THE SPLIT PRODUCTS OF THE TYPHOID BACILLUS.

AN EXPERIMENTAL STUDY OF THEIR ANTIGENIC AND TOXIC PROPERTIES.

by

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INTRODUCTION.

Immunity against disease is the ideal towards which all scientific medicine is striving.

It was long known that in the case of certain diseases, for example Typhoid fever, one attack protected against another for many years; on the other hand, it was not until the aetiology of these diseases had been definitely associated with the presence of certain bacteria, following the epoch making work of Pasteur and Koch, that the development of immunity was recognised as the result of the bacterial products introduced into the system, and not of the disease per se.

Furthermore, this truth led to the important discovery that by a suitable gradation of the doses of such products, or by the use of weakened toxins, a high degree of immunity could be attained without the occurrence of any symptoms whatever. Thus there arose/

arose the practice of artificial immunisation with vaccines and sera, which has been widely adopted in all parts of the world.

It cannot be gainsaid, however, that although this branch has furnished us with some of the most brilliant successes in the realm of experimental therapeutics, these have been counterbalanced by unaccountable failures - unaccountable in the sense that they occur in cases where by the principles which govern immunity, one should least expect them. The unbounded enthusiasm and faith, which characterised the introduction of vaccine inoculation have been replaced by a degree of scepticism, for in practice all the extravagant claims made for this new method of treatment have not been entirely justified. It is true that in the case of those organisms which elaborate an extracellular toxin, as for instance the Diphtheria Bacillus, a corresponding antitoxin can be produced which will effectually neutralise the toxin, and a most satisfactory therapeutic measure is thereby established. But the great majority of pathogenic bacteria are not known to form soluble toxins during their life processes, and the capacity of the latter to intoxicate has been explained by their setting free upon death a toxin/

toxin which during life is retained within the cell body. Organisms of the Coli-Typhoid Group belong to this class, and they are the members on which most investigation has been carried out.

The immunity which follows the introduction of these organisms into the body, consequent upon natural cases of the disease, or upon experimental inoculation, is associated with the appearance of antibodies in the blood of the nature of agglutinins, opsonins, lysins, and precipitins, but antitoxins are either absent altogether, or present only in very small amount. This is supposed to be dependent on the fact that the toxin being an endotoxin, and intimately bound up with the bodies of the bacteria, is incapable of producing an antiendotoxin of any strength, and in fact some authorities believe that no antibody is formed towards endotoxin. Though this latter statement is still lacking in proof, the fact remains that in infection by organisms of the Coli-Typhoid class, the mechanism of immunity, as far as can be made out, is mainly associated with the formation of lysins and opsonins which bring about the death and dissolution of the bacteria, either free in the tissue fluids or after ingestion by the phagocytes. The immunity is therefore anti-bacterial in nature rather than antitoxic. Thus it has/

has been found in practice, in the case of Typhoid Fever, that while prophylactic vaccination has been a highly successful procedure as evidenced by the statistics of recent campaigns, therapeutic measures directed against the established disease, either in the form of vaccines or antisera, have been singularly unsuccessful. This would appear to be due to the uncertain action of bacteriolytic immune bodies, Inasmuch as the bacteriolysins cause the death of the organisms, they would seem to strike at the very root of the evil; yet bacteriolysis is not always an advantageous phenomenon, for it is also responsible for the liberation of endotoxins. On the first invasion of the body by Typhoid Bacilli, a high titre of bacteriolysins may check the further development of the disease completely and the amount of endotoxin set free in the process is small and incapable of exerting any injurious action. On the other hand, once the disease is well established, and the organisms have proliferated to a considerable extent, a rapid and widespread dissolution of the bacteria may liberate so much endotoxin that the course of the disease is influenced unfavourably, and occasionally death may occur. It is as true in this field as in others, that any power of nature which serves humanity, may/

may also be directed against it, and the original contention of Wolff-Eisner[1], that the inoculation of immune serum in Typhoid Fever may occasionally cause death rather than recovery, is explained on these grounds.

It should be borne in mind however, that these pronouncements with regard to immunity in Typhoid Fever must not necessarily be regarded as strictly final. Most of the work on this subject has been done on animals, which are much less susceptible than man to organisms of the Coli-Typhoid group. This necessitates the inoculation of bacteria often in very large quantities, to produce the desired pathological effects, and there is less and less parallel between the experimental procedures, and the disease as it occurs in man. It was with this in view that the philosophic brain of Rosenbach invented the phrase: "Bacteriologists are concerned not with diseases of infection, but with diseases of injection", and though in consideration of the brilliant results achieved by immunologists, this crude statement is quite unjustified, at the same time it draws attention to the doubt which must always exist, when the conclusions derived from animal experimentation are applied to man.

THE SPECIFICITY OF VACCINES.

It has been long held that the antibodies developed towards the inoculation of a particular germ are specific. The brilliant researches of v. Behring, and the fascinating serological work of Ehrlich and Bordet, were advances made wholly on a background of the strictest specificity. Scientific vaccine therapy, therefore, has always postulated the isolation of the causal organism, and the manufacture of the vaccine from that organism, as essential to success. If the demonstrable antibodies in the blood are taken as representing the complete immunity mechanism of the body, certainly immunisation is strictly specific, and the apparent overlapping noticed in the case of the Colon-Typhoid bacteria is to be explained by a group reaction in closely allied members, and in nowise militating against the general principle.

On the other hand, during later years, presumptive evidence of collateral immunisation has been brought forward, chiefly by clinicians. For instance it is now established that a curative effect may be obtained in Gonorrheal rheumatism by inoculation of a B. Typhosus vaccine. One may go even further and face/

face other facts, since it appears to have been proved that good results have been obtained in certain bacterial diseases by the injection of non-bacterial proteins such as milk, proteoses, and other protein split products. (Jobling and Petersen[2], Bull[3], Dunkling and Jobling[4] and Petersen[5]) The apparent success of this non specific protein therapy has been explained by the theory of "protein shock", in that the shock induced by the injection of a foreign protein stimulates the body to produce more antisubstances towards any germ that may be attacking it. To the more rigid adherents of the doctrine of specificity, this development of non specific protein therapy is regarded as a step backwards in the progress of scientific immunisation. This outlook on the problem, however, would appear to be short sighted, for the foregoing facts simply demonstrate that our views on the mechanism of immunity as embodied in the humoral theory of Buchner and Nuttall, or in the cellular theory of Metchnikoff are still too limited.

SOME RECENT CONCEPTIONS OF THE MECHANISM OF PROTEIN THERAPY.

The increasing interest in the treatment of asthma and urticaria by various food and bacterial proteins, has paved the way in some measure for the reception/

reception and understanding of analogous principles applied to the employment of vaccines in bacterial infections. Furthermore, the more general application that phenomena of the nature of anaphylaxis directly follow under certain conditions the administration of proteins, emphasises the necessity of a revision or at least an amplification of our conception as to what takes place when vaccines, which consist chiefly of bacterial proteins, are introduced into the body.

It may be said at once that it has so far proved impossible to provide a complete chain of evidence as to what actually takes place when a foreign protein is introduced into the body. Certain well known facts stand out from a groundwork of theory, and in order to link up the former into a working hypothesis, workers on immunity problems have been compelled to draw to some extent at least on the imagination.

THE RELATION OF ANAPHYLAXIS TO VACCINE THERAPY.

One of the most important recent developments in vaccine therapy, has been the endeavour to correlate it with the extremely interesting subject of anaphylaxis or hypersensitiveness first described by Richet. There seems to be no doubt that anaphylaxis is/

is intimately connected with the general problem of immunity, and that a solution of the former would go far towards explaining the latter. At the same time, one must guard against the modern tendency to recognise the anaphylactic phenomenon in nearly every disease process, a tendency engendered to some extent by the great fascination which the subject has for research workers.

The main facts may be briefly summarised as follows:- An animal which has received an injection of foreign protein gradually develops a state of hypersensitiveness to that protein, so that after a certain period of incubation of about ten days, a second injection produces a condition, which owing to the violent symptoms of sudden onset, has rightly been compared to shock. An animal which has not received a preliminary or sensitising injection remains unaffected. Moreover this anaphylaxis is specific, and the substance used for sensitisation is alone capable of setting up the anaphylactic shock. Again the dose which causes the death of the animal, when inoculated at one time, is found to be harmless if introduced by fractional and intermittent injections. The animal which has received it in this fashion becomes like a new being; it is desensitised, and without suffering any harm, tolerates a large quantity

of the protein substance which previously would have killed it in the smallest doses. While sensitisation is only acquired after an interval, desensitisation is so to speak instantaneous, and constitutes anti-anaphylaxis, which is specific like anaphylaxis itself.

In man the classical features of anaphylactic shock comparable to what is produced in experimental animals, have been observed chiefly after injections of antitoxic horse serum in the treatment of Diphtheria and Tetanus, but a fatal issue is very rare, and it is the minor manifestations of the phenomenon, which may be represented by nothing more than a local urticarial wheal, with which we are mostly concerned.

The diseases Asthma and Urticaria furnish interesting examples of this. The rôle of protein substances either by ingestion, inhalation, or contact, in inducing an acute attack in susceptible subjects was early recognised, and the investigations of Chandler Walker[6], Freeman[7], and Auld[8] have resulted in a method of treatment being elaborated, the success of which in some cases has been very striking. Briefly stated, this method consists in testing by a skin reaction, the sensitiveness of the patients' tissues to a large number of proteins prepared from various foodstuffs, pollens, and bacteria. When definite reactions have been obtained, the patient is desensitised by repeated small/

small doses of that particular protein.

Again some success in the treatment of Asthma seems to have attended the use of injections of "Peptone", a combination of protein derivatives, and this is supposed to act as before by a process of desensitisation.

This brings us to the cognate problem of hypersensitiveness as applied to bacterial protein. The antibodies formed in the blood as a result of the inoculation of bacteria, are not demonstrable till about five to seven days after injection. On the other hand it has been found that in certain acute bacterial infections, the inoculation of a minute dose of vaccine may be followed by marked change for the better, within 6 to 12 hours. There may be fall of temperature and pulse, and sweating, and this may be followed by considerable alleviation in the general condition of the patient.

Such an effect can have nothing to do with the development of antibodies, such as agglutinins, opsonins, etc., and calls for a different explanation. An individual suffering from an acute infection becomes sensitive to the smallest dose of vaccine prepared from the causal organism, whereas the inoculation of doses of the same vaccine hundreds of times larger,/

larger, has little or no effect on a healthy individual, or on the same individual when completely recovered from the disease. This then is apparently an anaphylactic phenomenon associated with the bacterial protein contained in the vaccine. A striking example of this hypersensitiveness, and one long recognised, is furnished by the susceptibility of tubercular patients to the action of tuberculin.

It would appear that this element in vaccine therapy has hitherto received too little attention, and a failure to recognise it has led to the production of severe reactions in individuals recently recovered from an acute attack of disease, who have been treated with large prophylactic doses of vaccine. These severe reactions were regarded as being due to the unusual toxicity of the vaccine itself, while the change in the patient's susceptibility to vaccine was ignored. This has led to new methods of preparing vaccines, having for their aim the reduction of the toxicity. In how far this development is justified will be discussed later. It need only be said here, however, that the methods referred to, consist in the breaking up of the bacterial bodies by strong chemical solutions, aided by mechanical appliances, and employing a suspension of the residue as a vaccine. The argument against these vaccines is/

is that their antigenic value is much reduced in the process to which they are submitted, and the clinical results obtained no better than when small doses of an ordinary untreated vaccine are employed.

PRELIMINARY CONSIDERATIONS LEADING UP TO THE PRESENT WORK.

In the preceding pages, an endeavour has been made to give a generalised survey of the progress of vaccine therapy, or to employ the wider term, foreign protein therapy. It will be seen that though much has been discovered in the realm of immunity, the present state of knowledge does not appear to provide the known facts necessary to permit a great deal of the nature of dogma. Chief interest centres round the challenge of the strict specificity of vaccines by the rise of non specific protein therapy, and also round the toxicity of foreign protein per se, apart from the presence of preformed ecto-or endotoxins peculiar to different germs.

