEST SERIES (MENINGOCOCCAL CULTURE AND MUCIN).

	Polymorphs.	Large lymphocytes.	Small lymphocytes.	Monocytes.
3 hours	35% (70% damaged) 30% contained organisms.	9% (3% damaged)	48% (2% damaged)	8% (10% damaged)
6 hours	55% (75% damaged) 40% contained organisms.	5%	34%	6%
9 hours	68% (85% damaged) 38% contained organisms.	16%	9%	7%
24 hours	Excessive numbers of meningococci made an accurate count impossible.			
24-48 hours	All mice died of a blood infection.			

CONTROL SERIES (MENINGOCOCCAL CULTURE AND SALINE).

	Polymorphs.	Large lymphocytes.	Small lymphocytes.	Monocytes.
3 hours	62% (3% damaged) 52% contained organisms. Few organisms seen.	12%	20%	6%
6 hours	86% (2% damaged) 72% contained organisms.	3%	9%	2%
9 hours	88% (2% damaged) 70% contained organisms.	2%	8%	2%
24 hours	Very little exudate obtained. All cells normal. Very few organisms seen. All mice alive and well after five days.			

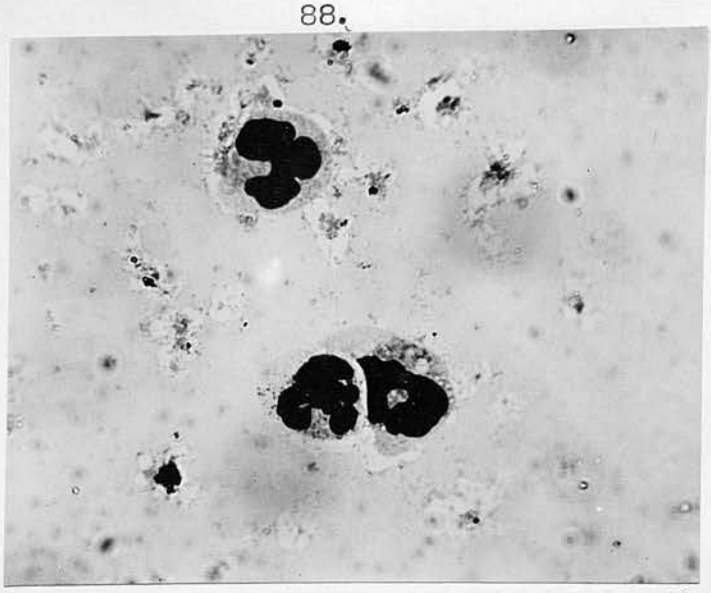

Fig. 1.

Mouse peritoneal exudate removed 6 hours after intraperitoneal injection of meningococcal culture and saline. The three polymorphs in the field are sharply defined marginally and consist of finely granular, easily visible cytoplasm, while their nuclei are densely stained and of variable lobation according to maturity. Few meningococci are present.

Leishman's stain X1000.

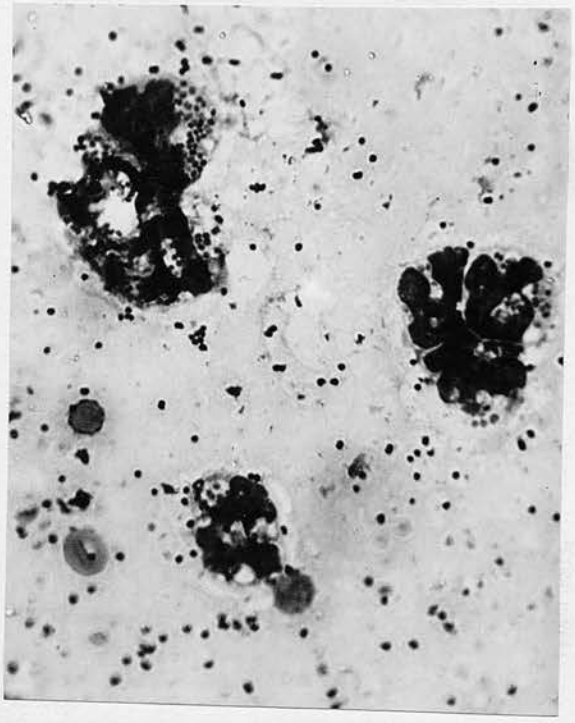

Fig. 2.

Mouse peritoneal exudate removed 6 hours after intraperitoneal injection of meningococcal culture and mucin (3% crude pH 7.2). The polymorphs show poor definition marginally and feeble staining, or almost complete lysis of their cytoplasm, while their nuclei are also more feebly stained than in the control. (Fig. 1). Many meningococci are present. The polymorph at bottom has a pseudopodium projecting from it's lower margin.

Leishman's stain X1000.

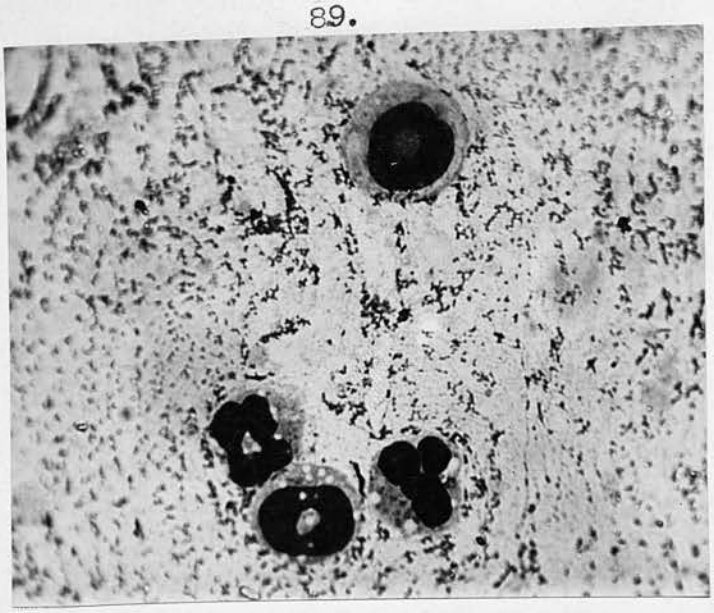

Fig. 3.

Mouse peritoneal exudate removed 24 hours after intraperitoneal injection of meningococcal culture and saline. The polymorphs are sharply defined marginally and consist of finely granular, easily visible cytoplasm. The nucleii are ring shaped or lobed, according as they are of immature or mature character. The background shows much debris, but no meningococci.

Leishman's Stain X1000.

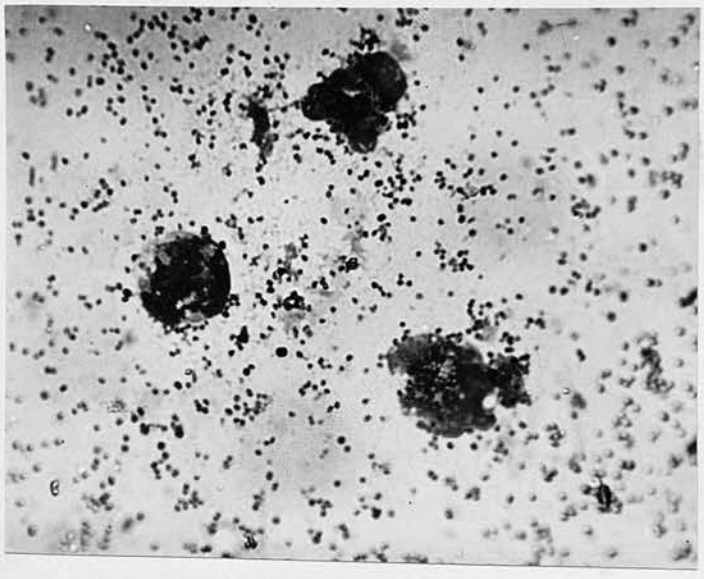

Fig. 4.

Mouse peritoneal exudate removed 24 hours after injection of meningococcal culture and mucin (3% crude pH 7.2). The polymorphs show poor definition marginally and feeble staining, rupture or complete lysis of cytoplasm, while their nucleii are less well defined than in control (Fig.3). Numerous organisms are present in the field.

Leishman's Stain X1000.

It was observed from the study of these peritoneal exudates, that the response of the control mice followed the normal physiological response to an infection caused by the meningococcus. In those mice inoculated with meningococcal culture and mucin, the polymorphs did not appear in any numbers until about four - six hours after inoculation, whereas in the control series, the polymorphs usually appeared earlier.

The type of polymorph in the exudates is worthy of note - in the test series there is not the marked 'left shift' seen in the Arneth count, as is observed in the control series.

All the exudates examined point to the damaged state of the polymorphs in the test series, and to the excessive numbers of organisms, the majority of which were extra-cellular. There are therefore three main features of the exudates from the mice inoculated with meningococcal culture and mucin, viz:

1. Damage to and destruction of polymorphonuclear cells.
2. Depression of phagocytosis.
3. Increasing numbers of organisms in the exudate as the infection advanced.

It is interesting to note that Miller and Castles (1934) in their studies of peritoneal exudates from mice inoculated with mucin and meningococci, noted that cells did not appear in the peritoneal

exudate in any considerable number until about four hours after inoculation. From then on the number of cells increased rapidly, two-thirds being polymorphs. As the infection progressed, an increased number of cells showed evidence of degeneration. The above findings are in agreement with this. They also found that when the strain was a highly virulent one, very few of the organisms were intracellular, irrespective of the number of organisms used. In the series of exudates studied by the writer, very few of the organisms were intracellular. Miller and Castles also observed that invasion of the blood occurred very shortly after intraperitoneal inoculation with large numbers of organisms, and when intraperitoneal inoculation exceeded a million organisms, cultures of blood withdrawn within fifteen minutes were positive, and bacteraemia persisted until death.

The same authors in 1936 examined peritoneal exudates at 3, 6, 9, 11 and 24 hours after inoculation and found that 75% of the cells at 6 hours were polymorphs, most of which were normal. No phagocytosis of meningococci was observed at 6 hours, and from then onwards very few organisms were seen within polymorphs, and many of the polymorphs were swollen, poorly staining, and vacuolated.

The findings in this study are practically the same as those of Miller and Castles, with the exception that the damage to the polymorphs was

observed at an earlier stage.

To ascertain whether the toxic effect on the leucocytes was primarily due to the mucin, two further experiments were carried out. 1. Mice were inoculated intraperitoneally with a suspension of mucin (5%) and saline, and 2. a lethal dose of meningo-coccal culture and saline. Peritoneal exudates were withdrawn and examined at 6 and 24 hours after injection.

	Mucin 5% and saline.			Lethal dose of meningococcal culture and saline.		
6 hours	Polymorphs.	Lymphocytes.	Monocytes.	Polymorphs.	Lymphocytes.	Monocytes.
	90%	4%	6%	58%	40%	2%
	60% of the polymorphs were damaged showing irregular cell outline and poorly staining. The cells were swollen, with pseudopodial projections of the cytoplasm. In many cases the cytoplasm had ruptured, and in some cases had actually disappeared. The nucleii were fragmented.			Only 15% of the polymorphs were damaged, and 77% contained ingested organisms.		
	15% lymphocytes damaged.			3% lymphocytes were damaged.		
	55% monocytes damaged.					
24 hours	Polymorphs.	Lymphocytes.	Monocytes.	Polymorphs.	Lymphocytes.	Monocytes.
	81%	7%	12%	95%	3%	2%
	68% of the polymorphs were damaged.			76% of the polymorphs were damaged.		
	20% " " lymphocytes " "			At this stage very few organisms were present.		
	60% " " monocytes " "					
	Mice alive and well after 5 days.			The mice died at 36 hours.		

The interesting point about these observations is the toxic effect of the mucin on the polymorphs which takes place early - six hours after injection, whereas in those animals receiving a lethal dose of organisms without mucin, the damage to the polymorphs is not apparent until 24 hours after injection. It would appear therefore, that one of the main actions of mucin is to break down the polymorphonuclear barrier in the peritoneum at an early stage, thereby permitting the access of large numbers of organisms into the blood stream.

TO ESTIMATE THE TIME OF ONSET OF BLOOD INFECTION IN MICE INOCULATED WITH MUCIN + MENINGOCOCCAL CULTURE.

TEST:- Mucin 3% + meningococcal culture (350 million) intraperitoneally in 3 mice.

CONTROL:- Buffer + meningococcal culture (350 million) intraperitoneally in 3 mice.

Tails were clipped and films and cultures made from the blood.

	Test		Control	
	Film	Culture	Film	Culture
10 minutes	-	-	-	-
15 "	-	-	-	-
30 "	-	-	-	-
1 hour	-	-	-	-
2 hours	+	+	+	+
3 "	+	+	+	+
4 "	+	+	+	+
5 "	+	+	+	+
6 "	+	+	+	+
24 " mice died (all with a blood infection)			-	-
30 "			-	-
48 "			-	-

This experiment was repeated with the same result. The organisms, therefore, appear in the blood in two hours, and in the case of the test mice, persist until death, in the control they disappear from the blood between 6 and 24 hours.

Wells and Johnstone (1907) found that when colon bacilli or streptococci were injected in large numbers into the peritoneal cavity of dogs or rabbits, they began to enter the blood through the thoracic duct in considerable numbers after from 15 to 30 minutes in the dog, and from 5 to 15 minutes in the rabbit.

Buxton (1907) in experiments with Salm. typhosa in rabbits, observed that organisms appeared in the blood in five minutes, and the numbers gradually

decreased from 30 minutes onwards, while Kyes (1916) found, that in pigeons, pneumococci were rapidly withdrawn from the general blood stream and localised in the spleen and liver.

PART III.

OBSERVATIONS ON THE TOXIC ACTION OF MUCIN TOWARDS LEUCOCYTES.

PART 111.

OBSERVATIONS ON THE TOXIC ACTION OF MUCIN TOWARDS LEUCOCYTES.

From the study of peritoneal exudates from animals inoculated with meningococcal culture and mucin it appeared that mucin might lower the resistance of the host by interfering with the leucocytic defence mechanism. With a view to throwing light on this question, a series of methylene blue reduction tests and leucocytic counting tests were carried out.

Neisser and Wechsberg (1901) were apparently the first to use the methylene blue reduction test to determine the viability of leucocytes, although it was Ehrlich who first recognised the fact that certain dyes were reduced by living cells and micro-organisms.

Neisser and Wechsberg attempted to explain the phenomenon on the basis that live leucocytes require oxygen and take it from their surroundings; thus, if into the liquid in which the leucocytes are suspended, an easily reducible colouring matter is introduced, it should be possible to demonstrate viable leucocytes by the decolourising of the dye, whereas the persistence of the colour indicated death of the cells. They proved conclusively that the leucocytes were responsible for the reduction of the dye; for by separating the leucocytes and using the supernatant

leucocyte-free fluid, no reduction took place. There was also no reduction if leucocytes were inactivated or killed by heating, or by the addition of quinine or alcohol.

The methylene blue reduction test has been extensively used as an indicator of the vitality of these cells and thus for demonstrating the action of bacterial leucocidins. It is probable however that a more complex oxidation-reduction mechanism is involved than that described by Neisser and Wechsberg.

The method of performing the test as used by Neisser and Wechsberg was as follows:- Equal parts of a 1% solution of sodium oxalate and an aleuronat exudate of leucocytes - (exudate obtained by injecting aleuronat, a vegetable protein preparation, into the pleural cavities of rabbits) - were made up to 2 c.c. with 0.85% sodium chloride. Five drops of a 1 in 2,500 solution of methylene blue were added, and a liquid paraffin seal superimposed. The tubes were then incubated for 2 hours at 37°C when readings were made.

In observations on the effect of certain leucocidal toxins the technique was:-

1. The minimum reducing dose of leucocytes was determined and found to be 0.25 c.c. In the test 0.5 c.c. was used.

2. Decreasing amounts of toxin were added.

3. The volume was made up to 2 c.c. with 0.85% of sodium chloride. The tubes were incubated for $1\frac{1}{2}$

hours at 37°C when two drops of methylene blue were added and a paraffin seal superimposed. The test was then read after a further incubation for two hours at 37°C.

The formula for the methylene blue solution was:-

Methylene blue	1
Absol.alcohol	20
Aqua dist.	29

to 1 c.c. of above 49 c.c. 0.85% Na.C1 was added.

The method of performing the reduction test used by the writer was similar to that of Neisser and Wechsberg.

TO ESTIMATE THE POSSIBLE LEUCOCYTE KILLING PROPERTY OF MUCIN.

Method of obtaining a preparation of leucocytes.

The method was similar to that used by Mackie, Finkelstein and Van Rooyen (1932).

