IMMUNISATION and ANTIBODY REACTIONS —

A Series of Experimental Studies

by

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INTRODUCTION

During the last quarter of a century, the knowledge of medicine has increased enormously. By far the most important factor in this increase was the discovery of the organismal cause of the majority of diseases. As a result, the study of infection and immunity was started on a scientific basis and, now in all fairness, it can be stated that the future development of diagnosis, prevention and treatment of disease will depend principally on the advancement in our knowledge of Immunology. The beginning of the science must be ascribed to Edward Jenner who, by inoculating human beings with the lymph of cows suffering from cow-pox, was able to produce immunity to small-pox. Many years elapsed before Pasteur, shortly followed by numerous investigators, produced, by many carefully controlled experiments, the evidence necessary to put the new subject of Immunology on a scientific basis.

It has been stated that all disease, directly or indirectly, is the result of infection. So wide a statement obviously requires explanation. The case is easily substantiated when dealing with infection/
infection as a direct cause; thus, infection with B. typhosus results in typhoid fever, B. diphtheriae in diphtheria, etc. In short, the majority of diseases are certainly the result of infection. The explanation of how infection is the indirect cause of disease is not so simple. It is generally agreed that one of the commonest causes of chronic ill health is auto-intoxication from the bowel. Clinicians admit that such diseases labelled in text books as arterio-sclerosis, chronic myocarditis, nephritis, etc. etc., are merely descriptions of the affected organs and are signs, not of a specific disease, but of a general poisoning of the system. Modern research has shown that the split-products of protein digestion in the intestinal tract, namely the amine derivatives, e.g. indol, skatol, guanidin and especially methyl guanidin, are the main causes of this intoxication. These are excreted in the urine and can be estimated accordingly. It can be proved by in vitro experiment that these poisonous products are the result of the action of putrefactive micro-organisms on the protein in the food. Immunity to these spore-bearing proteolytic micro-organisms can not be produced by vaccine therapy as they are really as/
as much outside the body as is the Staphylococcus albus outside the epidermis. If, however, we alter the hydrogen-ion concentration (pH) of the intestinal contents (by certain specific measures which need not be here stated) from the alkaline to acid, the flora of the intestine becomes changed to the simple non-putrefactive type of organism of the acidophilus group, and the putrefactive bacteria and their resulting toxic products are eliminated.

By indirect methods, immunity to the harmful putrefactive organisms has been produced. In this or similar ways, the future may show that all diseases are directly or indirectly the result of infection.

Slowly but surely is the information, collected by the study of infection and immunity, working its change on the minds of medical men. A much wider outlook, however, is necessary if medicine is to progress on a scientific basis. The treatment of symptoms by bottles of medicine containing minute quantities of drugs must be replaced by a diligent search for the true cause of disease in its earliest stage. Many learned clinicians have stated that at the most two dozen drugs would supply all that they required for the treatment of disease and that the/
the rest of the pharmacopoeia could probably be 
eliminated. There is a definite circumscribed 
place in medicine for certain specific drugs. Thus 
no one would deny the importance of salvarsan in 
syphilis, quinine in malaria, or digitalis in 
auricular fibrillation. But in many cases the main 
value, and one not to be lightly passed over, in the 
"bottle of medicine", undoubtedly lies in its psycho­
logical effect.

The future science of medicine will lie on 
a much higher plane. Prevention will be the keynote 
of the profession. But prevention is impossible 
without not only knowing the causes of disease but 
also the way in which infection takes place and the 
method by which the body meets the attack. Thus, 
since the causes of enteric fever, plague, etc. were 
discovered, by suitable preventive measures these 
diseases have been largely eliminated. Recently 
another step forward was made by the work of Schick 
and Dick on diphtheria and scarlet fever respectively. 
The natural immunity or susceptibility of individuals 
to these diseases can be estimated by the skin re­
actions following an injection of the toxin of B. 
diphtheriae and the haemolytic streptococcus. 
Individuals showing susceptibility can then be active­
ly/
actively immunised against the infection.

If similar tests for susceptibility to other diseases could be discovered, a great advance in prevention would result. The treatment of the future will develop along the following lines. Where the disease is acute the transference of antitoxin or antibacterial substances would be the logical therapeutic method.

Today the treatment has been remarkably successful in dealing with diseases caused by organisms which produce a soluble exo-toxin. Unfortunately, where the infection is of the nature of a bacteriæmia or due to an endo-toxin, similar satisfactory results do not occur: in other words, our knowledge of infection is at present only elementary, and the successful treatment of these latter types of disease will depend on the researches of the future. Where the case is chronic or where prophylaxis is the object, active immunisation by vaccine therapy should be attempted.

Today, as a result of the application of the above principles, diseases, which forty years ago were treated merely by ineffectual prescriptions of useless drugs, have either nearly disappeared or at least have had their mortality greatly decreased. Unfortunately, however,
however, the majority of disease still baffles the investigator. The reason of this is that though a vast number of facts have been collected as the result of both clinical and laboratory investigations, our knowledge is not sufficient to enable us to fit together all the pieces of the puzzle of immunity or even to estimate the value of the phenomena which we see. For instance, the relation which agglutination bears to true immunity is still not understood. Up to the last few years it was considered to be a serological index of immunity; today, owing to much new evidence having been brought forward, a definite statement cannot be made.

It is thus of the greatest importance that the medical student of the future should be well acquainted with the basic principles of immunity in order that he may himself help in adding to the store of knowledge and the elucidation of this great mystery.

The study of immunity can only be successful if preceded by the study of infection, for paradoxical as it may appear, immunity cannot exist without infection, natural or artificial. I therefore purpose to deal first with the facts relating to the mechanism of infection and later to state what is known of the defensive/
defensive forces which the body uses in repelling the attacks of infective agents.

**INFECTION**

If the effects of chemical and physical agents on the body are not included, it may be stated that all disease is due probably to vegetable or animal parasites or the products of their activity.

And since our knowledge of the mechanism of infection is based mainly upon the studies of the pathogenic bacteria, the statements which follow are made with special reference to these organisms.

**DEFINITION OF INFECTION.** Adami describes infection as follows:

"The abnormal state resulting from the deleterious local and general interaction between a host and an invading parasite with consequent tissue changes and symptoms, constitutes an infectious disease."

Infection must be clearly distinguished from contamination or invasion.

The surfaces of the skin and mucous membrane are contaminated with micro-organisms, but as these do not penetrate the tissues with resulting reactions, no infection occurs.

Again, micro-organisms normally present on the pharynx and tonsils may, and in fact, often do penetrate the tissues, where they are destroyed before producing/
producing any damage. Such a condition constitutes invasion and not an infection.

Two factors must always exist in an infection.

(1) The infecting agent.
(2) The defensive mechanism of the host.

When considering an infection, it is necessary to study the offensive forces of the infecting agent. These may be stated briefly to consist of

(a) Numbers. Where only a few microorganisms gain entrance to the host, the mechanism of defence may cause their destruction at once and in this case the condition is really only an invasion. Variation from this through mild cases up to rapid death of the host may depend on the actual numbers of the infecting organism.

(b) The Virulence of the organism. This is a matter of great importance. Thus one epidemic of diphtheria may be mild while the next may be very severe. Morphologically and culturally the infecting organisms appear identical yet their effects on the host may be widely different.

On prolonged artificial cultivation, organisms of the most pathogenic type may be rendered almost harmless. This is of great practical value as it will/
will be shown later that the best immunity can be produced by injecting a living culture of an organism (as opposed to a dead vaccine). This naturally can be done only if an avirulent strain is used. On the other hand, by the method of animal "Passage", strains can be exalted to great virulence. This can also be accomplished by cultivating the organism in animal fluid, e.g. blood.

(c) The power of the infecting agent of defending itself against the mechanisms of defence of the host.

Thus the formation of capsules is regarded as a means of defence. Again the aggressins, discovered and studied by Bail, may be of great importance. Certain organisms secrete substances which paralyse the protective forces of the host, especially the leucocytes. Bail was able to show that in the exudates of fatal infections, substances existed which, when injected together with sub-lethal doses of the micro-organism into another animal, rapidly produced death. The aggressins may be of the nature of liberated endo-toxins.

(II) The Defensive Mechanism of the Host.

Two main lines of defence exist:

(a) Non-specific
(b) Specific

(a) Non-specific - Under this heading should be included the

(1) Anatomical arrangement of the cells of/
of the epidermis, which act as an obstacle to the invading organisms.

(2) The sweeping action of ciliated epithelium.

(3) Surface discharges and secretion which act mechanically by washing away organisms, chemically as in the stomach and vagina with their acid secretions and antiseptically by their contained antibody content.

(b) Specific e.g. Antibodies - These are the host’s main line of defence and will be considered in detail in the chapter on Immunity.

These two factors, namely the infecting agent and the defensive mechanism of the host, are so closely associated that the one can not be considered without taking the other into account.

Immunity or susceptibility depends on the relative proportion of the one to the other.

Infection may be the result of excessive virulence or numbers of the invading parasite or to lessened vitality of the host with the consequent diminution of his defensive mechanism.

From the above it will be realised that the subject of Immunity is an extremely complex one. The problem may be focussed by comparing the subject to two countries at war, whose armies face each other on each side of the boundary. Army 'A' may gain a victory over army 'B' either by (a) increasing its efficiency/
efficiency by special training or by special warlike inventions e.g. tanks, aeroplanes, etc. or (b) by increasing its strength by new levies of men, or (c) it may defeat army 'B', not through any improvement of itself but by the weakening of 'B' by desertion, lack of discipline or loss of moral, etc. Conversely army 'B' may win or lose to army 'A' for similar reasons.

On the other hand, a draw may result in which neither army is able to cross the boundary. The last is what occurs in chronic disease. The host is able to restrain the parasite or to neutralise its toxins but not to destroy it, while the parasite is able to live in the host but cannot reduce the vitality to an extent at which acute infection occurs.

**SOURCES of INFECTION.** These may be described as

1. **Exogenous.**
2. **Endogenous.**

The former is by far the commoner.

(1) An **exogenous infection** is one in which the parasite reaches the tissues of the host from sources outside and unconnected with the host. Nearly all disease is of this type, e.g. -

(a) The intestinal infectious diseases are caused by the host partaking of infected water, milk or food.

(b) The respiratory infectious diseases are due to the inhalation of infected air.
An endogenous infection is the result of the activity of organisms normally present in or on the host. The condition is not the result of the virulence of the organisms but is rather a sign of lowered vitality or resistance of the host. An example of this is appendicitis following prolonged faecal stasis due to B. coli.

Channels of Infection

An infection may start locally at any point in the outer covering of the body. By the outer covering we must understand not only the skin and adjacent mucous membrane but also the covering of all surfaces and channels communicating with the exterior. It will be remembered that the only direct connection between the interior and the exterior of the body is the Fallopian tubes in the female. The infection may remain localised or become systemic by penetration. Two factors regarding the channels of infections must be considered -

(1) Certain organisms have inherent affinity for certain tissues and sites.

(2) Certain tissues and sites have an inherent susceptibility to certain organisms.

As a result of these two factors, most diseases have definite, regular avenues of infection. Where an organism is of extreme virulence, e.g. B. pestis, septicemia results whether the parasite is inhaled, or/
or enters through a local lesion in the skin. Again, when a tissue has received an injury, and, in consequence, is somewhat devitalised, it is more susceptible to infection.

But in addition to these facts, we are faced with the difficulty of explaining the selective affinity of certain tissue for certain organisms. Thus cholera, typhoid fever and dysentery are all gastro-intestinal diseases. Infection cannot take place via the skin. When the organisms have passed the stomach, they select special sites in the intestines; thus, B. typhosus attacks the lymphoid tissues in the small intestines, e.g. Peyer's patches while the B. dysenteriae has an affinity for the mucosa of the large bowel.

Numerous other examples might be given such as the immunity of the mucous membrane of the oesophagus to B. diphtheriae. The explanation of these phenomena is still wanting. We must assume that either all the tissues except those susceptible actually destroy the circulating organisms or else that the organisms, lacking some definite affinity for the non-affected tissues, passively circulate through them without stopping.

This subject is of great practical importance and/
and much work requires to be done on it. Nichols and Hough (1) have isolated strains of Treponema pallidum from the central nervous system of tabetics, which when injected into rabbits, have a selective affinity for the cornea, choroid and retina. Similarly Noguchi reports on certain selective strains of spirochaetes. It may be proved in the future that the reason why some syphilitics develop cutaneous and cardio-vascular disease while others are affected principally in the central nervous system is that there are definite selective types of spirochaetes. If we were able to recognise these types, the treatment and prognosis of syphilis would be vastly improved. Similar results with Streptococci are claimed by Rosenow (2).

GENERAL SUSCEPTIBILITY

A lowering of the host's general susceptibility is always liable to be followed by infection. A logical conclusion follows -- that the best way of reducing disease would be to raise the general standard of health of the nation. The medical statistics of the Great War show that only a very small percentage of the nation are in perfect health. The majority are classified as "C". To raise the general standard to "A", by means of better housing, feeding, sanitation, etc./
etc., must be the first aim of the country. This alone would reduce enormously infections of all types. The factors which predispose to disease must be understood before this can be accomplished. They can be divided into two groups -

(1) Inherited.
(2) Acquired.

(1) Inherited Susceptibility.

Although explanation of the facts is lacking, we know that certain animals and races have individual susceptibilities. Thus, while human beings may contract typhoid fever if infected water is ingested, animals enjoy complete immunity. Again, Gonococcus has a special affinity for the genital mucous membrane of human beings and has no effect on animals.

Dogs suffer from distemper while human beings are immune. Ordinary sheep succumb to anthrax while Algerian sheep are immune.

Races and even families may have special susceptibilities. Thus, Eskimos are reported to be extremely liable to measles and syphilis. This, of course, may be explained on the grounds of a lack of acquired immunity and not to a specific susceptibility.

(2) Acquired Susceptibility.

As already stated this is of great importance.
importance. It results from any cause which produces a lessened vitality:

(a) Exposure to wet and cold.

Although diseases of the respiratory passages are always of bacterial origin, there can be no doubt that excessive exposure to wet and cold is a predisposing cause of infection. Experimental proof of the lowering of vitality as the result of cold was demonstrated by Pasteur when he showed that fowls which normally are immune to anthrax could be infected if the body temperature was reduced by cold baths.

(b) Diet.

Not only must a diet be sufficient in calorific value but it must be properly balanced and contain sufficient amounts of vitamins. Any prolonged period of divergence from a properly balanced diet must result in lowering the powers of resistance. When the stage of famine is reached in a country, the nation is liable to suffer from terrible epidemics of disease. This has occurred repeatedly in Europe and Asia where famines have been followed by vast epidemics of cholera, plague and typhus fever.

(c) Overwork.

Either mental or physical results in a lowered/
lowered vitality. It is associated usually with insufficient sleep, mental and nervous worry, etc. It is the duty of the medical profession to reduce infection by explaining these problems to patients. When dealing with the working classes, overwork can be corrected only by Inspectors of the Public Health Department making careful enquiries in the factories and workshops. It should be clearly recognised that in every trade, depending on the amount of muscular or mental energy required, there is a definite limited number of hours per day in which efficient work is produced. Increasing the hours, not only reduces the output but lowers the vitality of the worker and thus starts a vicious circle.

(d) Previous infection.

While some diseases produce a resulting immunity, e.g. typhoid fever, measles, etc., others produce either no resistance to infection or an actual susceptibility. This is exemplified in pneumonia, influenza and acute rheumatic fever. No satisfactory explanation of this has ever been given but it is obviously of the greatest importance that further researches into the subject should be undertaken. Whether the second illness is the result of a new infection or of the previous infection never having been/
been completely eliminated, a subsequent recrudescence occurring when the host's vitality is lowered, must remain unsettled at present. In any case, even if the facts cannot be explained, the future workers on the subject must devise methods by which individuals, who have contracted a disease which is known to leave them more susceptible, shall be immunised actively and prophylactively against another attack.

When the invading parasites have entered the tissue and produced infection, disease is the result of one or more causes.

1. Toxaemia.
2. Bacteriaemia.

**Bacterial Toxins**

**Definition**

"Bacterial toxins are the poisonous products produced by bacteria in both living tissues and artificial culture-media."

Toxins are undoubtedly the parasite's main weapon against the host. In some diseases such as diphtheria, tetanus and botulism, toxaemia is the only factor of importance. In most diseases, however, infection is characterised by a combination of bacteriaemia and toxaemia.

Since the discovery of diphtheria toxin by Roux and Yersin in 1888, a vast amount of research work on these poisons has been done and as a result, some of the most important facts regarding immunity, and /
and the relation of antigen to antibody were discovered.

**Toxins** are divided into two great classes:

1. Extra-cellular.
2. Intra-cellular.

(1) *Extra cellular toxins* are produced and excreted by the living organisms into the tissues or artificial culture media, and hence are also called soluble or true toxins. They have been studied chiefly in diphtheria and tetanus but they occur also in botulism and dysentry and to a less extent are produced by the staphylococcus, streptococcus and B. welchii.

In these diseases the organisms remain at the site of infection, producing their poison which is removed by the blood lymph streams with resulting intoxication.

**General nature of extra-cellular toxins.**

Since toxins are extremely labile and are altered or destroyed by light and heat, it is impossible to obtain an accurate chemical analysis of them. Investigation has principally been in the field of animal experiments.

The following facts are, however, probably correct.

(1) They are soluble in water and dialyzable through thin membranes only.

(2) They are precipitated by alcohol and ammonium sulphate.

(3)/
(3) When injected into animals, anti-toxins can be demonstrated always in the serum. Thus they are absolutely specific and in this way differ from ptomaines which are non-specific cleavage products of the media upon which the bacteria have grown.

(4) They are colloids.

(5) They closely resemble enzymes or ferments both in the way in which they are produced, and in their mode of action and in their thermo-lability. They differ mainly in the fact that an enzyme acts as a catalyst. Small quantities by acting again and again can decompose large quantities of organic compounds, while a toxin after uniting with a compound loses its identity.

**Selective Actions of Toxins**

Just as it has already been shown that bacteria may have a special affinity for certain definite tissues of the body, so toxins may act specifically on certain cells. As a result of this, it has been found that a soluble toxin is usually a mixture of two or more toxins.

In the case of tetanus, there exists, according to Ehrlich,

(1) a neurotoxin-tetanospasmin.
(2) a haemotoxin-tetanolysin.

Flexner has clearly demonstrated that the symptoms of bacillary dysentery are mainly due to a soluble toxin which acts specifically on the intestine as can be shown by injecting the toxin alone into animals. Similarly the soluble toxin of B. botulinus has a selective affinity for the cells of the cranial nerves.
Soluble toxins would appear therefore to act in a manner similar to the poisons of certain plants and the venoms of some snakes, e.g. strychnine on the motor cells of the spinal cord, and cobra venom on erythrocytes.

Soluble toxins of the spore-bearing anaerobic bacilli

During the Great War, which was mainly fought on the highly manured agricultural countries of Belgium and France, a large number of gunshot wounds became infected with these spore-bearing organisms. Owing to previous work on tetanus, an anti-toxic serum was soon prepared in large quantities and all wounded soldiers received prophylactic injections. The results were eminently satisfactory as a case of tetanus was a comparatively rare occurrence. Unfortunately, this serum had no effect on the extremely serious condition called gas gangrene which was found to occur in muscular tissue which was seriously devitalised by lacerating gun shot wounds. It was soon discovered that B. welchii was a cause of gas gangrene and an antitoxic serum was prepared. Although this reduced the number of cases and was of some benefit in treatment, it did not eliminate them entirely and subsequent careful examination showed that the Vibrion septique and B. oedematium were also causes and anti sera were produced accordingly.
By 1918, a serum was in use, a few cubic centimetres of which would protect an animal infected with mixed cultures of B. tetani, B. welchii, Vibrion septique and B. oedematiens. This ended what may be termed a triumph of scientific investigation, the full benefits of which never were realised owing to the declaration of peace. It is along such lines as these that the future development of medicine must proceed.

**ENDO TOXINS.**

**Definition** - Pfeiffer defined endotoxins as follows:

"Endotoxins are the preformed toxic substances retained in the bodies of micro-organisms until released by disintegrating processes."

To obtain endotoxin, it is necessary that the organisms be disintegrated completely. Several methods are available. Thus, Cole (4) produced pneumococcal endotoxins by dissolving the organisms in dilute solutions of bile salts.

Rosenow (5) obtained similar results by alternately freezing and thawing the organisms suspended in saline.

Rowland and MacFayden secured typhoid endotoxins by first freezing the bacilli with liquid air and then grinding them. Unless a rapid disintegration/
disintegration of bacteria be secured, the toxic material may be of the nature of protein split products due to proteolytic enzymes acting on the bacterial protoplasm and not a true preformed endotoxin.

The Nature and Action of Endotoxins.

The chemical nature of endotoxins is unknown as, up to the present, the material has not been obtained in the pure state.

It is a matter of the greatest clinical importance that up to the present satisfactory antitoxins have never been produced for endotoxins. The marked success, which has been attained in the treatment of diseases due to soluble exotoxins, has already been stated. Until new factors are discovered to explain the failure of immune sera in the treatment of the much larger group of endotoxic diseases, serum treatment cannot progress to any great extent.

Although endotoxins do not produce antitoxins in the serum of an injected animal, they are nevertheless antigenic. That is to say antibodies of the nature of agglutinins and bactericidal substance are developed which are specific for the organism from which the endotoxin has been obtained.

The injection of large amounts of endotoxin into animals produces symptoms resembling acute or delayed anaphylaxis. When small immunising doses are given/
given, wasting and fever usually follows. In addition their action on leucocytes has been closely studied, and generally they produce a negative chemotaxis, and are anti-opsonic. Their action is similar or identical to the "aggressins" described by Bail.

**Bacterial Proteins.**

It is still unknown to what extent the toxic symptoms of the majority of diseases are due to the liberation of endotoxins or are the result of the toxic action of the split products of bacterial protein.

Vaughan \(^7\) has studied the problem extensively. He concludes that the greater part of bacterial protoplasm consists of true protein. By treating masses of organisms with absolute alcohol in which two per cent of sodium hydroxide had been dissolved, he was able to split the bacterial protein into two portions. One portion, which was soluble in alcohol, was poisonous while the other which was insoluble, was non-poisonous.

Vaughan believes that in an infection, specific "ferments" are produced in the body from leucocytes and tissue cells which act on the organisms in a manner similar to the chemical process just described with the liberation of the toxic protein split products. Friedberger \(^8\) in Germany, and Embleton and Teale \(^9\) /
Teale believe that the splitting of the bacterial protein is due to action of amboceptor and complement.

Grave doubts about these theories have more recently arisen since Bordet, Jobling and Peterson, and others have shown that identical toxic substances can be produced in vitro without the presence of bacterial protein by substituting such substances as kaolin and agar. In short, the most recent researches point to a view opposed to Vaughan and Embleton and Teale.

It seems probable that in infection, the toxic substances are derived from the blood constituents, the alterations being brought about by physical and chemical changes, in which bacterial protein may play the part of a catalyst but is otherwise inert. (Kolmer - Infection and Immunity - Page 114).

Sufficient has been written now to explain the main lines of attack of an invading parasite. The next step is to examine the defensive forces of the host.

**IMMUNITY**

The mechanism of defence consists of two portions:

(1) Specific.
(2) Non-specific.

The non-specific forces have already been described. They are only important in so far that they prevent a/
a contamination or an invasion of the body surfaces from becoming an infection. Once an infection has resulted, the body cells of the host react with the production of specific defensive measures known as antibodies in order to defend the system. The defensive forces of the host are as numerous and even more complex than the offensive weapons of the infecting organisms.

Before dealing in detail with these antibodies, it is advisable that a short survey of the theories of immunity should be made.

Historic. The science of immunity can be traced back to the earliest ages. Thus, Hippocrates taught that the factor that causes disease is capable of curing it. The savages of Africa practised immunization against snake venoms from the earliest times, while for many centuries Eastern races have made preventive inoculations against small-pox by exposing subjects to mild cases of infection.

To Edward Jenner is due the credit of being the first to make a scientific study of immunity. He produced immunity to small-pox by inoculating subjects with the lymph of calves suffering from cow-pox. Eighty years elapsed before Pasteur, by his investigations into fowl cholera, anthrax and rabies, finally/
finally established immunology on its present scientific basis. Pasteur believed that acquired immunity resulted when the organism invading the host had used up some substance essential for its existence, and for lack of proper nourishment ceased to multiply and finally died. This theory is untenable in the light of the present day knowledge. There exist at present two main theories regarding immunity:

(1) The cellular theory of Metchnikoff.
(2) The humoral theory of Ehrlich.

The Cellular Theory

Metchnikoff adduced this theory as the result of his researches into the amoeboid movements and phagocytic properties of unicellular organisms. In his original theory, immunity depended on the success or failure of the phagocytes in destroying the invading organisms.

He divided the phagocytes into two classes:

(1) Microphages, principally the polymorpho-nuclear neutrophile leucocytes.

(2) Macrophages, principally the large mononuclear leucocytes. These may be derived from the spleen or lymphatic glands, or may consist of the endothelial cells lining the serous cavities and lymph spaces.

CHEMIOTAXIS - The attractive force which bacteria, as well as other foreign substances exert on the migration of leucocytes, was called by Bordet, chemiotaxis.

These/
These forces are transmitted through the body fluids and are produced by injection of both living and dead organisms. The phenomenon of immigration may be explained, however, on a physical basis. There is evidence to show that as the result of the injection of foreign protein, or because of the injury to the cells at the point of injection, a lowering of surface tension results which causes the leucocytes to move towards the site of infection. Other substances may raise the surface tension and thus cause a negative chemiotaxis. A more probable explanation of negative chemiotaxis is that the endotoxins or aggressins liberated from the bacteria being anti-opsonic, paralyse the movement of the leucocytes.

When phagocytosis has taken place, the ingested organism is digested by means of intra-cellular ferments called endo-lysin. Occasionally leucocytes may engulf living bacteria and carry them into the deeper tissues where the cell dies and the micro-organisms are liberated with the consequent spread of infection.

When the advocates of the humoral theory of immunity demonstrated that bacteria could be killed by substances in the blood serum entirely independent of phagocytosis, Metchnikoff was forced to modify and expand/
expand his theory. Thus, he admitted the existence of bacterioclysins, bacterictropins and other antibodies in the serum but claimed that these substances are derived from the phagocytic cells. It is generally agreed to-day that antibodies are produced by the tissue cells and are poured into the blood stream which acts merely as a conveyance. It has never been proved that they are only produced from phagocytic cells and it is far more reasonable to believe that antibodies are the result of a general cellular activity.

Humoral Theory of Immunity

The humoral theory ascribes immunity to the effects of body fluids on the infecting organisms. The originator of the theory was Fodor (1) who in 1896 found that the blood of rabbits would kill anthrax bacilli in the test-tube independent of cells and phagocytosis. In 1894, Pfeiffer produced fresh support by his discovery of the fact that if cholera vibrios are introduced into the peritoneal cavity of a guinea pig previously immunised, bacterolysis takes place.

Three years later, Ehrlich published an explanation of these and other observations of supporters of the humoral theory in what he called the "Side-chain" theory.

It/
It depends primarily on the claim that the antigen introduced into an animal forms a chemical union with the body cells. The whole theory is hypothetical to a degree, as well as complicated. Apart from the fact that much fresh information on the subject of immunity came to light by the investigation of its possibilities, it has been of doubtful value in explaining the problem of immunity. Today it is best relegated to a back shelf and regarded as a fascinating though impractical theory. My own personal opinion is that a cellular theory in the broadest sense is probably correct. Immunity must not be said to depend on any special type of cell but rather on the reaction of all the tissue cells of the body.

**Antigens and Antibodies.**

Since antibodies are the specific results of the interaction between antigens and body cells, a preliminary summary of the subject of antigens is essential.

**Definition:** "Antigens are substances that cause the formation of antibodies in the body fluids."

They are essentially specific in their action in producing antibodies. The problem of non-specific antigens is dealt with in the chapter on non-specific immunity.
The nature of antigens is still an open question. They are probably colloids and in addition all evidence goes to prove them to be proteins. Proteoses may possibly show some weak antigenic value. Amino-acids and polypeptides are definitely non-antigenic.

The position of lipoids as antigens is still not definitely settled. Most of the evidence points clearly to their being non-antigenic. The fact that lipoids are of such importance in the Wassermann reaction and other complement fixation tests in no way proves them to be antigenic.

**Antibodies.**

**Definition:**

"Antibodies are the specific substances found in the body fluids as the result of the interaction of the body cells and the antigen."