It would appear that further progress towards the solution of these and similar problems in immunity, can only be made by research conducted on the protein molecule, which forms the basis of all antigens. It is rather unfortunate that between the/

the bacteriologist on the one hand, and the biochemist on the other, there has stretched a kind of 'No Man's Land', represented by the Chemistry of the colloids. This branch of physical chemistry has been as yet all too little explored, and there seems to be no doubt that it holds the key to the solution of much that is at present purely theoretical.

From the study of bacterial infection in man and the lower animals, evidence has accumulated that pathogenic organisms do not produce their effects by their mere physical presence, and in the case of those organisms which do not form a soluble toxin during their life processes, a disintegration of the bacterial protoplasm is a necessary preliminary to the liberation of those substances, either toxic or antigenic, which pass through the circulating medium of the body, and affect cells and organs at a distance.

If we inject a few organisms, in the form of a vaccine, under the skin, these organisms are found to swell up and gradually to pass into solution. This is brought about mainly by proteolytic ferments present in the body fluids, and the result is cleavage of the native protein of which those bacteria are mainly composed. This process, analogous to alimentary digestion and brought about by similar ferments, must go on until the native protein is reduced to its simplest components - viz. amino-acids, for only then can it be incorporated as part of the body protein.

The antigenic and toxic substances of the bacteria are liberated during this process of hydrolysis of the protein, and much of the experimental work to be described in the following pages, has been undertaken in an endeavour to find out the relationship between the cleavage of the bacterial protein molecule, and the production of antibodies in the blood.

For instance, (1) Are the antigenic substances which give rise to the various antibodies given off at the same period of digestion of the bacterial protein, or do they represent various phases in the digestive process?

(2) What is the antigenic value of isolated split products of the native bacterial protein?

(3) How far can the protein molecule be split before it ceases to have any value as an immunising agent?

(4) Does proteolytic cleavage result in the breaking off of a split product or products possessed of a high degree of primary toxicity, to which the inherent toxicity of the native bacterial protein is mainly due, and can such split product be isolated in any degree of purity?

All these problems, in view of our present knowledge, call for elucidation, not only because they constitute gaps in our understanding of the immunity process,/

process, but also because their solution would help to explain many points with regard to non specific protein therapy, and the detoxication of vaccines, both subjects of much recent controversy.

THE CHOICE OF A METHOD.

Inasmuch as the solution of bacteria in vivo is apparently the result of enzyme action, the endeavour was made in this investigation to imitate the body processes as closely as possible, and for this reason the digestion of the bacteria in vitro was carried out by Trypsin. It appeared to me that the methods previously employed by workers to obtain bacterial derivatives, - both mechanical and chemical as in the use of strong alkali of the nature of antiformin, - were so crude and so unlike any process that could occur in the animal body, that their employment could only yield disappointing results.

The organism employed for these investigations was the B. Typhosus, and the considerations which dictated this selection were first, that the Typhoid Bacillus is a pathogenic organism, non-sporing, grows readily on artificial media, and is capable of producing easily demonstrable antibodies when injected into laboratory animals.

Secondly/

Secondly, it is an organism which has been used extensively in vaccines, not only for the prophylaxis of Typhoid Fever itself, but also in the realm of non-specific therapy.

THE DIGESTIBILITY OF B. TYPHOSUS.

Nitrogenous substances form the basis of bacterial cells, and yet bacteria are not readily digested by strong proteolytic ferments, and are even capable of multiplying in a solution which would split up these proteins of which they have been shown to be composed. This resistant faculty is apparently not dependent on the fact that the protein is living, for bacteria are not digested to any extent even when killed by weak antiseptics or at low temperatures. It is owing to this property that bacteria are enabled to continue their existence when surrounded by the digestive juices of the intestinal canal, - a point of particular importance in the aetiology of Typhoid infection in man.

Kantorowicz,[9] one of the very earliest contributors to the literature on this subject, noted, that after heating to 70°C; gram negative bacilli are much less resistant to digestion, while the more recent work of Dukes[10] goes to show that a temperature of 70°C./

70°C. applied for thirty minutes is sufficient to destroy the ferment resistance of B. Typhosus. The resistance to digestion in all cases is associated with the presence of lipoids, which on account of their action on surface tension, are concentrated round the periphery of the bacterial cell. The effect of heat, according to Dukes, is to cause a redistribution of the lipoid protein complex, as a result of which the bacteria are capable of adsorbing pepsin or trypsin.

In my earliest experiments, I employed the method of Dukes, of heating to 70°C. for half an hour in order to increase the digestibility of the Typhoid Bacillus. I found however that employing 1% pepsin or trypsin, there was not much appreciable clearing in the turbid bacterial suspension even after 24 to 48 hours digestion at 37°C, and inasmuch as I did not wish to use strong ferment solutions to achieve this purpose, I tried the method employed by Douglas[11] in his work on bacterial digests, namely, the preliminary extraction of the bacilli by acetone. Utilising a suspension of killed Typhoid Bacilli, which had previously been extracted with acetone for 24 hours, I found that 1% Trypsin in 1% Sodium carbonate was sufficient/

sufficient to cause an almost complete clearing of the fluid in 4 to 6 hours. Furthermore the work of Douglas in comparing the antigenic properties of Typhoid bacilli simply dried, with bacilli extracted with acetone, proved conclusively that the latter lost none of their antigenic power by extraction, a conclusion which my own control in succeeding experiments largely confirms.

EXPERIMENTAL TECHNIQUE.

The laboratory strain of B. Typhosus was used. It was plated out on McConkey's bile salt agar to ensure freedom from contamination, and passed through the sugars to be certain that it remained typical. I had personally employed this strain during the twelve months previous to my undertaking this work, in the production of agglutinating antisera from rabbits, and as one could obtain fairly consistently an agglutinin titre of nearly 1 in 50000 following a course of six injections, it constituted a very satisfactory antigen. On the other hand, the strain had been isolated for some years, and by constant subculturing, its virulence for laboratory animals was very attenuated.

THE GROWTH AND EXTRACTION OF THE BACTERIA.

The organisms were grown on ordinary nutrient agar, both in Petri plates and in large tubes. Four litres of medium were used, and the inoculated plates and slopes were incubated for 48 hours at 37°C, when an abundant growth was obtained. The growth was scraped off with a minimal amount of saline, and for this purpose I found a strong wire bent in the form of a triangle, and fixed to an ordinary inoculating needle handle, very useful. Great care was exercised not to remove any of the medium in so doing, for the agar containing as it does about 1% peptone might have introduced a possible source of error in the later investigations.

The organisms were killed by heating to 60° in a water bath for thirty minutes.

The total bulk of bacterial emulsion was 120 cc.

This emulsion was enumerated by Wright's method against red blood corpuscles, and 1 cc. was found to contain approximately 50,000 million bacilli. 80 cc. of this emulsion were earmarked for the purposes of the digestion experiment, while the remaining 40 cc. were set aside for the preparation of controls.

To the 80 cc. of Typhoid emulsion, an equal bulk of acetone was added. This caused agglomeration and sedimentation/

sedimentation of the bacilli. Sedimentation was completed by centrifugalisation, the clear supernatant fluid was pipetted off, and the moist bacterial mass was transferred to a fat extraction thimble. After having been allowed to filter dry, the thimble was placed in a Soxhlet apparatus and extracted with acetone for 24 hours. The bacilli after acetone extraction were found to have retained their typical morphological appearance in film.

The thimble, containing the bacterial mass, was then dried in a dessicator over sulphuric acid. Its contents were then transferred to a small sterile glass stoppered bottle, and weighed. The weight of dried extracted Typhoid bacilli was 950 mg. From the previous enumeration of the bacilli in the emulsion, the number of bacilli corresponding to 1 mg. of the dried mass, was estimated. It was found that 1 mg. of the dried bacillary powder was equivalent approximately to 4,210 million bacilli.

The remaining 40 cc. of the original emulsion was divided into two equal parts. In the one case the bacilli were sedimented and extracted by acetone as before, while in the other, they were simply sedimented by centrifuging at high speed. In both cases after thorough dessication, the caked bacterial masses were transferred to sterile glass stoppered bottles,/

bottles, and weighed. Standardised emulsions of dried and acetone estracted Typhoid bacilli were made in Carbol saline, so that 1 cc. of the emulsion was equivalent to 5 mg. of the dried powder. These emulsions after preparation, were placed in sterile glass ampoules, sealed off in the flame, and stored in the ice chest until required. They were to be used as controls and standards of comparison, for the estimation of the antigenic properties of the split products isolated in the digestion experiments.

THE CHEMICAL PHYSIOLOGY OF PROTEINS AND PROTEIN DERIVATIVES.

Inasmuch as the processes to be described in connection with the hydrolysis of bacterial protein, are based on the analogy with ordinary proteins such as are contained in egg white and blood serum, a short description of the cleavage products of the latter, together with the principles of physiological chemistry involved in their separation, will help towards the proper understanding of the character of bacterial protein and its derivatives.

The proteins of blood serum and egg white are mainly albumens and globulins. These are known as simple or native proteins, and they consist of complex molecules,/

molecules, which in turn are built up of combinations of amino-acids. Chemically the proteins are relatively stable and inert, and are amphoteric, combining with both the hydrogen and the hydroxyl ion. They can be hydrolysed by acids and alkalis, as well as by enzymes, yielding a series of degradation products commonly referred to as protein split products.

Again these simple proteins, albumens and globulins, may be compounded with some nonprotein group usually acid in nature, and the most important members amongst these conjugate proteins are the nucleoproteins, in which the added radical is nucleic acid.

Bacterial protein is supposed to consist mainly of nucleoproteins.

The most important derivatives and split products of the native protein are:-

(1) The Metaproteins or Albuminates.

These are the earliest derivatives of proteins, and their formation depends upon the property which proteins possess, of acting as acids or bases, and combining with oppositely charged radicals. Thus according to the reaction of the solution, there are formed acid or alkali metaproteins.

(2)/

(2) Coagulated proteins.

These are irreversible, insoluble, products of proteins, produced by the action of heat or alcohol. Examples are coagulated albumen and globulin.

(3) Proteoses or Albumoses.

These are products of the partial hydrolysis of proteins, which are no longer heat coagulable, and can be salted out by concentrations of certain salts such as ammonium sulphate.

(4) Peptones.

These are further hydrolytic cleavage products, soluble in water, not coagulable by heat nor precipitable by ammonium sulphate.

(5) Polypeptids.

These are compounds of amino-acids, the latter constituting the ultimate derivatives of proteolytic cleavage.

The original protein molecule can be regarded then as a combination of amino-acids. The process of digestion would seem to consist in a progressive splitting off of amino-acids, or groups of amino-acids, with the production of smaller sized molecules of simpler structure.

Again the earlier derivatives of native protein, including/

including peptone, give certain definite colour tests by which their protein character may be recognised. The most important of these is the Biuret reaction, which consists in the production of a violet colouration in presence of strong alkali, and a drop or two of copper sulphate solution.

THE PROTEINS OF BACTERIA.

It has now become recognised that the proteins of bacteria are the substances most intimately related to the vital structures of the organism. Moreover, authorities are almost unanimous in affirming that the proteid constituents are closely allied to, if not actually identical with, the similar substances found in plant and animal tissues,[12] and referred to previously as albumens, globulins, and nucleoproteins. The percentage of proteid present in bacteria varies according to the type investigated, and also to some extent according to the composition of the culture medium. Zinsser states that it varies from 50% to 80% of the total weight. This proteid seems to be very closely associated with lipoidal bodies, just as is found in the higher cells.

THE DIGESTION OF THE TYPHOID BACILLI AND THE SEPARATION OF THE SPLIT PRODUCTS.

The ferment used was Trypsin in the form of Injectio Trypsini Co. (Allen and Hanbury). It is supplied in sterile glass ampoules containing 1 cc. Its activity on thin strips of coagulated egg white was tested in dilutions up to 1 in 1000. 1% Trypsin in saline, rendered definitely alkaline by the addition of 1% sodium carbonate, was used as the digesting fluid. The powdered Typhoid Bacilli, to the weight of 950 mg., were transferred to a large sterile glass tube. 50 cc. of the digesting fluid were added, and one or two drops of ether to prevent any growth of other organisms. Digestion was carried out at 37°C. At the end of two hours, 0.5 cc. of Trypsin was added to make good any ferment destroyed by the alkali. After four hours, the organisms were largely digested, and the solution was opalescent.