Sufficient 10% sodium citrate solution to give a final concentration of between 0.5 - 1% was added to a wide mouthed bottle of one litre capacity, fitted with a bakelite screw cap. The bottle with citrate solution was sterilised in the steamer. An ox or sheep at the abattoir was bled from the neck vessels direct into the bottle, the bottle being gently shaken to mix the blood and citrate. The blood was divided into four 250 c.c. centrifuge bottles and centrifuged for thirty minutes at R.C.F.1000 Xg. Most of the plasma was separated off and the remainder together with the 'buffy coat' (leucocyte layer)

drawn off by means of a Pasteur pipette into sterile test tubes. The tubes were centrifuged for fifteen minutes at approximately R.C.F. 1,700 Xg. The leucocyte layer was then separated off into a sterile test tube, and the cream washed twice with modified Locke solution (i.e. Locke sine calcium). It was then suspended in modified Locke to make a leucocyte suspension of 5%.

Test — 0.5 c.c. leucocyte suspension.
0.5 c.c. mucin of varying concentrations.

Controls (a) 0.5 c.c. leucocyte suspension
0.5 c.c. modified Locke solution.

(b) 0.5 c.c. mucin suspension.
0.5 c.c. modified Locke solution.

Both test and control series were incubated at 37°C for ½ - 1 hour. They were then transferred to agglutination tubes and 0.05 c.c. methylene blue (1 in 2,000) added. A paraffin seal was superimposed and the tubes incubated for approximately one hour, or until the leucocyte control showed complete reduction. A series of methylene blue reduction tests was carried out with commercial mucin and with certain fractions prepared by Dr. H.K. King. The method of preparing the fractions will be dealt with in part 6 of the thesis. The fractions tested were G 14, G 217, G 225, G 327 and 3 E2.

RESULTS.

Expt.	Animal from which leucocytes obtained	5%	4%	3%	2%	1%	0.5%	0.25%	0.125%	Leucocyte control	Mucin control 5%	Mucin control 1%
		of mucin preparations.										
1	Ox	-	-	-	+	++	++	+++	++++	++++	-	-
2	ox	-	-	-	-	+++	+++	+++	+++	+++	-	-
3	ox	-			-	++++	++++	++++	++++	++++	-	-
4	ox	-			-	-	+++	++++	++++	++++	-	-
5	ox	-								++++	-	-
6	ox	-								++++	-	-
7	ox			-	-	+++	+++			+++	-	-
8	ox			-	+	+	+++	++++	++++	++++	-	-
9	ox			-	+	+	+++	++++	++++	++++	-	-
10	sheep			-	-	-	+	+	+	++++	-	-
11	sheep					-	+++	+++	++++	++++	-	-
12	sheep					-	+++	+++	++++	++++	-	-
13	horse				-	+	++++	++++	++++	++++	-	-
14	ox	-	-	++	+++	++++				++++	-	-
15	ox	-	+	+++	++++	++++				++++	-	-
16	ox	-	-	-	±	+				+++	-	-
17	ox	-	-	±	++	++++				++++	-	-
18	ox					+++	+++	++++	++++	++++	-	-
19						+	+++	+++	++++	++++	-	-
20	(3E2)			-	++++	++++	++++			++++	-	-

Expt.	Animal from which leucocytes obtained	2%	1%	0.5%	0.25%	0.125%	0.0625%	0.03125%	0.0152%	Leucocyte control	Mucin control 5%
21 (G7)	Sheep		-	-	-	-				+++	-
22 (G14)	Sheep		-	-	±	±				+++	-
23 (G217)	Sheep	-	-	-	-	-	-			++++	-
24 (G225)	Horse			-	-	++	++			++	-
25 (G225)	Sheep				-	-	-	++++	++++	++++	-
26 (G327)	Ox		-	-	+	+	++	++	++	++++	-
27 (G327)	Ox		-	-	+	+	++	++	++	++++	-

++++ = complete reduction.

+++, ++ and + = varying degrees of partial reduction.

- = no reduction.

From these results it is observed that with a 3% suspension of the commercial mucin the reducing property of the leucocytes is definitely inhibited. The fractionated samples show an even greater activity than the commercial preparation, in being active in some cases at concentrations as low as 0.0625%.

Taking the whole series of leucocyte counting tests there is a statistically significant difference between the results with mucin, and those without.

To obtain further evidence of the leucocyte destroying power of mucin, a series of counting tests were carried out.

Leucocytes- Human, horse, ox or sheep.

Method- The leucocyte count was standardised to a convenient figure before the test. Constant volumes of leucocyte suspension were added to equal volumes of mucin. In the control series Locke's solution replaced mucin. Test and control tubes were incubated at 37°C for one hour when counts were carried out.

Expt.	Mucin	Leucocytes	Before incubation	After incubation
1	3% (crude) buffered to pH 7.2	Ox	4,700/cmm	2,550/cmm
	control	"	4,700 "	3,900 "
2	3% (crude) buffered to pH 7.2	Ox	7,475 "	4,700 "
	control	"	7,475 "	6,950 "
3	3% (crude) buffered to pH 7.2	Sheep	6,500 "	3,500 "
	control	"	6,500 "	5,000 "
4	1% (crude) in saline	Human	5,000 "	1,800 "
	control	"	5,000 "	4,500 "
5	1% (crude) in saline	Sheep	4,000 "	2,600 "
	control	"	4,000 "	3,400 "
6	1% (crude) in saline	Ox	6,000 "	2,500 "
	0.5% (crude) in saline	"	6,000 "	2,700 "
	control	"	6,000 "	4,700 "
7	1% (crude) in saline	Ox	5,600 "	2,200 "
	control	"	5,600 "	3,200 "
8	G217 2% pH 7.2	Sheep	10,000 "	9,000 "
	control	"	10,000 "	9,750 "
9	G225 0.5% pH 7.2	Horse	5,075 "	4,000 "
	control	"	5,075 "	5,000 "
10	G327 1% pH 7.2	Ox	4,700 "	2,250 "
	control	"	4,700 "	3,900 "
11	G327 1% pH 7.2	Ox	7,475 "	2,950 "
	control	"	7,475 "	6,950 "

The above series of counting tests illustrates that when leucocytes are left in contact with mucin for one hour at 37°C their numbers are markedly reduced. In all tests there was a much greater reduction of the leucocytes in the mixtures containing mucin as compared with those containing Locke's solution. That the control tubes did show a reduced count after incubation might be explained by damage sustained to the leucocytes in pipetting etc.

The greatest reduction in leucocytes was seen with fraction G 327 (1%) where the fall in the count after incubation was 4,525, although it was not always the case that refined fractions were more active than the crude preparation in this respect. In this type of test it will be appreciated that the leucocytes may show variations according to 1 the species of animal from which the blood is obtained and 2 the time of the year. Thus, in different experiments leucocytes from different animal species were used and the experiments in the series recorded were done at different times of the year.

TO ESTIMATE THE PERCENTAGE OF DAMAGED LEUCOCYTES AFTER CONTACT WITH MUCIN FOR ONE HOUR AT 37°C.

Mucin and leucocytes were allowed to interact for one hour at 37°C. In the control Locke's solution replaced mucin. Films were made from test and control with a platinum loop (Valentine, 1936) and stained by Leishman's method.

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The following points were taken as criteria of cell damage:-

(1) Irregular outline of cells.

(2) Pseudopodial projections of cytoplasm.

(3) Vacuolation of cytoplasm.

(4) Vacuolation and fragmentation of nucleus.

(5) Poor staining reaction.

Expt. 13.

Mucin	Leucocytes	Damaged cells	
3% crude pH 7.2	Ox	76%	200 cells were counted in all cases.
G 327 pH 7.2	Ox	84%	
Control	Ox	12%	

TO ESTIMATE THE NUMBER OF RECOGNISABLE LEUCOCYTES AND POLYMORPHS AFTER CONTACT WITH MUCIN FOR ONE HOUR AT 37°C.

	Mucin	Leucocytes	No. of recognisable leucocytes	No. of recognisable Polymorphs
Expt. 14	G225 (0.5%)	Horse	0.42 per field	
	control	"	1.02 " "	
Expt. 15	G217 (2%)	Sheep	1.89 per field	0.50 per field
	control	"	2.35 " "	0.88 " "

The differences in Experiments 13, 14 and 15 are statistically significant.

OBSERVATIONS ON THE ACTION OF MUCIN WITH REGARD TO THE PHAGOCYTIC PROPERTY OF LEUCOCYTES: EXPERIMENTS PERFORMED IN VITRO.

Expt. 16 Test series- Fresh rabbit serum+fresh ox leucocytes+mucin+staphylococci.

Control series- Fresh rabbit serum+fresh ox leucocytes+Locke's solution+staphylococci.

The mixtures were incubated for one hour at 37°C. Films were made and stained by Leishman's method.

Control (no mucin)	3% mucin (crude pH 7.2)	G327 1% (pH 7.2)
2.6 organisms/cell	0.38 organisms/cell	0.18 organisms/cell
84% cells contained organisms	30% cells contained organisms	18% cells contained organisms

OBSERVATIONS ON THE ACTION OF MUCIN WITH REGARD TO THE PHAGOCYTIC PROPERTY OF LEUCOCYTES: EXPERIMENTS PERFORMED IN VIVO.

Expt.17 Peritoneal exudates were examined at 3, 5 and 9 hours from mice inoculated with meningococcal culture and mucin, and from mice inoculated with meningococcal culture and saline.

	% cells of exudate with ingested organisms.			% cells of exudate without ingested organisms.		
	3 hours	5 hours	9 hours	3 hours	5 hours	9 hours
Meningococcal culture and mucin 4% (pH 7.2)	22%	18%	15%	78%	82%	85%
Meningococcal culture and saline	62%	60%	61%	38%	40%	39%

SUMMARY OF PART 111.

The experimental studies on the action of mucin on leucocytes illustrate:-

1. Inhibition of the reducing system of leucocytes.
2. Actual destruction of leucocytes, in particular of the polymorphonuclear cells.
3. Depression of phagocytosis.

The possibility of the destructive action on leucocytes being due to some chemical impurity in the commercial mucin must be considered. It was thought that, due to the method of manufacture, the mucin might contain some chemical e.g. a preservative, which might exert a toxic action on leucocytes or that in the process of extraction of the mucin from the pig's stomach some anti-leucocytic factor may have been extracted from the stomach.

Other factors such as hypertonicity or the presence of heavy metals were also considered.

Tests carried out on the crude mucin for the presence of heavy metals were negative, and from the information received from the manufacturers, no preservative was added to the crude mucin. Moreover, the ash obtained in the fractionated samples proved inactive on testing in mice.

The possibility of an osmotic effect on the leucocytes could be excluded when it was found that several fractions which had inorganic salts removed by dialysis all proved active.

PART IV.

OBSERVATIONS ON THE EFFECT OF MUCIN ON THE BACTERICIDAL ACTION OF NORMAL SERUM, PLASMA, AND WHOLE BLOOD.

PART IV.

OBSERVATIONS ON THE EFFECT OF MUCIN ON THE BACTERICIDAL ACTION OF NORMAL SERUM, PLASMA, AND WHOLE BLOOD.

The experimental studies so far pointed to an injurious effect of mucin on leucocytes, and it was therefore decided to attempt some assessment of the effect of mucin on the normal bactericidal action of whole blood, and for purposes of comparison, of plasma or serum, from the same specimen of blood.

The bactericidal action of blood constitutes a complex mechanism and depends on (a) the direct bactericidal properties of the plasma, and (b) the opsonisation of the organisms by plasma followed by their phagocytosis by leucocytes and destruction in the cytoplasm of these cells.

It seemed possible that the aggressin-like action of mucin, particularly its effect in promoting blood infection and septicaemia, might be due to an interference with one or more of the factors involved in these natural bactericidal mechanisms.

The results of a series of quantitative tests are first given to illustrate the normal bactericidal action of whole blood and plasma towards particular organisms, and these are followed by similar tests in which mucin was incorporated in the reacting system. Rabbits were selected for most of these tests in view

of the convenience of obtaining samples of their blood and plasma. In some cases, however, specimens from man and the horse were used.

The technique and system of recording results was as used by Mackie and Finkelstein (1931) with certain minor modifications regarding periods of incubation.

A given quantity of whole blood (or plasma) was mixed with a given volume of varying dilutions of bacteria made up in 0.85% sodium chloride solution, which however, was replaced in the later experiments by gelatin-Locke solution since it was found that even physiological sodium chloride solution affects the viability of certain organisms. A standard loopful of each mixture was inoculated by the single stroke method on a plate of the appropriate medium. This was done before incubation and at varying periods up to 24 hours after incubation at 37°C. In this way the bactericidal action of the blood could be determined by comparison of the end-points of growth after the various periods of incubation, with the end-point before incubation (this acting as a control). Lack of bactericidal action was indicated by coincidence of the two end-points. The bacterial dilutions were prepared from 24 hours cultures.

The mixtures were all carefully shaken before making transfers, to ensure uniformity of the bacterial suspension. In some cases the mixtures were shaken continuously between transfers.

The comparison of end-points was also checked by estimates of the approximate amount of growth resulting from each transfer.

The series of bacterial concentrations used for the test consisted of successive tenfold or twenty-fold dilutions prepared from a standard concentration determined by comparison with Brown's opacity standards. In order to obtain definite end-points, an extensive range of such dilutions was found necessary - S/1 to $S/10^9$ (or S/1 to $S/20^9$). (S = Standard concentration).

The initial standard varied (according to trial tests) for different organisms, the object being to arrange the series so that an end-point was obtained in the control at the fourth or fifth dilution. For Salm. typhosa Brown's opacity standard No. 2 represented a suitable initial suspension.

The mixtures of bacteria and blood (or plasma) were made in small sterile stoppered test tubes. The volume of bacterial suspension was usually 0.5 c.c. The volumes of blood varied from 0.5 to 0.125 c.c. and the volume of plasma corresponded to the relative proportion of plasma to blood in the specimen.

The anticoagulant for the blood was in most cases heparin; in a few experiments citrate was used. The blood was withdrawn with aseptic precautions and used within a few hours of withdrawal.

The transfers were made with a loop of 4 m.m. diameter and each loopful was stroked on to a marked division of the plate.

The degree of bactericidal action was recorded as follows:- for example, if in the platings carried out before incubation (control) growth resulted from all concentrations up to the fourth ($S/20^3$) while in the platings after incubation growth occurred only from the first ($S/1$) the result (a bactericidal effect) was stated as +3. i.e. the difference between the indices of the two dilutions.

In the detailed records the relative amount of growth from each transfer was recorded by + symbols as follows:- 5, 4, 3, 2 and 1, f.c. = a few scattered colonies only, 1c, 2c etc. = 1 colony, 2 colonies etc. only, + = bactericidal, - = growth promoting.

Organisms- Salm. typhosa strain Cole, ('S' form) and Salm. typhosa ('R' form).
Strength of standard suspension ('S')- Tube 2 (Brown's opacity standards).
Serial dilutions- Twentyfold. Diluent- Gel. Locke. Whole blood- Rabbit.
Anticoagulant- Citrate. Plasma- Rabbit Tubes shaken continuously.

BACTERIAL DILUTIONS.

Series	Time	S/1	S/20^1	S/20^2	S/20^3	S/20^4	S/20^5	S/20^6	S/20^7	S/20^8	S/20^9	Result.
Salm. typhosa ('smooth') 0.5 c.c. +	B	4	2	1	f.c.	3c	-	-	-	-	-	
Plasma 0.3 c.c. +	4 hrs.	4	1c	-	-	-	-	-	-	-	-	+3
Gel. Locke 0.2 c.c.	24 "	4	35c	-	-	-	-	-	-	-	-	+2
Salm. typhosa ('smooth') 0.5 c.c. +	B	4	2	1	f.c.	9c	-	-	-	-	-	
Whole blood 0.5 c.c.	4 hrs.	4	1c	-	-	-	-	-	-	-	-	+3
	24 "	4	-	-	-	-	-	-	-	-	-	+3
Salm. typhosa ('rough') 0.5 c.c. +	B	4	3	1	f.c.	9c	-	-	-	-	-	
Plasma 0.3 c.c. +	4 hrs.	4	13c	-	-	-	-	-	-	-	-	+3
Gel. Locke 0.2 c.c.	24 "	4	f.c.	17c	-	-	-	-	-	-	-	+2
Salm. typhosa ('rough') 0.5 c.c. +	B	4	2	1	f.c.	12c	2c	-	-	-	-	
Whole blood 0.5 c.c.	4 hrs.	4	2c	-	-	-	-	-	-	-	-	+3
	24 "	4	f.c.	f.c.	-	-	-	-	-	-	-	+1

B = before incubation.
f.c. = few scattered colonies only.
1c, 2c etc. = 1 colony, 2 colonies etc.
+ = bactericidal.
- = growth promoting.

Conclusions:- Both rabbit plasma and whole blood are bactericidal but show little significant difference, apparently the effect manifested by whole blood is largely dependent on the plasma. The results also show the effect may not be sustained over a period of 24 hours and in fact at this time surviving organisms are growing in the mixture.