The actual sites of antibody production remain an unsettled question. While it is likely that the leucocytes and blood forming organs are of the greatest importance as producers of antibodies, it is probably that the tissue cells as a whole must be regarded as the essential factor in immunity. In fact, the most recent researches point to the endothelial cells lining the capillary walls as being important factors in the production of antibodies. Once an antibody has been produced, it may be found in varying concentrations,"
concentrations, not only in the body fluids, but in
the normal secretions such as the urine, saliva and
milk, and also in inflammatory exudates. It is
present however in by far the greatest amounts in the
blood.

Since the chemical composition of antibodies
is unknown, the antigen-antibody reaction in the body
must be left unsettled until further investigation
reveals new facts. All the evidence points to the
reaction being of a colloidal nature. When antigen
and antibody are brought together in solution in
in vitro experiments, there is a union of the two with
demonstrable physical changes. But although union
has occurred, it exists in a loose combination.
Thus, when a neutral mixture of diphtheria toxin and
antitoxin are injected into an animal, antibodies are
produced in the serum thus proving that some of the
toxin must have become dissociated. This experimental
finding led von Behring to produce his exceedingly
valuable researches into active immunisation in diph­
theria.

The most recent investigations show that by
certain procedures the antibody-antigen combination
may be broken up with recovery of the two parts
apparently undamaged. Thus Huntoon and Etris (13)
have recovered the pneumococcal antibody from the
immune/
immune serum. They found that the antigen-antibody combination may be dissociated by treatment with ten per cent saccharose solution, normal salt solution and ammonium carbonate solution.

A new field in serum therapy is opening as a result of these researches. For not only will the antibody be concentrated into a small bulk for injection, but since the protein has been mainly eliminated, sensitization and anaphylactic reactions in patients will practically disappear.

Transmission of antibodies

The experience of investigators of the problem of the transmission of antibodies from the mother to the foetus is contrary to the idea held by the clinical section of the medical profession. As judged mainly by the agglutinin content of the foetal blood, Grunbaum, Schenk, Park, Reymann and many others state that antibodies are not transmitted to any degree from mother to foetus. When antibodies are found to exist in the foetal blood, the probable explanation is that the antigen passed to the foetus via the placental circulation where it produced antibodies by stimulation of the foetal tissue.

One definite exception exists in the case of diphtheria where there appears to be a natural transference of antitoxin with resulting immunity for at least a year. Infants under one year yield a negative Schick/
Schick reaction: from the recently published work by Dick, probably the same is true of scarlet fever. After birth there is definite evidence to show that the maternal antibodies are transferred in the colostrum and milk.

Specificity of Antibodies.
The reactions and principles of immunity depend absolutely on the basic law that antibodies are specific for their antigens. Non-specific immunity is considered by itself in a subsequent chapter.

By means of these biological reactions, sera of different animals which appear identical can be sharply differentiated. Wells states, however, that if these serum proteins are obtained pure, definite chemical difference exists. Hartly of the Wellcome Research Laboratories, in a paper read at the British Medical Association meeting at Bradford in July 1924, states that after the most careful chemical analysis of serum proteins of different animals, he was unable to find any chemical difference. This, however, is denied by other workers. The fact remains, that by these reactions, quantities of protein or antibody can be detected in amounts far too small for even attempting a chemical analysis. Thus by the intradermal method, 0.00000025 c.c. of a "high potency" diphtheria antitoxin can/
can be detected. O'Brien (Opening paper B.M.A. Bradford 1924) states that he has produced a pneumococcal serum of which 1 c.c. will protect a mouse against fifty million fatal doses of living pneumococci Type I. Yet this serum fails to protect a mouse injected with small numbers of pneumococci Type II. Morphologically and biochemically the two type of pneumococci are identical.

Similarly the different members of the B. botulinus and the paratyphoid organisms can only be differentiated by antibodies reactions.

It must be remembered that although antibodies are specific, it does not follow that they are protective. While antitoxins are both specific and protective, agglutinins are only specific and do not injure the organism. On the other hand, an animal may be immune although its body fluids apparently contain no antibodies.
Immunity is divided into two types:

1. Natural
2. Acquired

Definition: "Natural immunity is defined as the resistance to infection normally possessed, usually as the result of inheritance, by certain individuals or species under natural conditions."

As has already been stated in the chapter on infection, certain species and races are susceptible to certain infections. The converse is also found. Thus, human beings are immune to fowl cholera, swine fever and distemper, while animals are immune to cholera, typhoid fever, chickenpox, etc. This immunity exists in spite of the close association of human beings with domestic animals which furnishes ample opportunity for infection.

Racial Immunity is said to exist but is somewhat doubtful. Thus the negroes are supposed to be immune to yellow fever and Mongolians to scarlet fever. A more probable explanation is that the individuals have acquired the diseases in early infancy in an unrecognised form. Even in closely related species, great variation may occur. For instance, the white mouse is immune to anthrax, the house mouse somewhat susceptible, while the field mouse is extremely susceptible.

In short, natural immunity may be said to be relative/
Thus it is a fact that fowls are naturally immune to ordinary doses of tetanus toxin but it should be remembered that this immunity is only relative, larger doses readily proving fatal.

**Acquired Immunity**

Acquired immunity occurs in two forms:

1. Active
2. Passive

**Definition:** "Active acquired immunity is that form of resistance to infection which results from a person or animal having the actual disease or being inoculated with the causative organism." (Kölmer)

The essential feature of active immunity is the reaction of the living cells and tissues of the host in their endeavour to overcome the invading parasites.

Active immunity may be produced by one of three methods:

(I.) Accidental infection. This is the commonest way and is exemplified in such diseases as measles, scarlet fever, typhus, etc.

(II.) Producing the disease by inoculation of the living virus. The Eastern races have employed this method in dealing with smallpox. The object of this method is to produce a mild case of the disease. Undoubtedly the immunity resulting is more satisfactory than that produced by giving the dead virus. But, unfortunately, the method at present/
present must be held to be too dangerous to employ on human beings. It is, however, largely used in veterinary practice with very good results.

(III.) Inoculation with a modified or dead virus. This is called vaccination. Examples of a modified virus are the Jennerian vaccination against smallpox and Pasteurian vaccination against rabies. Examples of a dead virus are the inoculations against typhoid and paratyphoid fevers with dead cultures of the organisms.

Passive Acquired Immunity

Passive acquired immunity is produced by the injection of serum containing preformed defensive substances which are not the result of the host's own cellular activity.

As the name indicates, it is a passive transference and not an active production of defensive substances.

Passive immunity is quite as specific as active immunity. The immune serum will protect or benefit only a person who is suffering from an infection due to the same organisms as produced the antibodies in the immune serum. Passive immunity is produced immediately on injection of the immune serum, there being no negative phase. Hence it is of/
of the greatest benefit in acute severe infections and as a prophylactic means of preventing diseases which have a short incubation period.

In proportion to its quickness of action is its shortness of duration. Nor is the degree of immunity so high in passive as in active immunity. In diseases due to soluble toxins, e.g., diphtheria and tetanus, the passive immunity conferred by injecting the homologous antitoxic serum is of the greatest benefit, both to those exposed to infection as well as those actually suffering from it.

The results of injection of antibacterial sera are comparable in no way to those of antitoxic sera. Until this difference is corrected, antisera can only be of limited value. As soon as methods are devised by which potent antibacterial sera are produced, the whole field of serum therapy will be widened greatly, with results so beneficial that diseases which today are mainly beyond the control of the profession, will be greatly reduced both in morbidity and incidence.

**ANTIBODIES**

The defensive bodies which result from the interaction of antigen and tissue cells are called antibodies. They are believed to be the host's main line of defence against infection. This is undoubtedly true/
true of antitoxins, but with regard to the others, the relation which they bear to actual protection from infection is still unsettled. A vast amount of investigation has brought much information to light. Methods are known of detecting antibodies in the most minute quantities. And yet today, twenty eight years after Bordet first described the phenomenon of agglutination, we are unable to state whether agglutination is or is not an index of immunity.

Before describing each individual antibody, it may simplify matters if the various antigens and antibodies are shown in tabular form:

<table>
<thead>
<tr>
<th>ANTIGENS</th>
<th>ANTIBODIES</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Opsonigenous substances (bacterial endo-toxins ?)</td>
<td>Opsonins</td>
</tr>
<tr>
<td>2. Cytoligenous substances</td>
<td>Cytolysoins</td>
</tr>
<tr>
<td>(a) Vegetable cells (bacterial)</td>
<td>(a) bacteriolysins</td>
</tr>
<tr>
<td>(b) Animal cells (erythrocytes, spermatozoa, kidney-tissue, etc.)</td>
<td>(b) (Hæmolysins, spermolysins, nephrolysins, etc.)</td>
</tr>
<tr>
<td>3. Agglutinogenous substances (bacteria, erythrocytes, etc.)</td>
<td>Agglutinins</td>
</tr>
<tr>
<td>4. Precipitogenous substances (soluble animal and vegetable protein)</td>
<td>Precipitins</td>
</tr>
<tr>
<td>5. Enzymes or ferments (rennin, lipase)</td>
<td>Anti-enzymes (antirennin, antilipase)</td>
</tr>
<tr>
<td>6. Toxins.</td>
<td>Antitoxins</td>
</tr>
</tbody>
</table>

Definition/
Definition: "Opsonins are substances in normal and immune sera which act upon bacteria and other cells in such a manner as to prepare them for ingestion by the phagocytes."

Historic: Metchnikoff as early as 1893 showed that the body fluids contained substances which greatly facilitated phagocytosis. In fact, he proved that if leucocytes and bacteria are thoroughly washed, practically no phagocytosis results. He attributed this result to substances in the serum which stimulated the cells to phagocytic activity.

In 1903 Wright and Douglas showed that the substance in the serum acted directly on the bacteria and prepared or sensitized them for ingestion by the cells. They called the substance in the serum opsonin (from opsono - I prepare food for). At the same time but quite independently Penfold and Rimshaw came to the same conclusion and named the substance Bacteriotropin.

An opsonin consists of two parts:

1. Thermo-labile, which is similar to or identical with complement.

2. Thermo-stable, which is the true opsonin.

Although opsonins are described as natural and immune, it seems likely that natural opsonins are really complements, since they are non-specific and are absorbed by charcoal, chalk, etc. The most marked phagocytosis results when both the thermo-labile and/
and thermostable parts are present. True opsonins are highly specific and are of undoubted help to the host in resisting or recovering from certain infections.

In the pyogenic infection, in which phagocytosis is probably the main line of defence, opsonins must necessarily be of great importance. In other types of disease, opsonins are of doubtful value.

**OPSONIC INDEX**

Wright and Douglas must be given the credit of devising a technique which enabled an estimation of the opsonic content of a serum to be made. They defined the opsonic index of a patient as follows:

**Definition**: "The opsonic index is the ratio of the number of bacteria ingested by a given number of phagocytes in the presence of the patient’s serum, to the number ingested by the same number of phagocytes in the presence of normal serum."

**Technique**: By means of a capillary pipette, equal volumes of the patient’s serum, a suspension of washed leucocytes derived from a normal blood, and an even emulsion of the organisms are mixed together and incubated at 37°C. for 15 minutes. Films are spread and stained and the phagocytic count made.

Unless/
Unless the test is performed by competent bacteriologists with considerable experience of the technique, it is of little value, as in no other serological test is the personal error so prominent. Moreover, in diseases, in which the serum has high agglutination or bacteriolytic properties, the technique fails as it is essential that the organisms be in an even emulsion if regular phagocytosis is to take place.

In my hands it has been a complete failure when attempting to estimate the opsonic index of the sera of rabbits injected with B. typhosus and B. suipestifer.

As a routine measure, the test has fallen into disuse. This is due partly to the difficulties of the technique and the long period required for the test, but mainly to the fact that the resulting information has not the important significance which the originators of the process ascribed to it. Thus it is of doubtful value in diagnosis and prognosis. Wright placed the greatest value on the opsonic index as a guide to the size and frequency of the dose of bacterial vaccines in the treatment of disease. By means of it, he claimed to eliminate the injection of vaccines during the period called by him the negative phase. Recent investigations have thrown considerable doubt/
doubt on the occurrence of a negative phase. In any case, the best guide in vaccine therapy is the clinical evidences of reaction and the condition of the patient. No useful purpose would be served therefore by going into the question of opsonins in greater detail.

ANTITOXINS

Definition: "Antitoxins are antibodies that are capable of directly and specifically neutralising the dissolved toxins that caused their production."

Historic: In 1890 von Behring showed that the blood serum of animals actively immunised against diphtheria and tetanus would protect normal animals against these diseases. Furthermore, the immune serum did not possess bactericidal properties.

In 1891 Ehrlich found that specific antitoxins could be produced for the vegetable poisons, ricin and abrin. In 1894, Calmette produced antitoxins (antivenins) to snake poison.

The sites and methods of antitoxin formation is still unknown. It is generally agreed that when a toxin is introduced into circulation, it anchors itself to the tissue cells and stimulates them to produce antitoxins. These antitoxins cannot be manufactured by the serum, as a living cellular activity affords the only explanation of the following facts."
facts. An animal whose blood contains a stable concentration of antitoxin (tetanus) may lose by repeated bleedings as much antitoxin as was contained in the total blood at the commencement of the experiment; and yet without further injection of toxin, it may replace in the circulation as much antibody as was abstracted.

Glenny and Sudmersen showed that a guinea pig immunised with diphtheria toxin maintained the same concentration of antitoxin in its blood for a period of from one to two years. During this time, it almost doubled its weight and gave birth to eight young, the blood of which contained at birth the same concentration of antitoxin as that of the mother. It is therefore certain that a continuous cellular activity is the most important part of antibody formation.

**Nature of Antitoxins**

Since antitoxins have never been obtained completely free from serum proteins, their exact chemical nature has never been discovered. Brodie was the first to show that antitoxin is completely precipitated from solution by any means which removes the globulins. Antitoxins are far more stable than toxins and are scarcely affected by heat up to 62° C. Boiling completely destroys them. Antitoxic sera

when/
when kept in a cool place and protected from light and air keep with little deterioration for a period of from one to two years.

**Natural Antitoxins.**

These substances undoubtedly exist in the blood of both human beings and animals. With regard to natural diphtheria antitoxin, it is probable that it is transferred to the foetus from the mother via the placental circulation. Infants under one year give a negative Schick reaction and are very insusceptible to the disease. According to Dick, his intradermal reaction shows the same phenomenon occurs in scarlet fever. With regard to natural antibodies to other infections, they are more likely to have resulted either from a transference of antigen to the foetus during its uterine life or else from a uterine attack of the disease or even a mild unrecognised attack during early infancy.

**The Schick test for natural diphtheria antitoxin**

In 1913 Schick (19) published his researches into a skin test which enabled an estimation to be made of the presence of natural immunity to diphtheria in a human being. His discovery has proved to be of paramount importance. By means of this test the susceptible members of the community can be found and by active immunisation can be protected from infection.
A similar test for scarlet fever has been devised by the Dicks. By means of the intradermal injection of the soluble filtrate of a broth culture of a haemolytic streptococcus, isolated from the throat of a patient suffering from scarlet fever, susceptible individuals can be separated from those naturally immune and can be actively immunised by a streptococcal vaccine. This work, which was started some two years ago, has been examined sufficiently now to afford the belief that it will be of benefit in the eradication and prevention of scarlet fever. Since the Schick test, which is very similar, has had a much longer trial and is of equal importance, I propose to give the details of the methods employed in performing it.

The test consists of the intradermic injection of a minute dose of diphtheria toxin. If the individual possesses an amount of natural antitoxin equal to at least 1/30 of a unit in each c.c. of blood serum, the injected toxin is neutralised and no reaction follows. If, however, the individual has no antitoxin in his body fluids, the injected toxin acts as an irritant to the skin, producing in 24 - 48 hours a small area of redness and oedema. A positive reaction, therefore, indicates that he is susceptible to diphtheria while a negative suggests he is at least relatively/
relatively immune. Individuals who have been exposed to infection and show a positive reaction should be injected prophylactically with antitoxic serum. In the case of school children, among whom epidemics regularly occur, all positive cases should be actively immunised by injection of the toxin-antitoxin mixture.

**BACTERIAL AGGLUTININS**

**Definition**: "Agglutinins are antibodies that possess the power of causing bacteria suspended in a fluid to adhere together and form clumps."

**Historic**:

Grüber and Durham \(^{20}\) and Bordet \(^{21}\) were the first to discover the phenomenon of agglutination. Widal \(^{22}\) found that sera of patients suffering from typhoid fever produced agglutination of a suspension of the homologous organism and turned this fact to practical use by instituting the diagnostic test now called the Widal reaction.

Two years later Bordet discovered that agglutinins for red blood corpuscles existed in certain sera.

Agglutinins are of two types:

1. Normal
2. Immune

\(^{1}\) Normal agglutinins are found in the sera of animals or persons who have neither suffered from infection nor been actively immunised with the homologous organism./
organism. Since the serum of new born children is devoid of normal agglutinins (23), it is more than likely that the so-called normal agglutinins may be in reality acquired properties. Where normal sera have high agglutinating properties, it is certain that a previous unrecognised attack of the disease has occurred. Normal agglutinins are always of a relative low titre, seldom going as high as 1 in 10 dilution. Occasionally in the typhoid paratyphoid group of organisms, apparently normal serum possesses an agglutinating titre up to 1 in 30.

(2.) Immune agglutinins are the substances which are found in the serum of an animal or person as a result of infection or systemic immunisation with the organism.

Agglutinogens

Agglutinins are produced from substances contained in the micro-organism called agglutinogens.

Agglutinogens may be excreted by the bacteria or may be liberated by their disintegration. Thus the filtrates from old broth cultures when injected into animals produce specific agglutinins.

The nature of agglutinins.

Although found in all the secretions and fluids of an infected person, agglutinins occur in by far the highest concentration in the blood. They withstand heat up to 60°C for 30 minutes without deterioration.

They/
They resist drying and are best preserved in this state. Along with other antibodies they exist mainly in the globulin fraction of serum and are therefore precipitated by ammonium and magnesium sulphates.

They differ from bacteriolysins in two important respects. First, they are unaffected by heating to 55°C, while bacteriolysins are rendered inactive owing to this temperature destroying complement. Secondly, agglutinated organisms are uninjured while bacteriolysed ones are destroyed.

**Mechanism of Agglutination**

The exact manner by which agglutination takes place is still an unsettled question. Various theories exist.

(1.) It may be that the agglutinin makes the bacterial membrane more viscous, thus producing clumping of the organisms into masses. No visible changes, however, can be seen by microscopic examination.

(2.) Again it is suggested that the flagella of bacteria are the important factor in agglutination. This is probably partially correct but does not explain how agglutination occurs in non-flagellated organisms. It is of interest to note that Smith and Reagh (24) found that two agglutinins could be obtained from B. suipestifer, one of which acted on the bodies while the other acted on the flagella (25).
The theory which has most to recommend it may be called the Colloidal Theory. Bordet (26) found that if the salts were removed from the serum and from the bacterial suspension by dialysis, that when the two were mixed no agglutination occurred. He believes that the agglutinin acts on the bacteria by altering the relations of molecular attraction between them and the surrounding fluid. The loss of motility and clumping results from the salts altering the electric conditions of the colloidal agglutinin-bacterium combination with increase of their surface tension. To overcome this increased surface tension, clumping is necessary as a mass of organisms presents less surface tension than if the individual remained separate. Bordet gives as an analogy the case of potter's clay which, if made into a fine emulsion with distilled water, remains in suspension. If a little salt is added, the clay immediately clumps and falls to the bottom in a manner identical to agglutination.

It is probable therefore that the following effects take place:

First, a vital reaction between the antigen and tissue cell results in the production of the agglutinating antibody which is carried through the body in the blood stream. This reaction is essentially specific. When the serum comes in contact with the/
the organism, a result - agglutination - takes place which can be explained by the laws of molecular physics.

**Specificity of agglutinins**

When first discovered, agglutinins were considered to be highly specific. It is recognised now that an agglutinating serum for a specific organism (e.g. *B. typhosus*) will agglutinate other organisms that are morphologically, biologically and often pathologically closely related (e.g. *B. paratyphus A & B*).

These additional agglutinins are called group agglutinins and exist in the serum of the immunised animals in much less concentration than does the main agglutinin. Thus typhoid serum might agglutinate *B. typhosus* in 1 in 5,000 and *B. paratyphosus B* in 1 in 50. If a patient suffers from a mixed infection, for example typhoid and paratyphoid fever, his serum contains the main agglutinins and group agglutinins of both organisms. By means of the absorption test devised by Castellani (27) it is possible to differentiate between single and mixed infections.

**The Relation of Agglutination to True Immunity.**

It has already been stated that animals may be immune to an infection without possessing any agglutinins in their serum. Again, animals may have a high agglutinin content and yet be susceptible. The agglutinated bacilli/
bacilli are in no way injured and cultures from agglutinated organisms show them to be alive and as virulent as before agglutination took place.

The relation of agglutinins to immunity is doubtful therefore. They are, however, of great diagnostic value and can certainly be used as indicators of the vital reaction between antigen and living tissue cells on which the whole basis of our knowledge of immunity rests. In addition, agglutinins may play an important subsidiary role in preparing organisms for bacteriolysis and phagocytosis. An important observation by Bull is worth recording. He states that experimental agglutination may occur in vivo and the power of the blood to cause agglutination determines in a large measure whether, after their direct introduction experimentally, the bacteria are promptly removed from the circulation and bacteraemia avoided. Thus, the rapid disappearance of pneumococci from the blood of a rabbit, following the intravenous injection of the homologous antipneumococcal serum may be explained on this basis. If in vivo agglutination is incomplete and a few organisms escape, a fatal septicæmia results. Thus, according to Bull, agglutinins, opsonins and phagocytosis are closely related and exert an important rôle in the recovery from disease.

Technique/
Technique of the Bacterial Agglutination Reactions.

Two methods are available:

1. Microscopic
2. Macroscopic

**Microscopic method:**

Equal volumes of an even emulsion of the organism and various dilutions of serum are mixed on a slide, covered with a cover glass and examined microscopically. Slowing followed by loss of motility and finally clumping indicates a positive reaction. The microscopic method has the advantage of being quick and requiring small amounts of serum but otherwise can be compared in no way to the macroscopic method for scientific investigation. As the macroscopic method is the method of choice and is used practically always nowadays, there is no need to give further details of the microscopic methods.

**The Macroscopic Method:**

**Bacterial Suspension:**

This may be made by washing the growth off the surface of solid medium by normal saline solution or by using broth cultures of the organism. It is essential that young cultures be used and that an even emulsion be obtained.

Dreyer has found broth cultures, sterilised and killed by 0.1 per cent formalin to be the best.

**Serum**

The serum should be clear and free from cells.
The Test:

The test consists of adding equal volumes of the emulsion of organisms to the same volumes of different serum dilution in agglutinating tubes.

It is a matter of indifference whether the serum dilutions are made with a 1 c.c. pipette, a marked capillary pipette or one of Dreyer's graduated drooping pipettes, provided the same method is used through the whole investigation. Personally, I use a drawn capillary pipette, on which a blue circle is marked about half an inch up the stem, by means of a grease pencil. The volume contained up to this mark is usually about 0.5 c.c. Into each tube, this unit volume of saline is put. Into tube I, unit volume of undiluted serum (or whatever dilution is required) is placed and mixed thoroughly by aspirating up and down on the pipette by means of the rubber teat. Unit volume of the mixture is removed to tube II, where the process is repeated and unit volume removed to tube III, and so on. At the last tube, unit volume is discarded. As a result, we have dilutions of serum with tubes as follows:

- Tube I. 1 in 2
- Tube II. 1 in 4
- Tube III. 1 in 8 etc.

The next procedure is to put unit volume of the even suspension of the organism into each tube and cause/
cause a thorough mixture to be formed. The dilution of serum in each tube will therefore be doubled and will now be:

- Tube I: 1 in 4
- Tube II: 1 in 8
- Tube III: 1 in 16 etc.

Finally equal parts of bacterial suspension and saline are placed in a tube as a control. The tubes are then placed in the incubator either for two hours at 37°C or, as I prefer it, one hour at 55°C in the water bath, when the results of the test may be read.

When agglutination is complete, all the organisms will be at the bottom of the tube as a dense white sediment of clumped bacteria while the supernatant fluid will be perfectly clear. This may be denoted as follows ++++. Where there are both clumps of organisms in suspension as well as on the bottom of the tube, it can be designated as follows ++. While if definite clumping can be seen in the suspension but there is no sedimentation, it can be shown as follows +. A hand lens may help in reading this category. The control tube should remain unchanged as an even emulsion of the organisms.

Agglutinoids and Proagglutination

On examination of the tubes after incubation, it may occasionally be noted that the tubes containing
the serum in highest concentration show little or no agglutination while the more diluted sera have produced complete agglutination and sedimentation. This phenomenon is of practical importance as ignorance of the fact may cause an investigator to report that a serum has no agglutinins through not having made sufficient dilutions. It is known as the "Zone phenomenon" or "proagglutination." The explanation is uncertain but, according to Ehrlich's side chain theory, it is due to the action of agglutinoids, which have a stronger affinity for agglutinogen than has agglutinin. When the serum is diluted to the extent at which agglutinoids have no influence on the reaction, the agglutinins are still present in sufficient quantity to bring about agglutination.

From my own practical experience, I believe that though the "zone phenomenon" undoubtedly occurs, it does not deserve the prominence given to it in text books. During the past two years I have done several thousand agglutination tests with the typhoid-paratyphoid group of organisms and have never seen it occur. When doing pneumococcal typing, I have noticed that the tube containing undiluted serum often takes longer to produce agglutination than the tube containing 1 in 10 dilution. In all cases, however, both tubes eventually showed complete results.

PRECIPITINS/
Definition: "Precipitins are specific antibodies that develop in the serum of animals inoculated with bacteria or with solutions of animal or vegetable albumins which possess the power of producing a precipitate in a clear solution of the particular albumin or culture filtrate against which the animal has been immunised." (Kolmer)

Historic: In 1897 Kraus first described bacterial precipitins. He stated that when serum from animals immunised with cholera or typhoid bacilli was added to a clear filtrate of the broth cultures of the homologous organism, the solution became turbid and a precipitate formed.

The reaction was essentially specific. In 1899, Bordet found that the serum of rabbits inoculated with the serum of chickens gave a precipitin reaction when mixed with chicken serum.

In 1901 Wassermann and Schultz demonstrated the value of the precipitin test in differentiating the blood and secretions of man from other animals. These discoveries were of the greatest value to Forensic Medicine.

The Nature and Mechanism of Precipitation.

Except for the fact that in the precipitin reaction an albuminous solution is used, whereas, in agglutination a bacterial suspension is employed, the two/
two phenomena appear to be identical. Both are essentially specific but in addition show a group action for closely allied species. Both result from the interaction of the injected antigen and the tissue cells.

The antigen (bacterial emulsion) which produces the agglutinin is called an agglutinogen, while the albuminous solution which produces the precipitins in a serum is called the precipitinogen. The mechanism of both reactions is due probably to colloidal action. Both are thermo-stable to a temperature of 60°C. for half an hour. Both are precipitated in the globulin fraction of the serum.

The relation of precipitins to immunity is similar to that of agglutinins. They are of importance only indirectly, unlike antitoxins or bacteriolysins which actually neutralise the toxin or destroy the invading organism, they merely sensitise the antigen for final destruction by other agencies.

The Precipitate

The origin of the precipitate is of interest. Since a large albuminous precipitate may result from the addition of a greatly diluted precipitinogenous fluid to a high titre immune serum, it is certain that most of the albumins deposited cannot have come from the precipitinogenous fluid. Moreover, if the precipitating/
precipitating serum is diluted, the precipitate becomes smaller and smaller. For these reasons, Welsh and Chapman (34) consider that the precipitate originates mainly in the immune serum and believe that it consists of the insoluble modification of the previously soluble proteins of the precipitin. A portion of the precipitate, however, is derived from the precipitinogen.

**Practical application.**

From the practical point of view in diagnosis of disease or in the identification and differentiation of bacteria, I consider the precipitin test to be inferior in every way to the agglutination test. Satisfactory results are not obtained so easily and where a quantitative result is desired, not only do I find it not so delicate as agglutination, but also, it is difficult to read the results in the tubes containing high serum dilution. With the exception of the test as used for typing pneumococci, I have given up the method completely. On the other hand, when used as a test in forensic medicine or as a means of detecting the adulteration of food stuffs etc., it is of the greatest practical value.