The digest was now filtered through a sterile Berkefeld filter, until the filtrate was perfectly clear. In this respect it need only here be noted that it is advisable to test the filter before hand to determine its efficacy in keeping back whole bacilli. This can readily be done by filtering a living broth culture of B. Typhosus, and inoculating agar plates from the filtrate.

5 cc./

5 cc. of this filtered digest was removed with a pipette and standardised by the addition of saline until 1 cc. of the solution represented 5 mg. of dissolved bacilli. This standardised sample of digest was stored in a sterile sealed glass ampoule in the ice chest.

ISOLATION OF ACID METAPROTEIN.

The residue remaining on the surface of the filter candle was scraped off, and transferred to a sterile test tube containing 20 cc. of $\frac{N}{1}$ Hydrochloric acid, and extraction was carried out for 12 hours at 37°C. The solution was then filtered through a Berkefeld candle, and $\frac{N}{1}$ Sodium Hydroxide added from a burette until the reaction was neutral or very slightly alkaline to litmus. A colourless, glairy looking precipitate gradually separated out. It was isolated by filtration through a hardened filter paper in a Buchner funnel. It was dried, scraped off, weighed and made up in saline to the standard emulsion of 5 mg. per cc. The total weight of acid meta-protein isolated was 32 mg.

SEPARATION OF ALKALI METAPROTEIN.

The filtered digest was made neutral or slightly acid to litmus by adding $\frac{N}{10}$ Hydrochloric Acid. It was noticed that as the solution approached neutral point, it lost its clearness, became markedly turbid, and if allowed to stand for an hour or two in the ice chest, a white flocculent precipitate of alkali metaprotein separated out. This was removed by centrifuging at high speed. It was then dried, weighed, and emulsified in saline up to the standard of 5 mg. per cc. The weight of alkali metaprotein separated was about 40 mg.

ISOLATION OF THE REMAINING COAGULABLE ALBUMEN.

The neutral filtrate was now heated to 80°C in a water bath, rendered slightly acid by the addition of about 5 cc. of 10% acetic acid, and kept at just under boiling point for three minutes. The albumen thus coagulated was removed by centrifugalisation. It was dried, weighed, and emulsified in saline as in the previous instances. The weight of dried coagulable albumen thus isolated was 30 mg.

SEPARATION OF PROTEOSE FRACTIONS.

The volume of the filtrate was measured, and an equal quantity of neutral saturated ammonium sulphate solution added. After thorough mixing, a white flocculent precipitate corresponding to the primary proteose fraction began to separate out. Separation was not complete until after six hours standing in the ice chest. This primary proteose fraction was collected by filtration through hardened filter paper, and dried and weighed as before. It was found to be largely soluble in saline. The weight of Primary Proteose separated was about 60 mg.

SEPARATION OF SECONDARY PROTEOSE FRACTION.

In protein solutions, secondary proteoses are precipitated by complete saturation with ammonium sulphate. In my experience, the most important point in this procedure is to ensure the purity of the ammonium sulphate. The ammonium sulphate sold by manufacturing chemists as pure, is apt to contain a considerable quantity of impurity, and therefore should be recrystallised at least twice before use.

The filtrate was heated to 60°C. and poured into a warm sterile mortar containing rather more than the amount of ammonium sulphate required to completely saturate/

saturate the filtrate. It was then acidified slightly with 1% sulphuric acid (saturated with ammonium sulphate), mixed thoroughly and rapidly filtered through ordinary filter paper. A brownish white flocculent precipitate separated out on standing, and was collected on a hardened filter paper by filtration under negative pressure. It was dried, weighed, and put up in standardised solution in the manner previously described. The weight of secondary proteose isolated was 100 mg. It was largely but not entirely soluble in normal saline.

SEPARATION OF PEPTONE FRACTION.

The filtrate was now evaporated on a water bath to about one quarter of its bulk, cooled, and the ammonium sulphate which crystallised out, removed by filtration. The ammonium sulphate still remaining in solution was removed by adding Barium Hydrate solution, and the insoluble barium sulphate was filtered off. Excess of Barium Hydroxide was removed by passing a current of CO_2 through the fluid, and then filtering off the insoluble barium carbonate. Portions of this filtrate gave a faintly positive Biuret test, so separation of peptone was proceeded with. The fluid was evaporated down to small bulk - about 10 cc. - and double this amount of absolute alcohol/

alcohol was added. A well marked white precipitate separated out on standing, and was collected by high speed centrifugalisation. It was dealt with in the manner described for the other fractions. The weight of dried Peptone was 20 mg.

The technique just described for the separation of the split products of the Typhoid Bacillus was formulated as the result of preliminary trials, and is the method I have found most satisfactory. It is based on Milroy's[13] technique for the separation of the products of peptic and tryptic digestion, and the chief difficulties encountered, were dependent on the small bulk of material for digestion, and the still smaller bulk of the various fractions. It will be noticed also that while the original weight of dried B. Typhosus digested was 950 mg. the total weight of all fractions isolated was only about 300 mg. This loss, although partly accounted for by undigested residue, and partly by digestion having taken place beyond the peptone stage, is also strongly suggestive of some degree of clumsiness in the methods of separation of the various split products. It may be that in course of time more exact methods will be evolved than those at present in use.

THE SUBSTANCES REMOVED FROM THE BACILLI BY ACETONE EXTRACTION.

It has been mentioned above, that the Typhoid Bacilli were rendered capable of digestion by preliminary extraction by acetone in a Soxhlet apparatus. Acetone dissolves cholesterol, neutral fats and fatty acids, but the lipins, of which lecithin is an example, are not dissolved. Inasmuch as some recent American work has endeavoured to attribute to the fatty substances in the bacterial cell, a definite rôle in the immunity process even more important than that of the protein content, the opportunity was taken to determine whether any fatty material removed by acetone extraction was possessed of antigenic power, and per contra to learn if acetone extraction removed any element from the Typhoid Bacilli, whereby organisms so treated became less efficient antigens than the ordinary dried Typhoid Bacilli.

To this end, the acetone used for extraction was evaporated off on a water bath, and the fatty and waxy substances left, bright yellow in colour, were dried and weighed. The weight of the fatty material removed by acetone extraction from the bacilli was 44 mg.

ANIMAL INOCULATION.

The animals used were young rabbits all of the same age, and not varying much in weight. Ten rabbits were employed for the injections, while six others were retained as normal controls. Samples of blood were drawn off from all the rabbits as the first step in the investigations, and the agglutinating and bactericidal power of each serum separately tested against the typhoid bacillus. None of the sera showed agglutination against B. Typhosus in as low a dilution as 1 in 4, so that the possibility of natural agglutinins was excluded. Estimation of the bactericidal power of the sera, by Wright's method and by Douglas' serum dilution method (both to be described later) showed that some of the normal rabbits' sera could kill all organisms in a 1 in a million dilution of 24 hours' broth culture of B. Typhosus, when serum and broth were mixed in equal proportions. In no instance, however, was the bactericidal power of the normal rabbit's serum greater than this.

In the accompanying table the substances inoculated, and the doses in the case of the first two injections, are set down.

TABLE 1./

TABLE 1.

No. of Rabbit.	Weight.	Material Inoculated.	Amount.		Route.
			4.9.22.	6.9.22.	
1.	1800 gr.	Dried B. Typhosus	2.5 mg.	5 mg.	Intravenous.
2.	1790 gr.	Acetone Extracted B. Typhosus.	"	"	"
3.	1570 gr.	Filtered Digest (4 hours)	"	"	"
4.	1420 gr.	Coagulated Protein	"	"	"
5.	1480 gr.	Alkali Metaprotein	"	"	"
6.	1520 gr.	Acid Metaprotein	"	"	"
7.	1650 gr.	Primary Proteose	"	"	"
8.	1520 gr.	Secondary Proteose	"	"	"
9.	1710 gr.	Peptone	"	"	"
10.	1630 gr.	Acetone Soluble Lipoid Material.	5 mg.	10 mg.	Subcutaneous.

On 4.9.22 each rabbit received 2.5 mg. of the particular bacterial product, and 5 mg. on 6.9.22. These injections were made into the marginal ear vein, with the exception of Rabbit No.10, which received double doses of the acetone soluble lipoid subcutaneously. This was on account of the impracticability of making a fine emulsion suitable for intravenous injection.

Samples of blood were drawn off on 11.9.22 from the ear veins of the inoculated rabbits. The serum was separated by centrifuging.

AGGLUTININS.

Throughout all the estimations of agglutination titre, a formolised broth culture of B. Typhosus was used to ensure uniformity in readings. This culture was prepared according to the method of Dreyer[14]. B. Typhosus was grown in a flask of peptone broth for 24 hours. At the end of this period, sufficient formalin was added to give a final concentration of 0.1% of 40% formaldehyde. The flask of broth culture was then placed in the ice chest, and shaken up at intervals. Agar plates were inoculated from this culture at the end of 24, 48, and 72 hours. At the end of 72 hours the organisms were found to be all/

all killed. This sterile broth culture, closely stoppered with rubber, was kept in the ice chest, and retained its sterility and its efficiency during all the period of the experiments.

For the actual agglutination, the naked eye method, utilising glass sedimentation tubes, was employed. 100 cmm. of serum were added to tube 1 of the series, and 100 cmm. of saline to all the remaining tubes, with the exception of the last, which was to act as a control for spontaneous agglutination in the bacillary emulsion.

100 cmm. of serum was added to tube 2, thoroughly mixed with the saline, and 100 cmm. of this dilution transferred to tube 3, and so on. In this way, multiple dilutions of serum were obtained, 1 in 2, 1 in 4, 1 in 8, etc.

200 cmm. of formolised broth culture were then added to each tube, and thoroughly shaken. The tubes were incubated in a water bath for 2 hours at 55°C.

The highest dilution of the serum in which sedimentation was present naked eye, was accepted as the titre of the serum.

The following table gives the results obtained.

TABLE 2./

TABLE 2. 12.9.22.

ESTIMATION OF AGGLUTININS.

No. of Rabbit.	Material Inoculated.	Dilutions of serum 1 in 3	6	12	24	48	96	192	384	768	1536	3072	Control
1.	Dried B. Typhosus	+	+	+	+	+	+	+	+	+	+	-	-
2.	Acetone Extracted B. Typhosus.	+	+	+	+	+S	-	-	-	-	-	-	-
3.	Filtered Digest.	+	+	+	+	+S	-	-	-	-	-	-	-
4.	Coagulable Protein	+	+	-	-	-	-	-	-	-	-	-	-
5.	Alkali Metaprotein	-	-	-	-	-	-	-	-	-	-	-	-
6.	Acid Metaprotein	-	-	-	-	-	-	-	-	-	-	-	-
7.	Primary Proteose	-	-	-	-	-	-	-	-	-	-	-	-
8.	Secondary Proteose	-	-	-	-	-	-	-	-	-	-	-	-
9.	Peptone	-	-	-	-	-	-	-	-	-	-	-	-
10.	Acetone Soluble Lipoid.	-	-	-	-	-	-	-	-	-	-	-	-

A study of the above table reveals, that following two injections amounting to 7.5 mg. in all, the two controls injected with dried Typhoid bacilli and acetone extracted bacilli, showed well marked agglutinins, though the titre was highest in the first control. In the case of the rabbits inoculated with the various derivatives, only those having received the filtered digest and the coagulable protein, had demonstrable agglutinins in the serum.

ESTIMATION OF THE BACTERICIDAL AND LYSOGENIC POWER OF THE SERA.

It was held by Pfeiffer, and by many since his time, that perhaps the most important protective mechanism against the B. Typhosus, is associated with the appearance in the serum of substances capable of killing and dissolving the bacteria. As in the present state of our knowledge, the bactericidal action cannot be dissociated from the lysogenic, these antibodies are usually grouped under the generic term of bacteriolysins. The bacteriolytic action according to Ehrlich, and Bordet, is dependent upon a thermostable immune body, which however can only exert its influence in the presence of normal complement.

METHOD EMPLOYED.