Organisms- Salm. typhosa (strain Cole 'rough' form).
Strength of standard suspension ('S')- Tube 2.
Serial dilutions- Twentyfold. Diluent- Gel.Locke. Whole blood- Rabbit
Anticoagulant- Heparin.
Shaking- Continuous.

BACTERIAL DILUTIONS.

Series	Time	S/1	S/20^1	S/20^2	S/20^3	S/20^4	S/20^5	S/20^6	S/20^7	S/20^8	S/20^9	Result.
Salm. typhosa 0.5 c.c.	B	4	2	1	15c	2c	2c	1c	-	-	-	
+	3 hrs.	3	6c	-	-	-	-	-	-	-	-	
Whole blood 0.5 c.c.	6 "	3	-	-	-	-	-	-	-	-	-	+2
	9 "	3	-	-	-	-	-	-	-	-	-	+2
	24 "	4	4	2	-	-	-	-	-	-	-	+2
Salm. typhosa 0.5 c.c.	B	4	3	1	25c	5c	-	-	-	-	-	0- +1
+	3 hrs.	3	13c	-	-	-	-	-	-	-	-	
Whole blood 0.25 c.c.	6 "	3	7c	-	-	-	-	-	-	-	-	+2
	9 "	3	4c	-	-	-	-	-	-	-	-	+2
	24 "	4	4	4	4	1	1	25c	-	-	-	+2
												-3

Conclusions:- Whole blood is bactericidal at 3 hours and this is sustained up to 9 hours, but not after this time. This is more evident with the smaller quantity of blood (0.25 c.c.).

Organism- Salm. typhosa (strain Cole 'rough' form).
Strength of standard suspension ('S')- Tube 2.
Serial dilutions- Twentyfold. Diluent- Gel.Locke
Plasma- Rabbit. Anticoagulant- Heparin. Shaking - Continuous.

BACTERIAL DILUTIONS.

Series	Time	S/1	S/20^1	S/20^2	S/20^3	S/20^4	S/20^5	S/20^6	S/20^7	S/20^8	S/20^9	Result.
Salm. typhosa 0.5 c.c. + Plasma 0.3 c.c. + Gel.Locke 0.2 c.c.	B	2	1	4c	1c	-	-	-	-	-	-	
	3 hrs.	2	-	--	-	-	-	-	-	-	-	+1
	6 "	2	-	-	-	-	-	-	-	-	-	+1
	9 "	2	-	-	-	-	-	-	-	-	-	+1
	24 "	2	-	-	-	-	-	-	-	-	-	+1
Salm. typhosa 0.5 c.c. + Plasma 0.16 c.c. + Gel.Locke 0.085 c.c.	B	3	1	9c	1c	1c	2c	-	-	-	-	
	3 hrs.	3	-	-	-	-	-	-	-	-	-	+1
	6 "	3	-	-	-	-	-	-	-	-	-	+1
	9 "	3	-	-	-	-	-	-	-	-	-	+1
	24 "	3	-	-	-	-	-	-	-	-	-	+1
Salm. typhosa 0.5 c.c. + Plasma 0.08 c.c. + Gel.Locke 0.045 c.c.	B	3	1	f.c.	3c	1c	-	-	-	-	-	
	3 hrs.	3	-	-	-	-	-	-	-	-	-	+2
	6 "	3	-	-	-	-	-	-	-	-	-	+2
	9 "	3	-	-	-	-	-	-	-	-	-	+2
	24 "	3	-	-	-	-	-	-	-	-	-	+2

Conclusions:- Plasma is bactericidal at 3 hours, and although not increased beyond this time, the effect is sustained for 24 hours. The effect of the plasma is more sustained than that of whole blood - c.f. previous experiments.

Organism - Salm. typhosa (617 'smooth' form).
Strength of standard suspension ('S')- Tube 2.
Serial dilutions- Tenfold. Diluent- Gel.Locke.
Plasma- Rabbit. Anticoagulant- Heparin. Shaking - continuous.

BACTERIAL DILUTIONS.

Series	Time	S/1	S/10^1	S/10^2	S/10^3	S/10^4	S/10^5	S/10^6	S/10^7	S/10^8	S/10^9	Result.
Salm. typhosa 0.5 c.c.	B	4	3	2	1	f.c.	14c	2c	-	-	-	
+	3 hrs.	3	1	f.c.	18c	2c	-	-	-	-	-	+2
Plasma 0.3 c.c.	6 "	4	1	f.c.	3c	2c	-	-	-	-	-	+2
+	24 "	3	3	f.c.	-	-	-	-	-	-	-	+2
Gel.Locke 0.2 c.c.												
Salm. typhosa 0.5 c.c.	B	4	4	2	1	1	20c	-	-	-	-	
+	3 hrs.	3	1	f.c.	12c	2c	2c	-	-	-	-	+2
Plasma 0.16 c.c.	6 "	4	2	f.c.	6c	-	-	-	-	-	-	+2
+	24 "	3	3	1	1	-	-	-	-	-	-	+1
Gel.Locke 0.085 c.c.												
Salm. typhosa 0.5 c.c.	B	4	4	3	2	1	f.c.	4c	-	-	-	
+	3 hrs.	3	2	1	f.c.	4c	-	-	-	-	-	+2
Plasma 0.08 c.c.	6 "	4	1	f.c.	4c	-	-	-	-	-	-	+3
+	24 "	3	2	1	1c	-	-	-	-	-	-	+2-+3
Gel.Locke 0.045 c.c.												

Conclusions:- Plasma is bactericidal at 3 hours. No increase in bactericidal effect after 3 hours. The effect up to 24 hours is well maintained, and is as great with the smaller amounts (0.08 c.c.). - c.f. whole blood where decreasing amounts of blood showed a corresponding decreased effect.

Organisms- Salm. typhosa 617 ('smooth' form).
Strength of standard suspension ('S')- Tube 2.
Serial dilutions- Tenfold Diluent- Gel. Locke.
Whole blood- Rabbit. Anticoagulant- Heparin. Shaking - continuous.

BACTERIAL DILUTIONS.

Series	Time	S/1	$S/10^1$	$S/10^2$	$S/10^3$	$S/10^4$	$S/10^5$	$S/10^6$	$S/10^7$	$S/10^8$	$S/10^9$	Result.
Salm. typhosa 0.5 c.c.	B	4	3	2	1	f.c.	4c	2c	-	-	-	
+	3 hrs.	2	1	20c	1c	1c	-	-	-	-	-	+2-+3
Whole blood 0.5 c.c.	6 "	2	1	8c	3c	-	-	-	-	-	-	+2-+3
	24 "	4	3	2	3c	-	-	-	-	-	-	+2
Salm. typhosa 0.5 c.c.	B	4	3	2	1	f.c.	6c	2c	1c	-	-	
+	3 hrs.	2	1	f.c.	8c	-	-	-	-	-	-	+2
Whole blood 0.25 c.c.	6 "	2	f.c.	4c	1c	-	-	-	-	-	-	+3
	24 "	4	4	4	3	3	-	-	-	-	-	-1
Salm. typhosa 0.5 c.c.	B	4	4	3	2	1	14c	2c	4c	-	-	
+	3 hrs.	3	1	f.c.	10c	1c	-	-	-	-	-	+3
Whole blood 0.125 c.c.	6 "	3	1	5c	1c	-	-	-	-	-	-	+3
	24 "	4	4	4	4	3	2	1.	5c	-	-	-2

Conclusions:- Whole blood is bactericidal at 3 hours. With 0.5 c.c. blood, bacteriostasis is maintained to 24 hours, but is not maintained in the case of smaller amounts of blood (0.25 c.c. and 0.125 c.c.). Whole blood is slightly more bactericidal than plasma but the plasma effect is more sustained.

Organism- Staph. aureus M77614
Coag. +ve; haemolytic, pigment +++. Very difficult to emulsify;
Penicillin insensitive.
Strength of suspension- Tube 2.
Dilution- Twentyfold. Diluent- Gel.Locke. Plasma- Animal- Rabbit. Anticoagulant- Heparin.
Amount- 0.2 c.c.
Whole blood- Animal- Rabbit. Shaking- continuous between B and 4 hours.
Amount- 0.3 c.c. partial " 4 " 24 "

BACTERIAL DILUTIONS.

Series	Time	S/1	S/20^1	S/20^2	S/20^3	S/20^4	S/20^5	S/20^6	S/20^7	S/20^8	S/20^9	Result.
Staph. 77614 0.5 c.c. +	B	4	4	2	f.c.	14c	3c	-	-	-		
Plasma (0.2 c.c.) +	4 hrs.	3	1	1	f.c.	6c	-	-	-	-		0
Gel.Locke (0.1 c.c.)	24 "	4	4	4	4	4	4	4	-	-		-3
Staph. 77614 0.5 c.c. +	B	4	3	2	f.c.	11c	1c	-	-	-		
Whole blood (0.3 c.c.)	4 hrs.	4	3c	-	-	-	-					+3
	24 "	4	4	4	4	4	4	-	-	-	-	-2

Conclusions:- Plasma shows only slight bactericidal action at 4 hours and at 24 hours is growth promoting.
Whole blood shows distinct bactericidal action at 4 hours; but is not maintained after this time; and like plasma is growth promoting at 24 hours though not to the same degree.

Organisms- Strept. viridans L.S.C. and Strept. pyogenes 1246.
Strength of standard suspension ('S')- Tube 2.
Serial dilutions- Tenfold. Diluent- Gel. Locke. Anticoagulant- Citrate.
Plasma- Animal- Rabbit. Whole blood- Animal- Rabbit.
Amount- 0.2 c.c. Amount- 0.3 c.c. Shaking - continuous.

BACTERIAL DILUTIONS.

Series	Time	S/1	S/10^1	S/10^2	S/10^3	S/10^4	S/10^5	S/10^6	S/10^7	S/10^8	S/10^9	Result.
Strept. pyogenes 0.5 c.c.	B	4	2	1	f.c.	22c	1c	-	-	-	-	
+												
Plasma 0.2 c.c.	4 hrs.	4	2	1	f.c.	22c	-	-	-	-	-	0
+												
Gel. Locke 0.1 c.c.	24 "	3	f.c.	f.c.	f.c.	- 4c	-	-	-	-	-	0
Strept. pyogenes 0.5 c.c.	B	4	2	1	f.c.	38c	1c	-	-	-	-	
+	4 hrs.	4	2	1	f.c.	25c	1c	-	-	-	-	0
Whole blood 0.3 c.c.	24 "	4	2	f.c.	10c	-	-	-	-	-	-	+1
Strept. viridans 0.5 c.c.	B	3	2	1	f.c.	f.c.	- 12c	-	-	-	-	
+												
Plasma 0.2 c.c.	4 hrs.	3	2	1	f.c.	-	-	-	-	-	-	+1+
+												
Gel. Locke 0.1 c.c.	24 "	4	3	f.c.	f.c.	5c	3c	-	-	-	-	0
Strept. viridans 0.5 c.c.	B	4	2	1	f.c.	35c	-	-	-	-	-	
+	4 hrs.	2	1	f.c.	-	-	-	-	-	-	-	+2
Whole blood 0.3 c.c.	24 "	4	1	f.c.	40c	6c	-	-	-	-	-	+1

Conclusions:- With both these organisms whole blood shows a greater degree of bactericidal action than plasma.

Mucin- 1% crude (normal saline). Plasma- Animal- Horse 0.3 c.c.
Organism- Salm. typhosa (Cole 'rough') 0.5 c.c. Diluent- Normal saline.
Strength of standard suspension ('S')- Tube 3. Anticoagulant- Citrate.
Serial dilutions- Twentyfold. Shaking- Not shaken continuously.

BACTERIAL DILUTIONS.

Series	Time	S/1	S/20¹	S/20²	S/20³	S/20⁴	S/20⁵	S/20⁶	S/20⁷	S/20⁸	S/20⁹	Result.
Salm. typhosa 0.5 c.c. +	B	4	4	3	2	2	6c	6c	-	-	-	
Plasma 0.3 c.c. +	4 hrs.	3	3	2	1	1	2c	-	-	-	-	0
Saline 0.3 c.c.	24 "	4	3	3	8c	-	-	-	-	-	-	+2
Salm. typhosa 0.5 c.c. +	B	3	3	2	1	f.c.	1c	-	-	-	-	
Plasma 0.3 c.c. +	4 hrs.	3	3	2	1	8c	-	-	-	-	-	0
Mucin 0.3 c.c.	24 "	3	3	3	3	3	3	3	-	-	-	-3
Salm. typhosa 0.5 c.c. +	B	4	3	2	1	f.c.	f.c.	-	-	-	-	
Mucin 0.3 c.c. +	4 hrs.	4	3	2	1	f.c.	1c	-	-	-	-	0
Saline 0.3 c.c.	24 "	4	4	4	3	3	3	2	-	-	-	-3
Salm. typhosa 0.5 c.c. +	B	4	3	3	1	f.c.	-	-	-	-	-	
Plasma 0.3 c.c. (heated to 55°C ½ hour) +	4 hrs.	4	3	3	1	f.c.	-	-	-	-	-	0
Saline 0.3 c.c.	24 "	3	3	3	3	2c	-	-	-	-	-	0
Salm. typhosa 0.5 c.c. +	B	4	3	3	2	f.c.	4c	-	-	-	-	
Plasma 0.3 c.c. (55°C ½ hour) +	4 hrs.	4	3	2	1	f.c.	6c	-	-	-	-	0
Mucin 0.3 c.c.	24 "	3	3	3-	3	3	3	3	-	-	-	-3

Conclusions:-

Bactericidal action of horse plasma completely abolished by mucin.

Series 3 demonstrates the growth promoting action of mucin.

The series with heated serum and organisms demonstrates the bactericidal action of plasma to be dependent on complement, as in this series there was no killing observed, the complement being labile, was inactivated at 55°C.

Mucin- 1% crude (Locke).
Organism- Salm. typhosa (Cole 'rough')
Strength of standard suspension ('S')- Tube 3.
Serial dilutions- Tenfold.

Plasma- Rabbit.
Diluent- Locke.
Anticoagulant- Heparin.
Shaking- Not shaken continuously.

BACTERIAL DILUTIONS.

Series	Time	S/1	S/10^1	S/10^2	S/10^3	S/10^4	S/10^5	S/10^6	S/10^7	S/10^8	S/10^9	Result.
Salm. typhosa 0.5 c.c. +	B	4	3	3	1	f.c.	-	-	-	-	-	
Plasma 0.3 c.c. +	4 hrs.	3	-	-	-	-	-	-	-	-	-	+4
Locke 0.2 c.c.	24 "	3	-	-	-	-	-	-	-	-	-	+4
Salm. typhosa 0.5 c.c. +	B	4	3	3	1	8c	3c	2c	-	-	-	
Plasma 0.3 c.c. +	4 hrs.	3	18c	-	-	-	-	-	-	-	-	+3
Mucin 0.2 c.c.	24 "	4	3	-	-	-	-	-	-	-	-	+2
Salm. typhosa 0.5 c.c. +	B	4	3	2	1	f.c.	4c	-	-	-	-	
Mucin 0.2 c.c. +	4 hrs.	4	3	3	2	1	19c	-	-	-	-	0
Locke 0.3 c.c.	24 "	4	4	4	4	4	4	4	4	4	-	-4

Conclusions:- Bactericidal action of rabbit plasma reduced by mucin.
Series 3 demonstrates the growth promoting action of mucin.

Mucin- 1% crude (normal saline). Serum- Animal- Rabbit.
Organism- Salm. typhosa (Cole 'rough' form). Diluent- Normal saline.
Strength of standard suspension ('S')- Tube 3. Shaking- Not shaken continuously.
Serial dilutions- Tenfold.

BACTERIAL DILUTIONS.

Series	Time	S/1	S/10^1	S/10^2	S/10^3	S/10^4	S/10^5	S/10^6	S/10^7	S/10^8	S/10^9	Result.
Salm. typhosa 0.5 c.c.	B	4	3	3	2	1	f.c.	-	-	-	-	
+												
Serum 0.3 c.c.	4 hrs.	3	-	-	-	-	-	-	-	-	-	+5
+												
Saline 0.3 c.c.	24 "	4	-	-	-	-	-	-	-	-	-	+5
Salm. typhosa 0.5 c.c.	B	4	3	3	2	1	f.c.	-	-	-	-	
+												
Serum 0.3 c.c.	4 hrs.	4	-	-	-	-	-	-	-	-	-	+5
+												
Mucin 0.3 c.c.	24 "	4	4	-	-	-	-	-	-	-	-	+4
Salm. typhosa 0.5 c.c.	B	4	3	3	2	1	f.c.	6c	-	-	-	
+												
Mucin 0.3 c.c.	4 hrs.	4	4	3	3-	2-	1	4c				0
+												
Saline 0.3 c.c.	24 "	4	4	4	4	4	3	3	3	2	2	-4

Conclusions:- Serum was bactericidal at 4 hours and the effect was sustained for 24 hours. Mucin did not alter the bactericidal effect at 4 hours but in the presence of mucin it was not sustained. Mucin per se has a marked growth promoting effect.