**Bacteriolysins**

**Definition:** "Bacteriolysins are substances in the serum and body fluids that kill bacteria with or without lysis."

**Historic**
Historic:

In 1886 Fodor demonstrated the germicidal action of defibrinated rabbit's blood upon anthrax bacilli. In 1890 Buchner corroborated the finding of bactericidal bodies in blood and showed that they were destroyed by heating at 55°C. for half an hour.

In 1894 Pfeiffer demonstrated the lysing of cholera vibrios after injection into the peritoneal cavity of an immunised guinea pig. In 1899 Bordet showed that there were two factors in bacteriolysis, one which was thermo-labile and which he called "Alexin"; the other was thermo-stable which he called "Substance sensibilatrice". Later Ehrlich corroborated these findings and renamed the substances "Complement" and "Amboceptor", by which names they are usually called today.

The Origin and Nature of Bacteriolysins

According to Metchnikoff, bacteriolysins are derived from the leucocytes. That leucocytes contain a bacteriolytic substance is undoubtedly true.

Thus, Denys and Havet were the first to show that exudates rich in leucocytes show a higher degree of bacteriolytic power than does the corresponding serum. This was confirmed by Hiss and Zinsser. In addition, these authors state that the injection of autolyzed leucocytic exudates into animals infected with/
with pneumococci and streptococci produced beneficial results.

Gerrow (35) studied the question in great detail and came to the conclusion that the leucocytic extracts were definitely bactericidal but were different from complements in being really endolysins.

There is general agreement that true bacteriolysins are produced by a general cellular reaction and not particularly by any one type of cell.

Bacteriolysins are thermostable and resist heating to 60°C. but are destroyed by temperatures of 70°C - 80°C. Bacteriolysis can not take place in vitro without the presence of complement. Thus serum heated to 55°C. is without effect, not because the bacteriolysin is injured but because the complement is inactivated.

The mechanism of bacteriolysis is explained by Ehrlich as follows:- The bacteriolytic antibody acts as an interbody or connecting link (Amboceptor) which joins the bacterial antigen to the complement. The amboceptor is essentially specific for the antigen but does not injure it. Lysis takes place when the complement becomes attached. Metchnikoff and Bordet believe the bacteriolysin acts as a sensitizer preparing the bacterium for the action of complement, just as a mordant helps in the penetration/
penetration of a dye stuff. Thus apart from the leucocytic extracts, bacteriolysis depends on complement being present.

**COMPLEMENT**

**Definition:** "Complement is the substance present both in normal and immune serum, which is destroyed by heating to 55°C. for half an hour and which acts with an amboceptor or sensitiser to produce lysis."

**The nature of Complement:**

Complement is found to a greater or less degree in the fresh serum of all animals. It is derived probably from the circulating leucocytes either as a secretion or a result of their disintegration.

Since complement from a fresh serum will produce both bacteriolysis as well as haemolysis, the question arises whether one complement unites with all amboceptors or whether several complements are present in one serum and these act specifically with different amboceptors. Bordet showed that a complement which will activate either a haemolytic or a bacteriolytic amboceptor may be absorbed out of a serum by furnishing excess of either amboceptor. From this he states that only one complement exists in a serum.

Ehrlich/
Ehrlich and Morgenroth (37) believe that many different complements are present in varying amounts in different sera.

Their experimental results are very convincing, and the doctrine of multiplicity of complements is accepted generally today. From a practical point of view, the question is fortunately of little consequence, since in all tests, in which complement is required, whether haemolytic, cytolytic or bacteriolytic, the serum of a guinea pig furnishes not only the most satisfactory but also the largest quantity of complement.

When bacteriolytic sera are administered therapeutically, the patient's own serum supplies the complement.

While the true nature of complement is unknown, it is evident that it bears a very close resemblance to a ferment in every way except one. Thus, unlike enzymes, complement is used up during cytolysis. A definite quantitative relationship exists between the complement and the sensitized red cells on which it acts.

Role of Bacteriolysins in Immunity

Bacteriolysins are extremely specific. Kolmer states that a group action for closely allied species of organism does occur, but on a much smaller scale/
scale than in agglutinins and precipitins. Bacteriolysins are produced both by disease and by artificial immunisation but the amount produced depends greatly on the antigen. Thus the pathogenic cocci produce relatively small amounts of bacteriolysin while certain Gram-negative intestinal bacilli produce the most. They are therefore of greatest importance in the process of resistance and recovery from the diseases due to these latter organisms. In addition, it is supposed that the curative value of antipneumococcal and antimeningococcal sera lies in their bactericidal properties.

**Technique of Bacteriolytic Tests**

The first bacteriolytic test was made by Pfeiffer who introduced a culture of cholera vibrios into the peritoneal cavity of a previously immunised suinea pig, and by withdrawing and examining the peritoneal fluid, found that lysis of the organism had taken place. The same result was produced when the culture and some immune serum were introduced into the peritoneal cavity. In both cases, the animal supplied the complement.

This method is employed still in the identification of suspected cholera cultures, but is of little value for the typhoid-paratyphoid group of organisms.

The/
The laboratory methods of measuring the bactericidal power of the blood by in vitro experiments are of three kinds:

(1.) The Plate Culture Method of Stern and Korte

It consists of mixing different dilutions of the test serum with fixed quantities of bacterial emulsion and fresh complement and incubating for three hours at 37°C.

The contents of the different tubes are mixed with melted agar and poured into Petri dishes. The plates are incubated for twenty four hours and the colonies are then counted. Control plates containing normal serum, bacillary emulsion and complement are incubated in the same way.

If bacteriolysins exist in the immune serum, few or no colonies should be seen, while the control plates should show thousands.

(2.) The Capillary Pipette Method of Wright.

In this method, looped pipettes are used. Into them are sucked varying dilutions of culture with a constant quantity of the fresh unheated test serum. Mannite litmus broth is then aspirated into the bulb and the pipette incubated for twenty four hours at 37°C.

The formation of acid is shown by the litmus turning/
turning red, thus proving that some bacilli (typhoid test) have escaped bacteriolysis. The bactericidal power of the serum is measured by finding the largest number of bacilli which will be killed by a constant quantity of serum.

(3.) The Lacy-Heist Method of Determining the Bactericidal Activity of the Blood.

The method is especially valuable when examining the bactericidal content of pneumococcal or meningococcal immune sera.

Explained briefly, the method consists of drawing into capillary tubes of about 0.5 m.m. diameter equal volumes of different dilutions of a blood broth culture of pneumococci ranging from the pure culture to 1 in 1,000. The cultures are then gently blown out thus leaving some organisms adhering to the side of the capillary stem. The immune animal is bled and by capillarity a drop of blood is taken up into each tube. The ends are sealed and the tubes incubated at 37°C for twenty four hours. The ends are then broken, the contents expelled on to slides, stained and examined microscopically. A similar process is done with normal blood. By this means we can find if bacteriolysis occurs and, if so, what is the largest number of organisms that a fixed amount of immune blood can lyse.
The details of these methods will be found in the second half of this thesis.

Author's opinion

While bacteriolysis undoubtedly occurs as is testified by large numbers of investigators, in my hands I have found these tests to be universally unsatisfactory. All three methods were tried and the results are stated later.

I obtained no evidence of bacteriolysis whatsoever in the serum of rabbits immunised with B. typhosus or B. suipestifer, in spite of the fact that these sera were highly potent in agglutinins and in complement fixing bodies.

If bacteriolysis plays the important part in immunity which is ascribed to it, I consider that this must be due to the conditions in the body being quite different from those in vitro. That Pfeiffer's phenomenon exists is beyond question. There, however, the organisms are not merely in contact with an immune serum but are surrounded by living cells actually producing defensive measures. Bacteriolysis in vivo is probably influenced by the action of bacteriotropins and phagocytic cells.

The Complement Fixation Test

Historic:

While seeking evidence in favour of his theory that/
that only one complement existed in a serum, Bordet was led to the discovery of this most important serological test.

Bordet and his pupil, Gengou found that if, to a mixture of an emulsion of plague bacilli, heated antiplague serum and fresh guinea pig's serum (complement) which had been allowed to stand at room temperature for four hours, be added a mixture of rabbit's red corpuscle and anti-rabbit haemolytic serum, no haemolysis occurred. When heated normal serum was substituted for antipeet serum, haemolysis occurred.

He concluded that in immune serum, a specific sensitiser was present which united with the antigen and prepared it for direct union with the complement. No free complement being left, lysis of the red blood corpuscles did not take place, the heated sensitising haemolytic serum of itself being inactive.

Gengou showed later that all proteins were capable of producing complement fixation bodies.

Neisser and Sachs showed that by the Complement Fixation Test, the most minute traces of different proteins could be differentiated. They were the first to realise by this test, that if the antigen is known, the antibody can be found and vice versa — if the antibody is known, the antigen can be found.

Wassermann/
Wassermann and Sachs in 1906, encouraged by these results, investigated the sera and cerebrospinal fluids of syphilitic patients. Their antigen consisted of a saline extract of a syphilitic foetal liver. They examined 257 cases of syphilitic disease in Neisser's clinic and only obtained nineteen percent of positive reactions. Later by an improved technique in which alcoholic extract of a syphilitic liver was used as the antigen, very satisfactory results were obtained (Wassermann, Neisser and Bruck, Deutsche Med. Woch. 1906).

Up to this period the Wassermann test was believed to be a simple complement fixation test identical to the Bordet Gengou phenomenon. In Jan. 1907 Weygaudt (44) and Marie and Levaditi reported that positive results could be obtained using extracts of normal spleens and livers as antigens. These findings, together with the improper use of the test in unskilled hands, delayed for several years the appreciation of its great value in the diagnosis of syphilis.

Today it is recognised that, while a luetic serum contains specific antibodies, yet to perform the test, a specific antigen (Treponema pallidum) is unnecessary, and in fact, the most sensitive results are obtained/
obtained from using a cholesterolised alcoholic extract of beef heart muscle. A true explanation of these facts is still lacking. Thus the Wassermann reaction, though very similar, must not be regarded as a true complement fixation, in which antigen and antibody must be essentially specific.

**Nature of the Antigen and the Complement Fixing Antibody.**

The antigen must consist of protein molecules. Polypeptides and amino-acids produce no complement fixing antibodies. The possibility of lipoids acting as antigen is of great interest. Niyers, Bergel and Meinicke state that they find lipoids to be antigenic while Ritchie and Miller, Teale and many others hold the opposite view. Fitzgerald and Leathes injected rabbits with substances obtained from liver which were capable of acting as antigens in the Wassermann reaction. On testing the rabbits' sera, no complement fixing antibodies were found. It must not be taken for granted that because some lipoidal substances serve as so called "antigens" in the Wassermann test that lipoids can act therefore as true antigens.

The nature of the complement fixing antibody is not known. It must be similar to other antibodies such as bacteriolysins and precipitins. In fact, it has been claimed that complement fixation is really only/
only a more sensitive test for bacteriolytic amboceptor. Neufeld and Hendel (43) have produced experimental evidence to show that the bacteriolytic and complement fixing antibodies are separate substances. Hendel reports similar findings with typhoid and cholera immune sera and Torrey produced similar information from his investigations with antigonococcal serum.

In 1905 Moreschi and also Gay (44) showed that the precipitate formed from the precipitin and homologous antigen fixed complement and that the degree of complement fixation bore a relation to the amount of precipitate formed. This explanation was challenged by Neisser and Sachs, Muir and Martin and others. Dean (45) studied the question in great detail and came to the conclusion that the two reactions represent two phases of the same reaction and that "flocculent precipitation represents the final stage of a change which can be recognised in its earliest and incomplete stage by means of complement fixation reaction."

The question must remain unsettled at present but there can be no doubt that precipitation bears an important relation to complement fixation.

Although the nature of the complement fixing antibody is uncertain, all investigators agree that it is/
is highly specific for the antigen engendering its production. The complement fixation test, when properly performed, is the most delicate method known of detecting minute traces of antigen or antibody. Complement fixing antibodies can be detected in the serum of an immunised animal in smaller amounts and at an earlier date than agglutinins or precipitins. Those organisms which produce high titre agglutinating sera (e.g. the Gram-negative coli-typhoid group) produce well marked complement fixing antibodies, while the Gram-positive cocci, especially the pneumococcus, produces only traces of the antibody.

The Complement Fixation Test

Only the broad principles of the test will be described in this chapter as the details are given in the experimental investigations detailed in the second half of the thesis.

Four constituents are necessary in the test:

(1) Antigen.
(2) Immune serum.
(3) Complement.
(4) The Haemolytic system.

A satisfactory adjustment of the amounts of these constituents is essential; this can be done only by preliminary titrations before performing the actual test.

ANTIGEN:

Since/
Since the test is essentially specific, the antigen must consist of the same protein constituent as caused the formation of the antibody. For bacteriological purposes, this is bacterial protein in some form or other, usually a simple saline bacterial emulsion. Various extracts of the organism can be used. In the case of Besredka's tuberculous antigen, not only the tubercle bacillus but also the culture medium is included in the antigen. However produced, it must consist of a homogeneous even emulsion.

**Titration of the antigen.**

If too large amounts of antigen be used, non-specific absorption of complement occurs, giving a false positive reading in the test. The antigen is then said to be anticomplementary.

Too small quantities of antigen may fail to fix specific antibodies present, giving a false negative reading. To avoid these possibilities, it is essential that the antigen be carefully titrated. A preliminary complement titration is performed and the unit of complement estimated. Varying amounts of the antigen are titrated with unit volumes of complement and the largest amount of antigen that is not anticomplementary is chosen as the unit of antigen. Instead of the immune serum, saline or normal serum is used in order/
order that the total volumes in the tubes shall be the same as in the actual test. While not absolute necessary, it is better to use normal serum than saline as it is generally found that the serum and antigen fix less complement than the antigen alone. Thus, antigen and saline might fix three units of complement while antigen and normal serum fixed only one unit. It would therefore be correct to deduct one unit from the final reading in the actual test as being due to non-specific fixation of complement by the antigen.

**Titration of serum:**

The same principles apply to the titration of the immune serum. Too large quantities give a non-specific fixation apart from any specific antibodies present: while too small amounts contain insufficient antibodies to produce a definite positive reading. In syphilis and the coli-typhoid group of infectious diseases where large amounts of complement fixing bodies are present, minute quantities of immune serum produce excellent results. In tuberculosis and pneumonia where antibody production is small, the maximum amount of serum which is not anticomplementary should be employed. This is usually about 0.1 c.c. The serum titration is made by titrating varying quantities/
quantities of serum (heated to 55°C. for half an hour to inactivate natural complement) against unit volumes of complement, the antigen being replaced by the same volume of saline.

**COMPLEMENT**

From practical experience, it is found that the serum of a guinea pig furnishes the most satisfactory complement. The animal is bled the night before; the blood is placed in the ice chest overnight and the serum removed next morning. It should be clear and free from cells or deposit. To find the unit of complement, dilutions of guinea pig's serum from 1 in 10, 1 in 20 etc. up to 1 in 120 are made. Unit volumes of these dilutions are mixed with the same volumes of sensitised cells and two unit volumes of saline to replace the immune serum and antigen which are absent. The tubes are shaken and incubated for half an hour at 37°C. when the results are read. Unit volume of the dilution of guinea pig's serum which produces complete haemolysis is taken as the unit of complement. In a quantitative test, this is called the minimum haemolytic dose.

Complement deteriorates rapidly at first, but in a few hours the decline becomes much slower. Hence the complement titration done in the morning should
some fifteen hours after the animal has been bled is liable to very small change during the two hours during which the complement fixation test is being done.

While it can be taken as a general rule that complement steadily deteriorates, it does not always follow that this is the case. If kept in the ice chest, little or no change may be found twenty four hours later.

The Haemolytic System:

The Haemolytic System consists of:

1. Haemolysin
2. Red Blood Corpuscles

It has been found that the most satisfactory haemolytic system consists of sheep's red corpuscles and anti-sheep haemolytic serum.

The haemolysin is prepared by immunising rabbits with washed sheep's corpuscles. The intravenous injection of five doses of 5 c.c. each of a ten per cent suspension of washed sheep's cells every four days into a rabbit, will cause a high titre haemolytic serum to be developed. The serum must then be titrated against red cells. The haemolytic dose or unit is the smallest quantity of haemolysin which will produce complete lysis of the given quantity of sheep's red blood corpuscles.

In/
In the actual complement fixation test, it is best to use four or five haemolytic doses. For instance, if 0.2 c.c. of 1 in 1,000 dilution of the haemolytic serum produces complete lysis in the titration, it would be wise to use 0.2 of 1 in 250 dilution in the test proper. This represents four haemolytic doses of haemolysis.

Red Blood Corpuscles

Sheep's blood is collected in a sterile vessel containing glass beads. Shaking for ten minutes produces defibrination of the blood. The cells must be washed three times with an excess of saline until the supernatant fluid is practically colourless. All traces of serum should then have been removed. The washed cells must now be suspended in saline.

In the original Wassermann reaction, a five per cent suspension was used. The results are sharper and easier to read if a less heavy suspension is employed. In my experience, a two or three per cent suspension is the most satisfactory.

The haemolytic system acts the part of an indicator and demonstrates visibly whether complement has been fixed or is free, in the same way that litmus shows whether an acid is present or not.

The value and accuracy of the complement fixation test depends on the proper adjustment of complement, cells/
The technical details and the various quantities of the different constituents will be found in the description of the experimental studies in the second half of the thesis.

Practical Application of the Test

If the antigen is known, the nature of an unknown antibody can be found. On the other hand, if the antibody is known, an unknown antigen can be determined. As a rule, a known antigen is used to find out if a serum contains the homologous specific complement fixing antibody.

The test is most commonly employed in the diagnosis of syphilis but is also of value in numerous bacterial infections such as gonorrhoea, tuberculosis, whooping cough, etc.

In addition, it yields even more sensitive results than the precipitin reactions in the differentiation of proteins and is therefore of value in medico-legal work.

Non-Specific Immunity

During the past fifteen years, a considerable amount of clinical evidence has been brought forward to show that the injection of non-specific foreign protein into a patient may influence favourably the course/
course of an infection; this therapy is known as "protein shock therapy" and the beneficial results which are said to occur are due to a non-specific reaction. The non-specific agents most commonly employed are:

1. Non-specific vaccines (e.g. typhoid vaccine in gonorrhoea or B. coli vaccine in typhoid fever)
2. Normal or non-specific immune horse serum.
3. Cow's milk.
4. Peptone.

Sufficient amounts of these foreign proteins must be injected to produce a reaction, if beneficial results are to be obtained. On the other hand, the excessive production of protein shock must be avoided, as being not only harmful but actually dangerous.

**Non-Specific General Reaction**

Depending on the dosage, the route of administration, the disease and the physical state of the patient, the general reaction produced by the injection of any foreign protein is the same no matter which of the non-specific agents is employed.

Within a period of half an hour to several hours, the patient complains of shivering or even of a severe chill. At the same time, there is an exacerbation of the local symptoms. The chill is followed by/
by fever varying from 100° to 104° F. If pyrexia is already present, the temperature is further raised. Increase of the pulse rate, sweating, headache, nausea and even vomiting occur, their degree depending on the intensity of the reaction.

In properly regulated treatment, the patient should have returned to his normal state at the end of twenty-four hours.

The Alteration of the Constituents of the Blood.

In addition to the physical symptoms described, other changes take place within the body.

A series of leucocyte counts shows, that following the injection, there is first a temporary leucopenia followed in a few hours by a definite leucocytosis which is mainly due to increase of the polymorphonuclear leucocytes: in addition, Cowie and Calhoun (46) state that there is an increase of red cells and platelets. This is, however, doubtful.

Jobling, Peterson and Eggstein (47) state the injection of non-specific agents is followed by "a mobilisation of proteolytic enzymes as well as of lipases" which are summarised as follows in the case of human beings:

(1) Concentration of serum.
(2) Practically no changes in the non-protein nitrogen of the blood.
(3)
Progressive increase of serum protease
" " " pepsidase
Irregular changes in the lipases.
Increase of the antifcrment (antitryptic) activity of the serum.

The Influence of Non-specific Agents on Antibody Production.

The experimental evidence on this question is both contradictory and unsatisfactory. Thus, Hektoen, Bieling, Flascheder and Dolken state that in typhoid fever, the administration of non-specific agents increases the antibody production. On the other hand, Baluit, Ludke, Marck and many others state that they could find no increase of antibodies. Uddgreen observed that the intramuscular injection of milk in Wassermann negative syphilitic patients frequently acted as a provocative and resulted in the production of a positive Wassermann reaction.

Additional methods of production of non-specific effects.

In addition to the methods already described, the benefits of the following therapeutic measures are generally stated to be due to non-specific protein effects.

The production of a sterile artificial abscess following the injection of turpentine.

(2) /
(2) The introduction of malarial parasites into the bloodstream of patients suffering from general paralysis: the interposition of another injection is believed to be beneficial. It is stated that an attack of malaria or influenza will raise the agglutinating titre of the serum of a patient suffering from typhoid fever, or who has suffered from the disease and in whose serum the agglutinins have practically disappeared.

(3) It is possible that the benefits resulting from the action of counter-irritants, such as the cautery and the blistering fluids, are at least partly due to non-specific effects. Similarly, the therapeutic value of such drugs as the Iodides may be due to similar causes.

Experimental proof of non-specific immunity.

When considering this question, it is necessary to distinguish clearly between reactions which are only non-specific and reactions which are both non-specific and connected with immunity. Thus, the Wassermann reaction is not a true complement fixation test since the antigen may consist of the alcoholic extract of normal organs. This non-specific reaction/
reaction is, however, in no way directly connected with the production of immunity to syphilis.

Similarly, it has long been known that the serum of normal rabbits may give a positive complement fixation test with the ordinary Wassermann antigen.

In addition, I have found that normal rabbit's serum may fix ten units of complement in a pneumococcal complement fixation test using a broth culture of the organism as an antigen. This again has no connection with immunity as the rabbit is extremely susceptible to the pneumococcus.

Similarly the so called Weil-Felix reaction constitutes a useful anomaly by means of which the diagnosis of typhus fever is greatly facilitated. The serum of patients suffering from the disease contains agglutinins for Bacillus proteus strain X.19 which is acknowledged to bear no aetiological relationship to the disease.

Forssmann's Antibody

If the kidney of a guinea pig is emulsified in saline and injected into a rabbit, it will be found that the rabbit produces in its serum a specific haemolytic antibody for sheep's and goat's red corpuscles. The result only occurs in rabbits which already possess some degree of the specific haemolytic antibody. Forssmann's heterophil antibody therefore represents merely/
merely an increased output of the natural antibody.

According to Mackie, the intravenous injection of metallic salts, of sodium nucleinate, and of albumose, will increase the normal haemolytic antibody for sheep's cells in the rabbit.

Also he states that normal horse serum shows some protective action when injected along with diphtheria toxin into guinea pigs.

Harvey and Iyengar found that pigeons had a relative immunity to B. avispticus after vaccination with B. coli. The immunity, however, was much inferior quantitatively to that produced by the specific vaccine.

Evidence against the value of non-specific agents

The evidence against the value of non-specific therapy is clearly stated by Dr. R. A. O'Brien in his opening paper at the Section of Pathology and Bacteriology of the British Medical Association, Bradford, 1924. In the Wellcome Physiological Laboratory over 100,000 carefully controlled animal experiments had been made and yet Dr. O'Brien states that he could find no evidence of beneficial effects from non-specific agents. Evidence from experiments in which animals had been injected with lethal doses of diphtheria toxin, a culture of pneumococcus, and dysentery/
dysentery (Shiga) toxin followed twenty four hours later by the injection of peptone, showed that no difference was found between the treated and control animals.

Glenny found that the injection of peptone into animals previously immunised with diphtheria antitoxin did not produce any increase in the concentration of antitoxin in the animal's blood.

Conclusions

It must be admitted that it is extremely difficult for the clinician to produce scientific proof of the beneficial results of protein shock therapy. In the first place, the remissions that occur in all diseases confuse the results. In the second place, other auxiliary aids are employed in the treatment. Thus, when rheumatoid arthritis is being treated by injection of peptone, massage and radiant heat are employed locally as well in order to obtain the best results and thus the allocation of the benefits becomes exceedingly difficult. Although the majority of claims of success following this treatment will not stand scientific investigation at all, there still remains sufficient clinical evidence to afford the belief that a further investigation of the problem is of value.
The experimental evidence is mainly against the value of non-specific agents. It is not improbable, however, that if the antibody producing tissues have been previously sensitised by a specific antigen, non-specific agents may stimulate the production of specific antibodies. This is not however a proved fact. It can safely be said in conclusion that the beneficial effects of non-specific treatment can in no way be compared to those of specific treatment.
An examination of the subject matter in the preceding chapters shows that during the past thirty years an enormous amount of research work has been undertaken in the attempt to clarify the processes underlying immunity. While a great number of definite facts are known, the mechanism of their production and their relationship to one another and to true immunity is still doubtful. The elucidation of these phenomena must be undertaken along the lines of practical medicine. That is to say, our object must be to try and discover anything which will help us in the diagnosis, treatment or prevention of disease. It will focus therefore the position more clearly if we examine the state of clinical medicine as it stands today compared to what it was forty years ago.

**Diagnosis:**

Unquestionably the biggest advance has been made in diagnosis. Previous to the discovery of such serological tests as agglutination and complement fixation, the diagnosis of such diseases as syphilis and typhoid fever depended purely on the symptoms and physical signs and the doctor's ability to evaluate them. Today, a bacteriological and serological examination will result in the diagnosis of the majority of/
of acute infectious disease. In addition to the diagnosis of disease, the serological tests have enabled us to differentiate into groups and even strains, organisms, which although apparently identical on morphological grounds, are absolutely separate and distinct as judged by the production of disease. Finally, the power of differentiating accurately the different proteins is of the greatest value to Forensic Medicine.

Prophylaxis:

The position is not so satisfactory in this category. Prophylactic serum treatment has only been of definite value in diseases which are due to soluble toxins - e.g. diphtheria and tetanus. In addition, prophylactic active immunisation has been proved to be successful in the group of intestinal diseases of which typhoid and paratyphoid fever are the most common members. The latest developments in this department, namely the Schick and Dick reactions followed by active treatment, give rise to the most hopeful feelings. On the other hand, in the group of diseases which are bacterial as opposed to toxic, e.g. streptococcal and pneumococcal infections, little or no advance has been made in prophylactic treatment.

Treatment:
With the exception of the diseases due to the soluble toxins, we must admit that the result of a vast amount of research has resulted in only limited therapeutic benefit. What are the reasons for this failure of the treatment of bacterial disease by specific vaccines and sera? A satisfactory explanation would revolutionise the whole therapeutic field of medicine. Further investigations must be carried out, and I would suggest that along the following lines most benefit will accrue:

(1) An investigation into the relationship of antibodies to immunity. It is recognised that antitoxins are antibodies which are truly protective. What position, however, do the agglutinins, precipitins and complement fixing bodies, etc. bear to true immunity? We can admit that they are specific in the sense that they are the result of the reaction between the antigen and the body cells and as such, are of the greatest value in diagnosis. The important point is, however, their relation to protection. May not these bodies result from the injury to the tissue cells by the specific antigen, and in that case they would not be protective? An examination of the work on the pneumococcus in the next part of the thesis shows that a highly potent antipneumococcal serum may apparently contain
contain no antibodies. The matter must be allowed to remain unsolved until further investigations have been undertaken.

(2) **The intradermal method of diagnosis (Schick and Dick reaction)** should be investigated in other diseases. If the susceptible members of the community can be found, preventive immunisation should be of the greatest value: I would suggest that a beginning might be made with pneumonia. By means of the intradermal injection of a toxic filtrate of a pneumococcal broth culture, it might be possible to find out not only the susceptible members of the community but also the type of pneumococcus causing the disease.

(3) An investigation of the problem of concentrating the antibodies in antisera.

From the work on antipneumococcal sera done in the Rockefeller Institute, it is obvious that one of the reasons why the majority of antibacterial sera produce little or no benefit is that they are given in far too small amounts. To obtain the optimum results, 200 - 300 c.c. must be injected intravenously. Unfortunately this is followed by severe symptoms of serum sickness. If the antibodies could be concentrated in 5 or 10 c.c. of serum, a great advance would be made.