Bactericidal power of the sera was determined by Almwroth Wright's method,[15] with this modification that small sedimentation tubes were used instead of the looped capillary pipette. The aim in this method is to determine the number of bacteria which can be killed off by a given quantity of serum, and for this purpose, equal quantities of serum are brought in contact with equal quantities of varying dilutions of a 24 hour broth culture of B. Typhosus. The details of the technique are as follows:-

A 24 hour broth culture of B. Typhosus is taken, and enumerated against red blood corpuscles, according to Wright's method of counting bacteria. My experience of this latter method is that it gives rather lower counts than enumeration on a Thoma-Zeiss haemocytometer slide, but inasmuch as Wright's method was employed in all cases, there could be no relative error.

A capillary pipette with a bulb was drawn out in the blow flame, and calibrated with mercury so as to give volumes of 25 cmm. and 250 cmm. Using this pipette, dilutions of the broth culture from 1 in 10 up to 1 in 1,000,000 were made, the diluent being sterile peptone bouillon. A series of eight sedimentation/

sedimentation tubes, thoroughly sterilised by exposure to 160°C for 1 hour, was used for each serum, and from a calibrated capillary pipette, 100 cmm. of the varying multiple dilutions of the broth culture were added to each tube, except the last. This last tube was left empty, and served as a control. 100 cmm. of serum were then added to each tube, the tubes shaken to secure thorough admixture, and the battery was sealed with plasticine. Incubation was carried out over night at 37°C. In the morning, the plasticine was removed, and 200 cmm. of litmus peptone mannite added to each tube. The tubes were shaken, sealed again, and incubated for 24 hours.

Inasmuch as B. Typhosus produces acid in mannite, a change in colour to red indicated that all the bacteria had not been killed by the action of the serum, while no alteration in the reaction of the solution, was taken as evidence that the bactericidal action of the serum had been complete. Unheated sera were employed so that the bactericidal action was dependent on the complement present. For this reason, and in view of the deterioration of complement when kept for any length of time, the experiments were always put up within 4 hours after the blood had been withdrawn, the serum being separated by centrifuging.

Controls/

Controls were always put up as follows:- (1) Serum and mannite peptone. Staphylococcus aureus if present may produce acid and nullify results. (2) Mannite peptone - equal volume of 1 in 1,000,000 broth culture. This was to ensure that a sufficient number of organisms was present in the high dilution, to obtain an alteration in the reaction.

A table showing the results obtained is given below.

TABLE 3./

TABLE 3. - BACTERICIDAL POWER (WRIGHT) 13.9.22.

The number of bacilli in the original broth culture was 480 millions per cc.

Dilution of Culture.	Number of Rabbit. 1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	Control Normal.
in 1,000,000	Sterile.	Sterile.	Sterile.	Sterile.	Sterile.	Sterile.	Sterile.	Sterile.	Sterile.	Growth.	Growth.
in 100,000	"	"	"	"	"	"	"	"	Growth.	"	"
in 10,000	"	"	"	"	"	"	Growth.	Growth.	"	"	"
in 1,000	Growth.	"	"	"	"	Growth.	"	"	"	"	"
in 100	"	"	"	"	Growth.	"	"	"	"	"	"
in 10	"	Growth.	Growth.	Growth.	"	"	"	"	"	"	"
Undiluted	"	"	"	"	"	"	"	"	"	"	"

BACTERICIDAL POWER (SERUM DILUTION METHOD OF DOUGLAS)[16]

This method was used chiefly as a control. Sterile sedimentation tubes were used as before. 50 cmm. of saline were added to all tubes with the exception of the first, which contained 50 cmm. of undiluted serum. To tube 2, 50 cmm. of serum were added, giving 100 cmm. of dilution 1 in 2. 50 cmm. were taken from tube 2, and added to tube 3, giving a dilution of 1 in 4. By this method of multiple dilution, dilutions up to 1 in 128 were made. A 24 hour broth culture of B. Typhosus was diluted 1 in 100,000. 50 cmm. of this diluted culture were added to each tube, and the tubes mixed thoroughly. The battery of tubes was sealed with plasticine, and incubated for 2 hours at 37°C. Then 100 cmm. of peptone mannite were added to each tube, the tubes sealed with plasticine, and incubated over night. Any tube showing acid production indicated that in that particular dilution of the serum, the organisms had not all been killed. The protocol is given below.

TABLE 4./

TABLE 4. BACTERICIDAL POWER (SERUM DILUTION METHOD)

Broth Culture diluted 1 in 100,000.

Dilutions of Serum.	Number of Rabbit. 1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	Control Normal.
1 in 2	Sterile.	Sterile.	Sterile.	Sterile.	Sterile.	Sterile.	Sterile.	Sterile.	Sterile.	Growth.	Growth.
1 in 4	"	"	"	"	"	"	Growth.	Growth.	Growth.	"	"
1 in 8	"	"	"	"	"	"	"	"	"	"	"
1 in 16	Growth.	Growth.	"	"	Growth.	Growth.	"	"	"	"	"
1 in 32	"	"	"	"	"	"	"	"	"	"	"
1 in 64	"	"	Growth.	Growth.	"	"	"	"	"	"	"
1 in 128	"	"	"	"	"	"	"	"	"	"	"
1 in 256	"	"	"	"	"	"	"	"	"	"	"

It will be seen from a study of these two protocols, that the results in the experiments are relatively, if not absolutely comparable. By a repetition of these experiments, I have come to the conclusion that Wright's method gives more constant figures, and for that reason I have used it exclusively in succeeding estimations. Perhaps the anomalies that creep into the readings when Douglas' method is used, are to be explained by the lack of complement in the higher dilutions of the serum. On the principle that there is an optimum amount of immune body which gives the greatest bactericidal effect with a given amount of complement, and that increase of immune body beyond a certain point, with no increase of complement, may give diminished bactericidal action, it may be that here one is dealing with manifestations of the Neisser-Wechsberg phenomenon as it is called.

On analysing the results obtained from estimation of the bactericidal power, it is found that the rabbits receiving inoculations of filtered digest, and coagulated protein, showed the greatest concentration of bacteriolytic antibodies in the serum, if anything greater than the controls receiving whole Typhoid Bacilli. The earlier products of cleavage, alkali metaprotein and acid metaprotein, also produced bacteriolysins/

bacteriolysins in considerable quantity, but not to the same degree as the filtered digest, or the coagulated protein. The proteose fractions, while slightly antigenic from the point of view of the lysogenic antibodies, are apparently very weak antigens, and compare unfavourably with the earlier products of cleavage.

THE ESTIMATION OF THE PRECIPITATING POWER OF THE SERA.

In 1897 Kraus[17] showed in the case of the organisms of Typhoid and Cholera, that the antiserum not only caused agglutination, but when added to a filtrate of the culture of the corresponding bacterium, produced a cloudiness and afterwards a precipitate. To this substance in the immune serum, the name precipitin was given.

METHOD EMPLOYED.

As the antigen, a 4 hours digest of acetone extracted B. Typhosus was used. 50 mg. of the acetone extracted bacteria were digested in 10 cc. of 1% Trypsin solution. The digest was then filtered twice through a Berkefeld filter, until the filtrate was of crystal clearness. Samples of blood were drawn off aseptically/

aseptically from each rabbit, and the separation of the serum completed by centrifuging.

In carrying out the tests, it was found from experience that perhaps the most important detail in the technique, was to ensure absolute cleanliness of the glass sedimentation tubes employed. These were first boiled in water, and then cleansed individually with ether. They were dried, and sterilised in the hot air oven. The antigen was used undiluted, and put up against multiple dilutions of the various antisera. 100 cmm. of serum and 200 cmm. of antigen, were the volumes employed. The tubes were placed in the water bath for 4 hours at 50°C., and then allowed to stand over night at room temperature, protected from contamination. Readings were made in the morning. Controls of serum alone, and antigen alone, were put up. The protocol is given below:-

TABLE 5. PRECIPITATING POWER. 14.9.22.

No. of Rabbit.	Material Inoculated	Dilutions of Serum 1 in 3	6	12	24	48	96	192.
1.	Dried B. Typhosus.	+	+	+	+	+	-	-
2.	Acetone Extracted B. Typhosus.	+	+	+	+	-	-	-
3.	Filtered Digest.	+	+	+	-	-	-	-
4.	Coagulated Protein.	+	+	-	-	-	-	-
5.	Alkali Metaprotein.	+	+	+	-	-	-	-
6.	Acid Metaprotein.	+	+	-	-	-	-	-
7.	Primary Proteose.	+ S	-	-	-	-	-	-
8.	Secondary Proteose.	+ S	-	-	-	-	-	-
9.	Peptone.	-	-	-	-	-	-	-
10.	Acetone Soluble Lipoid.	-	-	-	-	-	-	-
Control Normal.	-	-	-	-	-	-	-	-

A study of the above table reveals well marked concentration of precipitins in the sera of rabbits receiving whole Typhoid bacilli, either in the form of dried B. Typhosus, or acetone extracted B. Typhosus. The earlier derivatives, including the filtered digest, have induced precipitin formation in the homologous serum, up to a dilution of from 1 in 6 to 1 in 12 of the serum. The sera of rabbits injected with the proteose fractions, showed haziness, but no definite granular precipitate in a dilution of 1 in 3 of the serum, and have been classed provisionally as slightly positive. The remaining fractions, peptone, and acetone soluble lipoid, induced no precipitin response that could be determined.

OPSONIC POWER OF THE SERA.

The researches of Denys and Leclef,[18] Wright and Douglas,[19] and Neufeld and Rimpau,[20] demonstrated that there are present in sera, normal as well as immune, substances capable of making organisms more sensitive to phagocytosis. In this country, following Wright, these substances in the serum are usually designated Opsonins, and two varieties are differentiated, namely:- (1) A non specific thermolabile opsonin found in normal serum: (2) A thermostable opsonin found in immune sera and having all the specific characters of antibodies/

antibodies in general. Inasmuch as specific opsonins increase greatly in amount in response to immunisation by the homologous organism, the determination of the opsonic power of the serum, has always been regarded as a valuable procedure in estimating the degree of immunity attained, though certain technical difficulties in carrying it out have prevented it being utilised to such an extent as the principles underlying it might have warranted.

TECHNIQUE EMPLOYED.

The determination of the opsonic indices of the various sera was carried out by Wright's method. The writer's own leucocytes were used in all the estimations. Blood was drawn off from the median basilic vein, and received into a centrifuge tube containing 1.5% citrate of sodium in saline. It was centrifuged carefully, washed three times with saline, and the supernatant fluid pipetted off. The bacterial emulsion was prepared by emulsifying portion of a 24 hour agar culture of B. Typhosus in saline. This was thoroughly shaken up in a mechanical shaker, and then centrifuged at high speed, to bring down all clumps. The emulsion was then diluted with saline until only a faint cloudiness could be made out.

The/

The serum in each case was heated previous to use for 10 minutes at 60°C. This procedure was rendered necessary on account of certain difficulties that were encountered during the preliminary trials. For one thing the bacteriolytic power of several of the sera was so strong, that lysis commenced, and the organisms stained poorly. By heating to 60°C., complement was destroyed, and bacteriolysis reduced to a minimum. Again, the strain of B. Typhosus used, being of very low virulence, was easily phagocyted even after contact with a normal control serum. This was in virtue of the nonspecific opsonin present. If the bacterial emulsion was diluted in order to get workable numbers of bacilli in the leucocytes, there was a tendency to dilute it below the numerical strength necessary to get the highest readings in the highly immune sera. Heating the sera to 60°C. destroyed the thermolabile opsonins, but the differences in amount of thermostable immune opsonin present in the various sera, could be the better made out. It followed of course from this, that the readings from the absolute standpoint were much lower than would otherwise have been the case.

Equal volumes of blood corpuscles, emulsion, and serum, were drawn up, in that order, into a Pasteur pipette, expressed on to a clean glass slide, and/

and thoroughly mixed. The mixture was then drawn up in the pipette, and the end sealed in the by pass of the Bunsen. The pipette was then placed in the incubator for 10 minutes at 37°C. It is usually recommended that 15 to 30 minutes is the time for incubation, but in the case of several of the sera, rapid intracellular digestion of the phagocyted organisms took place, and organisms partially digested stained badly, and were apt to be missed in counting.