Organism- Salm. typhosa ('R') form.
Strength of standard suspension ('S')- Tube 2.
Serial dilutions- Tenfold.

Whole blood- Rabbit.
Diluent- Gel. Locke.
Anticoagulant- Heparin.

Mucin- 3% crude (pH 7.2).
Shaking- Partial.

BACTERIAL DILUTIONS.

Series	Time	S/1	S/10^1	S/10^2	S/10^3	S/10^4	S/10^5	S/10^6	S/10^7	S/10^8	S/10^9	Result.
Salm. typhosa 0.5 c.c. + Whole blood 0.5 c.c. + Gel. Locke 0.5 c.c.	B	4	3	2	1	1	f.c.	f.c.	10c	8c	5c	
	4 hrs.	3	2	1	5c	-	-	-	-	-	-	+4
	24 "	4	3	3	2	1	f.c.	f.c.	f.c.	f.c.	f.c.	-3
Salm. typhosa 0.5 c.c. + Whole blood 0.5 c.c. + Mucin 3% crude 0.5 c.c.	B	4	3	2	1	1	10c	7c	5c	2c	-	
	4 hrs.	3	2	1	2c	2c	2c	-	-	-	-	+2
	24 "	4	4	3	2	2	1	1	1	1	f.c.	-5
Salm. typhosa 0.5 c.c. + Ge. Locke 0.5 c.c. + Mucin 3% crude 0.5 c.c.	B	4	4	3	2	1	1	10c	5c	2c	1c	
	4 hrs.	4	4	3	2	1	1	15c	14c	6c	5c	0
	24 "	4	4	4	4	3	3	3	3	2	1	-4

Conclusions:- Whole blood is bactericidal at 4 hours.
This effect is reduced by mucin.

Organism- Strept. pyogenes 1246. Source- Ac. Tonsillitis. Plasma- Human 0.15 c.c.
Strength of standard suspension ('S')- Tube 6. Mucin- 0.15 c.c. 1% in Ord. Locke.
Serial dilutions- Tenfold. Diluent- Saline. Anticoagulant- Heparin.
Shaking- Not shaken continuously.

BACTERIAL DILUTIONS.

Series	Time	S/1	S/10^1	S/10^2	S/10^3	S/10^4	S/10^5	S/10^6	S/10^7	S/10^8	S/10^9	Result.
Strept. pyogenes 0.5 c.c. +	B	4	3	2	1	11c	2c	-	-	-	-	
Plasma 0.15 c.c. +	4 hrs.	4	3	2	1	4c	-	-	-	-	-	0
Ord. Locke 0.15 c.c.	24 "	4	4	2	1	11c	-	-	-	-	-	0
Strept. pyogenes 0.5 c.c. +	B	4	3	2	1	14c	1c	-	-	-	-	
Mucin 0.15 c.c. +	4 hrs.	4	3	2+	2	1+	f.c.	-	-	-	-	-1
Plasma 0.15 c.c.	24 "	4	4	4	4	4	4	4	4	-	-	-4
Strept. pyogenes 0.5 c.c. +	B	4	3	2	1	19c	2c	-	-	-	-	
Mucin 0.15 c.c. +	4 hrs.	4	3	1	1	6c	1c	-	-	-	-	0
Ord. Locke 0.15 c.c.	24 "	4	3	1	1	1	f.c.	3c	-	-	-	-2

Conclusions:- In this experiment the plasma had no bactericidal action but was bacteriostatic at 24 hours, whereas the presence of mucin abolished this bacteriostatic effect.

Organism- Strept.pyogenes 1246.
Strength of standard suspension ('S')- Tube 6.
Serial dilutions- Tenfold. Diluent- Gel.Locke. Anticoagulant- Heparin.
Mucin- 1% in Locke. Plasma- Rabbit Shaking- Not shaken continuously.

BACTERIAL DILUTIONS.

Series	Time	S/1	S/10^1	S/10^2	S/10^3	S/10^4	S/10^5	S/10^6	S/10^7	S/10^8	S/10^9	Result.
Strept.pyogenes 0.5 c.c. + Plasma 0.15 c.c. + Gel.Locke 0.15 c.c.	B	4	2	1	f.c.	7c	-	-	-	-	-	
	4 hrs.	4	2	1	17c	15c	-	-	-	-	-	0
	24 "	4	3	f.c.	6c	1c	-	-	-	-	-	+1-
Strept.pyogenes 0.5 c.c. + Mucin 0.15 c.c. + Plasma 0.15 c.c.	B	4	2	1	f.c.	10c	-	-	-	-	-	
	4 hrs.	4	2	1	f.c.	10c	-	-	-	-	-	0
	24 "	4	4	4	4	4	3c	2c	-	-	-	-1- -2
Strept.pyogenes 0.5 c.c. + Mucin 0.15 c.c. + Gel.Locke 0.15 c.c.	B	4	2	1	f.c.	11c		-	-	-	-	
	4 hrs.	4	2	1	27c	2c	2c	-	-	-	-	0
	24 "	4	4	4	4	4	4	4	4	4	f.c.	-6

Conclusions:- Bactericidal action of plasma at 24 hours is reduced by mucin.

Mucin- Fraction G.16 (Locke) 0.5%.
Organism- Salm. typhosa (Cole) 'rough.
Strength of standard suspension ('S')- Tube 3.
Serial dilutions- Tenfold.
Diluent-Gel. Locke.
Plasma- Rabbit.
Anticoagulant- Citrate. Tubes- Not shaken continuously.

BACTERIAL DILUTIONS.

Series	Time	S/1	S/10^1	S/10^2	S/10^3	S/10^4	S/10^5	S/10^6	S/10^7	S/10^8	S/10^9	Result.
Salm. typhosa 0. 5 c.c. + Plasma 0.3 c.c. + Gel. Locke 0.7cc.c.	B	4	3	2	1	f.c.	23c	-	-	-	-	
	4 hrs.	3	f.c.	-	-	-	-	-	-	-	-	+3
	24 "	3	1	1	-	-	-	-	-	-	-	+2
Salm. typhosa 0.5 c.c. + Plasma 0.3 c.c. + Mucin 0.5 c.c. + Gel. Locke 0.2 c.c.	B	4	3	2	1	f.c.	5c	-	-	-	-	
	4 hrs.	4	1	f.c.	12c	1c	-	-	-	-	-	+2
	24 "	4	3	2	2	-	-	-	-	-	-	+1
Salm. typhosa 0.5 c.c. + Mucin 0. 5 c.c. + Gel. Locke 0.5 c.c.	B	4	3	2	1	f.c.	22c	1c	-	-	-	
	4 hrs.	4	3	3	1	f.c.	f.c.	13c	2c	-	-	-1
	24 "	4	4	4	4	4	4	3	2	2	1	-5

Conclusions:- Bactericidal action of rabbit plasma reduced by mucin.

Mucin- G.16 (Locke) 0.5%.
Organism- Salm. typhosa (Cole) 'rough' form.
Strength of standard suspension ('S')- Tube 3.
Serial dilutions- Tenfold Diluent- Gel.Locke.
Whole blood- Rabbit Anticoagulant- Heparin. Shaking- Not shaken continuously.

BACTERIAL DILUTIONS.

Series	Time	S/1	S/10^1	S/10^2	S/10^3	S/10^4	S/10^5	S/10^6	S/10^7	S/10^8	S/10^9	Result.
Salm. typhosa 0.5 c.c.	B	3	3	2	1	f.c.	23c	2c	-	-	-	
+												
Whole blood 0.5 c.c.	4 hrs.	2	32c	-	-	-	-	-	-	-	-	+4
+												
Gel.Locke 0.5 c.c.	24 "	4	3	1	f.c.	-	-	-	-	-	-	+1
Salm. typhosa 0.5 c.c.	B	4	3	2	1	f.c.	26c	1c	1c	-	-	
+												
Whole blood 0.5 c.c.	4 hrs.	3	1	f.c.	5c	4c	-	-	-	-	-	+2
+												
Mucin 0.5 c.c.	24 "	4	3	2	1	1						0-+1

Conclusions:- Whole blood bactericidal at 4 hours and mucin reduced this effect at 4 hours.

Mucin- 0.5 c.c. of 0.5% G.16 (Locke).
Organism- Staph.aureus L.S.C. coag +ve.
Strength of standard suspension ('S')- Tube 2.
Serial dilutions- Tenfold. Diluent- Gel.Locke.
Plasma- Rabbit 0.3 c.c. Anticoagulant- Citrate. Shaking- Partial.

BACTERIAL DILUTIONS.

Series	Time	S/1	S/10^1	S/10^2	S/10^3	S/10^4	S/10^5	S/10^6	S/10^7	S/10^8	S/10^9	Result.
Staph.aureus 0.5 c.c. +	B	4	4	2	1	f.c.	8c	-	-			
Plasma 0.3 c.c. +	4 hrs.	4	2	1	18c	-	-	-				+1
Gel.Locke 0.7 c.c.	24 "	4	4	3	3	3	2	1	f.c.	f.c.	f.c.	-5
Staph.aureus 0.5 c.c. +	B	4	3	2	1	f.c.	6c	-	-			
Mucin 0.5 c.c. +	4 hrs.	4	3	1	1	f.c.	18c					0
Plasma 0.3 c.c. +	24 "	4	4	3	3	3	2	2	2	2	1	-6
Gel.Locke 0.2 c.c.												
Staph.aureus 0.5 c.c. +	B	4	3	2	1	f.c.	4c					
Mucin 0.5 c.c. +	4 hrs.	4	3	2	1	f.c.	2c					0
Gel.Locke 0.5 c.c.	24 "	4	4	4	4	3	2	1	1	1	1	-6

Conclusions:- Slight bactericidal action of serum abolished by mucin.

Mucin- 0.5 c.c. of 0.5% G341 (pH 7.2)
Organism- Salm. typhosa ('rough' form).
Strength of standard suspension ('S')- Tube 2.

Serial dilutions- Tenfold.
Plasma- Rabbit.
Diluent- Gel.Locke. Shaking- Continuous.

BACTERIAL DILUTIONS.

Series	Time	S/1	S/10^1	S/10^2	S/10^3	S/10^4	S/10^5	S/10^6	S/10^7	S/10^8	S/10^9	Result.
Salm. typhosa 0.5 c.c.	B	4	3	2	1	f.c.	16c	7c	5c	-	-	
+												
Plasma 0.3 c.c.	4 hrs.	3	f.c.	-	-	-						+4
+												
Gel.Locke 0.7 c.c.	24 "	4	3	3	2	1	1	f.c.	-	-	-	-2
Salm. typhosa 0.5 c.c.	B	4	3	2	1	f.c.	f.c.	5c	1c	-	-	
+												
Plasma 0.3 c.c.	4 hrs.	4	f.c.	f.c.	3c	-	-	-	-	-	-	+3
+												
G341 0.5 c.c.	24 "	4	4	3	3	3	2	2	1	f.c.	f.c.	-4
+												
Gel.Locke 0.2 c.c.												
Salm. typhosa 0.5 c.c.	B	4	3	2	1	f.c.	f.c.	12c	4c	-	-	
+												
G 341 0.5 c.c.	4 hrs.	4	3	2	1	f.c.	f.c.	5c	1c	-	-	0
+												
Gel.Locke 0.5 c.c.	24 "	4	4	4	4	4	3	3	3	2	1	-4--6

Conclusions:- Plasma is bactericidal at 4 hours. This effect is reduced by mucin.

Mucin- 0.5 c.c. of 0.5% G.341 (pH 7.2).
Organism- Salm. typhosa ('R') form).
Strength of standard suspension ('S')- Tube 2.
Serial dilutions- Tenfold. Whole blood- Rabbit 0.5 c.c. Diluent- Gel.Locke.
Anticoagulant- Heparin Shaking- Partial.

BACTERIAL DILUTIONS.

Series	Time	S/1	$S/10^1$	$S/10^2$	$S/10^3$	$S/10^4$	$S/10^5$	$S/10^6$	$S/10^7$	$S/10^8$	$S/10^9$	Result.
Salm. typhosa 0.5 c.c.	B	4	3	2	1	1	f.c.	f.c.	10c	8c	5c	
+												
Whole blood 0.5 c.c.	4 hrs.	3	2	1	5c	-	-	-	-	-	-	+4
+												
Gel.Locke 0.5 c.c.	24 "	4	3	3	2	1	f.c.	f.c.	f.c.	f.c.	f.c.	-3
Salm. typhosa 0.5 c.c.	B	4	3	2	1	f.c.	f.c.	10c	3c	-	-	
+												
G341 0.5 c.c.	4 hrs.	4	2	1	-	-	-	-	-	-	-	+1-+3
+												
Whole blood 0.5 c.c.	24 "	4	3	3	2	1	1	1	1	f.c.	f.c.	-4
Salm. typhosa 0.5 c.c.	B	4	3	2	1	f.c.	f.c.	12c	4c	-	-	
+												
G341 0.5 c.c.	4 hrs.	4	3	2	1	f.c.	f.c.	5c	1c	-	-	0
+												
Gel.Locke 0.5 c.c.	24 "	4	4	4	4	4	3	3	3	2	1	-4--6

Conclusions:- Whole blood is bactericidal at 4 hours. This effect is reduced by mucin.

The series of tests recorded demonstrates the natural bactericidal action of whole blood and plasma (or serum). This bactericidal action is exhibited both towards Gram-negative and Gram-positive bacteria, but is much stronger towards the Gram-negative species. The mechanism in serum responsible for the bactericidal action towards Gram-negative bacteria has been called the 'α lysin' which has been shown to consist of complement acting along with a natural antibody-like substance, while the effect on Gram-positive bacteria is due to a different active principle designated 'β lysin' whose nature and origin is still unknown but which is independent of complement (Browning and Mackie, 1949).

In the tests recorded whole blood, plasma, and serum were all bactericidal towards the Gram-negative bacteria (e.g. Salm. typhosa; while only whole blood displayed this action to any degree against the Gram-positive micro-organisms (e.g. streptococcus). In general, plasma was only weakly bactericidal to the Gram-positive organisms selected for the tests and the bactericidal action of whole blood therefore depended largely on the leucocytes.

It was interesting to note during the carrying out of these tests at different times of the year, that the rabbits' blood displayed a more pronounced bactericidal effect during the months February/May.

The experiments showed that mucin either completely annulled or reduced the bactericidal action of whole blood, plasma, and serum.

Both the commercial mucin and the refined fractions were active in this respect. There was no appreciable difference between the two types of preparation although the refined fractions were used in lower concentrations than the crude product. In some cases the mucin suspensions were not accurately buffered to pH 7.2. This was not found to be necessary as the gelatin-Locke exerted an efficient buffering action.

Keefer and Spink (1938) have suggested that mucin depresses the bacteriolytic power of whole blood and of antigonococcal serum, while Pacheco and Peres (1940) have reported that mucin opposes the bactericidal action of a cholera antiserum in vitro.

Whether in the case of the bactericidal action of blood or serum towards Gram-negative bacteria, mucin interferes with the natural antibody or complement or both is difficult to establish. Complement however, is an extremely labile constituent of serum whereas antibodies are more stable to various adverse influences, and it might therefore be suggested that the effect is more likely to be on the complement than on the antibody. As it was only whole blood which in these tests exhibited a distinct bactericidal action towards Gram-positive organisms,

and as mucin reduced this effect it would appear that mucin exerted its action either by interfering with phagocytosis and intracellular killing of organisms in the phagocytic cells or with serum opsonins. In view of previous observations which demonstrated a directly injurious effect of mucin on leucocytes, the former hypothesis seemed the more likely. On the other hand, the interference with the bactericidal action of plasma shows that mucin may also have some effect on such active principles of serum as the normal opsonin which is either a complement-like substance or a complex of complement and natural antibody (Browning and Mackie, 1949).

It is also recognised that natural aggressins act through interference with serum opsonisation.

An additional observation in these tests was the growth promoting action of mucin, but this has been the subject of further study and will be dealt with in the next section.

PART V.

THE GROWTH PROMOTING ACTION OF MUCIN.

PART V.

THE GROWTH PROMOTING ACTION OF MUCIN.

It was observed in the series of bactericidal tests that mucin by itself exerted a growth promoting action on certain micro-organisms. To obtain more evidence of this effect, a series of experiments was carried out in which the growth of certain micro-organisms on ordinary media was compared with the growth of the same organisms on media containing mucin.

In general, crude commercial mucin was used for these comparisons, but in a few cases, a refined preparation (G 341), was substituted for the crude product. In the initial experiments, the pH of the crude commercial mucin was not accurately adjusted, as the pH of the medium after the addition of the mucin was approximately pH 7.2. The following series of media were tested, namely, Hoyle's blood tellurite medium, Löffler's serum medium, blood agar, and ordinary nutrient agar.