(4) /
All must agree that the results of vaccine therapy are far from satisfactory. The best immunity results from an actual attack of a disease. Under these conditions there are two facts to consider. First, the organism is in its natural state and is unchanged by any physical or chemical process such as is used in the manufacture of certain vaccines. Secondly, the body is reacting to a living virus. I am strongly of opinion that these two facts are of the greatest importance. I believe that a thorough investigation into the question of using either an avirulent strain of a pathogenic organism or an avirulent organism which is closely allied to the pathogenic member will lead to much useful information. At the same time, an investigation into other types of killed vaccines must be undertaken in the hope of finding some efficient stimulator of the processes underlying immunity.

An investigation into some of these problems will be found in Part II. of this thesis.
PART II.

INTRODUCTION

Part II. of this thesis consists of an account of my investigations into the problems of infection and immunity. Several hundred animals were used and many thousands of tests performed. A detailed account of attempts at producing both active and passive immunity is given. Many antigens were examined and an account of the antibodies produced and the serological methods of detecting them will be found in the following chapters.

In the broadest sense, my endeavour was to study the basic principles of immunity. In addition to this, I set myself the narrower task of discovering what effect physical and chemical agencies had on the antigenic values of vaccines. This is a matter of practical importance to the medical profession in view of the claims and the widespread use of certain types of vaccines which have come on to the market during the past few years - e.g., detoxicated and defatted vaccines.

In order to make as full an investigation as possible, vaccines were made from the three main organismal groups, namely Gram-negative, Gram-positive and/
and acid-fast. The organisms selected were the *B. suispestifer* XII (Lister Inst.), the pneumococcus (*Type I.*) and the tubercle bacillus respectively.

The investigations on the *B. suispestifer* will be found in the chapters entitled -

"The effects of physical and chemical agencies on bacterial vaccines."

The investigations into the pneumococcus are found in the chapters entitled -

"Immunity to the pneumococcus,"

while the work on the tubercle bacillus is given in the concluding chapters under the heading -

"Tuberculin treatment in experimental tuberculosis."

In these latter chapters, a description is given of the treatment of infected guinea pigs with detoxicated, defatted and ordinary vaccines of the tubercle bacillus. My object was to discover which of these antigens was the best. Since the result of treatment with all antigens was unsatisfactory, little information regarding the relative antigenic value of the test vaccines was obtained. On the other hand, the experiment was of great value in focussing clearly the reasons why tuberculin treatment in experimentally infected animals is likely to fail.

It must be admitted that no sweeping statements on tuberculin therapy can be made from this investigation.
investigation. Great variations, both in the original dose of living organisms and in the subsequent therapeutic doses of tuberculin, would need to be tried. In addition, in different animals, treatment would have to be started at different periods after the initial injection. To accomplish a thorough investigation, many animals and much time would be required. This question has been examined on many occasions and since Koch's original publication, no evidence has ever been published that guinea pigs can be cured by tuberculin treatment. I had no intention therefore of making a complete examination of the subject except in so far as it was necessary for a comparison of the effects of various antigens employed.

I am convinced, however, that the conclusions stated at the end of this investigation are correct and are warranted on the experimental facts given.
THE EFFECTS OF PHYSICAL AND
CHEMICAL AGENCIES ON BACTERIAL VACCINES.

Chapter I.

For some considerable time it has been my opinion that physical and chemical agencies when applied to a vaccine would result in a great part of its antigenic value being lost. This assumption appears to be justified in view of the fact that generally the most efficient and lasting immunity is developed when a person or animal suffers and recovers from a disease of bacterial origin. It would appear, therefore, that an organism in its natural state is the best antigen. This has become an important practical question since the introduction of certain newer methods of vaccine preparation, e.g. Detoxication and Defatting.

Some time ago, with a view to investigating this question, the following preliminary tests were carried out:

Three rabbits were immunised with a typhoid vaccine made by emulsifying in saline a twenty-four hours' growth on an agar slope.

Three rabbits were also inoculated with a "detoxicated" typhoid vaccine made from the same strain by/
by Dr. Thomson, the originator of this method of vaccine preparation.

After three graded inoculations, the sera of these six animals were tested by the agglutination and complement fixation test. As evidenced by antibody response to the different antigens, very marked diminution in antigenic value was noted in the case of the detoxicated vaccine. The small number of animals used in this experiment was insufficient to eliminate the fallacies which arise in such comparisons due to varying response of different individual animals to a particular immunising stimulus. The whole question was, therefore, reinvestigated on a much larger scale.

About this time Professor Dreyer of Oxford published his work on "Defatted" Vaccines, and in consequence, new experiments were planned to compare the antigenic values of the following three types of vaccines:

1. The ordinary heat killed bacterial emulsion in saline.
2. The detoxicated vaccine.
3. The so-called defatted vaccine.

The experiment was to include an example of the three main types of organisms, namely, acid-fast, Gram-negative and Gram-positive. The tubercle bacillus,
the bacillus suipestifer, strain XII of the Lister Institute, and the pneumococcus, were accordingly selected. In the short time at my disposal, it is impossible to give the details and the results of the whole of this experimental work, and I propose to confine my remarks to the effects of physical and chemical agencies on the antigenic value of the bacillus suipestifer. This organism was selected in virtue of its natural virulence for the rabbit, which was the animal employed in these experiments. Thus, in addition to the serological index of immunity, the demonstration of increased resistance, by means of infection experiments, was aimed at. It was necessary, therefore, to use an organism of fixed virulence to the test animal.

It is unnecessary here to deal with the theoretical aspects of antibody production, and I shall confine myself to merely stating what was done and the results found. I must, however, digress slightly from the practical to the theoretical on one point as it has an important bearing on these experiments.

The question arises -- what significance is to be attached to the finding of antibodies, such as agglutinins and complement fixing bodies, in the serum of immunised animals? Should these substances be considered/
considered criteria of immunity? In some cases antibodies undoubtedly constitute the mechanism of specific resistance, e.g. antitoxins; on the other hand, it is well known that an animal with a high content of specific agglutinins in its blood serum may show no actual immunity to the homologous infection. This may be explained on the grounds that the immunity, though increased, has not yet reached a point at which it can successfully cope with the particular infection. Again immunity to infection may occur in the apparent absence of demonstrable antibody. Our present knowledge of the mechanism of immunity is insufficient to give definite answers to all these questions. There can be no doubt, however, that the production of antibodies in the serum of an animal depends on the reaction or interaction between the antigen injected and the actual living tissue cells. This is the essential phenomenon underlying all immunity processes. For this reason antibodies may be considered as being indicators of the antigenic values of vaccines.
OBJECTS OF THE EXPERIMENT.

The objects of the experiment were fourfold. The first and foremost was to ascertain whether the physical and chemical agencies used in production of detoxicated and defatted vaccines reduced their antigenic value; secondly, to determine whether subcutaneous or intravenous injection is the more effective; thirdly, to compare the effects of varying doses; and fourthly, to find out if there was any relation between the body weight, the dosage, and the antibody production.

The actual experiment consisted in weighing and numbering 33 rabbits, which were divided into three groups (A.B.C.). Group A consisted of 12 rabbits injected with the ordinary killed bacillary emulsion, Group B 12 rabbits inoculated with the detoxicated vaccine, and Group C 9 rabbits injected with the defatted vaccine. Each group was divided into three sub-groups of four rabbits. Each sub-group contained one heavy rabbit weighing 2,000 grams or more, one small rabbit of less than 1,400 grams, and two intermediate ones of approximately 1,800 grams weight. One rabbit of intermediate weight in each sub-group was injected subcutaneously, the other three intravenously. Rabbits in sub-group A. one received what/
what was considered to be a small dose of the vaccine. This was approximately a total of 50 millions of the organisms administered in four weekly injections of 5, 10, 15 and 20 millions. Those in sub-group A. two received medium-sized doses totalling 500 millions in four weekly injections of 50, 100, 150 and 200 millions, while those in sub-group A. three received doses totalling 5,000 millions in four injections.

TABLE I.
TABLE I.

Showing amounts of "Bacillary Emulsion Vaccine" injected into rabbits in group A.

<table>
<thead>
<tr>
<th>Sub-Group</th>
<th>Weight in grammes</th>
<th>Feb. 20</th>
<th>Feb. 27</th>
<th>March 4</th>
<th>March 12</th>
<th>Total in millions</th>
<th>Route of injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.I</td>
<td>R. I. 1940</td>
<td>5</td>
<td>50</td>
<td>100</td>
<td>200</td>
<td>355</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td></td>
<td>&quot; II. 1820</td>
<td>5</td>
<td>10</td>
<td>15</td>
<td>20</td>
<td>50</td>
<td>Intravenous</td>
</tr>
<tr>
<td></td>
<td>&quot; III. 1350</td>
<td>5</td>
<td>10</td>
<td>15</td>
<td>20</td>
<td>50</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>&quot; IV. 2250</td>
<td>5</td>
<td>10</td>
<td>15</td>
<td>20</td>
<td>50</td>
<td>&quot;</td>
</tr>
<tr>
<td>A.II</td>
<td>R. V. 1950</td>
<td>50</td>
<td>100</td>
<td>150</td>
<td>200</td>
<td>500</td>
<td>Intravenous</td>
</tr>
<tr>
<td></td>
<td>&quot; VI. 1300</td>
<td>50</td>
<td>100</td>
<td>150</td>
<td>200</td>
<td>500</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>&quot; VII. 1920</td>
<td>50</td>
<td>100</td>
<td>200</td>
<td>400</td>
<td>750</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td></td>
<td>&quot; VIII. 2870</td>
<td>50</td>
<td>100</td>
<td>Died</td>
<td>-</td>
<td>-</td>
<td>Intravenous</td>
</tr>
<tr>
<td>A.III</td>
<td>R. IX. 1840</td>
<td>500</td>
<td>1000</td>
<td>1500</td>
<td>2000</td>
<td>5000</td>
<td>Intravenous</td>
</tr>
<tr>
<td></td>
<td>&quot; X. 1300</td>
<td>500</td>
<td>1000</td>
<td>1500</td>
<td>Died</td>
<td>-</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>&quot; XI. 1920</td>
<td>500</td>
<td>1000</td>
<td>Died</td>
<td>-</td>
<td>-</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td></td>
<td>&quot; XII. 2870</td>
<td>500</td>
<td>1000</td>
<td>1500</td>
<td>2000</td>
<td>5000</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

The object of this was to show that there is an optimum dosage below which smaller amounts of antibody are produced, and above which, owing to the toxicity of the foreign protein injected, there will be not only a decreased antibody production, but, in addition, illness and even death of the animals. An analogy might be drawn from a very severe case of pneumonia, in/
in which the overwhelming amount of toxin paralyses
the haemoblastic tissues in the bone marrow and thus
produces a leucopenia instead of the usual leucocytosis.
It is necessary to emphasise this point, because the
whole foundation of the theory of detoxicated vaccine
depends on the following supposition as published by
Dr. David Thomson (5) in his recent book on Gonorrhoea:
He states that when an amount of sheep's red blood
corpuscles equivalent in weight to the dose of a
bacterial vaccine is injected into a rabbit, no haemolytic antibody can be subsequently demonstrated. It
is only when large amounts, such as two or three cubic
centimetres, of red cells are introduced that a high
titre haemolytic serum is produced. From this he
draws the conclusion that if large quantities of
bacterial protein were inoculated into a human being,
a high degree of immunity would result. In order to
obviate the toxic effects which result when large
quantities of bacterial protein are injected, he
originated the process now known as "detoxication".
It can be proved experimentally, however, that when an
amount of bacterial protein is introduced into an
animal beyond the optimum, the antibody production
falls instead of rising.

**EXPERIMENTS WITH THE ORDINARY HEAT-KILLED
BACILLARY EMULSION.**

The/
The vaccine used in the injection of the 12 rabbits in Group A. was made by growing the organism on six-inch Petri dishes containing nutrient agar. The 24 hours' growth was removed by a special suction apparatus from the surface of the plate, dried, and weighed. An even emulsion in 0.5 per cent. carbol saline containing one mg. of dried organisms per c.c. was made. This was then standardised by Brown's Opacity method. The approximate number of bacilli per c.c. was found to be 1000 millions. This vaccine will in future be referred to as the bacillary emulsion. The remainder of the bacterial mass was used for making the defatted vaccine. Four injections of the bacillary emulsion were given at seven days' interval and before each injection, blood was removed from the ear vein of the animal and tested.

Tests Used: Practically all the antibody tests were tried and it was decided that the agglutination and complement fixation tests were the most satisfactory for a comparative estimation of antibody production.

Description of Tests:

I. The Agglutination Test was done with a marked capillary pipette, each transference from tube to tube resulting in a doubling of the serum dilution.

Dilutions/
Dilutions of serum were made from 1 in 10 to 1 in 20,000.

II. The Complement Fixation Test was carried out according to Harrison's method as published in the Medical Research Council's pamphlet on the Wassermann Reaction. This technique is used in the Royal Infirmary, Edinburgh, and has always been found to be absolutely reliable. The quantities used were:

- **Antigen**: 0.2 c.c. of an emulsion of the organisms in saline equivalent to about 500,000 per c.c.; this quantity gives good fixation without being anticomplementary.
- **Serum**: 0.1 c.c. of undiluted serum, plus 0.1 c.c. of saline, making a total quantity of 0.2 c.c.
- **Complement**: 0.2 c.c. of guinea-pig serum in various dilutions as found by previous titration to represent 2, 3, 5, 7, 9, 10 and 12 units of complement.
- **Red blood Corpuscles**: 0.2 c.c. of a two per cent suspension of washed sheep's cells sensitised with five haemolytic doses of B. & W. immune body; the total quantity in each tube was therefore 0.8 of a c.c.

The tubes were incubated at 37° C. for one hour to allow of fixation; sensitised sheep's cells were then added and the tubes returned to the incubator. Readings were taken half an hour and one hour later. In addition, serum and antigen controls were always included.
included. The results of these tests are shown graphically.

TABLE II.

Showing Agglutination titres of rabbit's serum seven days after each injection.

<table>
<thead>
<tr>
<th>Date</th>
<th>Feb.28</th>
<th>Mar.5</th>
<th>Mar.13</th>
<th>March 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit I.</td>
<td>Negative</td>
<td>1 in 80</td>
<td>1 in 200</td>
<td>1 in 200</td>
</tr>
<tr>
<td>&quot; II.</td>
<td></td>
<td>1 in 80</td>
<td>1 in 800</td>
<td>1 in 1600</td>
</tr>
<tr>
<td>&quot; III.</td>
<td></td>
<td>1 in 80</td>
<td>1 in 400</td>
<td>1 in 1600</td>
</tr>
<tr>
<td>&quot; IV.</td>
<td></td>
<td>1 in 80</td>
<td>1 in 800</td>
<td>1 in 1600</td>
</tr>
<tr>
<td>&quot; V.</td>
<td></td>
<td>1 in 160</td>
<td>1 in 3200</td>
<td>1 in 1600</td>
</tr>
<tr>
<td>&quot; VI.</td>
<td></td>
<td>1 in 160</td>
<td>1 in 3200</td>
<td>1 in 3200</td>
</tr>
<tr>
<td>&quot; VII.</td>
<td></td>
<td>1 in 160</td>
<td>Died</td>
<td>-</td>
</tr>
<tr>
<td>&quot; VIII.</td>
<td></td>
<td>1 in 160</td>
<td>1 in 400</td>
<td>1 in 200</td>
</tr>
<tr>
<td>&quot; IX.</td>
<td></td>
<td>1 in 400</td>
<td>1 in 18,800</td>
<td>1 in 6400</td>
</tr>
<tr>
<td>&quot; X.</td>
<td></td>
<td>1 in 400</td>
<td>1 in 800</td>
<td>Died</td>
</tr>
<tr>
<td>&quot; XI.</td>
<td></td>
<td>1 in 400</td>
<td>Died</td>
<td>-</td>
</tr>
<tr>
<td>&quot; XII.</td>
<td></td>
<td>1 in 800</td>
<td>1 in 16,000</td>
<td>1 in 6400</td>
</tr>
</tbody>
</table>

RESULTS OF AGGLUTINATION TESTS.

Turning to Chart I. - On examining the curves of the rabbits in sub-group A.I, it is noticed that the agglutination titres are low, reaching on the 28th day to approximately 1 in 1,500 in the case of rabbit IV. The lowest titre was found in rabbit I. which was inoculated subcutaneously, and which received a total of 355 million organisms as compared to 50 million injected intravenously.
intravenously in the other three. It is noticed also that the graph shows a steady rise in the titres up to the 28th day. Turning to sub-group A.2, which received medium-sized doses, it is seen that on the 21st day—that is after three injections—and before the fourth was given, rabbits V. and VI. had an end titre of more than 1 in 3000. Rabbit VIII. had died previously from a staphylococcal septicæmia due to infected scratches received from fighting, and in consequence is omitted from the experiment. Rabbit VII., which was injected subcutaneously with a total of 750 million organisms as compared with 500 million which the other animals in this sub-group received, had only reached a titre of 1 in 400. With the exception of rabbit VI., which had remained stationary, all the agglutination titres had fallen by the 28th day to one half, showing that three injections were sufficient, and the fourth had merely lowered the animals' immunity. In sub-group A. 3, it is observed that on the 21st day rabbit IX. showed an agglutination titre of 1 in 12,000, but on the 28th day it had fallen to half. Rabbit XII., which was injected subcutaneously, had reached a titre of 1 in 16,000 on the 21st day, which also had fallen to less than half by/
by the 28th day. Rabbit X. died after the third injection, and rabbit XI. after the second. One of these was replaced by another rabbit, and it also died after two injections. These animals were obviously suffering from toxaemia, and survived only two or three days.

The conclusions to be drawn from this graph are, I think, as follows: -

(I) Rabbits in Group A.1, which received the smallest dose were given less than the optimum amount.

(II) The rabbits in group A.2 had received the optimum dose, whereas in group A.3 three out of four rabbits injected intravenously died, showing that they had received more than the optimum dose. The fourth rabbit which withstood the injections, however, produced the best result of all, with the exception of rabbit XII. which received large doses subcutaneously.

(III) It is clear that, with the exception of group A.1, in which insufficient quantities had been given and in which the titre was still rising on the 28th day, all the other rabbits would have had a higher degree of immunity with three injections instead of four, and this is shown by the marked drop in the agglutination titre of the 28th day.

(IV) It is evident that subcutaneous injections
of small or moderate amounts do not produce as high a titre as do the injections of similar quantities intravenously. It would appear, however, that the best results are obtained when large quantities are inoculated subcutaneously, as it will subsequently be seen that rabbit XII was the only animal to survive a dose of the living organisms. It is necessary, however, to confirm this observation, as it is of importance to know definitely whether large subcutaneous doses produce the best true immunity.

These points are clearly brought out in Chart 2, in which the agglutination titres of the animals in each sub-group are added together and divided by the number of animals. The rabbits injected subcutaneously are shown separately.

RESULTS OF COMPLEMENT FIXATION TEST.

The results of the complement fixation test are shown in Chart 3. The graphs are made by adding the units of complement fixed in each sub-group and dividing the result by the number of animals. The results of the complement fixation test in rabbits injected subcutaneously are again shown separately. It is observed that in sub-group A.1 four units of complement were fixed on the 21st day, and this had fallen to three by the 28th day. Sub-group A.2 fixed eight/
eight units on the 21st day. The same fixation was found on the 28th day. Sub-group A.3 fixed only five units, and this had fallen to three on the 28th day. Rabbits X and XI were charted until their death. Rabbit XII, which had been inoculated subcutaneously with large doses, and which had produced the highest agglutination titre, showed a complement fixation of six units on the 21st day and over ten on the 28th day. This confirms to a striking degree the efficacy of the injection of large subcutaneous doses.

EXPERIMENTS WITH DETOXICATED VACCINE.

The second part of the experiment in which 12 rabbits were injected with the detoxicated vaccine will now be considered. A culture of the organism was sent to the Pickett-Thomson Research Laboratory, and a detoxicated vaccine was prepared by Dr. David Thomson. I should like to thank Dr. Thomson for his kindness in providing me with the large quantities of detoxicated vaccines used in these investigations, and for the continued interest which he has shown throughout the course of the investigation. After the original experiment with the B. typhosus vaccines referred to at the beginning of the paper, I informed Dr. Thomson of the effects of his process on the antigenic value of/
of his vaccine, and suggested that the chemicals employed were the cause of the deterioration. He himself had evidently come to this conclusion, because he had introduced a smashing and breaking machine which enables him to use much weaker chemicals. At one time detoxicated vaccines consisted of Alkali, Alcohol and Chloroform soluble fractions of the bacterial protein. Chloroform soluble fraction was first discarded, and later, as a result of investigations into the effects of alcohol on red blood corpuscles used in the production of haemagglutinins, he decided to dispense with the alcohol soluble fraction as well.

In order to demonstrate the effect of alcohol on bacterial antigens I obtained from Dr. Thomson two vaccines, one which contained the alkali soluble fraction only, and the other which was a mixture of the alcohol and the alkali soluble fraction. Since detoxicated vaccines are given in doses of from a hundred to a thousand times that of ordinary vaccines, the animals in group B. received a similar multiple compared to those in group A. In sub-group B. 1, rabbits XIII and XIV received a total of 35,500 million organisms of the alkali plus alcohol soluble detoxicated vaccine; rabbits XV and XVI received the same quantity of the alkali soluble portion. This was given in four weekly/
weekly injections of 500, 5,000, 10,000 and 20,000 millions respectively. Group B.2 consisting of rabbits XVII - XX received 355,000 million organisms; rabbits XVII and XVIII received the alkali plus alcohol soluble fraction, while XIX and XX received the alkali soluble fraction alone. Sub-groups B.1 and B.2 were all injected intravenously, whereas the four rabbits in sub-group B.3 were inoculated subcutaneously, all four receiving a total of 215,500 millions in four injections at weekly intervals, as follows: - 500 millions, 5,000 millions and 10,000 millions, and as no antibodies could be detected in the serum of the animals a fourth injection of 200,000 millions was given to each. Rabbits XXI and XXII received the alkali soluble vaccine, and rabbits XXIII and XXIV, the alcohol plus alkali soluble vaccine.

TABLE III./
TABLE III.

Showing amounts of Detoxicated Vaccine injected into rabbits in group B.

<table>
<thead>
<tr>
<th>Weight in grammes</th>
<th>Feb. 20</th>
<th>Feb. 27</th>
<th>March 5</th>
<th>March 12</th>
<th>Total in millions administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sub-group B.I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XII</td>
<td>1670</td>
<td>500</td>
<td>5000</td>
<td>10,000</td>
<td>20,000</td>
</tr>
<tr>
<td>XIII</td>
<td>1200</td>
<td>500</td>
<td>5000</td>
<td>10,000</td>
<td>20,000</td>
</tr>
<tr>
<td>XIV</td>
<td>1290</td>
<td>500</td>
<td>5000</td>
<td>10,000</td>
<td>20,000</td>
</tr>
<tr>
<td>XV</td>
<td>1790</td>
<td>500</td>
<td>5000</td>
<td>10,000</td>
<td>20,000</td>
</tr>
<tr>
<td>XVI</td>
<td>1670</td>
<td>500</td>
<td>5000</td>
<td>10,000</td>
<td>20,000</td>
</tr>
<tr>
<td>XVII</td>
<td>1340</td>
<td>5000</td>
<td>50,000</td>
<td>100,000</td>
<td>200,000</td>
</tr>
<tr>
<td>XVIII</td>
<td>3450</td>
<td>5000</td>
<td>50,000</td>
<td>100,000</td>
<td>200,000</td>
</tr>
<tr>
<td>XIX</td>
<td>1540</td>
<td>5000</td>
<td>50,000</td>
<td>100,000</td>
<td>200,000</td>
</tr>
<tr>
<td>XX</td>
<td>1500</td>
<td>5000</td>
<td>50,000</td>
<td>100,000</td>
<td>200,000</td>
</tr>
<tr>
<td>XXI</td>
<td>1870</td>
<td>500</td>
<td>5000</td>
<td>10,000</td>
<td>20,000</td>
</tr>
<tr>
<td>XXII</td>
<td>1320</td>
<td>500</td>
<td>5000</td>
<td>10,000</td>
<td>20,000</td>
</tr>
<tr>
<td>XXIII</td>
<td>3200</td>
<td>500</td>
<td>5000</td>
<td>10,000</td>
<td>20,000</td>
</tr>
<tr>
<td>XXIV</td>
<td>1850</td>
<td>500</td>
<td>5000</td>
<td>10,000</td>
<td>20,000</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Rabbits XV, XVI, XIX, XX, XXI, XXII were inoculated with a detoxicated vaccine consisting of an alkali soluble fraction alone. Rabbits XIII, XIV, XVII, XVIII, XXIII, XXIV were inoculated with a detoxicated vaccine consisting of an alkali and an alcohol soluble fraction.
RESULTS OF AGGLUTINATION TESTS.

In Chart 4 it is seen that the base line is the same as in the previous graph, each division representing a seven days' interval. Whereas in Chart 1 each division in the vertical line represents an agglutination titre of 1 in 1,000, in Chart 4 it represents a titre of 1 in 50 only. It is observed that of the twelve animals injected only four produced appreciable agglutinins, namely, rabbits XV and XVI in sub-group B.1, which showed titres of 1 in 340 and 1 in 150 respectively on the 21st day, and rabbits XIX and XX in sub-group B.2, which gave titres of 1 in 60 and 1 in 40 respectively. These four rabbits were injected intravenously with the vaccine made from the alkali soluble portion alone.

RESULTS OF COMPLEMENT FIXATION TESTS.

With regard to the complement fixation test it is only necessary to say that the number of units fixed was always so small as to be practically negative, the average being somewhere under one unit. The detoxicated vaccine itself when used as an antigen in the complement fixation test with the serum of rabbits in groups A, B, and C, was found to have practically no fixing power.

The/
The conclusions to be drawn from these results are, I think, quite clear:

(1) That in spite of the fact that these animals received the products of more than a thousand times the number of the organisms in the vaccine used in group A, the agglutination end titre was extremely low.

(2) The effect of the alcohol is distinctly injurious to the antigen as the rabbits injected with the alcohol plus alkali soluble vaccine produced no agglutinins at all.

(3) Three injections were sufficient and by giving the fourth the end titres were reduced to half.

(4) The subcutaneous injections of the detoxicated vaccines yielded completely negative results.

(5) Though large quantities of vaccine in almost semi-solid condition were injected, no ill effects whatsoever were produced on the animals, that is to say, it certainly was detoxicated.

Experiments with Defatted Vaccines.

The third part of the experiment consists of the injection of nine rabbits with the so-called "defatted" vaccine. The mass of dried organisms, already referred to in part I, was treated for four hours/
hours at 100 degrees centigrade with formalin and subsequently extracted for 24 hours with Acetone in a Soxlet apparatus, as described by Dreyer. The defatted material was dried and weighed, and an even emulsion in 0.5 per cent carbol saline containing 1 mg. per c.c. was made. When examined under the microscope, after staining with carbol fuchsin, the organisms were quite distinctly seen, and with the exception that they seemed slightly smaller than the untreated organisms, no appreciable difference was visible. It is thus possible to compare the effects of similar doses of defatted and untreated organisms on animals.

The nine rabbits in group C. were divided into three sub-groups as before. Animals in sub-groups C.1, C.2, C.3 received in four weekly injections totals of 0.05 mg., 0.5 mg. and 5.0 mgs. respectively.

TABLE IV./
TABLE IV.

Showing amounts of defatted vaccine injected into rabbits in group C.

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>R.</th>
<th>Weight in grammes</th>
<th>Route of administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.I</td>
<td>XXV</td>
<td>2210</td>
<td>Intravenous</td>
</tr>
<tr>
<td></td>
<td>XXVI</td>
<td>1690</td>
<td></td>
</tr>
<tr>
<td></td>
<td>XXVII</td>
<td>1250</td>
<td></td>
</tr>
<tr>
<td>C.II</td>
<td>XXVIII</td>
<td>1670</td>
<td></td>
</tr>
<tr>
<td></td>
<td>XXIX</td>
<td>3400</td>
<td></td>
</tr>
<tr>
<td></td>
<td>XXX</td>
<td>1340</td>
<td></td>
</tr>
<tr>
<td>C.III</td>
<td>XXXIV</td>
<td>1420</td>
<td></td>
</tr>
<tr>
<td></td>
<td>XXXV</td>
<td>2140</td>
<td></td>
</tr>
<tr>
<td></td>
<td>XXXVI</td>
<td>1850</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>R.</th>
<th>Feb. 20</th>
<th>Feb. 27</th>
<th>March 4</th>
<th>March 12</th>
<th>Total in mgs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.I</td>
<td>XXV</td>
<td>0.005</td>
<td>0.01</td>
<td>0.015</td>
<td>0.02</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>XXVI</td>
<td>0.005</td>
<td>0.01</td>
<td>0.015</td>
<td>0.02</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>XXVII</td>
<td>0.005</td>
<td>0.01</td>
<td>0.015</td>
<td>0.02</td>
<td>0.05</td>
</tr>
<tr>
<td>C.II</td>
<td>XXVIII</td>
<td>0.05</td>
<td>0.1</td>
<td>0.15</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>XXIX</td>
<td>0.05</td>
<td>0.1</td>
<td>0.15</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>XXX</td>
<td>0.05</td>
<td>0.1</td>
<td>0.15</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>C.III</td>
<td>XXXIV</td>
<td>1.0</td>
<td>1.0</td>
<td>1.5</td>
<td>2.0</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>XXXV</td>
<td>1.0</td>
<td>1.5</td>
<td>Died</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>XXXVI</td>
<td>1.0</td>
<td>1.5</td>
<td>2.0</td>
<td>5.0</td>
<td></td>
</tr>
</tbody>
</table>

One of the rabbits in this last group died from the toxic effects of the injection.