At the end of incubation, the contents of the pipettes were expressed on to clean class slides, and films made. These were stained by Leishman's method and the number of bacilli in 100 leucocytes counted. From this, the average number of organisms per leucocyte, was determined. The phagocyte index obtained by the pooling of the serum from four normal control rabbits, was used as the denominator. The serum in this latter estimation was unheated. The phagocyte index, obtained in the case of the test sera, divided by the phagocyte index for normal unheated rabbits' serum, gives the opsonic index. It should be clearly understood, however, that the figures given in the following table, do not represent the absolute opsonic indices of the sera, but are only relative figures based on the immune opsonin content, and calculated to bring out the differences between the various sera.

TABLE 6. OPSONIC POWER.

Sera heated for 10 minutes at 60°C.

Unheated pooled serum of four normal control rabbits as standard.

100 leucocytes counted in each case.

No. of Rabbit.	Material Inoculated.	Opsonic Index.
1.	Dried B. Typhosus	0.48
2.	Acetone extracted B. Typhosus.	0.37.
3.	Digest.	0.22
4.	Coagulated Protein.	0.36.
5.	Alkali Metaprotein.	0.24.
6.	Acid Metaprotein.	0.15
7.	Primary Proteose.	0.15
8.	Secondary Proteose.	0.20
9.	Peptone.	0.25
10.	Acetone soluble lipoid.	0.18.
Control Normal (heated serum)	-	0.17

From the above protocol, it is seen that there is a definite rise in the opsonic index of the sera of Rabbits 1, 2, and 4, inoculated respectively with dried B. Typhosus, acetone extracted B. Typhosus, and Coagulated Protein. In the case of Rabbits 3, 5, and 9, inoculated respectively with digest, alkali metaprotein, and peptone, there is a slight rise above the basal/

basal figure, represented by the heated serum of a normal control rabbit, but this is not sufficient at this stage to exclude marginal error.

It should be noted that leucocytes brought in contact with normal heated serum, are still capable of phagocytosis in small degree. This is apparently attributable to phagocytic activity of washed leucocytes independent of the presence of serum. Wright and Douglas in their first contribution to the study of Opsonins, pointed out that washed leucocytes are incapable of phagocytosis. From control experiments carried out by me, in which the bacterial emulsion was brought directly into contact with well washed leucocytes, a slight degree of phagocytosis could usually be made out. This seemed to bear out, that where the organisms were avirulent as in the present experiments, the leucocytes possessed a small degree of phagocytic activity, which was independent of the presence of serum. Again there is the suggestion, as stated recently by Ledingham[21] that all normal opsonin may not be removed from a serum by the usual inactivation temperatures.

The inoculations were continued on 22.9.22, each rabbit received an additional 5 mg. of the various products. Sample bleedings were carried out on 28.9.22 and on subsequent days, for the further determination of antibody titre with the following results:-

TABLE 7. 29.2.22.

AGGLUTINATING POWER.

No. of Rabbit.	Material Inoculated.	Dilution of serum 1 in 3	6	12	24	48	96	192	384	768	1536	3072	6144	12288	Control
1.	Dried B. Typhosus	+	+	+	+	+	+	+	+	+	+	+	+	-	-
2.	Acetone extracted B. Typhosus.	+	+	+	+	+	+	+	+	+	+	+	-	-	-
3.	Filtered Digest.	+	+	+	+	+	+	-	-	-	-	-	-	-	-
4.	Coagulable Protein	+	+	+	+	S+	-	-	-	-	-	-	-	-	-
5.	Alkali Metaprotein	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6.	Acid Metaprotein.	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7.	Primary Proteose.	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8.	Secondary Proteose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9.	Peptone.	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9.	Acetone Soluble Lipoid.	-	-	-	-	-	-	-	-	-	-	-	-	-	-

It will be seen that amongst the derivatives from Typhoid bacilli, only the filtered digest, and the coagulable protein, were capable of raising the agglutinin titre in experimental rabbits. The controls in which whole Typhoid bacilli were injected, show a high agglutinin titre of 1 in 6144 and 1 in 3072. respectively. The rabbits inoculated with the remaining split products, show no agglutinin formation. These results bear out the findings of 12.9.22.

BACTERICIDAL POWER.

Wright's method employed, with the modification of Smallman[22] of utilising litmus peptone mannite instead of the plating method.

24 hours broth culture of B. Typhosus contained 650 million bacilli per cc. Equal volumes of broth dilutions and serum mixed, incubated overnight, and an equal bulk of litmus peptone mannite added in the morning. Tubes incubated 24 hours.

TABLE 8. 30.9.22.

BACTERICIDAL POWER.

Dilutions of Culture.	Number of Rabbit. 1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	Control Normal.
1 in 1,000,000	Sterile.	Sterile.	Sterile.	Sterile.	Sterile.	Sterile.	Sterile.	Sterile.	Sterile.	Growth.	Growth.
1 in 100,000	"	"	"	"	"	"	"	"	Growth	"	"
1 in 10,000	"	"	"	"	Growth	"	"	"	"	"	"
1 in 1,000	Growth.	Growth.	"	Growth	"	Growth.	Growth.	Growth.	"	"	"
1 in 100	"	"	"	"	"	"	"	"	"	"	"
1 in 10	"	"	Growth	"	"	"	"	"	"	"	"
Undiluted.	"	"	"	"	"	"	"	"	"	"	"

From the accompanying protocol, it will be seen that while the bactericidal readings are if anything lower than those obtained on 13.9.22, the same features are brought out. All the Typhoid derivatives, with the possible exception of peptone, have the power of stimulating the formation of bacteriolytic and bactericidal antibodies to almost the same extent as the controls of whole Typhoid bacilli. It is further seen that the highest antibody titre was present in the rabbit which had received injections of filtered digest, and it would appear that a digest containing as it presumably does, all the split products in solution, is the most efficient antigen for raising the bactericidal power of the serum.

TABLE 9.

PRECIPITATING POWER.

Technique as previously described, using multiple dilutions of serum, and a filtered digest of B. Typhosus as antigen.

No. of Rabbit.	Material Inoculated.	Dilutions of serum 1 in					
		2	4	8	16	32	64
1.	Dried B. Typhosus.	+	+	+	+	+	-
2.	Acetone extracted B. Typhosus.	+	+	+	+	-	-
3.	Filtered Digest.	+	+	+	-	-	-
4.	Coagulated Protein.	+	+	+	-	-	-
5.	Alkali Metaprotein.	+	+	+	-	-	-
6.	Acid Metaprotein.	+	+	+	-	-	-
7.	Primary Proteose.	+	+ S	-	-	-	-
8.	Secondary Proteose.	+	+	-	-	-	-
9.	Peptone.	-	-	-	-	-	-
10.	Acetone soluble Lipoid.	-	-	-	-	-	-
Control Normal.	-	-	-	-	-	-	-

ESTIMATION OF PRECIPITATING POWER BY DILUTIONS OF THE ANTIGEN.

This method was carried out on the principle that the sera with the highest concentration of precipitins, will give a positive precipitin reaction with the higher dilutions of the antigen. Multiple dilutions of the antigen are made with saline, 1 in 2, 1 in 4 up to 1 in 32. 100 cmm. of antigen are added to each tube, and an equal bulk of undiluted serum. Incubation is carried out for 4 hours at 50°C. The tubes are then allowed to stand over night at room temperature protected from contamination, and readings are taken next morning. The antigen contained 5 mg. of digested bacilli per cc.

TABLE 10./

TABLE 10. 2.10.22.

PRECIPITATING POWER (ANTIGEN DILUTION).

No. of Rabbit.	Material Inoculated.	Dilutions of antigen 1 in					
		2	4	8	16	32	64
1.	Dried B. Typhosus.	+	+	+	-	-	-
2.	B. Typhosus. (Acetone extracted)	+	+	-	-	-	-
3.	Digest.	+	+	-	-	-	-
4.	Coagulable Protein.	+	-	-	-	-	-
5.	Alkali Metaprotein.	+	+	-	-	-	-
6.	Acid Metaprotein.	+	+	-	-	-	-
7.	Primary Proteose.	+ S	-	-	-	-	-
8.	Secondary Proteose.	+ S	-	-	-	-	-
9.	Peptone.	-	-	-	-	-	-
10.	Acetone Soluble Lipoid.	-	-	-	-	-	-
Control Normal.	-	-	-	-	-	-	-

Comparing this protocol with the preceding, where the antigen was used undiluted with varying dilutions of the serum, it is seen that the same sera give positive precipitin reactions, but in the latter instance in no higher dilution than 1 in 8 of the antigen.

It is noteworthy that the proteoses, according to these findings, are capable of leading to the production of precipitins in the animals into which they are inoculated.

OPSONIC POWER.

Sera heated to 60°C for 10 minutes. Standard normal derived from the pooled unheated serum of four normal rabbits. Sera incubated 10 minutes, with equal volumes of bacterial emulsion, and washed leucocytes.

TABLE 11./

TABLE 11. 5.10.22.

No. of Rabbit.	Material Inoculated.	Opsonic Index.
1.	Dried B. Typhosus.	1.56
2.	B. Typhosus (acetone extracted)	1.28
3.	Digest.	0.27
4.	Coagulated Protein.	0.45
5.	Alkali Metaprotein.	0.62
6.	Acid Metaprotein.	0.32
7.	Primary Proteose.	0.27
8.	Secondary Proteose.	0.23
9.	Peptone.	0.41
10.	Acetone soluble Lipoid	0.20
Control Normal (heated serum)	-	0.21

From the above table, it is seen that the controls receiving whole Typhoid bacilli, show by far the highest concentration of opsonins. The rabbits receiving coagulable protein and alkali metaprotein, both show considerable rise in opsonic power, but the remaining derivatives have up to this time caused practically no rise in the opsonic content. The rabbit receiving injections of peptone occupies an anomalous position, but without further confirmation, this isolated result cannot be put without the bounds of technical error.

The/

The injections were continued. On 11.10.22 15 mg. of the test antigens were inoculated intravenously. On 17.10.22, and subsequently, samples of blood were withdrawn.

TABLE 12./

TABLE 12.

AGGLUTINATING POWER.

No. of Rabbit.	Material Inoculated.	Dilutions of serum 1 in														
		3	6	12	24	48	96	192	384	768	1536	3072	6144	12288	24576	49152
1.	Dried B. Typhosus.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
2.	B. Typhosus (Acetone extracted)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
3.	Digest.	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
4.	Coagulated Protein.	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
5.	Alkali Metaprotein	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6.	Acid Metaprotein.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7.	Primary Proteose.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8.	Secondary Proteose.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9.	Peptone.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10.	Acetone Soluble Lipoid.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

TABLE 13. 19.10.22.

BACTERICIDAL POWER (WRIGHT'S METHOD)

Dilutions of Culture.	Number of Rabbit. 1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	Control
1 in 1,000,000	Sterile.	Sterile.	Sterile.	Sterile.	Sterile.	Sterile.	Growth.	Sterile.	Sterile.	Growth.	Growth
1 in 100,000	Growth.	Growth.	"	"	Growth.	Growth.	"	"	Growth.	"	"
1 in 10,000	"	"	Growth.	Growth.	"	"	"	Growth.	"	"	"
1 in 1,000	"	"	"	"	"	"	"	"	"	"	"
1 in 100	"	"	"	"	"	"	"	"	"	"	"
1 in 10	"	"	"	"	"	"	"	"	"	"	"
Undiluted.	"	"	"	"	"	"	"	"	"	"	"

Culture contained 580 living Typhoid bacilli per cc.

The above protocol shows that all the sera were found to have declined considerably in respect of bactericidal power. Only the rabbits receiving injections of digest, coagulable protein, and secondary proteose, remained appreciably above normal.

TABLE 14./

TABLE 14. 20.10.22.

PRECIPITATING POWER.

No. of Rabbit.	Material Inoculated.	Dilutions of serum 1 in 2	4	8	16	32	64	128
1.	Dried B. Typhosus.	+	+	+	+	+	+	-
2.	B. Typhosus Acetone extracted.	+	+	+	+	+	+	-
3.	Digest.	+	+	+	-	-	-	-
4.	Coagulable Protein.	+	+	+	-	-	-	-
5.	Alkali Metaprotein.	+	+	-	-	-	-	-
6.	Acid Metaprotein.	+	+	+	+	-	-	-
7.	Primary Proteose.	+	-	-	-	-	-	-
8.	Secondary Proteose.	+	+	-	-	-	-	-
9.	Peptone.	-	-	-	-	-	-	-
10.	Acetone soluble Lipoid.	-	-	-	-	-	-	-
Control normal.	-	-	-	-	-	-	-	-

The antigen contained 5 mg. of Typhoid bacilli per cc.