The technique of incorporating mucin and making the plates was as follows:-

1. Hoyle's medium. 30 c.c. of a 3% suspension of crude commercial mucin were added to 100 c.c. of the base (lab. lemco, peptone, sodium chloride, potassium tellurite, agar and water). Saponin, which had been previously added to the blood in appropriate amounts

and incubated at 37°C for ten minutes, was added to the mixture of the base plus mucin, and plates poured.

2. Blood agar. 30 c.c. of a 3% suspension of crude commercial mucin were added to 100 c.c. melted nutrient agar. 5 c.c. blood were then added and plates poured.

3. Nutrient agar. 30 c.c. of a 3% suspension of crude commercial mucin were added to 100 c.c. melted nutrient agar and plates poured.

4. Löffler's serum medium. 30 c.c. of a 3% suspension of crude commercial mucin were added to 100 c.c. Löffler's medium in the fluid state, the mixture was then added to sterilised tubes and the medium inspissated.

Expt. 1. Esch. coli, Salm. typhosa and Salm. Schottmülleri (B. paratyphosus B.), were inoculated on to nutrient agar, and nutrient agar plus mucin. The Meningococcus, Strept. pyogenes, Staph. pyogenes aureus, Staph. pyogenes albus and the Pneumococcus, were inoculated on to blood agar and blood agar plus mucin. B. diphtheriae gravis was inoculated on to Hoyle's medium and Hoyle's medium plus mucin. The inoculation in each case was one standard loopful of a 24 hours broth culture of the organism.

After 24 hours incubation at 37°C it was found that the resultant colonies were much larger and more mucoid on the mucin media, especially in the case of

B.diphtheriae gravis. More detailed comparisons were then carried out with the different types of the diptheria bacillus.

Expt. 2. A 24 hours' culture of B.diphtheriae gravis on Loffler's medium was suspended in the condensation water and one standard loopful inoculated into (1) nutrient broth, and (2) nutrient broth plus mucin 3% (equal parts). A 1 in 1000 dilution was made from each, and one standard loopful used as the inoculum in each case, on Hoyle's medium. The plates were incubated at 37°C for 18 hours and the resultant colonies counted.

	Broth.	Broth plus mucin.
Colonies	30	190

The number of colonies obtained from the standard inoculum was significantly greater when the organisms had been grown in the presence of mucin.

Expt.3. One colony of B.diphtheriae gravis was picked off a plate of Hoyle's medium after 36 hours incubation, and inoculated into 10 c.c. nutrient broth. Another colony was inoculated into a mixture of 5 c.c. broth and 5 c.c. 3% mucin. Broth and organisms were well mixed and incubated at 37°C for 18 hours. A 1 in 1000 dilution was made and one standard loopful used as the inoculum on Hoyle's medium. The plates were incubated at 37°C for 18 hours and the resultant colonies counted.

	Broth.	Broth plus mucin.
Colonies	29	450+

Again a marked difference was observed.

Expt. 4. In this experiment the meningococcus and pneumococcus were tested along with diphtheria bacilli. One loopful of a 24 hours' broth culture of each of the following organisms, meningococcus, pneumococcus, B.diphtheriae (gravis, intermedius and mitis) was inoculated into broth and broth plus mucin, and incubated at 37°C for 18 hours. The resultant growth was undiluted, diluted 1 in 100, or 1 in 1000; one loopful was inoculated on to the appropriate medium and the resultant colonies counted after 18 hours incubation at 37°C.

	Broth.		Broth plus mucin.	
Meningococcus	(undiluted)	no growth.	(undiluted)	900+
	(diluted 1 in 100)	" "	(diluted 1 in 100)	350+
Pneumococcus	" 1 " 1000	230	" 1 " 1000	320
B.diph.gravis	diluted 1 in 1000	192	diluted 1 in 1000	508+
B.diph.inter.	" 1 " 1000	170	" 1 " 1000	220+
B.diph.mitis.	" 1 " 1000	360	" 1 " 1000	575

Taking experiments 2, 3 and 4 as a group, there is a significant statistical difference between media with and without mucin, and these results clearly showed it's growth-promoting effect.

Expt. 5. A 48 hours' throat swab culture in broth was mixed with an equal volume of broth cultures of the three types of B.diphtheriae respectively, and one loopful from each was inoculated on to plates containing (1) Hoyle's medium, and (2) the same medium with mucin added. Cultures were examined after 18 hours incubation at 37°C.

Hoyle's tellurite medium is a selective medium used for the diphtheria bacillus, and on this medium the diphtheria bacillus grows as a round smooth dark or greyish colony attaining a size of 2 - 3 m.m. in diameter after 48 hours. The growth of such organisms as staphylococci, streptococci, pneumococci etc., is inhibited.

	Hoyle's medium.	Hoyle's medium plus mucin.
B.diphtheriae gravis and throat swab culture.	Very poor growth in 18 hours. Characteristic B.diph.gravis colonies not present. (colonies ½ m.m. in diameter).	Colonies of B.diph.gravis very well formed in 18 hours. Practically pure culture of B.diph.gravis with characteristic morphology. (2 - 3 m.m. in diameter).
B.diphtheriae intermedius and throat swab culture.	Colonies very small at 48 hours (probably not all diphtheria bacilli).	Colonies small but larger than control (probably not all diphtheria bacilli).
B.diphtheriae mitis and throat swab culture.	B.diph.mitis colonies small (less than ½ m.m.).	Mitis colonies large and shiny (1 - 2 m.m. in diameter).

The growth-promoting action of mucin is shown by the more luxuriant growth on the media containing mucin, and the cultural morphology of the organism is quite typical after a relatively short period of incubation.

Expt. 6. One loopful of a mixed throat swab culture (containing diphtheria bacilli) on Löffler's medium was inoculated on to Hoyle's medium and the same medium plus mucin. The plates were examined after 21 hours incubation at 37°C.

	Hoyle's medium.	Hoyle's medium plus mucin.
4 hours	No growth.	2 colonies.
21 "	Very poor growth, (type not identified).	Profuse growth, (type identified as mitis.)

The growth-promoting effect of mucin on the diphtheria bacillus is again demonstrated.

Expt. 7. A sterile throat swab was soaked in a mixture of broth cultures of B.diphtheriae gravis, Strept.viridans, Staph.pyogenes albus and B.xerosis in equal proportions and was left on the laboratory bench overnight, and then inoculated on Hoyle and Hoyle mucin plates. The plates were examined after 24 and 48 hours incubation at 37°C.

	Hoyle's medium.	Hoyle's medium plus mucin.
24 hours	Very faint suggestion of growth of B.diphtheriae gravis.	Growth fairly well developed and a few colonies easily identified as B.diphtheriae gravis.
48 "	Growth of B.diphtheriae gravis - fair.	Colonies of B.diphtheriae gravis twice as large as on medium without mucin.

The growth of B.diphtheriae gravis appears earlier, and after 48 hours is more luxuriant on the medium containing mucin, than on the medium without mucin.

Expt.8. Repetition of experiment 7 with slopes of Löffler's medium plus mucin instead of Hoyle's medium plus mucin. After 18 hours the growth on the mucin Löffler medium was much more abundant than on ordinary Löffler. Strept.viridans and staphylococci were inhibited on the mucin Löffler medium. At 18 hours the growth of B.diphtheriae gravis was much more abundant on mucin Löffler medium than on the ordinary medium.

TO OBSERVE THE EFFECT OF VARYING THE CONCENTRATIONS OF MUCIN IN THE MEDIUM.

Expt.9. Equal volumes of a 24 hours broth culture of B.diphtheriae gravis (dilution 1 in 100 in broth), were inoculated on to blood agar, and blood agar mucin media. The mucin was adjusted to pH 7.2 before incorporation in the medium. To 100 c.c. melted agar, the following amounts of 3% mucin were added:- 30 c.c., 20 c.c., 15 c.c., 10 c.c., 5 c.c. The blood was then added and plates poured. The plates were examined after 24 hours incubation at 37°C.

	Blood agar.	Blood agar mucin. Mucin volumes per 100 c.c. agar.				
		5 c.c.	10 c.c.	15 c.c.	20 c.c.	30 c.c.
Number of colonies	916	1457	1375	1137	1455	1694

The colonies on all mucin media were twice as large as on ordinary blood agar, and the growth-promoting action of mucin when incorporated in blood agar medium was demonstrated. Moreover, the number of colonies obtained from the standard inoculum was greater on all media containing mucin.

The largest number of colonies was on the medium which had a concentration of 30 c.c. mucin per 100 c.c. agar. There was no significant difference in the resultant number of colonies in concentrations of mucin from 5 - 20 c.c. per 100 c.c. agar.

Expt.10. The above experiment was repeated with plates of Hoyle's medium and Hoyle's medium plus mucin. In addition Hoyle's medium containing a buffer corresponding in volume to the volume of mucin in the medium containing mucin was also used. Counts were not carried out, but it was observed that the colonies on the medium containing mucin were $1\frac{1}{2}$ to 2 times larger than the colonies on the ordinary Hoyle's medium, and on the medium containing buffer.

Expt.11. In order to have the same concentration of tellurite etc. in the Hoyle's medium as in Hoyle's medium containing mucin, a number of plates containing Hoyle's medium plus water were made (the water corresponding to the volume of mucin). The plates of Hoyle's medium plus water were not so satisfactory as the ordinary Hoyle's medium. One loopful of a 1 in 1000 dilution of a 24 hours broth culture was used as the inoculum. The plates were examined after 24 hours incubation at 37°C.

	Hoyle's medium.	Hoyle's medium plus water.	Hoyle's medium plus mucin.
B.diph.gravis	++	+	++++
B.diph.inter.	±	±	++
B.diph.mitis	+	±	++++

Number of + marks indicates the relative amount of growth.

Films of the gravis colonies from the various media stained by Albert's method showed:-

Hoyle's medium plus mucin - involution forms common, metachromatic granules common. Most organisms smaller than normal.

Hoyle's medium plus water - involution forms common, few exhibiting granules.

Hoyle's medium - Size and shape fairly normal. Granules poor.

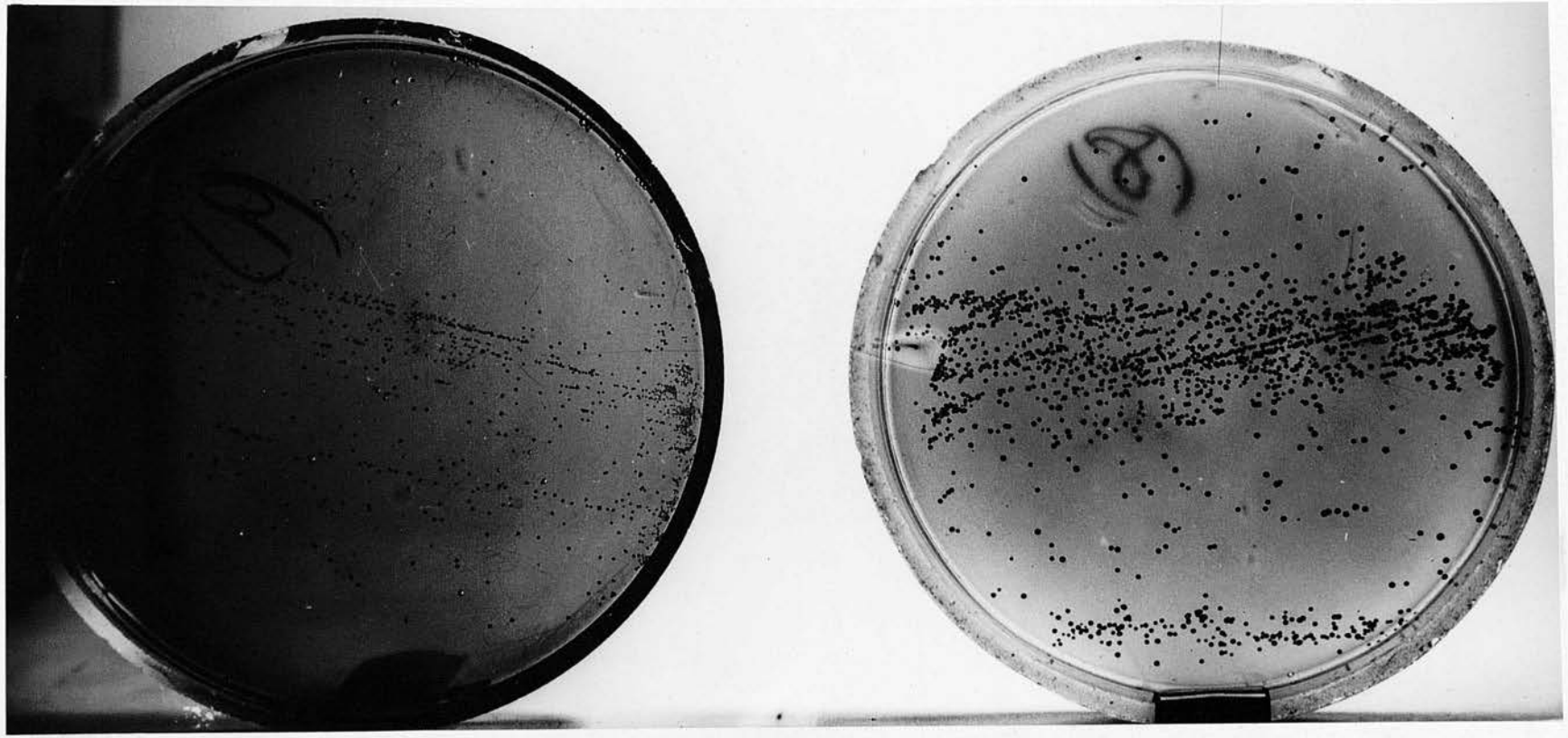
These experiments illustrate the practical advantage of incorporating mucin in media for culturing the diphtheria bacillus.

The growth-promoting effect of mucin is seen both when (1) the organisms are grown in mixtures of nutrient broth and mucin prior to plating on ordinary solid media, and (2) when the organisms are grown on media containing mucin subsequent to cultivation in nutrient broth alone. The mucin, therefore, appears to provide a stimulus for growth either when it is added to nutrient broth or to a solid medium.

In all cases there was a greater growth on mucin media than on ordinary media in routine use. All types of the diphtheria bacillus grew more luxuriantly on media which contained mucin, although the greatest effect of the mucin was seen with the "gravis" type, while the type least affected was "intermedius". It is interesting to note that in concentrations of mucin below 30% (30 c.c. per 100 c.c. medium) the growth promoting effect falls off considerably.

The possibility of the mucin containing one or more growth factors for the diphtheria bacillus, such as pimelic acid, nicotinic acid, and B.alanine, must be considered. If mucin was acting in this way, one would expect the growth-promoting action to be produced by minute quantities. Although this was not

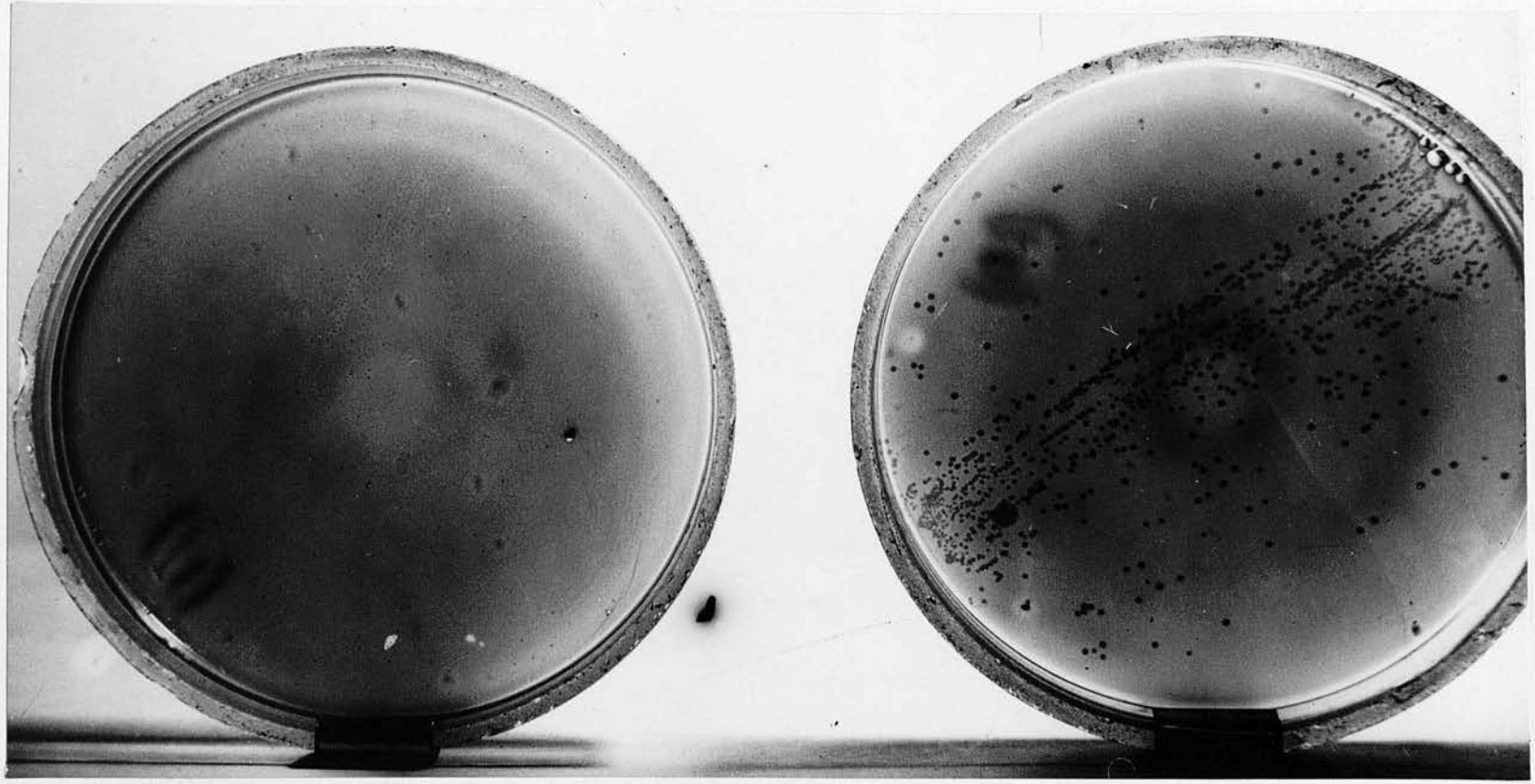
actually found with the crude commercial product, it will be observed in experiments to be recorded, that minute quantities of a refined fraction, G 341, produced a growth-promoting effect towards the gonococcus.