RESULTS OF AGGLUTINATION TESTS.

Chart 5 shows the graphs of the agglutination titres of the animals in group C. Each division on the base line represents an interval of seven days, while, in the vertical line, it represents a titre of 1/
On the 21st day rabbits XXV and XXVII had agglutination titres of 1 in 40 and 1 in 20 respectively, the remainder producing no appreciable agglutinins.

The conclusion to be drawn from this graph is, I think, that the physical and chemical processes employed in defatting the vaccine have practically destroyed its antigenic value.

RESULTS OF COMPLEMENT FIXATION TEST.

The sera of the rabbits in group C. gave a negative result, except for slight fixation in rabbit XXXIV and very good fixation in rabbit XXX. The serum of this animal fixed $\frac{3}{5}$ units after the second injection, $\frac{6}{5}$ units after the third injection and $\frac{7}{5}$ units a week after the fourth injection. The serum was repeatedly tested and was always found to give a markedly positive result. Why the serum of this animal contained such a large amount of complement fixing antibody I am unable to explain.

Chart 6 shows the combined agglutination curves of the animals in groups A, B and C. The agglutination titres in each group are added together and divided by the total number of rabbits. By the 21st day the rabbits in group A. had an average agglutination titre of 1 in 4,600, dropping on the 28th/
28th day to just under 1 in 3,000, while the maximum titre of the rabbits in group B. averaged 1 in 50 and in group C. 1 in 5. This, I think, is a very striking picture.

Chart 7 shows the combined complement fixation curves of groups A, B and C. The graphs are made by adding the number of units fixed and dividing by the number of animals tested. On the 21st day the average fixation of complement by animals in group A. was 5 units, while group B. fixed under 1, and group C. was practically negative. The interesting point about this graph is the remarkable way in which charts 6 and 7 agree. While it is not suggested that in any individual rabbit the graphs of agglutination and complement fixation will run exactly side by side, yet, if the average of a large number of animals is taken, the resulting curves will be very similar. An agglutination titre of 1 in 5,000 in group A. is equivalent to fixation of 5 units of complement. In groups B. and C. the very low agglutination titres are comparable to the practically negative results of fixation of complement.

PROTECTION EXPERIMENTS.

The experiment about to be described was undertaken to ascertain whether any active immunity to/
to a lethal dose had been produced. It was necessary first to establish the minimum lethal dose. Altogether 20 rabbits were used for this purpose.

**TABLE V.**

The pathogenicity of *B. suipestifer* for normal rabbits.

<table>
<thead>
<tr>
<th>Dose of living <em>B. suipestifer</em></th>
<th>Survival period in days.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit I 50,000</td>
<td>2.5</td>
</tr>
<tr>
<td>&quot;      50,000</td>
<td>3</td>
</tr>
<tr>
<td>&quot;      10,000</td>
<td>4</td>
</tr>
<tr>
<td>&quot;      10,000</td>
<td>4</td>
</tr>
<tr>
<td>&quot;      10,000</td>
<td>4</td>
</tr>
<tr>
<td>&quot;      10,000</td>
<td>5</td>
</tr>
<tr>
<td>&quot;      10,000</td>
<td>6</td>
</tr>
<tr>
<td>&quot;      5,000</td>
<td>4</td>
</tr>
<tr>
<td>&quot;      5,000</td>
<td>4</td>
</tr>
<tr>
<td>&quot;      5,000</td>
<td>5</td>
</tr>
<tr>
<td>&quot;      5,000</td>
<td>5</td>
</tr>
<tr>
<td>&quot;      5,000</td>
<td>8</td>
</tr>
<tr>
<td>&quot;      1,000</td>
<td>4</td>
</tr>
<tr>
<td>&quot;      1,000</td>
<td>4</td>
</tr>
<tr>
<td>&quot;      1,000</td>
<td>5</td>
</tr>
<tr>
<td>&quot;      1,000</td>
<td>5</td>
</tr>
<tr>
<td>&quot;      1,000</td>
<td>6</td>
</tr>
<tr>
<td>&quot;      1,000</td>
<td>6</td>
</tr>
<tr>
<td>&quot;      500</td>
<td>4</td>
</tr>
<tr>
<td>&quot;      500</td>
<td>6</td>
</tr>
</tbody>
</table>

**Note:** So virulent for rabbits is the *B. suipestifer* that no minimum Lethal Dose could be found.
Doses of organisms from 50,000 to 1,000 - the latter representing 0.000001 of a mg. of dried organisms - were injected intravenously. 1,000 organisms invariably produced death from septicæmia within a week.

In dealing with experiments in which rabbits are inoculated with an organism of such high virulence as the Bacillus suipestifer, the remarks made by Dr. Theobald Smith in a lecture on animal pathology in Edinburgh last year, may be recalled. He said that some animals were so susceptible to micobac diseases that we could hope for no benefit from vaccine treatment. Again other animals were so resistant that there was no need for vaccine treatment, while there remained an intermediary class from which good results might be expected. The action of B. suipestifer on rabbits comes under the first category. Pratt-Johnson, when working with this organism in the Lister Institute, was quite unable to produce any actual immunity. Even so minute a number as 200 living bacilli, when introduced intravenously into previously immunised animals, proved fatal. As a result of this extremely high virulence, it has been necessary to rely mainly on the serological phenomena of agglutination, complement fixation, etc., and not on the resistance of/
of immunised animals to lethal doses.

Six immunised rabbits from each of the groups A, B and C, were injected with living organisms; two in each group received 1,000 - two 5,000 and the other two 10,000. Of these 18 rabbits all, except one, died within a week and the organism was recovered from the heart blood in pure culture.

TABLE VI.
TABLE VI.

Experiment to test the immunity produced by immunisation with the "Bacillary Emulsion", the detoxicated and the defatted Vaccines of B. suipastifer.

<table>
<thead>
<tr>
<th>Rabbits in group A. immunised with the Bacillary Emulsion</th>
<th>Dose of living B. suipastifer</th>
<th>Period of Survival in days</th>
</tr>
</thead>
<tbody>
<tr>
<td>R: I</td>
<td>1,000</td>
<td>8</td>
</tr>
<tr>
<td>&quot; II</td>
<td>1,000</td>
<td>6</td>
</tr>
<tr>
<td>&quot; III</td>
<td>5,000</td>
<td>5</td>
</tr>
<tr>
<td>&quot; V</td>
<td>10,000</td>
<td>5</td>
</tr>
<tr>
<td>&quot; VII</td>
<td>5,000</td>
<td>5</td>
</tr>
<tr>
<td>&quot; XII</td>
<td>10,000</td>
<td>Survived</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Rabbits in group B. immunised with Detoxicated Vaccines.</th>
<th>Dose of living B. suipastifer</th>
<th>Period of Survival in days</th>
</tr>
</thead>
<tbody>
<tr>
<td>R: XIV</td>
<td>1,000</td>
<td>5</td>
</tr>
<tr>
<td>&quot; XV</td>
<td>10,000</td>
<td>5</td>
</tr>
<tr>
<td>&quot; XIX</td>
<td>5,000</td>
<td>5</td>
</tr>
<tr>
<td>&quot; XX</td>
<td>10,000</td>
<td>5</td>
</tr>
<tr>
<td>&quot; XXI</td>
<td>1,000</td>
<td>5</td>
</tr>
<tr>
<td>&quot; XXIV</td>
<td>5,000</td>
<td>5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Rabbits in group C. immunised with Defatted Vaccine.</th>
<th>Dose of living B. suipastifer</th>
<th>Period of Survival in days</th>
</tr>
</thead>
<tbody>
<tr>
<td>R: XXVI</td>
<td>1,000</td>
<td>5</td>
</tr>
<tr>
<td>&quot; XXVII</td>
<td>10,000</td>
<td>5</td>
</tr>
<tr>
<td>&quot; XXVIII</td>
<td>5,000</td>
<td>5</td>
</tr>
<tr>
<td>&quot; XX</td>
<td>10,000</td>
<td>4</td>
</tr>
<tr>
<td>&quot; XXXI</td>
<td>1,000</td>
<td>5</td>
</tr>
<tr>
<td>&quot; XXXIV</td>
<td>5,000</td>
<td>5</td>
</tr>
<tr>
<td>&quot; XXXVI</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Normal Rabbits (Controls)</th>
<th>Dose of living B. suipastifer</th>
<th>Period of Survival in days</th>
</tr>
</thead>
<tbody>
<tr>
<td>R: A</td>
<td>1,000</td>
<td>5</td>
</tr>
<tr>
<td>&quot; B</td>
<td>5,000</td>
<td>5</td>
</tr>
<tr>
<td>&quot; C</td>
<td>10,000</td>
<td>4</td>
</tr>
</tbody>
</table>

Note: Rabbit XII was the only survivor.

This/
This animal was in group A.3 and was injected subcutaneously with large doses of the bacillary emulsion. It had produced the highest agglutination titre, namely 1 in 16,000, and also the highest complement fixation, namely 10 units (v. Charts). It was a striking fact that this was the only animal that survived the test lethal dose. Moreover, it resisted an injection of 10,000 living organisms, which was 10 to 20 times the minimum lethal dose.

**THE CONCLUSION** to be drawn from this protection experiment is that the virulence of the test culture was of such high grade that the increased resistance produced by vaccine immunisation was still insufficient to protect these animals from lethal doses. The result obtained in the case of rabbit XII is of some interest, but no conclusions can be drawn until the experiments at present being undertaken to repeat and confirm the facts described are completed.

**CONCLUSIONS**
CONCLUSIONS.

(1) As shown by in vitro immunity reactions, the antigenic values of detoxicated and defatted vaccines are in no way comparable to those of the ordinary heat-killed bacillary emulsions in saline.

(2) It would, therefore, appear that the physical and chemical processes involved in the preparation of the detoxicated and defatted vaccines are responsible for the marked loss of antigenic value.
CHAPTER II.

Since no method of immunising rabbits with a B. suipestifer vaccine has ever been successful in saving them from a test lethal dose of living organisms, I decided to reinvestigate the problem, using as the immunising agent a living culture of a closely allied organism.

Pratt-Johnson found that although even so small a dose as 200 living suipestifer bacilli invariably produced a fatal result in rabbits immunised with a B. suipestifer vaccine, such was not necessarily the case in animals immunised with a living vaccine of B. para-typhosus B (Type "Mutton" Lister Institute).

Two of his experiments shown in Tables I. and II. deserve special consideration.

The following deductions may be drawn from Table I.

(1) Rabbits 81 - 86, which were injected with the killed hog cholera vaccine, possessed comparatively low titre agglutinating sera when tested against the homologous antigen. No cross agglutination with the "Mutton" antigen was found.

(2) Rabbits 97 - 92, which were injected with the killed "Mutton" vaccine, possessed comparatively low titre/
titre agglutinating sera when tested against the homologous antigen. No cross agglutination with the hog cholera antigen was found.

(3) Rabbits 93 - 98, which were injected with the living "Mutton" vaccine, possessed high titre agglutinating sera against the homologous antigen. In addition, a moderate degree of cross agglutination with hog cholera antigen was present.
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Killed &quot;Hog Cholera XII&quot; Vaccine</td>
<td>81</td>
<td>1 in 800</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>82</td>
<td>1 in 100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>83</td>
<td>1 in 100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>84</td>
<td>1 in 200</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>85</td>
<td>1 in 400</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>86</td>
<td>1 in 1600</td>
<td>0</td>
</tr>
<tr>
<td>Killed &quot;Mutton&quot; Vaccine</td>
<td>87</td>
<td>0</td>
<td>1600</td>
</tr>
<tr>
<td></td>
<td>88</td>
<td>0</td>
<td>1600</td>
</tr>
<tr>
<td></td>
<td>89</td>
<td>0</td>
<td>1600</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>0</td>
<td>1600</td>
</tr>
<tr>
<td></td>
<td>91</td>
<td>0</td>
<td>1600</td>
</tr>
<tr>
<td></td>
<td>92</td>
<td>0</td>
<td>1600</td>
</tr>
<tr>
<td>Living &quot;Mutton&quot; Vaccine</td>
<td>93</td>
<td>50</td>
<td>6400</td>
</tr>
<tr>
<td></td>
<td>94</td>
<td>50</td>
<td>3200</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>200</td>
<td>6400</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>50</td>
<td>6400</td>
</tr>
<tr>
<td></td>
<td>97</td>
<td>200</td>
<td>6400</td>
</tr>
<tr>
<td></td>
<td>98</td>
<td>50</td>
<td>6400</td>
</tr>
</tbody>
</table>

Immunisation: Three injections at intervals of seven days were given to each animal.

Doses of killed vaccines were - 2,000 millions, 2,000 millions and 6,000 millions.

Doses/
Doses of live "Mutton" vaccine were 30 millions, 30 millions and 300 millions.

### TABLE II.

(Pratt-Johnson)

<table>
<thead>
<tr>
<th>Test dose of Living Hog Cholera XII</th>
<th>Killed H.C.XII</th>
<th>Killed &quot;Mutton&quot; vaccine</th>
<th>Living &quot;Mutton&quot;</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>April 27.</td>
<td>Rabbit Day of death</td>
<td>Rabbit day of death</td>
<td>Rabbit Day of death</td>
<td>Rabbit Day of death</td>
</tr>
<tr>
<td>200 organisms</td>
<td>81 May 4</td>
<td>87 May 2</td>
<td>93 survived</td>
<td>101 May 2</td>
</tr>
<tr>
<td></td>
<td>82 &quot;9</td>
<td>88 &quot;2</td>
<td>94 May 4</td>
<td></td>
</tr>
<tr>
<td>1000 organisms</td>
<td>83 May 3</td>
<td>89 Survived</td>
<td>95 survived</td>
<td>102 May 2</td>
</tr>
<tr>
<td></td>
<td>84 &quot;2</td>
<td>90 May 2</td>
<td>96 May 3</td>
<td></td>
</tr>
<tr>
<td>1,000,000 organisms</td>
<td>85 May 2</td>
<td>91 May 2</td>
<td>97 survived</td>
<td>103 May 1</td>
</tr>
<tr>
<td></td>
<td>86 &quot;2</td>
<td>92 &quot;2</td>
<td>98 May 4</td>
<td></td>
</tr>
</tbody>
</table>

The deductions to be drawn from Table II. are that:

1. Since three rabbits survived the injection of living *B. suispestifer* a definite degree of immunity had been produced by immunisation with living "Mutton" vaccine.
(2) The survival period of the animals that died was from 4 to 7 days. No relation appears to exist between the survival period and the test dose.

(3) Since rabbit 97 which received a test dose of 1,000,000 living bacilli survived while rabbit 94 which received only 200 living organisms succumbed, it must be assumed that the individual response to active immunisation is a matter of paramount importance.

The confirmation of Pratt-Johnson's experiments is a matter of great importance. If it can be proved that immunity to excessively virulent infections can be produced by vaccination with living cultures of closely related organisms of low virulence, a new field in vaccine treatment will be opened to the medical profession.

The following investigation was therefore undertaken:

Six rabbits were injected first with dead cultures and then with living cultures of B. para-typhosus B (type "Mutton" Lister Institute.)

TABLE III./
TABLE III.

<table>
<thead>
<tr>
<th>Days :</th>
<th>1</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
<th>Total in Millions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dead</td>
<td>Dead</td>
<td>Living</td>
<td>Living</td>
<td>Living</td>
<td></td>
</tr>
<tr>
<td>Rabbit 31</td>
<td>50</td>
<td>500</td>
<td>50</td>
<td>Died</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>&quot; 32</td>
<td>50</td>
<td>500</td>
<td>50</td>
<td>Died</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>&quot; 33</td>
<td>50</td>
<td>500</td>
<td>50</td>
<td>50</td>
<td>-</td>
<td>550 100</td>
</tr>
<tr>
<td>&quot; 27</td>
<td>200</td>
<td>500</td>
<td>15</td>
<td>50</td>
<td>100</td>
<td>700 165</td>
</tr>
<tr>
<td>&quot; 50</td>
<td>200</td>
<td>500</td>
<td>15</td>
<td>50</td>
<td>150</td>
<td>700 215</td>
</tr>
<tr>
<td>&quot; 53</td>
<td>200</td>
<td>500</td>
<td>20</td>
<td>100</td>
<td>200</td>
<td>700 320</td>
</tr>
</tbody>
</table>

Rabbits 31 and 32 died from the effects of the injection of living bacilli. B. para-typhosus ("Mutton") is therefore only of relative low virulence. The four remaining rabbits were injected with living B. supestifer XII as follows:

TABLE IV./
TABLE IV.

<table>
<thead>
<tr>
<th>Dose of living B. suipastifer XII in millions</th>
<th>Period of survival</th>
<th>Normal Rabbits Controls</th>
<th>Period of survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit 33 5,000</td>
<td>11 days</td>
<td>R.1 - 1,000</td>
<td>5 days</td>
</tr>
<tr>
<td>27 10,000</td>
<td>9 days</td>
<td>R.2 - 5,000</td>
<td>5 days</td>
</tr>
<tr>
<td>50 1,000</td>
<td>9 days</td>
<td>R.3 - 10,000</td>
<td>4 days</td>
</tr>
<tr>
<td>53 5,000</td>
<td>survived</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Result of protection experiment:

Rabbit 53 survived while rabbits 33, 27 and 50 lived twice as long as the control animals. A definite degree of immunity was therefore produced to a highly virulent organism by active immunisation with a closely related organism of low virulence, thus confirming Pratt-Johnson results.

Agglutination Tests.

The sera of rabbits 27, 50 and 53 were examined for their agglutinin content, using B. para-typhosus B ("Mutton" Type) as the antigen. The end titre of the serum of each animal was 1 in 6,400. This agrees exactly with Pratt-Johnson's observations. When cross agglutination/
agglutination tests were made using B. suipestifer XII as antigen, the end titre was 1 in 640; thus the cross agglutinins were one tenth the amount of the specific.

Since Pratt-Johnson suggests from his investigation (Table I.) that the benefits following immunisation with living para-typhoid bacilli lay in the production of cross agglutinins for Hog Cholera XII, I decided to examine the question from another point of view. Accordingly, rabbit XII, which was the only animal to survive the test dose of living culture of B. suipestifer XII (Table VI - Chapter I.) was used as the test animal.

Rabbit XII was alive and well six months after the test lethal injection. The agglutination titre of its serum had fallen in those months from 1 to 6,400 to 1 in 320. Three doses of 250, 500 and 1,000 million of killed B. suipestifer XII at weekly intervals were accordingly given intravenously.

The agglutination titre rose to 1 in 25,000 using the homologous organism as antigen. This was the highest end titre against B. suipestifer produced in these experiments. When tested for cross agglutination against B. para-typhosus ("Mutton") the end titre was 1 in 2,480 (Table V). Here again, the cross agglutinins were one tenth the amount of the specific agglutinins.
agglutinins. Such a high degree of cross agglutination is rarely seen. Owing to the toxic effect of the immunising vaccine, rabbit XII began to waste rapidly and, before the test lethal dose of living B. suipesifer was injected, was seriously ill. It was therefore not surprising that this animal failed to withstand a dose of 10,000 living B. suipesifer and died in five days.

**BACTERIOLYTIC EXPERIMENT with B. PARA-TYPHOSUS B ("MUTTON" STRAIN) and HOG CHOLERA XII.**

The bactericidal action of the serum of rabbit 53 was tested by a method similar to that used by Neisser and Wechsberg. Three small test tubes were taken, and into the first was put 1 c.c. of a 1 in 10 dilution of immunised rabbit's serum inactivated at 55 °C. for half an hour -- into the second 1 c.c. of a 1 in 100 dilution of the inactivated immunised rabbit's serum, and into the third 1 c.c. of a 1 in 500 dilution of the inactivated immunised rabbit's serum. To each of these tubes were added 1 c.c. of 1 in 10 rabbit's complement and .05 c.c. of a 1 in 10,000 dilution of a 24 hours' agar slope emulsion of B. para-typhosus B ("Mutton" strain).

The/
The tubes were well shaken and incubated at 37 C. for three hours. At the end of that time the tubes were again shaken, 1 c.c. was added from each tube to a MacConkey's plate, and the plates were incubated overnight.

In Plate I (i.e. the plate made from the tube containing 1 c.c. of 1/10 immune serum) there was 1 colony.

In Plate II (i.e. the plate made from the tube containing 1 c.c. of 1/100 immune serum) there was 1 colony.

In Plate III (i.e. the plate made from the tube containing 1 c.c. of 1/500 immune serum) there were 5 colonies.

As a control, a similar experiment was done at the same time in which normal rabbit's serum was substituted for immunised rabbit's serum. This gave the following results:

Plate I ... 784 colonies
" II ... 749 "
" III ... 688 "

The results may be expressed shortly as follows:

1/10 .. 1 colony .... N10 .. 784 colonies
1/100 .. 1 " .... N100 .. 749 "
1/500 .. 5 colonies .... N500 .. 688 "

The experiment was repeated, but only two dilutions of immune serum were used. The first tube contained 1 c.c. of 1/10 dilution of inactivated immune serum;
serum; the second, 1 c.c. of 1/1000 dilution of inactivated immune serum. To each tube were added 1 c.c. of 1 in 10 rabbit's complement and .02 c.c. of 1/80,000 dilution of a 24 hours' agar slope growth of "Mutton" para-typhoid.

The tubes were well shaken and incubated at 37°C. for three hours. As before, a control was put up in which normal rabbit's serum was substituted for immune serum.

At the end of three hours a shake culture was made in a MacConkey's plate of the contents of each tube, and the plates were incubated overnight.

The results were:

<table>
<thead>
<tr>
<th></th>
<th>1/10</th>
<th>N10</th>
<th>1/1000</th>
<th>N1000</th>
<th>1/500</th>
<th>N500</th>
</tr>
</thead>
<tbody>
<tr>
<td>colonies</td>
<td>1</td>
<td>648</td>
<td>103</td>
<td>171</td>
<td>95</td>
<td>121</td>
</tr>
</tbody>
</table>

The bacteriolytic action of the serum of Rabbit 53 on Hog Cholera XII was tested in a similar way.

The number of colonies on each plate were:

<table>
<thead>
<tr>
<th></th>
<th>1/10</th>
<th>N10</th>
<th>1/100</th>
<th>N100</th>
<th>1/500</th>
<th>N500</th>
</tr>
</thead>
<tbody>
<tr>
<td>colonies</td>
<td>220</td>
<td>250</td>
<td>204</td>
<td>197</td>
<td>35</td>
<td>121</td>
</tr>
</tbody>
</table>

These experiments show that the serum of the surviving Rabbit 53 shows no bacteriolysis to Hog Cholera XII, but complete bacteriolysis against "Mutton" para-typhoid. 

TABLE V. /
TABLE V.

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>Rabbit serial number</th>
<th>Hog Cholera XII agglutination titre</th>
<th>Para-typhoid B. (&quot;Mutton&quot;) agglutination titre</th>
<th>Relative proportion of &quot;group&quot; agglutinins to &quot;Main&quot; agglutinins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Killed B. suipastifer XII vaccine.</td>
<td>R. XII</td>
<td>1 in 25,000</td>
<td>1 in 2,480</td>
<td>1:10</td>
</tr>
<tr>
<td>Killed followed by living Para-typhoid (&quot;Mutton&quot;) vaccine</td>
<td>R. 27</td>
<td>1 in 640</td>
<td>1 in 6,400</td>
<td>1:10</td>
</tr>
<tr>
<td></td>
<td>R. 50</td>
<td>1 in 640</td>
<td>1 in 6,400</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R. 53</td>
<td>1 in 640</td>
<td>1 in 6,400</td>
<td></td>
</tr>
</tbody>
</table>

CONCLUSIONS.

(1) A higher degree of immunity, as judged by protection to the test lethal dose of B. suipastifer XII, is obtained by immunisation with a living culture of a closely related organism of relatively low virulence than by immunisation with a dead vaccine of the homologous organism.

(2) The evidence that the production of cross agglutinins is of importance, is not convincing, since animals immunised with killed vaccines of B. suipastifer/
suipëstifer XII showed a high degree of cross agglutination with para-typhoid B ("Mutton") and yet were never able to withstand a test dose of living organisms.

It must be assumed that the efficiency of para-typhoid B. vaccine in producing immunity to B. suipëstifer XII lay in the introduction of living as opposed to dead organisms. The introduction of living organisms apparently stimulates in some unknown manner the production of protective substances.
A detailed examination of the antibody content of the serum of animals immunised with *B. suipestifer* XII and *B. para-typhosus* B ("Mutton" Lister Institute.)

An examination of the sera of the 33 test animals was made for a qualitative and quantitative estimation of the following antibodies:

1. **Agglutinins.**
2. **Complement fixing bodies.**
3. **Precipitins.**
4. **Bacterioclysins.**
5. **Opsonins.**

Blood was drawn off each rabbit after each of the four injections and again seven days after the last injection. Thirty three rabbits were immunised with different *B. suipestifer* vaccines and six with *para-typhoid B* vaccines. It will therefore be evident that many hundreds of tests had to be carried out. As explained in Chapter I. - Part II. - the production of agglutinins and complement fixing bodies was chosen as the criterion of the antigen value of a vaccine.

1. **The agglutination test.**

---

**CHAPTER III**

---
Blood was drawn from the ear vein of the rabbits and allowed to clot over night in the ice chest. It was then pipetted into sterile Wassermann tubes.

Antigen:

An 18 hour agar slope culture of the test organism was emulsified in saline and diluted so as to contain about 500 million organism per c.c. as judged by opacity. This density was found to give good agglutination: the bacillary emulsion was heated for half an hour at 55°C.

A drawn capillary pipette was marked with a grease pencil about half an inch up the stem. The volume contained was about $\frac{1}{2}$ c.c. and will be referred to as unit volume. Suction was obtained by attaching a rubber teat.

The tubes used were the ordinary agglutination tubes which were dry and spotlessly clean. Where, from previous examination, it was known that a serum produced agglutination in a dilution of 1 in 100 or over, the first tube in the test contained serum dilut­ed to 1 in 50. Where the examination of the previous week showed agglutination in under 1 in 100, the original serum dilution was 1 in 10.
Method employed:

A row of agglutination tubes were placed in a rack. Into all except Tube I, unit volume of normal saline was placed. If it had been decided to start with a serum dilution of 1 in 50, 0.1 serum was added to 4.9 c.c. of saline in a test tube and a good mixture made by rotating the tube between the palms of the hands. Unit volume was removed to Tubes I. and II. respectively. Tube II. now contained two unit volumes of a 1 in 100 dilution of serum. An even mixture of saline and serum was made by drawing the fluid up and down the pipette. Unit volume of the fluid was transferred to Tube III. where the process was repeated: as many dilutions as were considered necessary were made. Unit volume from the last tube was squirted away.

The following dilutions of serum were usually made:

1 in 50 1 in 100 1 in 200 1 in 400
1 in 800 1 in 1600 1 in 3200 1 in 6400
1 in 12,400 1 in 24,800

Unit volume of the antigen was added to each tube; the serum dilutions were therefore doubled. Unit volumes of antigen and saline were placed/
placed in a separate tube to act as the antigen control, in case of spontaneous agglutination of the antigen resulted from non-specific causes.

A thorough admixture of serum dilutions and antigen having been made, the tubes were placed in the incubator for one hour at 55°C. when the results were read.

If complete sedimentation were present, it was denoted by the following symbol - ++

If there were a degree of sedimentation but in addition the supernatant fluid showed flocculation, it was denoted by - +

If flocculation alone were present, it was denoted by - +

Table I. gives the result of the agglutination tests done on March 13.