TABLE 15.

OPSONIC POWER. 23.10.22.

No. of Rabbit.	Material Inoculated.	Opsonic Index.
1.	Dried B. Typhosus.	1.25
2.	B. Typhosus (acetone extracted)	0.87
3.	Digest.	0.41
4.	Coagulated Protein.	0.62
5.	Alkali Metaprotein.	0.46
6.	Acid Metaprotein.	0.55
7.	Primary Proteose.	0.17
8.	Secondary Proteose.	0.12
9.	Peptone	0.18
10.	Acetone soluble lipoid	0.15
Control normal (heated serum)	-	0.13

The earlier products of cleavage are seen to have produced a marked rise in the opsonic power in the sera of the rabbits, but the later products of digestion have produced no appreciable change. The two control rabbits injected with the whole Typhoid bacilli, demonstrate the value of the intact and unaltered protein molecule for the production of opsonins.

On 26.10.22, the rabbit which had been receiving injections of acid metaprotein died. Post-mortem examination/

examination revealed liver changes of the nature of cysts. The death of the animal was apparently in no way associated with the inoculations it had received.

ANTIBODY DETERMINATION BY THE METHOD OF FIXATION OF COMPLEMENT.

An endeavour was made to determine antibody formation in response to the injections of the Typhoid split products by this method, first described by Bordet and Gengou. The principle underlying the test is, that if an antibacterial immune body is present in a serum heated to 55°C., the serum when added to the corresponding bacterium leads to the fixation of complement, and thus prevents haemolysis when sensitised corpuscles are added.

PREPARATION OF THE ANTIGEN.

The antigen employed was a 4 hours digest of acetone extracted B. Typhosus. 100 mg. of dried acetone extracted Typhoid bacilli were added to 50 cc. of 1% Trypsin in normal saline. 1% sodium carbonate was added, and digestion was carried out for 4 hours at 50°C. in a water bath. The digest was then filtered through a Berkefeld filter, and a portion of the/

the filtrate - 5 cc. - was titrated with $\frac{N}{10}$ Hydrochloric acid, against phenol red as indicator, until the yellowish tinge appeared. From this, the amount of $\frac{N}{10}$ HCl required to bring the whole bulk of antigen to a hydrogen ion concentration of about 7, was determined.

SERA.

All sera to be tested were diluted 1 in 5, and heated to 56°C. for thirty minutes.

COMPLEMENT.

Fresh Guinea pig serum was used. It was kept in the ice chest over night, and titrated just before use in the morning.

HAEMOLYTIC SYSTEM.

Equal parts of 4% washed sheep corpuscles, and antisheep serum (Immune Body) 1 in 125. This admixture gives a 2% solution of corpuscles in 1 in 250 immune body. This was placed in the incubator at 37°C. for 30 minutes.

TITRATION OF COMPLEMENT.

In this, as in all subsequent titrations, I employed the technic as carried out at the Royal College of Physicians' Laboratory by Dr Wright. The reagents were used in a standard volume of 0.2 cc., the total bulk being 0.8 cc. In the titration of complement, 0.2 cc. of the various dilutions of fresh serum was employed. To this was added 0.4 cc. of 0.85% saline, and 0.2 cc. sensitised sheep corpuscles. Incubation was carried out at 37°C. for half an hour. The Minimal Haemolytic Dose of complement was determined as 0.2 cc. of 1 in 80 dilution.

TITRATION OF AMBOCEPTOR.

The haemolytic amboceptor gave haemolysis up to 1 in 1000, so that in the sensitisation of the sheep corpuscles, 4 doses of Immune Body were employed.

TITRATION OF ANTIGEN.

Antigen was put up, in varying dilutions, against normal rabbits' serum, and against a known immune rabbits' serum. 2 doses of complement were added to each. There was only partial haemolysis with antigen alone, up to dilution 1 in 4 of the antigen.

In/

In 1 in 8 dilution of antigen, there was complete haemolysis with antigen alone, and the combination of antigen and immune serum fixed complement, but there was almost complete haemolysis with antigen and normal control rabbits' serum.

Below is given a protocol of an experiment which has been repeated, with practically the same findings each time.

TABLE 16./

TABLE 16.

COMPLEMENT FIXING POWER OF SERA.

	Number of Rabbit.										
	1	2	3	4	5	6	7	8	9	10	Control Normal.
Antigen Control.	H	H	H	H	H	-	H	H	H	H	
Antigen Serum Complement (2 doses.	C.F.	C.F.	C.F.	C.F.	C.F.		C.F.	C.F.	P.H.	P.H.	
Antigen Serum Complement (4 doses)	C.F.	C.F.	C.F.	C.F.	C.F.		H	H	H	H	
Antigen Serum Complement (8 doses)	P.H.	P.H.	H	H	H		H	H	H	H	
Serum Control Complement 2 doses.	H	H	H	H	H		H	H	H	H	
Complement Control.	H	H	H	H	H		H	H	H	H	

Incubated 1 hour at 37°C. Haemolytic couple added.
Incubated 1½ hours at 37°C.

C.F. = Complete Fixation. H = Haemolysis. P.H. = Partial Haemolysis.

As the combination of antigen and normal serum was capable of fixing 1 dose of complement, only the sera fixing 4 doses of complement are accepted as possessing immune antibodies.

It will therefore be seen that in addition to the controls, the sera of the rabbits injected with coagulable protein, filtered digest, and alkali metaprotein, were capable of fixing 4 doses of complement. The lower split products showed complete haemolysis with this amount of complement. On the other hand, the sera of the controls receiving whole Typhoid bacilli, were able to fix almost 8 doses of complement. In the case of the animals receiving proteoses, peptone, and acetone soluble lipoid, complement fixing antibodies were not demonstrated.

THE HEALTH OF THE INOCULATED ANIMALS.

With the exception of the rabbit injected with acid metaprotein, which died from intercurrent disease, the rabbits remained well during the whole period of the inoculations, and steadily gained in weight.

THE PROTECTIVE POWER DEVELOPED AS A RESULT OF THE INOCULATIONS.

An endeavour was made to test the degree of immunity developed in the rabbits against inoculation with the living germ. For this purpose, the minimal lethal dose of living B. Typhosus per kilogram of body weight was determined, and two minimal lethal doses were inoculated intravenously into each rabbit on 1.11.22. In all cases, the rabbits died in from 12 to 48 hours, and post-mortem examination revealed the presence in every case of the lesions associated with Typhoid intoxication in animals, and which will be referred to later.

In view of the high titre of antibodies present in the sera, especially of the control rabbits inoculated with whole Typhoid bacilli, these findings are of considerable interest, and demonstrate how the titre of antibodies in the serum cannot be taken as an accurate criterion of the protection developed against the living germ. It is only fair to state, however, that when those lethal doses of living B. Typhosus were administered, the concentration of antibodies as a whole had begun to decline, and only the agglutinins and opsonins showed no falling off from the highest figures attained during the course of the immunising injections.

SPECIFICITY OF ANTIBODIES FORMED AGAINST THE TYPHOID DERIVATIVES.

In a preliminary series of experiments carried out during March and April 1922 for the purpose of elaborating a technique for the separation of the split products of the Typhoid bacillus, acid meta-protein, coagulated protein, primary and secondary proteose, were separated from a pepsin digest of the organism.

These were suspended in 10 cc. of saline, and injected in graduated doses into four rabbits, to determine if there was any antibody response. Blood was drawn off subsequent to these injections, and tested for agglutinins and precipitins. The rabbit injected with coagulated protein developed agglutinins in the serum up to a titre of 1 in 20, but there was no agglutinin formation in the other sera. On the other hand, the rabbits injected with acid metaprotein, coagulated protein, and secondary proteose, all showed the presence of precipitins in the sera, using as antigen an old bouillon culture of B. Typhosus filtered through a Berkefeld filter. To determine the specificity of this precipitin reaction, the various sera were tested against antigens derived from B. Coli, and B. Paratyphosus B, with negative results in all cases. Again the agglutinins developed/

developed against the injection of coagulated protein, were without effect on B. Coli and B. Paratyphosus B.

These experiments demonstrated that the antibodies, at least agglutinins and precipitins, formed as a result of the injection of the Typhoid split products, were strictly specific against the B. Typhosus, and were without effect on other members of the group.

I have not given the protocols of these experiments as the weights of the cleavage products inoculated were not determined, nor were the experiments adequately controlled by injections of the unaltered bacillus. Qualitative results only were aimed at, the quantitative estimations forming part of the subsequent series of experiments.

A SUMMARY OF THE EXPERIMENTAL FINDINGS.

Typhoid bacilli, when the lipoid envelope surrounding them was removed by acetone extraction, were capable of being easily digested by pepsin and trypsin.

From these digests, by suitable biochemical methods, various products could be isolated, representing progressive phases of the cleavage of the bacterial protein molecule. The earlier products were represented/

represented by alkali and acid metaprotein, and coagulated protein, while the later derivatives, no longer heat coagulable, were primary and secondary proteose, and peptone. In addition a digest, containing all the protein derivatives in solution, and unaltered by chemical methods, was tested as an antigen.

Injections of the protein fractions were carried out in rabbits over a period of five to six weeks, 27.5 mg. being the total weight of the test antigens inoculated in each case. At the same time controls were furnished by injecting the same amounts of whole Typhoid bacilli, both dried, and acetone extracted, into rabbits. Agglutinins, bacteriolysins, precipitins, and opsonins, were estimated from time to time, and finally the complement fixing antibodies.

AGGLUTININS.

Only the rabbits injected with filtered bacterial digest, and coagulated protein, showed any development of agglutinins. In the case of the rabbit receiving inoculations of the digest, the highest titre obtained was 1 in 768, while in the case of the rabbit receiving coagulated protein, the highest titre was 1 in 96.

The sera of the two control rabbits showed an agglutinin titre of about 1 in 25,000.

BACTERICIDAL POWER.

All the Typhoid derivatives, with the exception of peptone, produced a rise in the bactericidal power of the blood. The strongest antigen in this respect was the filtered digest of Typhoid bacilli, which raised the concentration of bacteriolysins above that present in the controls. The earlier derivatives, acid and alkali metaprotein, and coagulated protein, showed a greater bacteriolytic response than the proteoses, following the first injections, but subsequently there was little difference to be made out between them.

It appeared as if the bacteriolysins were only capable of being stimulated up to a certain point, a subsequent large injection of antigen being followed rather by a decline in the bactericidal power, which progressed until the sera were almost within the normal limits.

PRECIPITINS.

All the products, with the exception of peptone, were capable of inducing the formation of precipitins in the blood of the experimental animals, but the concentration fell considerably short of that in the controls, which gave a positive precipitin reaction up

to/

to 1 in 64 dilution of the serum. None of the remaining antigens furnished a precipitating serum stronger than 1 in 16. The proteoses were less effective than the coagulable antigens in this respect, and only gave a definite positive reaction in the lowest dilution of the serum.

OPSONINS.

The earlier derivatives, including the digest, all caused a rise in the opsonic power. The later derivatives, proteoses and peptone, produced no change. Of the coagulable antigens, coagulated protein was the most efficient in raising the opsonic power of the serum, but it fell considerably short of the controls - especially the rabbit receiving dried Typhoid bacilli, which showed an opsonic index (calculated on immune thermostable opsonin only) of 1.25, while coagulated protein raised the index to 0.62.

COMPLEMENT FIXING ANTIBODIES.

These were demonstrated in the sera of rabbits injected with acid and alkali metaprotein, coagulated protein, and digest. They could not be demonstrated in/

in the sera of rabbits injected with proteoses and peptone. Both controls showed the presence of complement fixing antibodies, and these were present in greater concentration than in the case of the other antisera giving a positive reaction.

ACETONE SOLUBLE LIPOID SUBSTANCES.

This material inasmuch as it is not a protein derivative has been considered by itself. It was injected into a rabbit in a total dosage of 40 mg. but there was no recognisable antibody formed - neither did the animal develop any degree of immunity against the living germ. This acetone soluble lipoid material consisted of fats, waxes, and fatty acids. Apparently its removal from the bacterial cell did not result in any material loss to the cell from the antigenic standpoint.