(a) (b)

C.diphtheriae mitis on (a) Hoyle's blood tellurite medium, and (b) on Hoyle's blood tellurite medium plus mucin, after 18 hours incubation at 37°C.
(a) The colonies of the organism are small and black.
(b) The colonies are large and black.

(a)

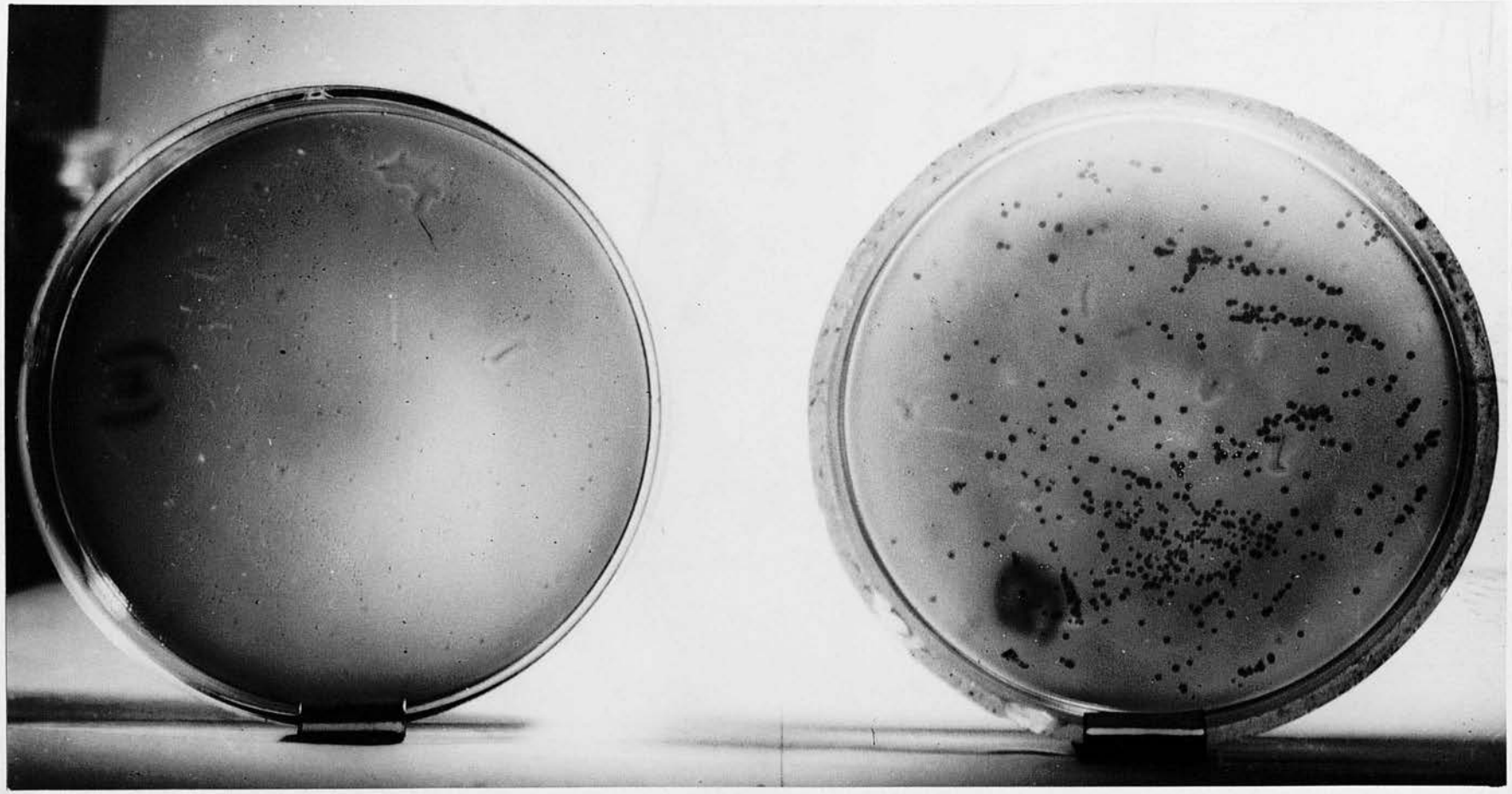


(b)

C.diphtheriae mitis on (a) blood agar and (b) mucin blood agar, after 18 hours incubation at 37°C.
(a) The growth is scanty and the colonies are minute and greyish in colour.
(b) There is a more luxuriant growth than on (a), and the colonies are large and grey in colour.

(a) (b)

C. diphtheriae gravis on (a) Hoyle's blood tellurite medium, and (b) Hoyle's blood tellurite medium plus mucin, after 18 hours incubation at 37°C.
(a) The colonies of the organism are small and black.
(b) The colonies are large and black.



(a) (b)

C.diphtheriae gravis on (a) blood agar and (b) blood agar plus mucin, after 18 hours incubation at 37°C.

(a) The growth is scanty and the colonies of the organism minute and greyish in colour.

(b) The growth is more luxuriant than on (a) and the colonies are large and greyish.

(a) (b)

C. diphtheriae intermedius on (a) Hoyle's blood tellurite medium and (b) Hoyle's blood tellurite medium plus mucin, after 18 hours incubation at 37°C.
(a) The colonies of the organism are minute and dark grey in colour. NOTE. The large raised circular clear spots are due to air bubbles in the medium.
(b) The colonies are small and dark grey in colour, much larger than in (a).

THE GROWTH OF VARIOUS ORGANISMS ON A MUCIN BLOOD AGAR MEDIUM COMPARED WITH THE GROWTH OF THE SAME ORGANISMS ON ORDINARY BLOOD AGAR.

Expt.12. One standard loopful of a 24 hours broth culture of each of the following organisms was inoculated on to blood agar and mucin blood agar. The plates were examined after 24 hours incubation at 37°C.

Organisms.	Blood agar.	Mucin blood agar.
Staph. aureus	+++ (haemolysis)	++++ (no haemolysis)
Staph. albus	+++ "	++++ "
Strept. pyogenes	+++ "	++++ "
B. xerosis	+++	++++
Esch. coli	+++ (haemolysis)	+++ (no haemolysis)

Mucin showed a growth-promoting effect on Staph. pyogenes aureus, Staph. pyogenes albus, Strept. pyogenes and B. xerosis. It will be observed that the haemolysis was not seen on the mucin blood agar plates. This may have been due to the opacity caused by the mucin.

THE EFFECT OF MUCIN ON THE GROWTH OF SALM. TYPHOSA.

Expt. 13. Equal volumes of mucin 3% (crude) and Salm. typhosa in broth culture were incubated overnight at 37°C. One standard loopful was inoculated on to blood agar. Growth was observed at hourly intervals. The control consisted of organisms in normal saline.

	Mucin and organisms.	Control (organisms in saline).
4 hours	Definite early growth.	No growth.
8 hours	Profuse growth.	Slight growth commencing.

Mucin exerted a definite growth-promoting effect on Salm. typhosa.

Experiments 12 and 13 demonstrate the growth-promoting effect of mucin on staphylococci, streptococci, B. xerosis and Salm. typhosa.

Expt.14. As in most of the animal experiments the meningococcus was the test organism, some observations were made to ascertain whether mucin enhanced the in vitro growth of this organism.

Meningococcus (strength of suspension equivalent to tube 2 Brown's opacity standards) diluted 1 in 500 in nutrient broth. Constant volumes were inoculated on to plates of mucin blood agar, and blood agar containing buffer. The mucin suspension was adjusted to pH 7.2 before use.

Mucin blood agar.

	1 c.c.	2 c.c.	4 c.c.	8 c.c.	16 c.c.	32 c.c.
24 hours	+++	+++	+++	+++	+++	+++

Blood agar plus buffer.

Volumes of buffer/100 c.c.agar.

	1 c.c.	2 c.c.	4 c.c.	8 c.c.	16 c.c.	32 c.c.
24 hours	+++	+++	+++	+++	+++	+++

After 24 hours incubation there was no difference between the growth of the meningococcus on the blood agar medium containing mucin and on the blood agar medium plus buffer. At 48 hours, however, the colonies on the mucin blood agar were $1\frac{1}{2}$ times as large as on blood agar medium containing buffer.

Expt. 15. Repetition of experiment 14. The inoculum in this case was one standard drop of a 1 in 1000 dilution of a 24 hours broth culture.

	Mucin blood agar.	Blood agar.
Number of colonies on medium after 24 hours incubation at 37°C.	932	554

In this case where a higher dilution of the inoculum was used (1 in 1000, compared with 1 in 500 in experiment 14), there was observed a growth-promoting effect of mucin towards the meningococcus.

TO OBSERVE THE EFFECT OF EXTREME HEAT ON MUCIN IN RELATION TO IT'S GROWTH-PROMOTING ACTION ON THE MENINGOCOCCUS.

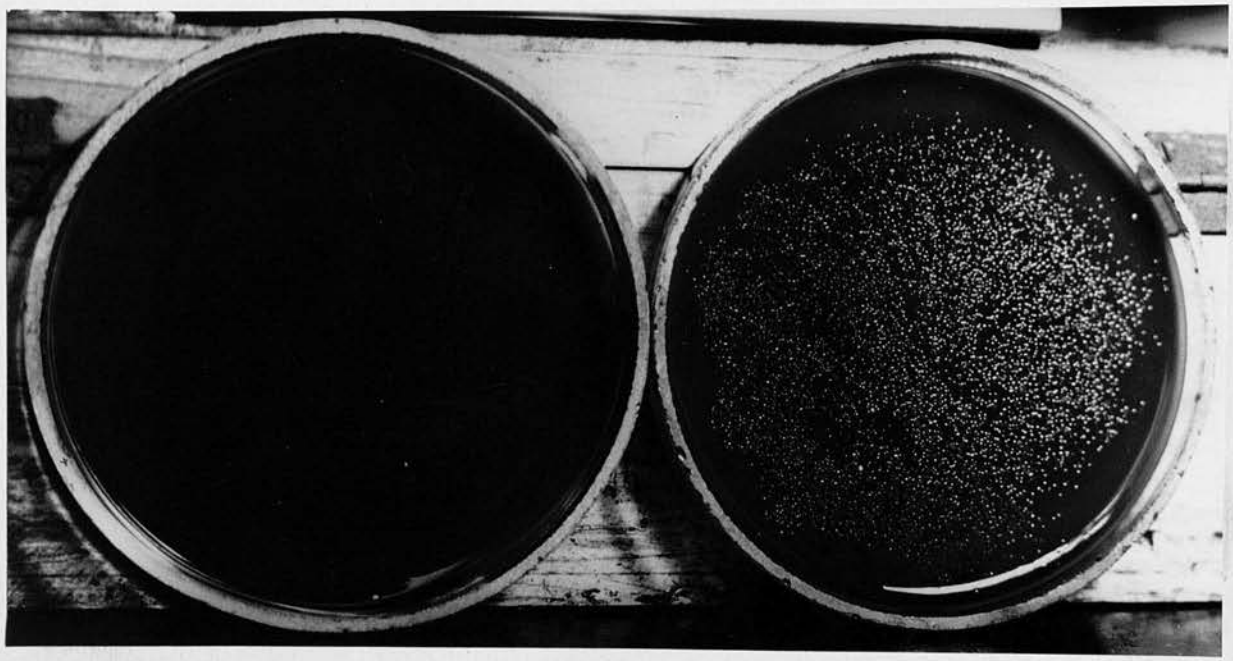
Expt.16.

Meningococcus. One standard loopful of a 24 hours broth culture was used as the inoculum.

	BLOOD AGAR.	MUCIN BLOOD AGAR. (mucin sterilised once).	MUCIN BLOOD AGAR. (mucin sterilised twice, i.e. autoclaved and subsequently heated at 100°C for 20 minutes.
24 hours	+++	+++	+++
		Colonies twice as large as on blood agar.	Colonies as on blood agar.

The above demonstrates that the addition of mucin to the blood agar medium produces much larger colonies of the meningococcus, and that if the mucin is previously subjected to excessive heat, this effect is abolished.

(a)



(b)

Meningococcus on (a) blood agar medium and (b) blood agar medium plus mucin (G341), after 20 hours incubation at 37°C.
(a) The growth is scanty, and the colonies very small and greyish.
(b) A luxuriant growth, the colonies are large and grey.

OBSERVATIONS ON THE GROWTH REQUIREMENTS OF THE GONOCOCCUS.

Expt.18. 24 hours serum broth culture in an atmosphere containing approximately 8% carbon-dioxide of the gonococcus. Equal volumes were inoculated on to the indicated media. One series was incubated in the presence of carbon-dioxide.

INCUBATED WITH CARBON-DIOXIDE.

	Nutrient agar.	Nutrient agar + buffer.	Nutrient agar + mucin.
After 24 hours incubation at 37°C.	No growth.	No growth.	No growth.

INCUBATED WITHOUT CARBON-DIOXIDE.

	Nutrient agar.	Nutrient agar + buffer.	Nutrient agar + mucin.
After 24 hours incubation at 37°C.	No growth.	No growth.	No growth.

Thus the addition of mucin to ordinary nutrient agar, did not promote the growth of the gonococcus.

COMPARISON BETWEEN ORDINARY BLOOD AGAR AND MUCIN BLOOD AGAR MEDIA FOR THE GROWTH OF THE GONOCOCCUS, AND THE EFFECT OF INCUBATION WITH AND WITHOUT CARBON-DIOXIDE.

Expt.19 A 24 hours culture on blood agar in carbon-dioxide of the gonococcus was washed off with sterile normal saline into serum broth. The strength of the suspension was made corresponding to tube 2 (Brown's opacity standards). 1 in 10, and 1 in 100 dilutions of this suspension (in serum broth) were inoculated on to ordinary blood agar and mucin blood agar media, incubated at 37°C and examined after 24 hours. One series was incubated in carbon-dioxide, the other without carbon-dioxide. The pH of the mucin was adjusted to pH 7.2 before adding to the media.

	Incubated in CO_2	Incubated without CO_2
Blood agar (1 in 10)	+ - + +	-
Blood agar (1 in 100)	+	-
Mucin blood agar (1 in 10)	+ +	+ - + +
Mucin blood agar (1 in 100)	+ +	+

The experiment demonstrated the growth-promoting effect of mucin towards the gonococcus when incorporated in a blood agar medium. It is also shown, that, whereas the gonococcus does not grow before 24 hours on an ordinary blood agar medium unless incubated in the presence of carbon-dioxide, this organism will grow on a blood agar medium containing mucin, irrespective of the presence of carbon-dioxide.

Expt. 20.

1. Gonococcus incubated at 37°C for 24 hours in a mixture of equal parts of nutrient broth and mucin 3% (pH 7.2) in the presence of carbon-dioxide. Subinoculation (1 in 10) was made on blood agar and mucin blood agar and incubated in carbon-dioxide.

2. Gonococcus incubated at 37°C for 24 hours in a mixture of equal parts of nutrient broth and mucin 3% (pH 7.2) in the presence of carbon-dioxide. Subinoculation (1 in 10) was made on blood agar and mucin blood agar and incubated without carbon-dioxide.

3. Gonococcus incubated at 37°C for 24 hours in a mixture of equal parts of nutrient broth and mucin 3% (pH 7.2) without carbon-dioxide. Subinoculation (1 in 10) was made on blood agar and mucin blood agar and incubated for 24 hours with carbon-dioxide.