TABLE I. /
TABLE I.

<table>
<thead>
<tr>
<th>Tubes:</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>End Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Dilution</td>
<td>1/100</td>
<td>1/200</td>
<td>1/400</td>
<td>1/800</td>
<td>1/1600</td>
<td>1/3200</td>
<td>1/6400</td>
<td>1/12800</td>
<td>1 in 200</td>
</tr>
</tbody>
</table>

Serial number of rabbit

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
<th>VIII</th>
<th>IX</th>
<th>X</th>
<th>XI</th>
<th>XII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

Note: Since the serum of rabbits IX and XII gave a positive result in tube 6, an additional test was done using higher dilutions.

TABLE II.

<table>
<thead>
<tr>
<th>Tubes</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>End Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Dilution</td>
<td>1/12,800</td>
<td>1/16,000</td>
<td>19,280</td>
<td>25,600</td>
<td>1/12,800</td>
</tr>
<tr>
<td>Rabbit IX.</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1/12,800</td>
</tr>
<tr>
<td>XII.</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>1/16,000</td>
</tr>
</tbody>
</table>
The end titre of rabbits IX and XII were therefore 1 in 12,800 and 1 in 16,000 respectively.

The sera of the twelve rabbits injected with detoxicated vaccine and the nine rabbits injected with defatted vaccine were tested in exactly the same way except that the original serum dilution in tube I. was 1 in 10. The end titres were as follows:

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>End Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>1 in 320</td>
</tr>
<tr>
<td>16</td>
<td>1 in 160</td>
</tr>
<tr>
<td>19</td>
<td>1 in 40</td>
</tr>
<tr>
<td>20</td>
<td>1 in 80</td>
</tr>
<tr>
<td>25</td>
<td>1 in 40</td>
</tr>
<tr>
<td>27</td>
<td>1 in 20</td>
</tr>
</tbody>
</table>

The serum of the other fifteen animals gave a negative result.

The Complement Fixation Test.

The method employed gives both a qualitative and quantitative estimation of the complement fixing bodies in a serum.

It has been found to be completely satisfactory in the many thousand Wassermann tests done in the Pathological Department of the Royal Infirmary, Edinburgh, during the past five years.

The four constituents necessary in the complement/
complement fixation test are:

I. Serum.
II. Complement.
III. Antigen.
IV. Haemolytic system.

I. SERUM. 0.1 c.c. of serum was chosen as the test amount. This amount is seldom anticomplementary to the extent of more than one unit of complement and usually fixes less than one unit. The natural complement in the serum is destroyed by heating for half an hour at 55°C. In addition, this heating reduces any anticomplementary action that is present. Equal volumes of serum and normal saline are mixed so that 0.2 c.c. of the mixture contains 0.1 neat serum.

II. COMPLEMENT: Fresh guinea pig's serum constitutes the best complement. The animal is bled the night before the test is performed, since complement is found to deteriorate very slowly after 18 hours. The haemolytic unit of complement is found by titration as follows:

TITRATION OF COMPLEMENT.

Dilutions of complement in saline from 1 in 10, 1 in 20, etc. up to 1 in 120 are made. 0.2 c.c. of each/
each dilution is removed in a 1 c.c. pipette to Wassermann tubes in a rack. 0.2 c.c. of sensitised red cells are added to each tube. 0.4 c.c. of normal saline is placed in each tube so that the total volume per tube may be the same as in the actual test. The tubes are shaken and then placed in the incubator at 37°C. for exactly half an hour when the results are read. 0.2 c.c. of the dilution of complement which produces complete haemolysis is the unit or haemolytic dose of complement. Table III. shows the titration of complement on March 14.

TABLE III./
### TABLE III.
**COMPLEMENT TITRATION.**

<table>
<thead>
<tr>
<th>Tubes :</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of each dilution of Complement</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Sensitized R. B. Cs.</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Normal Saline</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
</tbody>
</table>

**Incubation for half an hour at 37°C.**

| Result | H. | H. | H. | H. | H. | - | - | - | - | - | - |

**Note:** *H* = Haemolysis.

**Result:** The haemolytic dose of complement was 0.2 of a 1 in 60 dilution of the guinea pig's serum.

### III. Antigen:

The antigen used was an emulsion of the *B. suippestifer* in saline. An 18 hours' agar slope culture was emulsified in saline and standardised by the opacity method so that each c.c. contained 1,000/
1,000 millions of the organisms. To find the test dose of antigen, the following titration was performed.

Antigen titration.

Three rows of three Wassermann tubes were placed in a rack.

Row I. - Into each tube was placed 0.2 of the neat antigen (1,000 million organisms per c.c.)

Row II. - Into each tube was placed 0.2 of a 1 in 2 dilution of the antigen.

Row III. - Into each tube was placed 0.2 of a 1 in 3 dilution of the antigen.

0.2 of saline was placed in each tube to replace the serum which would be present in the test proper.

Into tubes 1, 2 and 3 were placed 1, 2 and 3 haemolytic doses of complement. Since by titration one haemolytic dose was known to be 0.2 c.c. of a 1 in 60 dilution of the guinea pig's serum, obviously two haemolytic doses were 0.2 c.c. of a 1 in 30 dilution and three doses were 0.2 c.c. of 1 in 20 dilution.

The tubes were shaken and placed in the incubator for one hour at 37°C. 0.2 of sensitized red cells were then added. The tubes were again shaken and incubated for another hour at 37°C, when the results were read.

Results/
Results of the Antigen Titration.

<table>
<thead>
<tr>
<th>Tubes:</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 Neat Antigen</td>
<td>0</td>
<td>0</td>
<td>H</td>
</tr>
<tr>
<td>0.2 of 1 in 2 dilution of Antigen</td>
<td>Trace</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>0.2 of 1 in 3 dilution of Antigen</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
</tbody>
</table>

Note:  
H = Haemolysis.

Result of test:  
0.2 of neat antigen could not be used since it was anticomplementary up to 2 units of complement.

0.2 of 1 in 2 dilution of antigen showed fixation of only a part of one unit of complement.

0.2 of 1 in 3 dilution of antigen gave no anticomplementary action at all.

The optimum dose of antigen is the largest amount that can be used without being anticomplementary.

Accordingly/
Accordingly 0.2 of a 1 in 2 dilution of the antigen was chosen as the antigenic unit.

IV. The Haemolytic System:
This consists of two parts -
(1) Washed sheep's Red Corpuscles.
(2) Antisheep haemolytic serum.

The Red Cells:
Sterile defibrinated sheep's blood was washed with saline from three to five times until all traces of serum were removed. A three percent suspension of washed cells was used throughout these investigations.

Haemolytic Serum
Burroughs & Wellcome's antisheep immune body was used. This is standardised so that 0.2 of a 1 in 1,000 dilution is one haemolytic dose. Between four and five haemolytic doses were used to sensitise the red cells.

If 100 c.c. of sensitised cells were required for the test, the following proportions of cells, immune body and saline would be used:

Saline .. 96.5 c.c.
Immune body .. 0.5 c.c.
(B. & W. antisheep
Haemolytic serum)
Washed sheep's
Red Corpuscles .. 3.0 c.c.

Total: 100.0 c.c.
The constituents must be well mixed and at least a quarter of an hour should be allowed for sensitisation to take place before the cells be used.

The COMPLEMENT FIXATION TEST

Having satisfactorily concluded these preliminary and essential tests, the complement fixation test proper may then be started.

The serum of each of the 35 rabbits in the experiment was examined for complement fixing bodies as follows:

(The serum of rabbit II. is shown as an example.)

<table>
<thead>
<tr>
<th>Tubes</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum rabbit II.</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>(1 in 2 dilution) Complement</td>
<td>3 units</td>
<td>5 units</td>
<td>8 units</td>
<td>12 units</td>
<td>15 units</td>
</tr>
<tr>
<td>Antigen (1 in 2 dilution.)</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Incubation one hour at 37°C.

<table>
<thead>
<tr>
<th>Sensitised Red Cells</th>
<th>0.2</th>
<th>0.2</th>
<th>0.2</th>
<th>0.2</th>
<th>0.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Result after one hour at 37°C</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>H</td>
<td>H</td>
</tr>
</tbody>
</table>

In addition to the test, antigen and serum controls must always be done.

Antigen control/
ANTIGEN CONTROL

<table>
<thead>
<tr>
<th>Tubes</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigen</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>1 in 2 dilution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complement</td>
<td>1 unit</td>
<td>2 units</td>
</tr>
<tr>
<td>Saline (to replace serum)</td>
<td>0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Incubation one hour at 37°C.

<table>
<thead>
<tr>
<th>Sensitised Red Cells</th>
<th>0.2</th>
<th>0.2</th>
</tr>
</thead>
</table>

Result after one hour at 37°C.

| Result after one hour at 37°C. | 0 trace | H |

Result: Non-specific fixation by the antigen of about 1 unit of complement.

SERUM CONTROL/
## SERUM CONTROL

<table>
<thead>
<tr>
<th>Tubes:</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum 1 in 2 dilution</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Complement Saline (to replace antigen)</td>
<td>1 unit</td>
<td>2 units</td>
<td>3 units</td>
</tr>
<tr>
<td>Incubation at one hour at 37°C.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitised Red Cells</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Result after one hour at 37°C.</td>
<td>0</td>
<td>H</td>
<td>H</td>
</tr>
</tbody>
</table>

**Result:** Non-specific fixation by the serum of 1 unit of complement.

**The TEST PROPER contd.**

Examination of the tubes in the actual test one hour after the sensitised cells had been added shows that the serum of rabbit II. had fixed 8 units of complement.

The next tube containing 12 units of complement showed complete lysis. Since both the antigen and the serum fixed by non-specific means one unit/
unit of complement each, it is necessary to deduct this from the eight units fixed in the test proper. It can therefore be recorded that the serum of rabbit II fixed six units of complement.

The sera of the 33 rabbits inoculated with ordinary, detoxicated and defatted vaccines were examined after each weekly injection exactly in the same way as has been given in detail in the case of rabbit II., and the results are shown in the graphs and chart in Chapter I.

In addition, detoxicated and defatted vaccines were used as antigens against the serum of rabbits injected with these as well as ordinary vaccines. No evidence was forthcoming that they were of any value as antigens in the complement fixation test or as producers of antibodies.
The Measurement of the Bactericidal Power of the Blood in vitro

Two methods are available:

(1) The Plate Culture Method of Stern and Korte.
(2) The Capillary Pipette Method of Wright.

(1) The Plate Culture Method

It is essential that all the vessels, diluting fluids and sera employed should be absolutely sterile. The technique consists of mixing dilutions of the test serum, inactivated by heating at 55°C. for half an hour with complement and an emulsion of the organism. The mixture is incubated at 37°C. for three hours when it is poured into test tubes of melted agar at 45°C. The contents are then plated in Petri dishes and incubated for 24 hours at 37°C., when the colonies are counted. A similar process is carried out using normal serum as a control.

The sera of rabbits immunised with B. suipastifer were examined as shown in Table I.

TABLE I.
TABLE I.

<table>
<thead>
<tr>
<th>1 c.c. of dilutions of immune (Inactivated) serum</th>
<th>24 hours culture of B. suipestifer dilution 1 in 500</th>
<th>Complement Fresh normal rabbit's serum 1 in 5</th>
<th>Plates poured after 3 hours at 37°C. counted after 24 hours at 37°C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 in 10</td>
<td>0.5</td>
<td>0.5</td>
<td>Thous- Thousands: ands: ands.</td>
</tr>
<tr>
<td>1 in 50</td>
<td>0.5</td>
<td>0.5</td>
<td>&quot;</td>
</tr>
<tr>
<td>1 in 100</td>
<td>0.5</td>
<td>0.5</td>
<td>&quot;</td>
</tr>
<tr>
<td>1 in 500</td>
<td>0.5</td>
<td>0.5</td>
<td>&quot;</td>
</tr>
<tr>
<td>1 in 1000</td>
<td>0.5</td>
<td>0.5</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

Result: No evidence of bacteriolysis could be found.

The test was repeated using 0.5 c.c. of a 1 in 2,000 dilution of a 24 hours' broth culture of B. suipestifer.

Result: About 500 colonies were found in all plates. Again no evidence of bacteriolysis could be found.

In addition to the test, the following controls were put up:

CONTROLS/
## CONTROLS

<table>
<thead>
<tr>
<th>Control</th>
<th>Description</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control I</td>
<td>0.5 c.c. of culture dilution</td>
<td>Hundreds of colonies</td>
</tr>
<tr>
<td>Control II</td>
<td>0.5 c.c. of complement + 0.5 of culture dilution</td>
<td>Hundreds of colonies</td>
</tr>
<tr>
<td>Control III</td>
<td>0.5 c.c. rabbit's serum (complement) alone</td>
<td>Sterile</td>
</tr>
<tr>
<td>Control IV</td>
<td>0.1 c.c. immune serum alone</td>
<td>Sterile</td>
</tr>
</tbody>
</table>

The controls were therefore satisfactory.

### The Capillary Pipette Method (Wright)

Quantitative titration is accomplished by furnishing varying dilutions of culture with a constant quantity of the test serum. The largest number of bacteria that a constant quantity of serum has been able to kill furnishes a measure of its bactericidal power. No complement is used in this test, the test serum being used fresh; thus supplying its own complement.
complement.

The following dilutions of a 24 hours' broth culture of B. suipestifer were made:

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 in 10</td>
<td>1 in 500</td>
</tr>
<tr>
<td>1 in 50</td>
<td>1 in 5000</td>
</tr>
<tr>
<td>1 in 500</td>
<td>1 in 50,000</td>
</tr>
</tbody>
</table>

Into looped pipettes, marked about 2 cm. up the stem, litmus mannite broth was drawn until the bulbs were about two-thirds full. The test serum is now allowed to run up to the fiduciary mark. An air bubble is aspirated and the dilution of culture is then sucked in up to the mark. The two volumes of culture and serum are then gently blown into a sterile tube. By drawing up and blowing out the fluid several times, a thorough mixture is made. Care must be taken that an air bubble supervenes between the mannite broth and the mixture in order to avoid infecting the broth at this stage. The mixture of culture and serum is drawn into the middle portion of the capillary stem and the end sealed in the flame. The pipette is incubated for 24 hours to allow bacteriolysis, if present, to take place. The mixture is then drawn into the broth and the pipette incubated for a further 24 hours. If any bacilli are alive, fermentation of the mannite takes place with the production of acid. The colour of broth is therefore changed from blue to red.

Controls:
Controls: The test is done with normal serum and the same culture dilutions.

In addition, the culture with no serum is examined in an identical manner.

Result: Even such dilutions of culture as 1 in 50,000 were unaffected by the serum of rabbits immunised with B. suipéstifer.

Conclusions:

Repeated investigations by the Plate Culture Method and Looped Pipette Method fail to demonstrate the presence of bacteriolysins in the serum of animals immunised with B. suipéstifer vaccines.

Opsonins:

The method of the test and pipette (Wright) was employed. Equal volumes of washed leucocytes, bacterial emulsion and serum were drawn into the stem of the pipette. Each ingredient was separated from the next by an air bubble. By blowing out and drawing in, the three constituents were thoroughly mixed, after which the end of the pipette was sealed in the flame. The mixture was incubated at 37°C for ten minutes, to allow of phagocytosis taking place. A similar test was made with normal serum. Films were spread and stained with Leishman's stain.
stain and a phagocytic count made.

Results:

Several attempts to obtain the opsonic index of the serum of animals immunised with *B. suippestifer* were made. According to Wright, it is essential that on microscopic examination of the films, the organisms should be evenly distributed over the field.

Since the test immune serum containing such large amounts of agglutinins that even in the ten minutes allowed for phagocytosis to take place, clumping of the organism occurred, it was impossible to obtain an even distribution of the bacilli. Because of this clumping, it was impossible to estimate the opsonic index of the test sera.

Conclusion:

No evidence could be obtained regarding the presence or absence of opsonins.
CHART 1.

Showing Graphs of Agglutination Titres of Rabbits I-XII Injected with the Bacillary Emulsion of Swinestifer.

NOTE:

Rabbits I, VII & XII were injected subcutaneously. The Graphs showing their Agglutination Titres are drawn with dotted lines.
The Average Agglutination Titres of Animals in Sub Groups A, B and C are shown. The Graphs of Animals inoculated subcutaneously are drawn with dotted lines.
CHART 3.

Showing Graphs of the Average Complement Fixation of Animals in Sub groups A, B, C. Animals inoculated subcutaneously are shown by dotted lines.
CHART 4.

Graphs of the Agglutination Titres of Rabbits 13-24 injected with B Suscetefor Detoxicated Vaccine

Note:
Rabbits 15, 16, 19, 20, 21, 22 were inoculated with a Detoxicated Vaccine consisting of an Alkali soluble fraction alone.

Rabbits 13, 14, 17, 18, 23, 24 were inoculated with a Detoxicated Vaccine consisting of an Alkali and an Alcohol soluble fraction.

Note:
Rabbits 13, 14, 17, 18, 21, 22, 23, 24 produced no Agglutinations.
CHART 5.

Graphs of the Agglutination Titres of Rabbits R5-36 injected with Defatted B. Suipstifer Vaccine.

Note:
Rabbits 26, 28, 29, 30, & 34, 35, 36, produced no Agglutinations.
Showing Graphs of Agglutination Titres of Rabbits injected with
1) Baxillary Emulsion
2) Detoxicated Vaccine
3) Defatted Vaccine.

CHART 6.


**CHART 7.**

Graphs showing Average Complement Fixation in Rabbits injected with,

1. Bacillary Emulsion.
2. Detoxicated Vaccine.
3. Defatted Vaccine.
Since 1891 it has been recognised that it is possible to produce a highly immune anti-pneumococcal serum which is capable of protecting a susceptible animal, such as the mouse, against many lethal doses of the organism. The administration of such immune sera to patients suffering from pneumonia was, however, followed by little or no therapeutic benefit. The publication, in 1917, of the researches of Avery, Chickering, Cole and Dochez, of the Rockefeller Institute, explained the reasons of this failure. These workers injected a large number of different strains of pneumococci into animals and by means of a serological test were able to classify the organism into four types. Organisms of Types I, II and III were agglutinated by their own homologous serum, but showed no cross agglutination with the other Type sera. The organisms classified as Type IV consisted of pneumococci which bore no serological relationship to each other or to members of Types I, II, or III. In addition, it was found that protective sera of any potency could only be produced against Type I pneumococcus.
pneumococcus. To obtain beneficial results clinically, it was necessary to inject intravenously large amounts of this immune serum. The failure of former immune sera was partly due to the injection of too small amounts, but mainly to the ignorance of the fact that several types of pneumococci exist which are serologically as separate from one another as they are from the streptococcus.

The Incidence of Types of Pneumococci in Lobar Pneumonia.

By means of the agglutination test, the organisms causing all cases of acute pneumococcal lobar pneumonia in the Hospital of the Rockefeller Institute were typed. The following table shows the incidence of the various types and the resulting mortality:

<table>
<thead>
<tr>
<th>Type of Pneumococcus</th>
<th>Incidence</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I.</td>
<td>33%</td>
<td>25% - 30%</td>
</tr>
<tr>
<td>Type II.</td>
<td>32%</td>
<td>32%</td>
</tr>
<tr>
<td>Type III.</td>
<td>9%</td>
<td>45%</td>
</tr>
<tr>
<td>Type IV.</td>
<td>20%</td>
<td>16%</td>
</tr>
</tbody>
</table>

Result of Serum Treatment in Type I. Cases.

The result of the injection of 200-300 c.c. of immune serum is certainly very striking. In 107 cases treated in the Hospital of the Rockefeller Institute,
the mortality rate fell from 25% to 7.5%. Reports from other Hospitals on 495 cases treated with serum showed a mortality rate of 10.5%.

The Advantages and Disadvantages of the Serum Treatment of Pneumonia.

No more need be said in regard to the advantage of this treatment if it reduces the mortality rate from 25% to 7.5%. From the point of view of the general practitioner there are, however, certain serious disadvantages:

I. The cost of large amounts of immune serum is a heavy item:

II. The technical difficulties which beset the general practitioner in giving several hundred cubic centimetres of serum intravenously:

III. The production of serum sickness which invariably follows within a fortnight:

IV. The necessity of having the close co-operation of a trained bacteriologist who can type the infecting agent.

The Identification and Typing of the Pneumococcus.

Before serum treatment can be started, it is essential to know

(1) If the causal organism is a pneumococcus;
(2) If so, to which type it belongs.

Only when it is established that the causal organism is a Type I. pneumococcus should serum treatment be instituted.
The following description contains all the evidence necessary for diagnosing the causal organism as being a pneumococcus:—

The pneumococcus is a Gram-positive, lanceolate-shaped diplococcus which is bile-soluble, ferments inulin, possesses a capsule, is pathogenic for mice, and on blood agar forms a flat ringed colony surrounded by a greenish zone of methaemoglobin. Moreover, with the exception of a Type IV pneumococcus, it reacts specifically with its homologous immune serum.

**The Typing of Pneumococci.**

A piece of fresh sputum about the size of a bean is finely emulsified and the emulsion is injected intraperitoneally into a white mouse. In a period ranging from 6-24 hours, depending on the virulence and numbers of the organisms in the sputum, the mouse will sicken and die from a pneumococcal peritonitis and septicæmia. The peritoneal cavity is then washed out with 3-4 c.c. of sterile saline and the washings, which are of milky opacity, are removed to a sterile tube. A microscopic examination shows a mixture of fibrin, cells, and large numbers of pneumococci. The washings should be centrifuged slowly for a few minutes to remove the cells and fibrin. The suspension/
suspension of organism is then tested against suitable dilutions of the different typing sera, the mixture being incubated at 37.5°C. A positive agglutinating reaction should occur within one hour if the pneumococcus belongs to Type I, II, or III. Gross agglutination never occurs if the proper serum dilutions are used. The result of the test is simply and easily read. As a general rule, it can safely be said that the bacteriologist can report the type of the organism within twelve hours of receiving the sputum.

During the past nine months, with a view to discovering what proportion of pneumococcal infections in Edinburgh were due to Type I. organism, I have carried out this technique in a number of cases. Many more cases would have been tested if I had not been delayed for three months by contracting the disease myself. Owing to the limited number of cases investigated, the following report must be considered only as a preliminary investigation into the subject:-

Table I. gives the results of typing the organism in different pneumococcal infections. The two periods February-April and October-November are given separately in order to show that in different epidemics the proportion of the different types may vary. Only three points deserve consideration:-

I./
I. That during the period October-November, seventeen cases of different pneumococcal infections were examined and in every case the organism was found to be Type I.

II. An examination of the organism in fifteen cases of acute pneumococcal lobar pneumonia during the period February-April shows that the Type I organism was responsible for only 40 per cent of the cases. This figure is approximately the same as that of the Rockefeller workers.

III. Seventeen cases of pneumococcal empyema were investigated, two of which were in children. In every case the organism was Type I.

CONCLUSIONS.

This preliminary investigation into the types of pneumococci prevalent in infections in Edinburgh affords sufficient evidence to justify the consideration of serum therapy, especially in cases of empyema occurring in children.

TABLE I /
TABLE I.

<table>
<thead>
<tr>
<th></th>
<th>Type</th>
<th>Feb.-April No. of Cases</th>
<th>Oct.-Nov. No. of Cases</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pneumonia</strong></td>
<td>I.</td>
<td>6</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>II.</td>
<td>4</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>III.</td>
<td>3</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>IV.</td>
<td>2</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td><strong>Children</strong></td>
<td>I.</td>
<td>7</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>II.</td>
<td>3</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>III.</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>IV.</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Empyema Adults</strong></td>
<td>I.</td>
<td>7</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>II.</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>III.</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Otitis Media</strong></td>
<td>I.</td>
<td>-</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>II.</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>III.</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td><strong>Brain Abscess</strong></td>
<td>III.</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td><strong>Meningitis</strong></td>
<td>I.</td>
<td>-</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>Endocarditis</strong></td>
<td>I.</td>
<td>-</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Total number of pneumococcal infections examined = 44

Total number of Type I. pneumococci found = 33 = 75 per cent

Total number of pneumonia cases examined between February and April = 15 cases
Percentage of Type I. pneumococci = 40 per cent.

Total number of empyema cases examined = 17 cases.
Percentage of Type I. pneumococci = 100 per cent.

Note: 17 Cases of different pneumococcal infections examined during Oct.-Nov. were all Type I.

Chapter II.
A Comparison of the Antigenic Values of Detoxicated, Defatted and Ordinary Heat-Killed Saline Pneumococcal Vaccines.

Before any therapeutic remedy is tried clinically, evidence of its beneficial effects should be forthcoming from experimental laboratory investigation.

For purposes of active immunisation in the past, heat-killed cultures prepared in the form of a saline emulsion of micro-organisms have been generally relied on and have constituted the usual type of vaccine employed in medical practice. More recently, newer types of vaccine preparations have been advocated, e.g., the so-called detoxicated and defatted vaccines. It is of undoubted practical importance to the practitioner to know what is the actual and relative immunising properties of these preparations.

In a paper read to the British Medical Association at Bradford in July, 1924, I showed that, as judged by the production of antibodies, detoxicated and defatted vaccines were in no way comparable to the ordinary heat-killed saline emulsions of the organism. In that experiment, the test organism was B. suipastifer, which is a Gram-negative organism of the paratyphoid C group of intestinal bacilli. B. suipastifer, which at one time was thought to be the causal agent in swine/
swine fever, produces an excellent antibody response in rabbits. When injected in the living state, it is so excessively virulent that no minimum lethal dose can be established. Owing to this intense virulence, no method of immunisation has ever been successful in protecting rabbits from even the most minute doses of the living bacilli.

The following Table shows a comparison of the antibody production in rabbits injected with detoxicated, defatted and ordinary saline vaccines of B. suipestifer:

<table>
<thead>
<tr>
<th></th>
<th>Number of Rabbits Injected</th>
<th>Average Agglutinating Titre</th>
<th>Average of Units in Complement Fixation Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ordinary Vaccine</td>
<td>12</td>
<td>1 in 5000</td>
<td>5</td>
</tr>
<tr>
<td>Detoxicated Vaccine</td>
<td>12</td>
<td>1 in 50</td>
<td>1</td>
</tr>
<tr>
<td>Defatted Vaccine</td>
<td>9</td>
<td>1 in 5</td>
<td>-</td>
</tr>
</tbody>
</table>

The detoxicated vaccine was given in doses of at least 1,000 times those used in the defatted and ordinary vaccines.

The question arises - What relation do antibodies bear to true immunity as judged by protection to a lethal dose of the organism? With the exception of the antitoxins, it must be admitted that we are unable definitely to state the relationship of antibodies to immunity.

While/
While it is probable that they form part of the host's defences against bacterial invasion, in the present state of our knowledge we are only justified in assuming that since antibodies are the specific result of the antigen injected and the host's body cells, that they may at least be regarded as indicators of the antigenic value of a vaccine.

The real test of a vaccine is therefore the production of immunity to a lethal dose of organisms. Accordingly a new series of experiments was undertaken, the pneumococcus being chosen as the test organism since its minimum lethal dose for rabbits and mice can be estimated accurately.

**Vaccines used:**

A recently isolated Type I pneumococcus was selected as the test organism. 1 c.c. of a living broth culture injected into a rabbit caused death.

The following vaccines were made from this organism:

**I.**

(a) Ordinary saline bacterial emulsion killed by heating for 1 hour at 60°C. Each c.c. contained 1 mgm. of dried organisms.

(b) Heat-killed 24 hours broth culture.

(c) Living 24 hours broth culture.

**II. Detoxicated Vaccine**

A subculture of the organism was sent to Dr. David.
David Thomson, the originator of the process. The detoxicated vaccine which he returned contained the products of 50 thousand million pneumococci per c.c.

My thanks are due to Dr. Thomson for the trouble he has taken in making this as well as other special detoxicated vaccines.

III. Defatted Vaccine

Pneumococci were treated with formalin at 100°C for four hours and were then extracted with acetone in a Soxlet apparatus for 24 hours. Microscopical examination showed that the organism had lost the power of retaining Gram's stain. The defatting process was carried out exactly as published by its originator, Professor Dreyer. The vaccine contained 1 mgm. of defatted pneumococci per c.c.

The Active Immunisation of Rabbits.

Each rabbit received six injections of vaccine at five days' intervals. Table II shows the quantities employed. It should be remembered that immunisation of rabbits with the pneumococcus is attended with great difficulty. The endo-toxin contained in the bacterial protoplasm produces a wasting and in time death of the animals.