PROTECTIVE POWER CONFERRED BY IMMUNISATION WITH TYPHOID BACILLI, AND THEIR DERIVATIVES.

The rabbits injected with the split products, including the digest and acetone soluble lipoid, were unable to stand the administration of 2 minimal lethal doses of living B. Typhosus. Even the controls, which developed a high concentration of antibodies, did not possess a protective immunity against this amount of the living germ.

TABLE 17.

TABULAR REPRESENTATION OF THE ANTIGENIC PROPERTIES OF TYPHOID DERIVATIVES.

Concentration of antibodies formed is roughly indicated by the number of plusses.
- indicates no antibody demonstrated. ∓ indicates doubtful result.
N.D. indicates "not determined".

No. of Rabbit.	Weight.	Material Inoculated.	Agglutinins.	Bacterio-lysins.	Precip-itins.	Opsonins.	Complement and Fixing Antibodies.	Protection (2 M.L.D)
1.	1800 gr.	Dried B. Typhosus.	+ + +	+ +	+ + +	+ + +	+ +	-
2.	1790 "	B. Typhosus (A.E.)	+ + +	+ ∓	+ + +	+ + +	+ +	-
3.	1570 "	Filtered Digest.	+ +	+ + +	+ +	+	+	-
4.	1420 "	Coagulated Protein.	+	+ +	+ +	+ +	+	-
5.	1480 "	Alkali Metaprotein.	-	+ +	+ +	+	+	-
6.	1520 "	Acid Metaprotein.	-	+ +	∓ +	+	N.D.	-
7.	1650 "	Primary Proteose.	-	+ +	+	-	-	-
8.	1520 "	Secondary Proteose.	-	+ +	+	-	-	-
9.	1710 "	Peptone.	-	∓	-	∓	-	-
10.	1630 "	Acetone soluble Lipoid.	-	-	-	-	-	-

PREVIOUS WORK ON THE ANTIGENIC VALUE OF BACTERIAL DERIVATIVES.

In 1894, Pfeiffer[23] who did the first important work on the Typhoid bacillus, found that in old cultures of the organism, toxic substances passed into solution, and the filtrates from these cultures were not only toxic, but also produced bactericidal substances in treated animals. Pfeiffer believed that the Typhoid bacilli, when acted on by lytic substances in the body fluids, liberated a specific endotoxin as a result of the destruction of the bacterial bodies. This he called a toxalbumin, and he considered that this endotoxin acted as an antigen on natural cases of the disease, giving rise to the antibodies in the blood of convalescents.

In 1902, Brieger[24] prepared antigenic substances from 3 to 4 day cultures of B. Typhosus, by saturation with ammonium sulphate, allowing the mixture to stand in a dark place for from one to four days, collecting the precipitate, shaking it in water for several hours, and then passing the water through a Berkefeld filter. The final filtrate gave Millon's reaction, and produced agglutinins and precipitins in treated animals.

In/

In considering Brieger's most important work, it is evident that some degree of autolysis was necessary before a soluble antigen was obtained. Autolysis permitted the native bacterial protein to pass into solution and it was precipitated by ammonium sulphate. Extraction with water would result in solution of substances of the nature of albumins and these albumins were antigenic. To my mind therefore, Brieger's antigen was unaltered bacterial native protein - only modified by the chemical precipitation methods necessary for its separation.

The next important contribution to the subject came from the Vaughans[25] in America. They found that organisms after thorough extraction with dilute salt solution, alcohol and ether, leave as a residue a proteid body still possessed of the form of the living cell, and poisonous on injection into animals. This can be split by alkali into two portions, one of which is soluble in alcohol, and very poisonous, the other insoluble in alcohol and inert. Experiments carried out with this non-toxic protein fraction produced an immunity in guinea pigs equivalent to sometimes as much as eight times the minimal lethal dose of the living organism, and in rabbits an immunity to quantities of the living germ that killed the controls in five hours. When the colon bacillus was used, the immunity developed from inoculation with the non-

toxic/

non-toxic residue was specific, and similar fractions derived from egg white and B. Typhosus, gave no immunity to the colon bacillus. In Vaughan's work, no investigation was carried out with regard to the presence or absence of antibodies in the serum of animals inoculated, nor were proper controls carried out with the whole bacilli weight for weight. Again Vaughan's process for separation of poisonous and non-poisonous fractions is a very complex one, and apart from the fact that his antigen was protein in nature there is little information given as to its true biochemical composition.

Jobling[26] in 1912 using ferments obtained from leucocytes (leucoproteases) came to the conclusion that the cleavage products of pneumococci were capable, when injected into animals, of giving rise to specific opsonic increase.

Somewhat similar researches to those of Vaughan, were carried out in this country in 1919, by Thomson[27] in his endeavour to reduce the toxicity of Gonococcal vaccines. Thomson dissolved the gonococcus in alkali, and precipitated the alkali soluble metaprotein with acid. According to him this precipitate was fifty times less toxic than the untreated germ and in large doses appeared to confer more immunity than smaller doses/

doses of the untreated germs. Using his detoxicated vaccine in doses equivalent to fifty times the amount of the ordinary gonococcal vaccine, Thomson claimed to produce twice the degree of immunity, and his test for immunity was the complement fixation reaction. Since his first work, he has elaborated his method of preparation of these antigens, so that acid metaprotein and proteoses are included in the final detoxicated product. Furthermore he has extended these principles to include all the ordinary organisms used for vaccines, including the Typhoid bacillus, in the belief that massive doses of the detoxicated germ substance result in a greater immunising response than the smaller doses of ordinary untreated vaccine.

Sanarelli[28] in 1920 working with cultures of vibrio cholera digested with Trypsin (1% in 1% sodium carbonate) found that the antigenic property of the digests was almost as great as equivalent quantities of 24 hour culture of V. Cholera in saline killed at 56°C. He however only determined the agglutinating power of the rabbits' sera. Sanarelli got a titre of 1 in 1000 to 1 in 1500 in the sera of rabbits injected with digests, and 1 in 3000 in the case of rabbits injected with equivalent doses of a 24 hour culture of vibrio cholera.

In 1921, Douglas[29] investigated the cleavage products/

products of bacteria with a view to formulating a method for obtaining more potent antibacterial sera. Using trypsin digests of B. Dysenteriae (Shiga) and B. Typhosus, he not only showed in the case of the former that the specific endotoxin is liberated by proteolytic cleavage of the bacteria in vitro, but he also determined the antigenic power of the digests.

In the case of B. Typhosus he found that acetone extracted bacilli, which had been digested overnight with 10% Trypsin, produced on injection into rabbits, a marked increase in the bactericidal and precipitating power of the sera, but he could make out no increase of the agglutinating or opsonic power. He suggested that the antigens giving rise to the agglutinating and opsonic properties are produced during the early stages of the solution of the bacteria, while the antigens in response to which the body produces the bactericidal and precipitating powers, are formed during a comparatively late period of the lytic process.

DISCUSSION AND CONCLUSIONS.

As has been stated earlier in this work, the rationale underlying the separation of bacterial derivatives in vitro, is based upon what is known to be the sequence of events following upon the subcutaneous/

subcutaneous inoculation of a bacterial vaccine or a sub-lethal dose of living bacilli. The injection of a small dose of dead Typhoid bacilli under the skin of a man, is followed in a short time by a considerable outpouring of lymph at the site of inoculation, and even in a previously uninoculated person, this lymph has a considerable power of breaking down the bacteria and completely dissolving them. In view of my preliminary work on the digestibility of bacteria, it seems likely that this lytic effect of serum is as much due to the presence of a lipase as a protease. Trypsin and pepsin produced very little digestion of Typhoid bacilli simply killed by heat, but on acetone extraction, the fatty envelope of the cell, which previously prevented the adsorption of ferment, was removed, and proteolysis readily took place.

The solution of the dissolved bacteria in vivo, is carried away in the lymph stream, and if rapidly absorbed causes well recognised symptoms such as fever, headache, and malaise. These symptoms soon subside, and as has been frequently shown, are followed by an increased agglutinating, opsonic, and lytic power of the serum, and under certain circumstances less well investigated by protective power against the living organism, and by the power to cause a precipitate when brought in contact with a bacterial filtrate.

One/

One essential then for the production of intoxication and symptoms, appears to be this lytic action of the serum or lymph, which enables the broken down bacteria to be carried in solution into the general circulation, and it seems reasonable to believe, that **only** when the organisms of a vaccine are dissolved, can they produce toxic symptoms, or lead to the formation of antibodies.

Again up to this time, it has been generally held that all antibodies are produced together. So strong has been this belief, that the measurement of one antibody in the serum has been considered an adequate criterion for the total concentration of antibodies. For instance, the titration of the agglutinating power of anti-meningococcus serum is supposed to be a means of measuring its content of antitoxin.

The present endeavour to arrive at a solution of problems associated with antibody formation and immunity in general by a series of digestion experiments in vitro such as have been described, lays one open to very obvious and justifiable criticism. Though bacterial protein, in common with all proteins is relatively stable, one cannot but feel that the Chemical processes associated with the separation of its derivatives, result in a denaturisation of the products so isolated. Furthermore, can one affirm with/

with any confidence, that these products separated artifically in vitro by the Chemical methods described, bear a true relationship to the derivatives set free in vivo by progressive proteolytic cleavage of bacterial protein? It is presumably because of this uncertainty, that Douglas in his work on the cleavage products of bacteria, worked simply with organisms digested for different periods of time. Such digests contained presumably all the split products in solution, and to a large extent unaltered by chemical interference. Such importance however has lately been attached to individual protein derivatives in the realm of clinical immunity, both specific and non-specific, that the study of these derivatives, even by the imperfect biochemical methods such as are in use to-day, is surely vindicated on the ground of urgency.

Comparing in the first place, the antigenic response of the controls - namely dried B. Typhosus, and acetone extracted B. Typhosus, it can be said with a considerable degree of certainty that the bacteria extracted with acetone for 24 hours, are as effective antigens as the ordinary dried Typhoid bacilli. The antibody response in the case of acetone extracted bacilli, though it appeared to lag behind that of untreated dried bacilli during the earlier period of immunisation, was ultimately the same.

This /

This conclusion is strengthened by the fact that the fatty and waxy material extracted by acetone from the bacilli was entirely devoid of antigenic properties, though administered in quantities much larger than the other bacterial derivatives.

It is believed that many of the bacterial fatty substances exist in the form of combinations with proteins, and such combinations have been called lipoproteins. The lipoproteins have been considered relatively loose compounds, and in virtue of their ready dissociation, have been regarded, especially by American workers, as of the greatest importance in cellular processes. Again, American workers have put forward the suggestion that specific fats play the principal rôle in the immunising process. My investigations go to show at least, that the large amount of fatty and waxy substance, capable of being extracted by acetone from Typhoid bacilli, is incapable of producing any demonstrable antibody against the Typhoid bacillus, and I have not been able to show that there is any increased immunity developed to it. Moreover the bacterial bodies, freed of fats and fatty acids, lose none of their antigenic power, compared with bacteria not so treated.

THE RELATIONSHIP BETWEEN AGGLUTININ AND BACTERICIDAL TITRE.

It is seen that in response to the control inoculations with whole Typhoid bacilli, all the recognised antibodies have been produced. On the other hand, a study of the protocols bringsout the the want of parallel between the bactericidal substances and the agglutinins. It is seen that while the agglutinin titre steadily increased to reach its maximum of 1 in 25,000 after the last injection. The bactericidal titre was greater after the first two inoculations, and then declined until ultimately it was little above normal limits. This lack of association between bacteriolysins and agglutinins has been previously pointed out by Wollstein,[30] and Douglas[31] has also emphasised the fact that repeated injections of untreated bacteria tend towards an ultimate diminution of the lytic power of the body fluids, even when comparatively small doses are employed. This phenomenon receives further confirmation, when the bacteriolytic response to inoculations of a 4 hours digest of Typhoid bacilli is examined. The digest, containing in solution all the protein derivatives, from coagulable native nucleoprotein on the one hand to peptone on the other, was capable of producing an even/

even greater concentration of bacteriolytic and bactericidal antibodies than the controls of dried Typhoid bacilli. This would suggest that in the digest, the bacteriolytic antigens were presented to the body in a manner in which they could be immediately utilised, while in the case of undigested Typhoid bacilli this stage had to be reached by proteolysis in vivo, and as a result repeated injections of the whole bacilli, might be considered to bring about a fatigue of the cleavage mechanism, whereby as a result of the body being unable to split the bacterial protein to the stage required for the production of bacteriolysins, the titre of these antibodies would diminish. On the other hand, the digest, presenting these antigenic substances preformed, is less likely to bring about exhaustion of the mechanism of antibody formation, and therefore leads to a higher titre at least as far as bacteriolysins are concerned.