4. Gonococcus incubated at 37°C for 24 hours in a mixture of equal parts of nutrient broth and mucin 3% (pH 7.2) without carbon-dioxide and subinoculated (1 in 10) on blood agar and mucin blood agar and incubated without carbon-dioxide.

	Mucin blood agar	Blood agar buffer
1. Primary inoculation in CO_2. Subinoculation in CO_2	+++	++ to +++
2. Primary inoculation in CO_2. Subinoculation without CO_2.	+++	± to +
3. Primary inoculation without CO_2. Subinoculation with CO_2.	+++	+++
4. Primary inoculation without CO_2. Subinoculation without CO_2.	+++	+

The above results show how carbon-dioxide aids the growth of the gonococcus on blood agar plates. The mucin also enhanced the growth equally with carbon-dioxide, and the latter did not further augment growth on mucin blood agar plates.

Expt. 21. A strain of gonococcus was inoculated into serum broth and incubated for 48 hours in carbon-dioxide at 37°C. Equal volumes were subinoculated on to the following media, and plates examined after 18 hours at 37°C.

Incubated with carbon-dioxide.			Incubated without carbon-dioxide.		
Ordinary blood agar.	Blood agar + buffer	Mucin blood agar.	Ordinary blood agar.	Blood agar + buffer.	Mucin blood agar.
+	+	++	-	±	+++

\- = No growth.
± = Very scanty growth.
\+ = Scanty growth.
++ = Fairly good growth.
+++ = Good growth.

SUMMARY AND CONCLUSIONS.

1. In the series incubated in the presence of carbon-dioxide, the best growth resulted on the mucin blood agar.

2. Of the plates incubated without carbon-dioxide, the mucin blood agar produced the best growth.

3. The only plate showing satisfactory growth (when incubated without carbon-dioxide) was the mucin blood agar. While carbon-dioxide is essential for the growth of the gonococcus on blood agar, this is not so when mucin is incorporated.

TO ESTIMATE THE TIME OF APPEARANCE OF GROWTH OF THE GONOCOCCUS ON ORDINARY BLOOD AGAR AND BLOOD AGAR MEDIUM CONTAINING MUCIN.

Expt.22. A 1 in 100 dilution of a 24 hours culture of the gonococcus was made in serum broth. Standard volumes were inoculated on to the respective media and incubated (1) with carbon-dioxide, and (2) without carbon-dioxide. Plates were examined at varying times up to 24 hours.

Incubated without carbon-dioxide.

	Blood agar.	Mucin blood agar (3% crude).	Mucin blood agar (G341).
3 hours	-	-	-
6 "	-	-	-
10 "	-	±	±-+
24 "	±	+++	+++

Incubated with carbon-dioxide.

3 hours	-	-
6 "	-	-
10 "	±	±
24 "	+-++	++++

\- = No growth.
± = Faint suggestion of growth.
\+ = Slight growth.
++++ = Very heavy growth.

The above observations suggest that mucin blood agar to be a more efficient medium for growing the gonococcus and again that when using a mucin medium, the presence of carbon-dioxide is not necessary.

TO OBSERVE THE EFFECT OF DECREASING THE CONCENTRATION OF MUCIN IN MUCIN BLOOD AGAR MEDIA ON THE RESULTANT GROWTH OF THE GONOCOCCUS.

Expt. 23. A 1 in 100 dilution of a 24 hours culture of the gonococcus was used. Plates were examined after 24 hours incubation at 37°C.

Mucin per 100 c.c. agar.	Mucin blood agar (crude).	Mucin blood agar (G341).
30 c.c.	+++	++++
15 c.c.	+	+++
7.5 c.c.	+	+++
3.75 c.c.	+	+-++

Expt. 24. Pus from a case of gonorrhoea was inoculated directly from the swab on to blood agar medium and blood agar containing mucin.

	Blood agar.	Mucin blood agar.
Plate 1.	No growth.	Profuse growth of gonococcus.
" 2.	" "	" " " "
" 3.	" "	" " " "
		After 18 hours incubation.

The above result may be of some interest in view of the fact that the gonococcus thrives well in those areas of the body where there is an abundance of mucin.

The experimental studies with the gonococcus illustrate the growth enhancement effect of mucin towards the gonococcus. The gonococcus is one of the most difficult organisms to cultivate, blood or serum incorporated in the medium is a first essential, and to obtain a reasonably quick growth an atmosphere

containing carbon-dioxide is also necessary. It was observed when using a blood agar medium containing mucin, carbon-dioxide could be dispensed with, as the growth of the organism on a mucin blood agar medium was as good as the growth on a normal blood agar medium incubated with carbon-dioxide. On a mucin blood agar medium growth appeared in ten hours, whereas on an ordinary blood agar medium, even in the presence of carbon-dioxide, satisfactory growth did not appear for 24 hours. In fact, the mucin medium incubated without carbon-dioxide produced a growth almost equal to that on the blood agar (incubated in carbon-dioxide) at 24 hours. The presence of carbon-dioxide did not appear to make any appreciable difference to the growth on the mucin media. Whereas with crude mucin the growth promoting effect was not marked below a concentration of 30 c.c. (3%) per 100 c.c. agar, the refined fraction was effective at a concentration of 3.75 c.c. (0.5%) per 100 c.c. agar. This observation may suggest that the refined fraction to be acting as a growth factor, although if this were the case, the mucin should be effective in concentrations below this.

(a)

(b)

Gonococcus on (a) blood agar medium and (b) blood agar medium plus mucin after 48 hours incubation at 37°C.
(a) There are few colonies which are small and light grey in colour.
(b) The colonies are much more numerous and much larger than in (a).

PART VI.

FRACTIONATION OF GASTRIC MUCIN CARRIED OUT IN COLLABORATION WITH DR.H.K.KING.

PART VI

FRACTIONATION OF GASTRIC MUCIN CARRIED OUT IN COLLABORATION WITH DR.H.K.KING.

Several different methods were employed in an attempt to concentrate the active material present in the commercial gastric mucin. The first method used was that of Gould and King (1947), which consisted of centrifuging a 1% solution of the mucin at R.C.F. 2000xg and discarding the sediment which had been found inactive. The supernatant was then centrifuged for 1 - 2 hours at R.C.F. 20,000xg, the active material being spun down, while the viscous and slightly opalescent fluid showed no activity. The gummy deposit represented about 10% of the original material and had about ten times the specific activity. It was treated with N/10 sodium carbonate, when about half passed into solution and could be reprecipitated on acidification. This reprecipitated material contained the active substance or principle.

The fraction prepared in accordance with Gould's technique - G7, was obtained in 5.5% yield; and was tested for virulence enhancement at 0.25% concentration. It gave anomalous results. It did however, show powerful inhibitory action on the methylene blue reducing properties of leucocytes (see part 3 page 101).

It was observed that if a solution of mucin in water was adjusted from the normal pH of mucin solution (about pH 5) to pH 7.0 to 7.5 and allowed

to stand overnight in the refrigerator, a change in the colloidal condition occurred, which could be seen with the naked eye. As a result, high speed centrifugation (60 minutes at R.C.F. 20,000 xg) now deposited at least the major part of the active material. Two fractions were prepared by this method, G 216 (supernatant) and G 217 (deposit), the supernatant G 216) being inactive at 6%, whereas the deposit (G 217) was approximately ten times as active as the crude starting material, in that mice were killed by 0.5 c.c. of 0.25%, as against the 0.5 c.c. of 2 - 3% required with the crude material.

G 216 (supernatant) at 0.519 g Yield 87%.

No. organisms millions	G 216	Mice	Deaths	Survivals
350	10%	3	2 (+)	1
350	6%	3	1 (+)	2

G 217 (deposit) at 0.053 g. Yield 9%.

No. organisms millions	G 217	Crude Mucin Prep.	Mice	Deaths	Survivals
350	0.5%		3	3 (+)	-
350	0.25%		3	3 (+)	-
350		3%	3	3 (+)	-

(+) = Death from septicaemia.
(-) = No septicaemia.
(±) = Culture from spleen positive.
Culture from heart blood negative.

Fraction G 217 showed powerful inhibitory action on the methylene blue reducing properties of leucocytes (see part 3 page 101), and also showed activity in the leucocyte counting tests (see part 3 page 103)

This method was repeated (G 225) which also showed activity at 0.25%, and in inhibiting the reducing properties of leucocytes and in the leucocyte counting tests (see part 3 pages 101 and 103).

No.organisms millions	G 225	Crude mucin prep.	Mice	Deaths	Survivals
350	0.5%		3	3(+)	-
350	0.25%		6	4(+)	2
350		3%	6	6(+)	-

This preparation is nevertheless, far from being homogenous - 31% is non-volatile ash.

Fractionation with ammonium sulphate was carried out, but was not effective in separating an active fraction.

Several fractions were prepared by the method of fractionation by solution in phenol, (e.g. G 51). In one case a higher specific activity than the original crude mucin was obtained, mice being killed at 0.5%

No.organisms millions	G 51	Crude mucin prep.	Mice	Deaths	Survivals
350	0.5%		2	2(+)	-
350	0.25%		2	1(+)	1
350	0.125%		2	-	2
350		3%	2	2(+)	-

Fractionation from formamide was another method used but the material did not appear to have a higher specific activity than the original material.

One method of fractionation which appeared to give concentration of active principle, was high speed centrifugation, followed by treatment with formamide. This method will now be described in detail.

Preparation of G 286 and G 302.

Aqueous suspension of the mucin was adjusted to pH 7 - 7.5 and allowed to stand at 0°C overnight. It was then centrifuged at high speed (1 - 2 hours at R.C.F.20,000 xg, using a Blitz or Sharples centri - fuge). The viscous supernatant was discarded and the solid particulate matter dried and then treated with formamide. After allowing several hours for the material to disperse in the solvent, the insoluble matter was centrifuged down (15 minutes on an ordinary centrifuge - R.C.F. 2000 xg), and discarded. The formamide solution was dialysed against water to remove the formamide and the remaining suspension freeze-dried.

This product represented 4% of the original mucin and exhibited at least six times its virulence enhancing potency.

It dissolved in water at slightly alkaline pH to give an almost non-viscous solution. It is not identical with the human blood group A factor known to be present in hog gastric mucin (Morgan and King 1943): this remains in the first viscous supernatant. It retained after prolonged dialysis, 11% of mineral

matter, and contains 7.7% N (Kjeldahl). It is probably still not homogenous, and therefore no detailed chemical investigation has been undertaken. The Molisch and Sakaguchi (arginine) reactions were strongly positive, the biuret and Millon reactions positive, the tryptophane reaction weak.

Organisms millions	G 286	Crude mucin prep.	Mice	Deaths	Survivals
10	0.25%		3	3 (±)	-
	G 288	(extraction with hot formamide).			
10	0.5%		3	3 (2+) (1-)	-
	G 302				
10	0.5%		5	1 (+)	4
10	0.25%		5	1 (+)	4
10	0.125%		5	-	5
10		3%	5	5 (+)	-

Preparation of G 327 - High speed centrifugation.

9g of crude mucin was suspended in 300 c.c. distilled water by continuous mechanical stirring. The pH of the viscous opaque suspension was adjusted to pH 7.2 by addition of 4.5 c.c. of N sodium carbonate. The material was then left at 0°C for 48 hours and then centrifuged in the closed bowl of a Sharples supercentrifuge for 4 hours at 25,000 R.P.M. corresponding to R.C.F. 20,000 xg. The almost clear supernatant was siphoned out of the bowl and a portion freeze dried (fraction G 328, corresponding to 81% of the original material). The

deposit remaining in the bowl was removed and suspended in a small volume of water for freeze-drying. The product, fraction G 327, weighed 1.4 g. and thus represented 15% of the original crude material. It was a friable dry powder, insoluble in water, but readily forming a suspension.

Organisms millions	G 327	Crude mucin prep.	Mice	Deaths	Survivals
10	0.5%		5	5(+)	-
10	0.5%		5	4(+)	1
10	0.25%		5	1(+)	4
10		3%	5	5(+)	-

The following fractions were prepared by the high speed centrifugation technique, G 261, G 300, G 321. These fractions were centrifuged at 1%

Organisms millions	G 261	Crude mucin prep.	Mice	Deaths	Survivals
350	0.5%		3	2(+)	1
350	0.5%		3	3(+)	-
350	0.5%		3	3(+)	-
350	0.25%		3	-	3
350		3%	3	3(+)	-
	G 300				
350	0.5%		3	1(+)	2
350	0.5%		3	3(+)	-
350	0.25%		3	2(+)	1
10	1%		5	4(+)	1
10	0.5%		5	1(+)	4
10		3%	5	5(+)	-

No.organisms millions	G 321	Crude mucin prep.	Mice	Deaths	Survivals
10	1%		5	2(+)	3
10	0.5%		5	2(+)	3
10	1%		5	5(+)	-
10	0.5%		5	5(+)	-
10		3%	5	5(+)	-
	G 326 (formamide fraction from G 321)				
10	0.5%		5	1(+)	4
10	0.25%		5	3(+)	2
	G 302 (formamide fraction from G 300)				
10	0.5%		5	1(+)	4
10	0.25%		5	1(+)	4
10	0.125%		5	-	5
	G 283 (formamide fraction from G 261)				
10	0.5%		3	3(+)	-
10		3%	3	3(+)	-

G 341

This was prepared by high speed centrifugation. Although so far it has not been fully tested re its activity in mice experiments, it has given satisfactory results when incorporated in culture medium.

CONCLUSIONS.

Of the many fractions which have been prepared, the experiments with which have been repeated several times, only one method of fractionation can be claimed to give a true concentration of the active material. This is high speed centrifuging after adjusting to pH 7 and allowing the material to stand at 0° C. The product however, is non-homogenous, containing for example 30% non-volatile ash. Many attempts to fractionate this material further have been made; including partial solution in alkali- both sodium carbonate and disodium hydrogen phosphate solution, solution in 90% aqueous phenol, and solution in anhydrous formamide. There has not been any increased activity demonstrated by these methods. By solution in formamide however, a product was obtained which was no less active and probably less mixed with impurities - the mineral ash content being reduced to below 10%.

In view of the fact that the concentrated and refined material was still quite impure chemically, any further investigation of the chemical nature of the active substance was not practicable. Viscosity determinations seemed out of the question with a preparation containing particles of insoluble matter, since the latter were found to obstruct the viscometer.

The refined material is relatively non-viscous, and does not correspond in its characters to a complete muco-protein. It is however, mainly protein, and the question arises whether the active substance itself is the protein fraction of mucin proper as has been suggested by Anderson and Oag (1939). This still requires further and more detailed investigation.

DISCUSSION AND CONCLUSIONS.

DISCUSSION AND CONCLUSIONS.

The virulence-enhancing action of mucin-containing products has been demonstrated for the meningococcus by Miller (1933), Miller and Castles (1934), Cohen (1936), Anderson and Oag (1939), Keefer and Spink (1938), and McLeod (1941); the gonococcus by Miller (1933); Staph.pyogenes aureus and some strains of Strept.pyogenes, by Anderson and Oag (1939), Salm.typhosa, by Rake (1935), and Anderson and Oag (1939); dysentery bacilli, by Bramham and Carlin (1948); B.anthracis, by Nungester, Jourdonnais and Wolf (1936); H.influenzae by Chandler, Fothergill and Dingle (1937); V.comma, by Griffitts (1942); B.fusiformis, by Tunnicliff and Hammond (1937); the virus of influenza, by Wheeler and Nungester (1942).

From the experimental data provided in this thesis there is adequate evidence that preparations of hog's gastric mucin, both commercial and laboratory preparations, enhance the virulence of the meningococcus, Salm.typhosa and B.mycoides, while towards B.anthracoides and B.subtilis a similar virulence-enhancing action has been observed though less pronounced. The greater part of the work, however, was carried out with the meningococcus, and it was in experiments with this organism that the detailed investigation regarding the action of mucin

was made.

The effect of intraperitoneal injection of non-lethal doses of meningococci along with mucin into mice was most striking. In most cases death occurred within 24 - 48 hours. In all cases the dead mice showed signs of a blood infection, cultures from the spleen and heart blood yielding a direct growth of meningococcus on blood agar.

There appeared, therefore, to be present in the mucin preparation, some form of 'aggressin'-like substance which could break down resistance when introduced into the body along with the meningococcus, the mucin alone, even in a concentration of 15%, and the organisms by themselves in the doses employed, were quite innocuous. It was also shown that the active substance could be concentrated and refined by chemical methods.