On November 12, seven days after the last injection, blood was drawn from all surviving animals.

TABLE II.
**TABLE II.**

### (A) ANIMALS INJECTED WITH ORDINARY VACCINES.

#### A.I. Heat-killed Saline Bacterial Emulsion.

<table>
<thead>
<tr>
<th>Date</th>
<th>Oct.</th>
<th>15</th>
<th>20</th>
<th>25</th>
<th>30</th>
<th>Nov.</th>
<th>Total in mgs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit 82</td>
<td>0.1</td>
<td>0.2</td>
<td>0.4</td>
<td>0.6</td>
<td>1.6</td>
<td>3.2</td>
<td>6.3</td>
</tr>
<tr>
<td>Rabbit 83</td>
<td>0.2</td>
<td>0.4</td>
<td>0.6</td>
<td>1.6</td>
<td>3.2</td>
<td>6.4</td>
<td>12.6</td>
</tr>
</tbody>
</table>

**Route of Administration:**
- Intravenous
- Subcutaneous

#### A.II. Heat-killed Broth Culture.

| Rabbit 80 | 0.2 | 0.4 | 0.6 | 1.6 | 3.2 | 6.8 |
| Rabbit 81 | 0.2 | 0.4 | 0.6 | 1.6 | 3.2 | 8.2 |

**Route of Administration:** Intravenous

#### A.III. Living Broth Cultures.

| Rabbit 82 | 0.2 | 0.4 | 0.6 | 1.6 | 3.2 | 3.0 |
| Rabbit 83 | 0.2 | 0.4 | 0.6 | 1.6 | 3.2 | 3.6 |

**Route of Administration:** Intravenous

**Note:** Rabbit 92 died on 8:11:24. Rabbit 93 died 13:11:24.

### (B) ANIMALS INJECTED WITH DETOXICATED VACCINE.

**Total in Millions**

| Rabbit 80 | 500 | 1000 | 2000 | 5000 | 10,000 | 20,000 | 38,500 |
| Rabbit 81 | 500 | 1000 | 2000 | 5000 | 10,000 | 20,000 | 38,500 |
| Rabbit 82 | 1000 | 2000 | 5000 | 10,000 | 20,000 | 50,000 | 88,000 |
| Rabbit 83 | 1000 | 2000 | 5000 | 10,000 | 20,000 | 50,000 | 88,000 |

**Route of Administration:**
- Intravenous
- Subcutaneous

### (C) ANIMALS INJECTED WITH DEFATTED VACCINE.

| Rabbit 84 | 0.1 | 0.2 | 0.4 | 0.6 | 1.6 | 3.2 | 8.2 |
| Rabbit 85 | 0.1 | 0.2 | 0.4 | 0.6 | 1.6 | 3.2 | 8.2 |
| Rabbit 87 | 0.2 | 0.4 | 0.6 | 1.6 | 3.2 | 6.4 | 13.8 |

**Route of Administration:** Intravenous

**Note:** Rabbit 85 died 8:11:24.
The pooled serum from the rabbits in groups A, B and C respectively was examined for protective value by means of the following experiment:

Sixty mice of approximately the same size and age obtained from the same dealer were placed in six cages; each cage therefore contained ten mice. Each mouse was marked by means of coloured dyes.

Dilutions of a 24 hours' broth culture of living pneumococcus were made so that 0.5 c.c. of each dilution contained the following quantities of the culture:

- 0.1 c.c., 0.01, 0.001, 0.0005, 0.0001, 0.00005
- and 0.00001 c.c.

0.5 c.c. of each dilution was drawn into a 1 c.c. luer syringe followed by 0.4 c.c. of the test serum. The mixture of culture and serum was immediately injected intraperitoneally into the mouse.

In addition to the pooled serum from rabbits injected with ordinary, detoxicated and defatted vaccines, Rockefeller immune serum, normal rabbit's serum, and the dilutions of culture with no serum were tested.

Table III gives the quantities injected and the results of the experiment.

Table III.
**Table III**

<table>
<thead>
<tr>
<th>Case</th>
<th>Serum</th>
<th>Culture</th>
<th>Serum</th>
<th>Culture</th>
<th>Serum</th>
<th>Culture</th>
<th>Serum</th>
<th>Culture</th>
<th>Serum</th>
<th>Culture</th>
<th>Serum</th>
<th>Culture</th>
<th>Serum</th>
<th>Culture</th>
<th>Serum</th>
<th>Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>III</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Note**: Survived = S  Died = D
The Result of the Experiment:

This experiment may be considered as being eminently satisfactory since a definite clear cut result is obtained.

I. The serum from animals injected with ordinary vaccines (Cage III.) and the Rockefeller Immune Serum (Cage IV.) gave complete protection against 10,000 lethal doses of pneumococci. From another experiment I am able to state that protection was afforded to at least 50,000 lethal doses.

II. The serum from animals injected with detoxicated (Cage I.) and defatted (Cage II.) vaccines showed no protective properties, since normal rabbit’s serum (Cage V.) gave similar negative results.

III. It is to be noted that when a protective experiment of this type is being done, the control animals should receive normal serum + the culture, and not the culture alone. The effect of introducing foreign protein into an animal is to produce temporarily a lowering of resistance. This is probably a non-specific toxic effect. In the above experiment the M.L.D. of the broth culture alone lay between 0.001 and 0.0005 c.c., while the M.L.D. of culture + normal serum lay between 0.0001 and 0.00005. Hence an immune serum, to be efficient, must not only be able to overcome/
overcome the infecting agent but, in addition, must counteract the temporary increased susceptibility produced by the toxic foreign protein.

It seemed not unlikely that the beneficial results following the injection of the pooled serum of rabbits immunized with ordinary vaccines were due to the protective bodies existing in the serum of Rabbit 93 since this animal had been injected with a living broth culture of the test organism. Moreover, this was the only animal to possess a serum containing agglutinins.

To test this hypothesis, the following experiment was performed:

Blood was withdrawn from Rabbits 82, 90 and 91, which had been injected with an ordinary killed vaccine, and the serum pooled.

Serum from Rabbit 93, which had been injected with a living broth culture, was used separately.

0.4 c.c. of these sera with different dilutions of broth cultures were injected into mice. In addition, normal serum + culture was injected into control animals. Table IV. shows the quantities injected and the results:

TABLE IV.
TABLE IV.

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Pooled Serum from Rabbits 88, 90, 91</th>
<th>Serum from Rabbit 93</th>
<th>Controls Pooled Normal Rabbit's Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Culture Serum Result</td>
<td>Culture Serum Result</td>
<td>Culture Serum Result</td>
</tr>
<tr>
<td>1</td>
<td>0.1 ± 0.4 S.</td>
<td>0.1 ± 0.4 D.</td>
<td>0.1 ± 0.4 D.</td>
</tr>
<tr>
<td>2</td>
<td>0.1 ± 0.4 S.</td>
<td>0.1 ± 0.4 D.</td>
<td>0.01 ± 0.4 D.</td>
</tr>
<tr>
<td>3</td>
<td>0.01 ± 0.4 S.</td>
<td>0.01 ± 0.4 D.</td>
<td>0.001 ± 0.4 D.</td>
</tr>
<tr>
<td>4</td>
<td>0.01 ± 0.4 S.</td>
<td>0.01 ± 0.4 D.</td>
<td>0.001 ± 0.4 D.</td>
</tr>
<tr>
<td>5</td>
<td>0.001 ± 0.4 S.</td>
<td>0.001 ± 0.4 D.</td>
<td>0.0001 ± 0.4 D.</td>
</tr>
<tr>
<td>6</td>
<td>0.001 ± 0.4 S.</td>
<td>0.001 ± 0.4 D.</td>
<td>0.0001 ± 0.4 D.</td>
</tr>
<tr>
<td>7</td>
<td>0.0001 ± 0.4 S.</td>
<td>0.0001 ± 0.4 D.</td>
<td>0.00001 ± 0.4 D.</td>
</tr>
<tr>
<td>8</td>
<td>0.00001 ± 0.4 S.</td>
<td>0.00001 ± 0.4 D.</td>
<td>0.000001 ± 0.4 D.</td>
</tr>
</tbody>
</table>

Results of the above experiment:

The result was again absolutely definite. The serum of the animals injected with the ordinary heat-killed vaccine contained the protective substances.

It is not to be assumed from the results shown in Table IV, that immunisation with living organisms is less effective than with dead organisms. In fact, I have evidence from other experiments that the contrary is the more probable. Rabbit 93 was practically moribund from the toxic effects of the repeated injections of living organisms.

Hence a fair comparison can be made between the protective values of ordinary vaccines, detoxicated vaccines and defatted vaccines.

In order to ascertain the degree of immunity
of the rabbits injected with ordinary, defatted and
detoxicated vaccines, three animals from each group
were injected with a living broth culture of the
pneumococcus.

Table V. shows the quantities injected, and
the results:

<table>
<thead>
<tr>
<th>Rabbits immunised with Ordinary Vaccines</th>
<th>Rabbits immunised with Detoxicated Vaccine</th>
<th>Rabbits immunised with Defatted Vaccine</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose of Culture</td>
<td>Period of Survival</td>
<td>Dose of Culture</td>
<td>Period of Survival</td>
</tr>
<tr>
<td>R.88 1/3 c.c.</td>
<td>Survived</td>
<td>R.80 1/3 c.c.</td>
<td>2</td>
</tr>
<tr>
<td>R.91 1 c.c.</td>
<td>Survived</td>
<td>R.81 1 c.c.</td>
<td>2</td>
</tr>
<tr>
<td>R.93 1 c.c.</td>
<td>Days</td>
<td>R.82 1 c.c.</td>
<td>1 day R.84 1 c.c.</td>
</tr>
</tbody>
</table>

NOTE: R. 93 was in a dying condition before the
test injection.

Results of Table V.

The minimum lethal dose of the broth culture
was 1/3 c.c. This killed a normal rabbit in four days.

I. Rabbits 80, 81 and 82, which had been
immunised/
immunised with detoxicated vaccine, showed no immunity to the pneumococcus. Moreover, they appeared to have an increased susceptibility since they survived only from 1 - 2 days, as compared to a four day survival period of normal rabbits.

II. One rabbit out of three immunised with defatted vaccine survived the test lethal dose.

III. With regard to the three rabbits immunised with ordinary vaccines, two survived the lethal dose. Rabbit 93, which succumbed, was in such an emaciated condition before the test injection that it was a matter of surprise that it survived for five days even without the introduction of living pneumococci. Its death should be ascribed to the toxic effects of the living vaccine with which it had been immunised and not to the test lethal dose of micro-organisms.

**General Condition of the Rabbits at the end of the period of immunisation.**

The rabbits injected with detoxicated vaccine were in a fat and healthy condition when examined seven days after the sixth injection. No evidence of local inflammation at the sites of subcutaneous injection was apparent.

The rabbits injected with defatted vaccine were/
were only in fair health and showed a degree of wasting.

Large hard lumps, the size of a bean, could be felt at the sites of subcutaneous injection.

The animals immunised with ordinary vaccines showed a considerable degree of wasting. Those injected with the living organisms were in the poorest health. Two out of three died before the test experiments could be performed. The survivor, Rabbit 93, was in a dying condition. It is indeed significant that the animals in the best state of health showed the least resistance while animals showing definite toxic symptoms could still contain serum of high protective value.

**GENERAL CONCLUSION.**

As judged by I. Production of antibodies;

II. Protection to lethal doses of the micro-organism,
detoxicated and defatted vaccines have no immunological value comparable to that of ordinary heat-killed bacterial vaccines.

Chapter III./
An investigation into the antibody content of immune antipneumococcal sera.

Since the pooled serum of rabbits immunised with ordinary heat-killed pneumococcal vaccines protected mice from 50,000 lethal doses of the living organism, it was necessary to discover, if possible, the nature of the contained protective substances.

Accordingly, the serum from all the immunised rabbits as well as Rockefeller immune serum, Burroughs and Wellcome immune serum and normal rabbit's serum were examined for the following antibodies:

I. Agglutinins.

II. Complement fixing bodies.

III. Bacteriolysins.

IV. Antiblastic action.

V. Opsonins.

I. Agglutinins:

The details of the technique employed need not be repeated in any of these tests as they were the same as described in the previous chapters. The sera were examined after the third, fourth, fifth and sixth injections of vaccines.

Table I. shows the result of the agglutination tests.
tests done one week after the sixth injection. The antigen used was a 24 hours' broth culture of pneumococcus Type I. - strain 659, which was the organism from which the vaccines were prepared. Since there is considerable difficulty in producing a heavy growth in ordinary bouillon, without the addition of either serum or blood to it, an investigation into the various ingredients of broth and the methods of standardising the pH was undertaken. With the help of information from Dr. H. Wright, University College, London, the following method was found to produce a thoroughly satisfactory medium:

Phosphate Broth/
PHOSPHATE BROTH for Growth of Pneumococcus.

I. Take some VEAL, remove most of fat, and mince.

II. Place 500 grms. of minced veal in pot, add 1000 c.c. of distilled water and allow to stand in cool place overnight.

III. Filter off fluid through muslin. Steam fluid for 1½ hours to coagulate albumins, etc. Filter through coarse grey filter paper and make quantity up to 1000 c.c. with Dist. Water.

IV. Steam twice for 20 mins. on two consecutive days: this can be kept as Stock Extract.

V. To 1000 c.c. Extract add:

- 10 grms. Peptone (De Fresnes);
- 2 " Di Sodium Phosphate;
- 20 c.c. N Caustic Soda.

VI. Steam for 1 hour: standardise to P.H. 7.8.

VII. Steam again for 20 mins. Test reaction must be 7.8. Filter through grey filter paper. Test reaction must be P.H. 7.8.

VIII. Tube and Autoclave for 10 mins. at 10 lbs. pressure.

Test Reaction should be P.H. 7.6.

If reaction drops more than two points at this stage, it usually means too little heating has been used previously and should be re-adjusted.
Veal is used instead of beef and a special peptone is employed. The effect of the phosphate is to produce an efficient buffering of the fluid, while the repeated heatings finally result in the production of a pH which does not alter even if the medium is kept for several weeks.

When dealing with the pneumococcus, the question of the hydrogen-ion concentration of the medium is probably the most important aspect of the problem.
<table>
<thead>
<tr>
<th>Serial Immunizing Number of Rabbit</th>
<th>Dilutions of Serum</th>
<th>Final agglutination titre.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 in 2</td>
<td>1 in 4</td>
</tr>
<tr>
<td>80 Detoxicated vaccine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>81 &quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>82 &quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>83 &quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>84 Defatted vaccine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>85 &quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>86 &quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>87 &quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>88 Heat-killed ordinary vaccine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>89 &quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>90 &quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>91 &quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>93 Living ordinary vaccine</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Normal Rabbit Serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rockefeller Serum</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Result:
Result:

With the exception of rabbit 93 which was immunised with a living pneumococcal vaccine, no agglutinins were present in the serum of any of the immunised animals.

II. Complement Fixation Test:

The technique employed was the same as previously described:

Serum 0.1 c.c.
Antigen 0.2 c.c. of an 18 hours' broth culture of pneumococcus.
Complement 0.2 c.c. of dilutions of guinea pig's serum which, by titration, were found to equal 1, 2, 3, 5, 7, 9 and 12 haemolytic doses of complement.

Haemolytic system 0.2 c.c. of a 3 per cent suspension of washed sheep's red corpuscles sensitised with five haemolytic doses of B. & W. antisheep immune body.

Since it is recognised that only small quantities of antibodies are produced by immunisation with pneumococcal vaccines, 2, 3, 4 and 5 units of complement were used in the first investigation. The antigen (broth culture) was heated for 30 minutes at 70°C to kill the organisms. Serum and antigen controls against 1, 2 and 3 units of complement were always made.

Result:

Examination of the tubes at the end of 1 hour
at 37°C. showed that complete fixation up to five units was present in all cases including the Rockefeller serum. Examination of the antigen control showed that 0.2 c.c. of the antigen alone fixed one unit. Examination of the serum controls showed that 0.1 c.c. of the serum of immunised rabbits fixed one or less than one unit of complement. 0.1 c.c. of Rockefeller serum, however, fixed two units.

It was obvious that the test would have to be repeated using larger amounts of complement before a complete estimation of complement fixation could be made. Deducting the non-specific fixation of the antigen (1 unit) and of the serum (under 1 unit except in the case of the Rockefeller serum), the readings recorded showed that the serum of the immunised rabbits fixed 3 + units of complement and that the Rockefeller serum fixed 2 + units.

The test was therefore repeated using 5, 7, 9 and 12 units of complement. Serum and antigen controls against 1, 2 and 3 units were also put up. The antigen (broth culture) was used unheated.

Result after 1 hour at 37°C.:

With the exception of the serum controls which showed fixation of one unit of complement only and which were of the normal red colour of blood corpuscles, the corpuscles in all the remaining tubes were of greenish-yellow colour and showed no signs of lysis.

This puzzling result could only be due to the antigen, since the serum controls being normal, obviously the serum, complement and sensitised red cells were not/
not the causal agents.

It was therefore concluded that this extraordinary result was due to the living pneumococci in the antigen producing, during the two hours in which they were in the incubation, a ferment which acted on the red cells in such a way as to produce methaemoglobin. The antisheep haemolytic serum was rendered inactive, either because of the action of the ferment on itself or more probably, because the corpuscles themselves had been altered; in consequence of this result, it was necessary in future tests to heat the broth culture at 70° C. for half an hour in order to kill the organisms and destroy any ferment already present.

The test was repeated, using a killed broth culture of antigen. In addition, normal rabbit's serum was tested. 3, 5, 7, 9 and 12 units of complement were used. Serum and antigen controls were put up as before.

Result:

0.2 c.c. of antigen fixed two units of complement.

0.1 c.c. of serum fixed no complement except in the case of the Rockefeller serum, which fixed two units.

After deducting these non-specific fixations of complement, I found that
I. The serum of rabbits immunised with detoxicated vaccine fixed nine units of complement.

II. The serum of rabbits immunised with defatted vaccine fixed ten units.

III. The serum of rabbits immunised with ordinary vaccines fixed ten units.

IV. The Rockefeller serum fixed two units.

V. Pooled serum of normal rabbits fixed ten units.

It was a matter of great importance to repeat the experiment, using normal rabbit's serum, since, if this non-specific fixation occurred, the test was valueless. Accordingly, pooled serum from six apparently normal rabbits was examined and non-specific fixation was found to exist.

In addition, since it is recognised that alteration of the hydrogen-ion concentration of the fluids employed may produce non-specific effects in the test, the phosphate broth used for cultivating the organism was employed as the antigen. The broth itself was found to fix between two and three units of complement when used in the test itself i.e. when mixed with serum and complement.

The non-specific fixation of normal rabbit's serum in the pneumococcal complement fixation test is extremely interesting, and I can find no record of this fact having been previously noted. It is of the same/
same nature as the non-specific fixation of normal rabbits, mice and pig's serum with the Wassermann antigen. An explanation of the phenomenon is wanting but it can safely be said that it is in no way connected with immunity.

Finally, since normal rabbit's serum fixed the same number of units of complement as did the serum of immunised rabbits, it must be concluded that no evidence of specific complement fixing bodies can be shown to exist.

III. Bacteriolysins:

The serum of rabbits immunised with ordinary heat-killed vaccine was tested for its bactericidal action. In addition, Rockefeller serum and Burroughs and Wellcome's serum were examined.

Two methods were employed:

I. The Plate Culture Method.

II. The Lacy-Heist Method.

I. The Plate Culture Method:

From a preliminary series of tests, it was found that not only was it difficult to count pneumococcal colonies under the surface of the culture medium, but also the organism failed to grow satisfactorily in the deeper layers. Accordingly, only a surface inoculation/
Technique employed:

(1) Serum:
The test serum was inactivated by heating for half an hour at 55°C. The following dilutions of serum in broth were made:

- 1 in 10
- 1 in 100
- 1 in 500
- 1 in 1000

(2) Complement:
Fresh serum from normal rabbits was used. The test amount was 0.5 c.c. of a 1 in 5 dilution.

(3) Organisms:
An 18 hours' broth culture was used. The test amount was 0.1 c.c.

Into four sterile Wassermann tubes 2 c.c. of sterile broth were placed. Into each tube 0.1 c.c. of culture and 0.5 c.c. of complement dilution were pipetted. Into tubes 1, 2, 3 and 4 were placed 1 c.c. of 1 in 10, 1 in 100, 1 in 500 and 1 in 1,000 dilutions respectively of the test serum. The tubes were then placed in the incubator at 37°C. for three hours to allow bacteriolysis to take place. 0.2 c.c. was removed from each tube to the surface of numbered blood agar/
agar plates and spread with sterile glass rods. From experience it was found that 1 c.c. of sterile rabbit's blood mixed with 9 c.c. of melted agar and poured into Petri dishes made a satisfactory culture medium. If, in addition, the plates were heated for one hour at 60°C., it not only ensured sterility but in addition, it turned the medium the colour of chocolate. The colonies were surrounded by a yellowish-green zone of discolouration due to the production of methaemoglobin, and by means of this it was simple to avoid mistaking pneumococcal colonies for those of any adventitious organisms which might be present.

Having inoculated the surfaces of four plates with 0.2 c.c. of the contents of tubes 1, 2, 3 and 4, the plates were incubated for 24 hours when the colonies were counted.

The same procedure was carried out with Rockefeller and Burroughs & Wellcome's immune serum, and normal rabbit's serum as a control.

Result:

The surfaces of all the plates were covered by thousands of colonies. The colour of the medium was greenish-yellow all over. No difference could be made out between any dilution or any of the test sera. Obviously no visible sign of bacteriolysis was present. The serum and complement controls were sterile.

In order that an actual count of the number
of colonies might be made, a much smaller amount of the 18 broth culture would need to be used. Accordingly, the test was repeated, using a 1 in 200 dilution of the culture. 0.1 c.c. of this dilution was added to 2 c.c. of broth, together with 1 c.c. of the test serum dilution and 0.5 c.c. of complement dilution. After three hours' incubation at 37°C, 0.2 c.c. was removed from each tube to a correspondingly numbered plate and spread as before. The plates were incubated for 24 hours when the colonies were counted.

In all dilutions of both normal and immune serum, a good growth of organisms resulted. From 300 to 500 colonies were present on the surface of every plate. If any comparison could be made, it seemed as if there were more colonies on the plates spread with the 1 in 10 dilutions than the 1 in 1,000 dilutions.

From experiments on the antiblastic reaction detailed later on, this was probably correct.

CONCLUSIONS:

No evidence of any bactericidal action could be found.

II. The LACY-HEIST method of determining the bactericidal activity of coagulable blood.

By means of this method, Heist and Cohen have shown that the pneumococcus is able to grow in the/
The technique:

(1) This consists of drawing out heavy walled glass tubing into lengths of 15 cms., having an inside diameter of about 0.5 to 1.0 mm. Five of these tubes are placed side by side, palisade fashion, and a pellet of plasticine moulded round them near one end. A short piece of glass tubing is placed over the protruding ends, thus making a convenient handle. Pressure on the plasticine spreads the capillary tubes out like a fan. With a wax pencil a fiduciary mark upon each tube, about 5 cms. from its free end, is made. Each tube in addition is marked by dots as in diagram -

(2) Into five sterile Wassermann tubes are placed the following dilutions of a 24 hours' broth culture of pneumococci:

\[
\begin{align*}
&\text{1 neat culture} \\
\end{align*}
\]
(3) By capillarity, each capillary tube is filled with the corresponding dilution of culture up to the fiduciary mark. The culture is then gently blown out again. Some organisms are left adhering to the inside walls of the tubes.

The ear vein of the test rabbit (in this case rabbits immunised with heat-killed pneumococcal vaccine) is pricked and a drop of blood is allowed to run up to the mark in each tube. The ends are sealed by dipping them into melted paraffin (thus avoiding the effects of heat which might occur if sealed in the flame).

The tubes are incubated for 24 hours at 37°C. The ends are then broken off and the contained material blown on to slides where it is spread and stained by Gram's method. If the blood is bactericidal, cells and fibrin will be seen but no organisms. While, if there is no bacteriolysis, large numbers of organisms will be visible, scattered through the debris.

The test is done with normal rabbit's blood as a control.

Result:
A smear showing growth of Pneumococci, illustrating that the serum had no bactericidal action.

Grams Stain. x 500.
A heavy growth of organisms was found in every film. No evidence was therefore forthcoming from this method that the blood of the immunised rabbits had any bactericidal properties.

IV. Antiblastic Immunity:

While it is generally believed that immunity to disease depends upon phagocytic activity and antibodies as already described, Ascoli (53) believes that immunity may sometimes be due to forces antagonistic to the growth of the micro-organism in the body. Dochez and Avery (54) state that anti-pneumococcal serum exerts an inhibitory influence upon the growth of pneumococci which they believe is directed against the ferments produced by these bacteria. By this means the nutritional processes, on which the growth of the organism depends, are unfavourably influenced.

From the investigations already described, it is clear that highly protective antipneumococcal serum appears to be deficient in antibody content, and it was therefore highly important to see if the protective value of the immune serum was due to its antiblastic action.

Rockefeller and Burroughs & Wellcomes' immune serum/
serum, both of which are highly protective, were chosen as the test sera. Normal rabbit's serum was used as the control.

The Test:

Six sterile Wassermann tubes were placed in a rack. Into each of them was put 2 c.c. of sterile broth and 0·1 c.c. of a 24 hours' culture of pneumococci. Into tubes I. and II. were pipetted 0·5 c.c. and 0·1 c.c. of inactivated sterile Rockefeller serum; into tubes III. and IV. were placed 0·5 c.c. and 0·1 c.c. of inactivated sterile Burroughs & Wellcome serum; into tubes V. and VI. were placed 0·5 c.c. and 0·1 c.c. of inactivated normal rabbit's serum.

The tubes were incubated for 24 hours at 37°C.

Result:

A naked eye examination appeared to confirm the conclusions of Dochez and Avery. Tubes V. and VI. containing normal rabbit's serum, showed a heavy growth of organisms, the broth being turbid and opaque, while the tubes containing the immune sera showed the broth to be perfectly clear. At the bottom of the tubes a heavy white sediment was lying.

Films were made of the supernatent layers of broth/
broth and examined microscopically. The naked eye observations were confirmed, since no organisms were found in the films made from tubes I. to IV. while the films of tubes V. and VI. were showing a heavy growth of pneumococci. Films were made from the sediment at the bottom of tubes I. to IV.; a microscopic examination made the situation clear. Enormous numbers of pneumococci were seen in large masses and clumps: the organisms were growing in chains. Chain formation is found to occur when any organism is grown in its own homologous anti-serum. (e.g. B. typhosus)

An explanation of these phenomena is obvious. The immune sera had agglutinated the organisms but had in no way affected their growth. Instead of growing evenly through the whole of the broth, they were growing at the bottom of the tube. Cultures made by pipetting some of the sediment on to coagulated blood slopes showed that the organisms were as viable as those growing in the normal rabbit's serum broth. Moreover, the secretion of ferments by the organism with the production of methaemoglobin was in no way altered.

The experiment was repeated several times.

CONCLUSIONS:

No/
No evidence could be found that the presence of immune serum inhibited the growth of pneumococci.

V. OPSONINS:

The technique of Wright was employed.

In this experiment the three constituents were:

1. Human leucocytes.
2. Emulsified 18 hours' chocolate slope culture of pneumococci.
3. Serum:
   (a) Rockefeller.
   (b) O'Brien.
   (c) "Immune" from Rabbits 88, 90, 91.
   (d) Normal Rabbit's serum.

Equal volumes of each of the above three constituents, thoroughly mixed and incubated in a drawn capillary pipette for 20 minutes at 37°C. films were made from the blood and stained by Leishman's method. The numbers of cocci ingested were as follows:

1st. Experiment:

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Rockefeller</th>
<th>O'Brien</th>
<th>&quot;Immune&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>per 100 leucocytes</td>
<td>148</td>
<td>156</td>
<td>107</td>
<td>139</td>
</tr>
<tr>
<td>Opsonic Index</td>
<td>1.0</td>
<td>1.05</td>
<td>0.72</td>
<td>0.34</td>
</tr>
</tbody>
</table>

2nd. Experiment:

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Rockefeller</th>
<th>O'Brien</th>
<th>&quot;Immune&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>per 100 leucocytes</td>
<td>128</td>
<td>160</td>
<td>80</td>
<td>128</td>
</tr>
<tr>
<td>Opsonic Index</td>
<td>1.0</td>
<td>1.25</td>
<td>0.32</td>
<td>1.0</td>
</tr>
</tbody>
</table>

The opsonic index of the serum of rabbits immunised with heat-killed vaccines appeared to be the same.
same as that of normal rabbit's serum.

When Rockefeller immune serum was tested, there appeared to be a definite increase in the numbers of ingested pneumococci per leucocyte than in the controls. The phagocytosis, however, was not markedly increased and the conclusion I came to was that if opsonins were present they exist in very small amounts.

CONCLUSIONS:

I. The serum of the immunised rabbits, though highly protective, was devoid of all antibodies on which protection is generally supposed to depend.

II. Since dead pneumococcal cultures are highly toxic, it seems not unlikely that a partial explanation of the protective efficiency of the serum lies in its being antitoxic powerfully.
In view of the claims made by certain clinicians in favour of tuberculin therapy, the absence of experimental proof in support of this form of treatment is specially significant.

In 1891 Koch published his fundamental researches on tuberculins which were the starting point and are still today the basis of tuberculin therapy. He claimed to have cured experimentally infected guinea pigs by injections of tuberculin. Thirty-three years have elapsed and these results have never been confirmed. Koch's claim that he had at last found a specific cure for tuberculosis was received with hopeful anticipation. Owing to indiscriminate use and excessive dosage in its clinical trials, the greatest disappointment resulted. Many years of clinical investigation was necessary before tuberculin treatment was placed upon the scientific foundation upon which it now rests. The apparent discrepancy between the clinical and laboratory findings may be explained by a study of experimental tuberculosis in laboratory animals.
The failure of tuberculin as a specific and certain cure is now universally admitted. In fact, it is open to question whether tuberculin therapy possesses any real value and whether the favourable results claimed from its use are not derived from general sanatorium regime. The majority of tuberculosis specialists, however, state that tuberculin treatment is of undoubted value, but even its most ardent supporters advance its claims with certain important reservations. Clinical experience is essential if satisfactory results are to be obtained. The physician must take into consideration

(1) The site and extent of the lesion

(2) The type of the disease, whether acute or chronic, stationary or spreading, febrile or afebrile.

(3) The general health of the patient.

Having assessed all the available evidence, he may then be able to judge the correct dosage. After each dose the local, focal and general reactions must be considered before estimating the next. No two cases can be treated alike and success is essentially a function of experience: for no benefit will accrue if the case is unscientifically handled, but actual harm, or even death may result from an excessive focal/
focal reaction with aggravation of the lesion and consequent serious systematic effects.

The difficulties of the laboratory worker, who attempts the tuberculin treatment of experimentally infected animals (e.g. guinea pigs), can thus be understood.

The appropriate initial dose, the subsequent ones and the intervals between them can only be estimated empirically. Little or no information is known regarding the reactions of the animals to the antigen. Moreover, for any immediate conclusions to be drawn from the experimental results, in order to eliminate the fallacies due to wide individual variations, a considerable number of animals must be used. Thus in any scheme of experiments in which batches of animals are treated with the same doses at fixed intervals, the consideration of each individual case on its own merits is lacking.

In addition, there are further difficulties. While it is well recognised that the guinea pig is extremely susceptible to tuberculosis, only those who have actually carried out experimental work on the subject can realise to what extent individual guinea pigs may vary. Thus in the publication of the Royal Commission/
Commission on Tuberculosis, it is stated that in one batch of animals injected with the same dose at the same time, the survival periods varied from 39-244 days. In the experiments about to be described, it varied from 21-180 days. In other words, a definite minimum lethal dose acting within a reasonable time cannot be established. Another fallacious statement which is constantly being made is that if a guinea pig has tuberculosis, it progressively loses weight. This only occurs either when the animal is extremely susceptible or when the dose is excessive. In the event of the animal having a moderate resistance, or when the dose of tubercle bacilli is small, it may steadily gain in weight. These two states can be seen in the charts published in this paper. Eventually, however, when the tubercle bacilli have overcome the resistance of the animal and the infection has become widespread, there is progressive loss in weight, followed by death. Erroneous conclusions, therefore, may be drawn if these facts are not realised.

A further important consideration in evaluating experimental results is the following:

It must not be taken for granted that, because a discharging ulcer dries up, or a lymphatic gland, previously soft, becomes hard and shotty to the touch,
or fibrosis is seen on microscopic examination in an organ removed at a post-mortem, that these observations indicate general healing, much less cure. Kettle clearly showed in his paper on the criteria of cure in experimentally infected animals that, in untreated guinea pigs one gland might be fibrosed while the next was caseous: that even in one organ, such as the spleen, one part might exhibit signs of healing while in another area there was active spread: that on careful microscopic examination, patches of active disease might be found in areas of fibrosis. In short, it is typical of tuberculosis that the disease heals in one area while it spreads in another.

In consequence of these pathological findings in untreated guinea pigs, fibrosis indicates only that a certain amount of cure has taken place in the area examined. It does not contra-indicate the evidence of active tuberculosis elsewhere. In my experience of the disease in guinea pigs such active lesions will invariably be found. The only proof of cure is the survival of treated animals with death of the controls. These considerations indicate the difficulties and fallacies/

*Reference: The criteria of cure in tuberculosis of the Guinea Pig. Lancet, 1924. 1. 68.
fallacies in any attempt to estimate the value of tuberculin treatment in experimentally infected animals.

The observations to be described form part of a more extensive investigation into the effect of physical and chemical processes on the antigenic values of bacterial vaccines.

Object of the Investigation.
Guinea pigs were injected with living tubercle bacilli and then treated by various vaccines of the tubercle bacillus in order to ascertain

(1) whether benefit or cure resulted, and, if so, which type of vaccine was the most efficient.

or (2) if actual harm resulted.

Vaccine used.

It was originally intended that three types of vaccine should be tested:

I. The ordinary heat-killed saline bacillary emulsion;

II. A detoxicated vaccine;

III. A defatted or diaplyte vaccine (Dreyer).

As no undiluted diaplyte antigen could be obtained from the Oxford laboratory, the work on this vaccine was held over. Since then, however, Kettle has investigated this question, using undiluted diaplyte antigen, and has kindly allowed me to quote his results.
The Ordinary Vaccine (Bacillary Emulsion).

This was prepared by growing a recently isolated strain of the human type on Dorset's egg medium. The surface growth was removed, weighed and evenly emulsified in saline so that each cubic centimetre contained 1 mg. of heat-killed tubercle bacilli. By means of dilutions, the requisite doses were made. This vaccine will be referred to as the "Bacillary Emulsion".

Detoxicated Vaccine.

A sub-culture of the same strain used for the "Bacillary Emulsion" was detoxicated by Dr. David Thomson, the originator of the detoxicating process. He prepared a vaccine containing the alkali soluble products of the organism, each cubic centimetre representing 100,000 million bacilli. I am indebted to Dr. Thomson for making this and other detoxicated vaccines and for his continued interest in these investigations.

Experimental Animals.

Forty-five guinea pigs obtained from the same source were weighed and divided into three groups (A, B, and C.) of fifteen each. On December 15, the animals in group A received $\frac{1}{100}$ of mgm. of living tubercle/
tubercle bacilli subcutaneously in the groin. Likewise group B. received \( \frac{1}{500} \) of a mgm. and group C. \( \frac{1}{1000} \) mgm. Owing to the severe cold about Christmas time, several animals in each group died. This was in no way connected with the injections as numerous stock animals also died at the same time. Only those animals surviving for a minimum of three weeks are included in these results. On January 18, two guinea pigs died, showing definite evidence of tuberculosis. Treatment was then started, i.e., one month after the injection of living bacilli. Eight guinea pigs were left untreated as controls. Nine were treated with detoxicated vaccine and eight with the bacillary emulsion. The animals were weighed and injected with progressively increasing doses at weekly intervals. The results are shown graphically.

\textbf{RESULTS}.

In every case tuberculous disease was present either when an animal died after progressively losing weight or was killed after continually gaining weight. The guinea pigs which died in a comparatively short time showed very advanced disease in every organ, while the animals killed at the end of the investigation only showed early lesions. The investigation was concluded/
concluded on March 25., i.e. about 100 days after the injection of living tubercle bacilli. With the exception of three animals which were kept to ascertain the length of their survival, all remaining guinea pigs were killed and their organs subjected to microscopic examination. These animals, despite the fact that palpable inguinal glands were present, appeared to be in good health and had increased in weight.

**Examination of the Weight Charts.**

Examination of the weight curves of the 17 treated guinea pigs shows that no benefits have accrued and no immunity has been produced. In fact, the reverse is the case, as the eight control animals show the largest proportional gain in weight. The following table clearly indicates the results:

<table>
<thead>
<tr>
<th></th>
<th>Average Weight in grams</th>
<th>Average Gain or Loss</th>
<th>Percentage Gain or Loss</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before Treatment</td>
<td>After Treatment</td>
<td></td>
</tr>
<tr>
<td><strong>Controls.</strong></td>
<td>357</td>
<td>487</td>
<td>+ 130</td>
</tr>
<tr>
<td>8 Guinea Pigs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Detox. Vaccine</strong></td>
<td>438</td>
<td>461</td>
<td>+ 23</td>
</tr>
<tr>
<td>9 Guinea Pigs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Bacillary Emulsion.</strong></td>
<td>423</td>
<td>465</td>
<td>+ 42</td>
</tr>
<tr>
<td>8 Guinea Pigs</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
On examination of the table of weights of control animals compared to animals treated with diaplyte antigen, published by Kettle, corresponding results are seen:

<table>
<thead>
<tr>
<th></th>
<th>Average Weight in grams</th>
<th>Average Gain or Loss</th>
<th>Percentage Gain or Loss</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before Treatment</td>
<td>After Treatment</td>
<td></td>
</tr>
<tr>
<td>Diaplyte.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 Guinea Pigs</td>
<td>445</td>
<td>394</td>
<td>-51</td>
</tr>
<tr>
<td>Controls.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 Guinea Pigs</td>
<td>394</td>
<td>424</td>
<td>+30</td>
</tr>
</tbody>
</table>

Thus, in Kettle's series the controls are 19 per cent better than the animals treated with diaplyte antigen. In my series, the controls are 31 per cent better than the animals treated with detoxicated vaccine and 26 per cent better than the animals immunised with the bacillary emulsion.

Microscopic Examination.

No microscopic examination was necessary in any of the animals that actually died from the disease, as the disease, seen by the naked eye, was in a very advanced state. Careful microscopic examination was made/
SPLEEN 1

LYMPH GLAND 2

of Control Guinea Pig inoculated with Tubercle Bacilli.

SPLEEN 3

LYMPH GLAND 4

of Guinea Pig treated with bacillary emulsion tubercle vaccine and then inoculated with Tubercle Bacilli.
made of the organs of all the animals that were killed on March 25. It will be remembered that, although all these animals had palpable inguinal glands, their general health was apparently good.

The lesions found in every animal, treated or control, were identical. The livers were the least affected, the lung next, while the lymphatic glands at and adjacent to the site of infection, and the spleen, were the most involved. Fibrosis was absent and the pathological changes were of the proliferative cellular type. It may be safely said that the microscopic evidence points to the lesions being of recent origin, the infection having been localised to the seat of infection for a considerable period. Shortly before the animals were killed, the infection had become systemic and probably during the next 4-8 weeks a steady loss of weight followed by death would have occurred.

A comparison of the sections of organs of treated and untreated animals shows identical lesions.

Photographs of sections of lymphatic gland and spleen of (1) a control guinea pig, and (2) animals treated with a detoxicated vaccine and a bacillary emulsion are shown to illustrate this fact.

The animals, when killed, all weighed the same, namely, 570 grams.

CONCLUSIONS
Spleen of Guinea Pig treated with Detoxicated Vaccine and then inoculated with Tubercle Bacilli.

Lymph Gland
CONCLUSIONS

As judged by body weight, the period of survival, and microscopic findings, tuberculin treatment in guinea pigs with experimental tuberculosis is not only ineffective in controlling the infection but is even harmful.
It is generally admitted that the diagnosis of tuberculosis disease in its early stages is a matter of the greatest difficulty. Furthermore, since the arrest and cure of the disease depends to a great extent on early diagnosis, any laboratory method which will help the clinician in arriving at a definite decision is worthy of the most careful trial.

Much benefit was expected from the complement fixation test, and it was hoped that just as the Wassermann reaction when performed by competent serologists may be regarded as giving definite proof of the presence or absence of syphilis, so the complement fixation test in tuberculosis would show whether a patient was infected with the tubercle bacillus or not.

Unfortunately, the results have not come up to expectation. The explanation of this lies in the fact that the tubercle bacillus produces only a very limited amount of antibodies. The detection of small quantities of complement fixing antibodies is liable to be fraught with many fallacies. Nimmo Smith after examining the published results of the leading authorities on this test, states that the position as regards/
regards the value of the complement fixation test in tuberculin may be summed up as follows:-

I. That in clinically active cases in which bacilli are found in the sputum, a positive reaction will be found in 85 to 95 per cent.

II. In doubtful cases, only 40 to 50 per cent give a positive reaction.

III. Repeated negative results are presumptive evidence either of the absence of tuberculosis or that the lesions are inactive.

IV. Repeated positive results are presumptive evidence of tuberculosis.

V. The intensity of the reaction bears no relation to the patient's power of resistance or to the degree of the severity of the infection.

Early active cases may give a strong positive reaction, while patients dying of the disease may give a negative result. It must, therefore, be admitted that the test has been unsatisfactory since it gives little information in the very class of patients in which diagnosis is difficult.

Since the tuberculous complement fixing body exists only in small amounts, the investigators, from whom the above conclusions were drawn, used as their test amount of complement 2, 2½ and 3 haemolytic doses. The fixation of three units of complement is regarded as a strongly positive result.

While admitting the difficulties, I must emphatically/
emphatically state from personal experience that completely fallacious results are liable to occur when the fixation of two units of complement is considered to be definite evidence of tuberculous disease.

In order to obtain good fixation, it is necessary to use the most sensitive antigen possible. Accordingly the following investigation into the value of different antigens was undertaken:

On Dec. 15 - 10 rabbits were weighed and injected intravenously with \( \frac{1}{100} \) mg. of living tubercle bacilli (human type).

On Jan. 29 - 4 animals were treated at weekly intervals with a heat-killed saline emulsion of tubercle bacilli.

3 animals were treated with detoxicated vaccine made by Dr. David Thomson.

3 animals were left untreated as controls.

Table I. shows the doses of vaccines injected.

<table>
<thead>
<tr>
<th>TABLE I.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
</tbody>
</table>
### TABLE I.

<table>
<thead>
<tr>
<th>Serial number of rabbits</th>
<th>Immunising vaccine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heat-killed bacillary emulsion.</td>
</tr>
<tr>
<td>I.</td>
<td>1 mg</td>
</tr>
<tr>
<td>II.</td>
<td>&quot;</td>
</tr>
<tr>
<td>III.</td>
<td>&quot;</td>
</tr>
<tr>
<td>IV.</td>
<td>&quot;</td>
</tr>
<tr>
<td>V.</td>
<td>Detoxicated vaccine</td>
</tr>
<tr>
<td>VI.</td>
<td>&quot;</td>
</tr>
<tr>
<td>VII.</td>
<td>&quot;</td>
</tr>
<tr>
<td>VIII.</td>
<td>Controls</td>
</tr>
<tr>
<td>IX.</td>
<td>Controls</td>
</tr>
<tr>
<td>X.</td>
<td>Controls</td>
</tr>
</tbody>
</table>

Table II. shows the weights of the animals from Dec. 15. to March 10.
### TABLE II.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1900</td>
<td>1790</td>
<td>1700</td>
<td>1970</td>
<td>2000</td>
</tr>
<tr>
<td>II</td>
<td>1470</td>
<td>1520</td>
<td>1420</td>
<td>1420</td>
<td>1500</td>
</tr>
<tr>
<td>III</td>
<td>1340</td>
<td>1350</td>
<td>1350</td>
<td>1350</td>
<td>1450</td>
</tr>
<tr>
<td>IV</td>
<td>1320</td>
<td>1370</td>
<td>1450</td>
<td>1820</td>
<td>1890</td>
</tr>
<tr>
<td>V</td>
<td>1250</td>
<td>1300</td>
<td>1300</td>
<td>1420</td>
<td>1540</td>
</tr>
<tr>
<td>VI</td>
<td>1700</td>
<td>1740</td>
<td>1700</td>
<td>1600</td>
<td>1590</td>
</tr>
<tr>
<td>VII</td>
<td>1100</td>
<td>1270</td>
<td>1170</td>
<td>1190</td>
<td>1300</td>
</tr>
<tr>
<td>VIII</td>
<td>2240</td>
<td>2350</td>
<td>2400</td>
<td>2200</td>
<td>2470</td>
</tr>
<tr>
<td>IX</td>
<td>1650</td>
<td>1680</td>
<td>1600</td>
<td>1770</td>
<td>1840</td>
</tr>
<tr>
<td>X</td>
<td>2850</td>
<td>2520</td>
<td>2500</td>
<td>2370</td>
<td>2870</td>
</tr>
</tbody>
</table>

**Note:** Weight in grammes.

On March 10., as all the animals were in perfect health and no appearance of tuberculous disease was present, one animal from each group was killed and its organs examined for evidence of tuberculosis. No lesions of any description could be found, every organ being in a healthy condition.

Accordingly, \( \frac{1}{2} \text{ c.c.} \) of an emulsion of living tubercle bacilli containing about 1000 million per c.c. (as judged by opacity) was injected subcutaneously into/
into the abdomens of the seven remaining rabbits. The strain of tubercle bacilli employed was the same as used in the injection of the guinea pigs and rabbits already described. The organism was a recently isolated strain of tubercle bacillus of the human type. Examination of the sites of injection at the end of 24 hours showed the formation of a lump surrounded by oedema and redness. In 48 hours the oedema had disappeared and there remained a lump the size of a small hazel nut which became progressively harder and harder. Two months later, the animals were still apparently in perfect health. They were killed and examined for signs of internal lesions. No evidence of disease could be found. Sections of the lump at the site of injection showed enormous numbers of tubercle bacilli lying in dense fibrous tissue.

This experiment shows the high degree of immunity which rabbits possess to the human type of tubercle bacillus.

Examination of the serum of the immunised rabbits for antibody content.

I. Agglutinins: Neither the serum of the rabbits or guinea pigs immunised with the various tuberculins showed any agglutinin content even when used undiluted.

II./
II. **Complement Fixing Bodies:** Using Beardedka's antigen, the serum of both rabbits and guinea pigs was tested by the complement fixation test.

Previous to Jan. 29, when treatment started, negative results were obtained. The tests performed on Feb. 11, 19 and 25, showed that fixation of three units of complement occurred after allowing for the anticomplementary action of serum and antigen. This production of specific antibody was due to the immunisation and not to actual disease since post-mortem examination failed to bring any tuberculous foci to light.

From more recent work on the serology of normal rabbits, I feel, however, that these results would need to be repeated and more careful controls instituted. I have already pointed out how normal rabbit's serum may give a positive Wassermann reaction and a positive pneumococcal complement fixation reaction. I have also obtained evidence of this occurring in the complement fixation test for tuberculosis. Until this matter is cleared up, no great weight should be attached to the finding of fixation of three units of complement in the above experiments.

An investigation into various antigens in the complement fixation test for tuberculosis.

Since the difficulties and fallacies of this test lie in the fact that the limited amounts of antibody produced/
produced fix only small quantities of complement, it is essential that the antigen employed should be as sensitive as possible. It is recognised that not every strain of tubercle bacilli possesses equally good fixing properties. In addition, various observers recommend different methods of making the antigen. Some recommend that the organism be used in the living state, believing it to be more sensitive in this condition. Others believe in allowing much longer time for fixation to occur and hence recommend what is called the "ice chest method".

I determined, therefore, to investigate some of these problems.

**Antigens**: Miller (67) classifies the various antigens in use into four groups:

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Comprises those which consist of the whole or ground up tubercle bacilli.</td>
</tr>
<tr>
<td>II</td>
<td>Antigens which may be classified as tuberculins.</td>
</tr>
<tr>
<td>III</td>
<td>Antigens consisting of extracts or derivatives of tubercle bacilli.</td>
</tr>
<tr>
<td>IV</td>
<td>Extracts or preparations of normal and tuberculous tissues.</td>
</tr>
</tbody>
</table>

It is generally agreed that antigens belonging to Group IV. are unsatisfactory in every way and are now seldom used. Antigens in Group II. are likewise
likewise unsatisfactory as they are neither sensitive nor reliable. In addition, Miller states that non-specific results are liable to occur, especially with the sera of non-tuberculous syphilitics.

Most observers believe that antigens of Group I. are the best.

The following antigens were examined:

I. The strain of human tubercle bacilli, used for the purpose of immunisation in the investigation already described, was subcultured alternately on Dorset's egg medium and glycerine potato.

After incubation for six weeks, the growth was removed to an agate mortar and ground up with saline which was added drop by drop until an even emulsion resulted: This antigen will be known as "Dillon".

II. A subculture of the "Inman" strain of tubercle bacillus was obtained. It was emulsified in a similar manner. This strain is stated to have especially good fixing properties.

III. Besredka's antigen: This is made by growing the bacilli on special egg-yolk medium. It was supplied by the Zeeland Trading Company. The antigen contains both the medium and the bacilli. To the naked eye it has the appearance of water, being quite clear.
clear. On microscopic examination, the bacilli are seen to occur in moderate numbers.

Antigens I. and II. were tested unheated and after heating for half an hour at 80°C.

Serum: Serum was obtained from three patients with clinically active tuberculosis of the lungs. Tubercle bacilli were present in their sputa. Examined by the Wassermann reaction, they all gave negative results. This is important and necessary as non-specific fixation is very apt to occur between the two diseases. As high as 30 per cent of positive Wassermann sera of patients with no clinical signs of tuberculosis give a positive complement fixation test. From preliminary investigations, 0.1 c.c. of serum was found to be the optimum amount that could be used in the test. This amount was rarely anticomplementary to more than one unit of complement.

Complement and Haemolytic System.

The same method and materials as previously described were employed, namely, guinea pig's serum and a 3 per cent suspension of washed sheep's cells sensitised with five haemolytic doses of Burroughs & Wellcome immune body.

The Test Proper
I. Having finished a preliminary complement, serum and antigen titration, the complement fixation test was performed. The serum from the three patients was pooled and 0.1 c.c. was the test amount used.

Table III. shows the quantities used and the result:

**TABLE III.**

<table>
<thead>
<tr>
<th>Row 1</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubes:</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Pooled Human Serum 1 in 2 dilution</td>
<td>0.2 c.c.</td>
<td>0.2 c.c.</td>
<td>0.2 c.c.</td>
<td>0.2 c.c.</td>
<td>0.2 c.c.</td>
</tr>
<tr>
<td>Complement</td>
<td>2 units</td>
<td>3 units</td>
<td>4 units</td>
<td>6 units</td>
<td>8 units</td>
</tr>
<tr>
<td>Antigen (Dillon)</td>
<td>0.2 c.c.</td>
<td>0.2 c.c.</td>
<td>0.2 c.c.</td>
<td>0.2 c.c.</td>
<td>0.2 c.c.</td>
</tr>
</tbody>
</table>

Incubation 1 hour at 37°C.

<table>
<thead>
<tr>
<th>Sensitised cells</th>
<th>0.2 c.c.</th>
<th>0.2 c.c.</th>
<th>0.2 c.c.</th>
<th>0.2 c.c.</th>
<th>0.2 c.c.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Result after One Hour at 37°C.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>H</td>
</tr>
</tbody>
</table>

**Antigen control:** 0.2 of antigen fixed 1 unit of complement.

**Serum control:** 0.2 of 1 in 2 dilution of the test serum fixed just under 1 unit.

**Result**

6 units were fixed in the test. Since antigen and serum fixed 1 unit each non-specifically, these must be deducted/
Specific fixation by the serum of four units of complement.

The test was repeated with the "Inman" antigen and "Besredka" antigen.

The final readings were:

I. Specific fixation with Dillon's antigen - 4 units.
II. " " " Inman's antigen - 5 units
III. " " " Besredka's antigen - 6 units.

The test was repeated with different sera from active cases of tuberculosis. The result was invariably the same.

In this test the Inman and Dillon antigens had been heated for half an hour at 80°C to kill the bacilli.

CONCLUSION:

Besredka's antigen is the most sensitive. In addition, it is the least anticomplementary of any antigen I have tested.

II. Does a living organism make a more sensitive antigen? This question was answered by repeating the test using Inman and Dillon antigen in the living state.

Result:

No/
No difference in fixation between the living and dead antigens could be found. Dillon and Inman's antigens fixed four and five units of complement respectively whether alive or dead.

Since no object was gained, and as there is considerable danger of infection when grinding up living bacilli, I always used the antigen dead.

III. Does the "Ice Chest Method" increase the degree of complement fixation?

To test this theory, two racks of tubes containing serum, complement and antigen in the amounts stated in the previous pages were incubated at 37°C.

Rack I. was then removed to the ice chest. At the same time half the sensitised cells were placed in the ice chest.

To the tubes in rack II., sensitised cells were added. After one hour in the incubator, the results were recorded. Next morning, some fifteen hours later, rack I. was removed from the ice chest and 0.2 c.c. of the sensitised cells was added to each tube. The rack was then placed in the incubator. The tubes were examined at five minute intervals. As soon as the serum and antigen controls showed complete lysis, the results for the test proper were recorded.
This usually occurred in from 15 to 30 minutes.

Result: The "ice chest method" intensified the fixation by at least one unit of complement. It was found that where the tube containing eight units of complement showed only a trace fixation by the usual method, the "ice chest method" gave complete fixation.

In definitely positive cases, it was unnecessary, since a satisfactory reading could be made with the ordinary method. In the majority of cases no benefit, in proportion to the extra time and trouble required, was forthcoming from the "ice chest method". On the other hand, it might be of undoubted value in those cases in which only small amounts of complement were fixed.

CONCLUSIONS:

I. The complement fixation test must be considered to be only of limited value in the diagnosis of tuberculous disease.

II. As judged by all standards, Besredka's antigen appears to be the most satisfactory.
CHART 1

Inoculated with 100 mg of living Bacillus
Inoculated with 50 mg of living Bacillus
6.0 mg of Guinea Pig
6.0 mg of Guinea Pig
4.5 mg of Guinea Pig
4.0 mg of Guinea Pig
0.5 mg of Guinea Pig
0.5 mg of Guinea Pig
0.0 mg of Guinea Pig

1 cc of Detergent = 1,000,000 Million
1 cc of Detergent = 1,000,000 Million

\[ \frac{\text{mg of Guinea Pig}}{\text{cc of Detergent}} \]
GENERAL CONCLUSIONS.

I. By means of many animal experiments, a thorough investigation has been made into the principles upon which our knowledge of immunity is based. The general conclusion I have drawn from a consideration of the investigation as a whole is, that the question of immunity is of such great complexity that only a few of the most elementary details are understood. If no benefit, other than the realisation of our ignorance and the difficulties with which we have to contend, resulted from this work, I should still be satisfied.

I have heard well known clinicians lecture to students on the subject of infection and immunity in such a way that the audience must have believed that the subject had been completely investigated and that no doubts now remained about the methods by which a patient overcomes an infection. From the researches described in Part II. the real truth is, that we are not in a position to dogmatise on even the simplest immunological reaction. For instance, from the experiments prepared with B. suipestifer vaccines, we saw that immunised animals could possess large amounts of antibodies and yet show no immunity to a lethal dose of living organisms. On the other hand, in/
in the pneumococcal investigation it was shown that animals could possess a high degree of immunity with an apparent absence of antibodies. While lastly in the tuberculin investigations, animals which had been immunised with large quantities of tuberculin appeared to be susceptible both to the infection and also to produce very limited amounts of antibody. The correlation of such facts is at present beyond our powers. My plea is that we should reinvestigate the whole problem of immunity from the very beginning. Let us freely admit our ignorance and the difficulties of the subject. Carefully controlled researches will unravel the problems and, when a solution is forthcoming, the whole therapeutic field of medicine will be revolutionised.

II. Apart from a general investigation into the problems of immunity, I set out to discover if the physical and chemical processes employed in the production of detoxicated and defatted vaccines affected the antigenic values of these vaccines. Fortunately, a definite clear-cut answer to this question can be given which is based on the experimental results described in Chapters I—V. Part II.

I can state with every degree of confidence that/
that as judged by

I. The production of antibodies and
II. Protection to a lethal dose of organisms

the physical and chemical processes employed in the
manufacture of detoxicated and defatted vaccines have
destroyed their antigenic value.
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