To investigate the mode of action of mucin, the first step was to examine and compare the peritoneal exudates from both the test and control mice at regular intervals after injection. These comparisons revealed some very striking results. In the case of the test mice (inoculated with organisms plus mucin) the cells of the peritoneal exudate were found in a degenerate state soon after inoculation, whereas those of the control animals presented a more normal picture. It was the polymorphonuclear cells which appeared to exhibit the greatest change. These cells did not appear in large numbers until 4 - 6 hours

after injection, whereas in the control series they appeared earlier. From the study of the Arneth counts it was observed that the polymorphs of the peritoneal exudate in those mice inoculated with organisms plus mucin were more mature than in the case of the control animals, which presented a more normal leucocytic response to the infection, a high percentage of the polymorphs being young forms. As the infection progressed in the test mice, increasing numbers of polymorphs showed evidence of degeneration as indicated by their poor staining quality, rupture and vacuolation of cytoplasm, distortion and fragmentation of nucleus. Not only did the polymorphs show signs of degeneration, but their powers of phagocytosis were depressed - few organisms were seen within the cells. Again, the exudates from the test animals contained increasing numbers of organisms up to the time of death, whereas in the control animals, there were extremely few organisms present after 24 hours. The picture, therefore, in those mice inoculated with organisms plus mucin, was one of degeneration of polymorphonuclear cells and depression of phagocytosis, the organisms multiplying in the peritoneum in very large numbers. In the case of those animals not receiving mucin, the response to the presence of bacteria in the peritoneal cavity was the normal physiological one. Polymorphs, mostly young forms, were present in large numbers, the organisms were quickly removed from the peritoneum

and those that remained were phagocytosed by the cells of the exudate. The organisms in both test and control animals appeared in the blood from 1 - 2 hours after inoculation; in the control series they disappeared between 6 - 24 hours, whereas in the test series they persisted until death. Miller and Castles (1934, 1936) examined peritoneal exudates from mice inoculated with meningococci plus mucin and glucose; eleven hours after inoculation, the polymorphs were seen to be vacuolated, swollen and poorly staining, while very few organisms were observed within these cells. They did not remark on any change in the nucleus of the cell. The degenerative changes observed by the writer commenced much earlier than noted by Miller and Castles.

It appeared from the initial experimental observations that when mucin was injected along with meningococci into the peritoneum of the mouse, the normal physiological response to the infection was interfered with; the cells of the exudate exhibited various signs of degeneration and their phagocytic activities were depressed, the bacteria multiplying without inhibition. In addition, mucin itself may have promoted growth; this effect will be discussed later. These processes therefore would tend to keep up a continuous flow of bacteria into the blood stream, with resulting septicaemia and death. It appeared then, as suggested by Miller and Castles

(1934) although their evidence did not seem altogether adequate, that mucin injured the phagocytes and allowed the organisms in large numbers to pass the barrier between the peritoneal cavity and the vascular system.

Although previous workers had observed a degree of degeneration in the cells of the peritoneal exudates from mice inoculated with meningococci and mucin, these observations were apparently not confirmed by *in vitro* experiments. The apparent toxic action of the mucin on leucocytes noted *in vivo* was further investigated by setting up the following *in vitro* experiments, viz:- methylene blue reduction tests, leucocyte counting tests, and phagocytic experiments. The incorporation of mucin in the methylene blue reduction test with leucocytes has not been previously investigated. Although the exact rationale of this test has never been fully explained, it was, nevertheless, pointed out by Neisser and Wechsberg (1901) that only leucocytes in the living state have the power of reducing the dye. The results of the writer's tests were so consistent that further evidence was thus obtained of an interference by mucin with the vital properties of these cells. It is also worth noting that the refined fractions of the mucin in all cases were active in the methylene blue reduction test in much lower concentrations than the crude product. The experiments on phagocytosis furnished evidence of

interference by mucin with that important property. In the leucocyte counting experiments, evidence was obtained of a destructive action by the mucin on leucocytes. From these *in vitro* experiments it appears that mucin preparations (1) inhibit the vital properties of leucocytes, (2) interfere with their power of phagocytosis, and (3) may actually destroy them. The latter two observations are confirmatory of the *in vivo* findings.

Nungester, Jourdonnais and Wolf (1936) concluded from *in vitro* experiments that concentrations of mucin which would be effective in raising the virulence of bacteria in the peritoneal cavity, did not decrease phagocytosis. The writer's observations are therefore not in agreement with such findings. These workers, however, demonstrated the survival and multiplication of bacteria in mixtures of whole blood, bacteria and mucin, which led them to postulate, that although mucin did not interfere with phagocytosis *in vitro*, it did prevent destruction of bacteria in the phagocytic system, and might act by inhibiting intracellular digestion in the phagocytes. This work was not repeated by the writer, for the results of the phagocytic tests *in vitro* fully confirmed the *in vivo* observations that mucin *did* inhibit phagocytosis. It is conceivable, however, that in those few cells whose phagocytic properties were not completely abolished, the intraphagocytic digestion of organisms might have been impaired.

From examination of peritoneal exudates McLeod (1941) observed depression of phagocytosis and inhibition of intracellular digestion of organisms.

The question might arise whether these effects of mucin preparations towards leucocytes could be due to some chemical impurity in the preparation. The manufacturers stated that no chemical preservative had been added in the process of manufacture, and tests for heavy metals were negative. The mineral ash from refined preparations injected with meningococci did not render them pathogenic. The possibility of an osmotic effect being responsible for the action on the leucocytes was excluded when several fractions which had inorganic salts removed by dialysis, all proved active. Moreover, a laboratory preparation of mucin showed the same effects as commercial products. Again, refined preparations were active in much lower concentrations than the crude product. The adjustment of the mucin suspension to pH 7.2 excluded any acidity factor.

The results of bactericidal tests showed that mucin either annulled or reduced the bactericidal action of whole blood, serum and plasma. There is no record in the literature of this particular effect of mucin having been tested by the technique employed by the writer. The results of the tests recorded showed that it was only whole blood which exhibited a distinct bactericidal action towards

the Gram-positive bacteria tested. Additional evidence is therefore furnished of the apparent interference by mucin with phagocytosis and intracellular killing of the organisms in the phagocytic cells. It is also possible that mucin may interfere with the serum opsonins, as suggested by Keefer and Spink (1938).

Although mucin, in most cases, reduced the bactericidal action of whole blood, in very few cases was this property of the blood completely abolished. This effect, interesting as it was, could not be considered so striking as the anti-leucocytic action. One is therefore of the same opinion as McLeod (1941), that this action of mucin is of secondary importance.

In the case of the Gram-negative organisms killing by plasma as well as by whole blood is in many cases a marked natural phenomenon, due to complement acting along with a natural antibody-like substance. Whether mucin interfered with the action of antibody, complement, or both, has not been investigated, as the effects did not seem to be of primary importance. However, as complement is a more labile constituent of serum than antibody, it would be more easily inactivated than the latter.

As has been pointed out, organisms in the presence of mucin appeared to multiply in very large numbers in the peritoneal cavity. This observation suggested the possibility of mucin supplying some

factor promoting bacterial growth, and absent from the body fluids and tissues of the animals used for the experiments. Several *in vitro* experiments were carried out in order to investigate this question. It was found that when mucin was incorporated in culture media, various organisms grew more luxuriantly than on ordinary media. This was particularly observed in the case of *C.diphtheriae*, *Streptococcus pyogenes*, *Staphylococcus pyogenes aureus*, *Staph. pyogenes albus*, the pneumococcus, the gonococcus and the meningococcus. It was interesting to note that a refined fraction G 341 was more active than the crude product.

It might be suggested that this effect takes place also in the animal body, but it is appreciated that as conditions affecting bacterial growth in animal tissues and blood are very different from those in artificial culture media, such a suggestion is open to criticism. Nevertheless, even on a medium containing animal blood, e.g. blood agar, which would appear to contain most of the growth factors necessary for such strictly parasitic organisms as the meningococcus and gonococcus, the addition of mucin still substantially augmented growth. The possibility of mucin acting in this way in the peritoneum cannot therefore be excluded.

It might be suggested, that because the organisms multiply so freely in the mucin, the leucocytes are overwhelmed merely by the excessive

numbers of organisms present and their products. This, however, can only be part of the process, as the in vitro studies adequately confirm the damaging effect of the mucin on leucocytes.

Another possibility is that the mucin may neutralise some toxic product in the medium, or may, by reason of its chemical structure protect the organism from such toxic products should they be present. Thus, it has been recognised that culture media may contain such bacterial antagonists which are nevertheless neutralisable by other constituents of the medium. (O'Meara 1937).

The growth-promoting action of mucin towards the gonococcus was of special interest. Keefer and Spink (1938) found mucin to be favourable for the survival of the gonococcus but a poor medium for the continuous growth of the organism. It was observed by the writer that a refined fraction, G 341, produced this growth-promoting effect in quantities as low as 3.75 c.c. per 100 c.c. agar, whereas the crude product was not really effective below 30 c.c. per 100 c.c. agar. The gonococcus is an organism whose growth is greatly aided by the presence of carbon-dioxide (8 - 10%) in the atmosphere. In fact, an atmosphere containing carbon-dioxide is necessary in order to obtain a satisfactory growth of the organism within 24 hours. The effect of carbon-dioxide on those organisms which grow well in its

presence is not fully understood. It may provide an additional source of carbon, neutralise some toxic products present in the medium, or contribute to the synthesis of an accessory growth factor. (Glass and Kennett, 1939; Bigger and Nelson, 1943; Wherry and Ervin, 1918; Rockwell and McKhann, 1921; Rockwell, 1923, 1924; Rockwell and Highberger, 1926, 1927; Wood and Workman, 1936; Hes, 1938; Gladstone, Fildes and Richardson, 1935; Wood et al, 1940; Pappenheimer and Hottle, 1940; Krebs and Eggleston, 1941;).

When using a mucin-containing medium for growing the gonococcus it was not necessary to incubate the culture in an atmosphere containing carbon-dioxide. The findings of Gould, Kane and Mueller (1944) are interesting in this connection. They obtained a growth of several strains of the gonococcus on a medium consisting of glutamic acid, histidine, glucose, starch, glutathione, magnesium and iron salts, phosphates, sodium chloride and agar. They also found that casein hydrolysate and meat infusion contained unknown factors which greatly stimulated the growth of the gonococcus on this basic medium, although these were not essential. The function of the starch was, according to these workers, as a protection against the inhibiting effect of certain samples of agar. They found several substances including gastric mucin, and the insoluble fraction of whole yeast autolyzate

were capable of completely or partially replacing starch and bone charcoal. The incorporation of mucin in the medium may be of practical value in growing the gonococcus, as it dispenses with the use of carbon-dioxide.

The in vitro growth of organisms in the presence of mucin does not appear to exalt their virulence, (Rake, 1935; Nungester, Jourdonnais and Wolf,1936). The observations of the writer are in agreement with the above. Nungester, Jourdonnais and Wolf (1936) however, pointed out that anthrax bacilli recovered from the peritoneal cavity of a rat which had died of an infection with these organisms, aided by mucin, were more virulent when titrated in saline than organisms from a stock culture. Miller and Castles (1936) however, did not obtain a similar result in their experiments with meningococci in mice.

The question arose whether mucin might promote infection by acting as a 'spreading factor', that is by increasing the permeability of the tissues. It is now well recognised that extracts of certain organs, for example testis (Duran-Reynals 1942; Chain and Duthie 1939) and products of various bacteria, for example the Clostridia (McLean 1936), and certain streptococci (Meyer, Hobby, Chaffee and Dawson, 1940, 1941) act in this way, by virtue of hyaluronidase. This enzyme by its action on the

cement substance of the tissues, composed of hyaluronic acid (Meyer and Palmer, 1934) increases their permeability and may allow bacteria to spread more readily from a focus of infection.

The method used by Duran-Reynals for measuring the 'spreading factor' was by intradermal injection in rabbits of a dilution of India ink and saline, the areas of the blebs or spots resulting from the injection and the density of the discolorations being measured immediately and at intervals after injection. Results were recorded after 24 hours. Forced spreading, whereby pressure was applied to the inoculation bleb immediately after injection was also employed. Thus the spontaneous and forced spreading could be determined. The writer used this technique in testing mucin as a possible 'spreading factor', but no significant results were obtained.

It has also been shown that mucin preparations remain active in enhancing virulence even after autoclaving, which would contra-indicate an enzymic agent being responsible for the biological effect.

The relation between the virulence of bacteria and capsule formation is well established. The possession of a capsule by micro-organisms is a characteristic of young active cells, and capsulated organisms are more virulent than non-encapsulated ones. Virulent pneumococci, for example, when isolated from pathological conditions are always in

the encapsulated form. The capsule displays an 'aggressin-like' action. It protects certain organisms such as pneumococcus and staphylococcus (Lyons, 1937) against phagocytosis. It was also pointed out by Danyz (1900) how encapsulated organisms were able to withstand the lytic action of sera, whereas non-encapsulated organisms could not. It is also established that the toxic 'O' antigens of the 'smooth' forms of the typhoid bacilli, which are associated with virulence, cause an intense leucopenia when injected into animals.

The action of mucin might appear to be similar in certain respects to this 'aggressin-like' action of encapsulated organisms. There also appeared to be a similarity in chemical structure between mucin and the polysaccharide isolated from group A haemolytic streptococci by Kendall, Heidelberger and Dawson (1937), which was composed of N-acetyl glucosamine and glucuronic acid. This particular polysaccharide resembled the specific polysaccharide of the pneumococcus in its encapsulated form. It might be possible that mucin provided a covering for the organisms in the form of a capsule, thereby protecting them from phagocytosis. However, in a few experiments carried out by the writer, no capsular structure was detected around organisms (injected along with mucin) in peritoneal exudates stained with India ink.

The localisation of organisms in injured tissue being due to the liberation by the tissue of histamine-like substances was pointed out by Finlay (1928). The possibility of such substances being present in the mucin preparations was at one time considered, but as the mucin preparations per se were innocuous to mice even at 15% and as these animals are in any case relatively resistant to histamine (Clark 1933) this possibility was excluded. Moreover, in those preparations where dialysis was employed, any histamine present would be removed.

There are conflicting opinions regarding the importance of viscosity in relation to the action of mucin. Nungester et al (1936) believed the relative viscosity of mucin was the important factor in its action, while Anderson and Oag (1939) were of the opinion that the viscosity of the mucin seemed to be of little importance. McLeod (1941) maintained that although viscosity, along with other factors such as concentration etc., determined the effectiveness of mucin in establishing infection with the meningococcus, nevertheless, it was not as important as concentration. The writer is of the opinion that viscosity is not the determining factor in the virulence-enhancing action of mucin, for it was found that the most refined and active fractions were relatively non-viscous. The concentration is of far greater importance than viscosity.

The important question still remains as to whether the virulence enhancing action of the various mucin preparations used are due entirely to mucin or to some unknown factor present in the preparation.

Though it has not been possible in the chemical studies referred to in this thesis to isolate in a pure state the active principle responsible for the infection-promoting action of mucin, partially purified fractions have been prepared from commercial mucin, possessing the same type of activity and in a higher quantitative degree than the crude product. These active fractions which are protein in character do not appear to possess the same physical or chemical properties of mucin as it is usually defined, although they may contain the protein fraction of the muco-protein complex which constitutes mucin. It is therefore impossible to state whether the active principle responsible for the infection-promoting action is derived from mucin proper or is some independent and as yet unknown factor derived from mucous membranes or mucous tissues. The results however, of the experimental work show the action to be a chemical one, and the activity appears to reside in the protein moiety, the carbohydrate part being inactive, a view also held by Anderson and Oag (1939).

The infection-promoting action of mucin is of

special biological interest and importance in view of the fact that mucin is a physiological constituent of the secretions of mucous membranes which often constitute avenues of microbic infection.

The results of the work on experimental pneumonia in dogs (Hamburger and Robertson, 1940) are significant in that pneumonia can be induced by introducing pneumococci into a terminal bronchus along with mucin, which appears to protect the organisms from the natural defences.

The natural functions of mucous secretions in the body are protective, by acting as a lubricant to mucous membranes and removing mechanically foreign matter, micro-organisms, and debris from various sites. Florey (1933) has shown clearly how an increased flow of mucous dislodges bacteria from the depths of the crypts of the mucous membranes of the colon, though the same organisms grow freely in the mucous secretion, the normal flow being insufficient to remove them from the crypts. Moreover, antibacterial constituents of mucous secretions e.g. lysozyme, and the hydrochloric acid of gastric secretion contribute to their protective function.

All the experimental work which has been done with mucin in promoting bacterial infection shows how mucous secretions contain a substance or substances which, under certain conditions, may

actually reverse the protective action of these secretions; and it can only be concluded, though quite tentatively in the present state of our knowledge, that certain qualitative and quantitative variations in these secretions, together with the degree of activity of their flow from the mucous glands, are the factors which determine whether such secretions will exert their natural protective function against bacterial infection or have a reverse effect in promoting infection.

Much more work is required to elucidate this complex problem which appears to have an important bearing on the course of bacterial infection.

It is hoped that the work recorded has helped to clarify some aspects of the problem and will open the way to further research into a subject of great fundamental importance in relation to infective disease.

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