With regard to the other antibodies, agglutinins, precipitins and opsonins, the digest constituted a less effective antigen than the controls of undigested Typhoid bacilli. Especially was this manifest in the case of the agglutinins and the opsonins. The inoculations of digest produced an agglutinin titre less than one thirtieth of that in the controls, while the opsonic power was only about one quarter of that/

that present in the controls. It is obvious therefore that the choice of a digest of the organisms as a vaccine, would depend upon the importance one attaches to particular antibodies. If, as is widely held, the immunity against the Typhoid bacillus is mainly bacteriolytic, then digests of the organisms could be recommended as a most effective form of vaccine for inoculation. On the other hand, if a maximum rise of the whole antibodies is aimed at then a vaccine composed of digested bacilli, presents no advantages over the ordinary vaccine, and in any case no mention has yet been made of the relative toxicity of these digests, a factor which is considered later in this work.

THE METAPROTEINS.

The metaproteins represent the first phase in the dissociation of protein. They are amphoteric, like native protein, and can combine with either a hydrogen or a hydroxyl ion. They are known as alkali or acid metaprotein, and inasmuch as they constitute the main bulk of the detoxicated vaccine, as prepared by Thomson, their antigenic properties are of considerable practical significance. Though these metaproteins as shown by the protocols, are definitely antigenic, still they appear to act only as partial antigens./

antigens. Agglutinins are not produced, and the other antibodies are present in much less concentration than in the controls. There is no difference to be made out as between alkali and acid metaprotein, and though these substances are the earliest recognisable derivatives of the unchanged bacterial protein, their combination with acid or basic radicals brings about a very marked deterioration in their antigenic power. It is obviously a very difficult problem to express the antigenic power of such derivatives as a fraction of that produced by the controls, for it is impossible to estimate the relative significance of each antibody in the production of general immunity. To what degree, for instance, the lack of agglutinin response is to be considered as marring an antigen, cannot as yet be determined, but it is obvious that lack of agglutinins in a serum cannot be taken as a criterion that no antibodies have been formed.

That metaproteins are capable of producing immunising response, has been held by other workers. Ball and Korns[32] found that if red cells were dissolved in weak Na.OH. and the protein substances precipitated with weak HCl, this protein precipitate when injected into rabbits was still capable of producing specific haemolysins. Again Kolmer[33] states that specific immunity is produced against certain tissue cells, when/

when animals are inoculated with the metaprotein of these cells prepared as described above.

Thomson's conclusions with regard to bacterial metaprotein will be discussed more fully, when the rationale, and efficacy of the detoxicated vaccine is considered.

COAGULATED PROTEIN.

The coagulated protein fraction represents soluble native protein not isolated by precipitation with acid or alkali. Its separation from the digest, ensured that in the subsequent precipitation of the lower split products by ammonium sulphate, one would be dealing only with protein derivatives digested beyond the coagulable stage. Experimental inoculation with this fraction showed, however, that while coagulation, as was to be expected, lessened the antigenic power of the protein, on the other hand all the recognisable antibodies were produced and the response differed from that of unaltered Typhoid bacilli only quantitatively, and not qualitatively. It is significant too that the coagulated protein proved an even more efficient antigen than the metaproteins, in spite of the fact that the latter had not been submitted to the alteration in molecular grouping which is believed to be associated with coagulation.

THE LOWER SPLIT PRODUCTS.

The proteoses and peptones constitute the split products proper. They are representative of protein derivatives which have been hydrolysed beyond the coagulable stage. As suggested earlier in this work, they are by no means clearly defined chemical entities, and it is certain that prepared by the precipitation methods previously described, they include mixtures of molecules and molecular aggregates varying to some extent in size. The proteoses are divided into primary and secondary proteoses, the former precipitated by half saturation with ammonium sulphate, and the latter by full saturation with the same salt. The peptones are further hydrolytic cleavage products, not precipitated by ammonium sulphate, but precipitated by alcohol.

The proteoses separated from bacterial protein, gave the positive Biuret test, but in the case of peptone the solution was so weak that the Biuret reaction was doubtful.

As seen from the protocols of antibody estimation, the proteoses act as weak antigens. No agglutinin or opsonic response could be made out following their inoculation, but there was a distinct albeit slight rise/

rise in the bacteriolytic titre. Similarly a positive precipitin response could be made out in the lowest dilutions of the serum. It would appear then that proteoses are capable of leading to the production of precipitins towards the homologous products in the antigen, and there is probably an advantage in using as an antigen in the precipitin tests, a 4 hour digest containing in solution those identical split products which have been used for the immunising experiments.

With regard to the increase in bacteriolytic titre following the inoculation of proteoses, it should be remembered that some of the rabbits previous to the immunising experiments, showed the presence of normal bacteriolysin in the serum.

The injection of proteoses however was followed by a rise in the bacteriolysin content considerably beyond the normal limits. This effect could not have been produced by complement alone, for complement does not increase in amount during immunisation. It would have been interesting to determine if these bacteriolysins were specific for the Typhoid bacillus alone, and were not of the nature of a non-specific response to cell activation such as is supposed to occur when proteoses are employed in non-specific therapy.

It is interesting to point out that in spite of those findings of bacteriolysin and precipitin formation/

formation to proteose inoculations, a definite positive complement fixation reaction could not be obtained. On the other hand, a positive reaction was obtained in the case of control rabbits, and those inoculated with the earlier protein derivatives, but there again, marked individual differences as regards the degree of immunity attained could not be brought out.

Peptone proved of no value for the production of specific antibodies towards the Typhoid bacillus, and it would appear that if peptone has any value in the production of artificial immunity, it must be in the realm of non-specific therapy.

The literature on the antigenic properties of proteoses does not help to elucidate this problem. Apart from Thomson's[34] work, there is no mention in the literature either for or against specific antigenic properties of bacterial proteoses, and most of the work relating to proteoses has been done with Witte's Peptone. Since the composition of this commercial product varies, results reported are always of doubtful value.

Zunz,[35] who reviewed the literature some years ago, came to the following conclusions.-

(1) Precipitin reactions with proteose and peptone fractions are negative.

(2) There is no statement with regard to complement fixation antibodies.

(3) Attempted demonstration of anaphylactogenic properties in proteoses derived from animal proteins, has usually given negative results. Zunz, however, believed that anaphylaxis could be produced with hetero- and protoalbumose, but not with secondary proteoses. This however is not confirmed by Friedberger and Joachmoglu[36] or by Wells[37]. The most recent contribution is by Fink[38]. He found that the proteoses from Witte's Peptone were unable to stimulate precipitins or complement fixation antibodies. On the other hand, experiments with the proteoses from egg white, led him to believe that the secondary proteoses were capable of producing precipitins and complement fixation antibodies, but to nothing like the same extent as the original egg white.

Thomson states that an injection of gonococcal proteose is capable of producing a provocative reaction at the focus of disease and he believes that proteoses - both primary and secondary - are antigenic, not only because after repeated injections into a patient the proteoses lost their local provocative reaction, but also because they could be used as antigens in the complement fixation test for gonorrhoea.

THE RÔLE OF AGGLUTININS IN THE IMMUNITY PROCESS.

One of the most remarkable features brought out in the preceding protocols, is the lack of agglutinin formation in response both to the earlier and later bacterial derivatives, as compared with the controls where undigested Typhoid bacilli were employed. The question may therefore be asked, "Are agglutinins of real importance in the production of immunity, and does the inability to produce a considerable titre of agglutinins, constitute a serious drawback to the use of bacterial derivatives as immunising agents'?

By some, agglutination has been considered as an accidental phenomenon associated with altered surface tension, and inasmuch as clumped bacilli are not thereby rendered less capable of multiplication, agglutinins have been regarded by them as unimportant antibodies. Furthermore it has been observed that a low titre, and, occasionally a complete absence of agglutinins, may be associated with a high grade immunity.

On the other hand, Carrol Bull[39] has investigated the fate of Typhoid bacilli when injected into normal rabbits, with the following results. The bacilli disappear from the blood within half an hour, and are to/

to be found in the organs. The bacilli were observed to be quickly agglutinated, removed by the organs, especially liver and spleen, and so destroyed. Bull concluded from these observations that agglutination, far from being an incidental phenomenon, was essential in association with phagocytosis for protection. Furthermore, he believed that the inagglutinability of some bacilli from the blood of Typhoid patients, might explain the persistence of the organisms. Injection of organisms was followed by their digestion; merely agglutinated bacilli were capable of further multiplication, but were soon removed by the liver and spleen. Single organisms tended to remain in the blood stream.

From Bull's work one must conclude that agglutination is an important feature in immunity against Typhoid, and that a partial antigen, which does not produce this antibody, must be less effective than the whole bacillus.

THE RELATION OF THE TITRE OF ANTIBODIES TO PROTECTIVE IMMUNITY.

A reference to the tables giving the concentration of the various antibodies in response to the inoculation of Typhoid derivatives, shows the want of parallel between antibody titre, and protective power against the/

the living organism. It is surely significant that even the control rabbits whose blood showed good concentration of all the recognised antibodies, were unable to withstand the intravenous inoculation of two minimal lethal doses of living bacilli. It is true that at the period when the injections were made, the antibodies were diminishing, while again the lack of virulence of the particular strain of Typhoid bacillus employed, necessitated the use of large doses; for instance the minimal lethal dose of the strain used, amounted to the growth from four agar slopes per kilogram of body weight. In spite of this, however, the fact remains that the degree of protective immunity attained was not high, and this would point to the lack of antitoxin or antiendotoxin as being an important factor. It would appear as if this has been the difficulty all along, where the production of potent antibacterial sera has been attempted, and for the same reason the use of serum in a case of Typhoid fever is of very questionable therapeutic value.

I would emphasise again, therefore, that there is no justification for the conclusion that clinical immunity can be determined absolutely by the measure of immune bodies in the blood. Besredka's[40] production recently of artificial immunity in men and animals against Typhoid and Dysentery, by the oral route,/

route, has shown that immunity may be mainly cellular, and that so called humoral immunity probably represents only one of the body mechanisms, and not the whole picture.

THE RELATION OF ANTIBODY PRODUCTION TO THE CLEAVAGE OF THE BACTERIAL PROTEIN MOLECULE.

A survey of the experimental work carried out, demonstrates that contrary to what has been previously held, antibodies are probably not all produced together, but apparently in response to different phases of digestion of the bacteria. From the fact that agglutinins were found only in response to unaltered Typhoid bacilli, and in a much lesser degree to the inoculation of filtered digest and coagulated protein, it may be concluded that unaltered native protein is necessary for their production, and that the specific substances giving rise to agglutinins are set free at the commencement of digestion. None of the remaining bacterial derivatives was capable of giving rise to agglutinin formation, and though the metaproteins represent a very slight transition from the state of unaltered soluble native protein, their combination with hydrogen and hydroxyl ions, destroys apparently their capacity for producing agglutinins.

Douglas[41] found that inoculations of digest led

to/

to no production of agglutinins, and that if agglutinins were formed, they were due to bacilli which had escaped the action of ferment. Inasmuch however as the digest used in my experiments was filtered through a Berkefeld filter, I have excluded the action of whole bacilli and can be dealing only with protein in a soluble form. Douglas, however, in his experiments used 12 hour digests, and moreover the strength of trypsin employed by him was 10%. In my experiments, on the other hand, 1 to 2% trypsin was employed, and digestion was only carried out for four hours. It is probable therefore that in the digests used by Douglas, the protein was all digested beyond the stage of soluble native protein, and as such was incapable of producing agglutinins. The digests used by Sanarelli[42] produced agglutinins in treated animals, but there is no statement in his work that the digests were submitted to filtration to remove all traces of undissolved bacilli.

OPSONINS.

The substances giving rise to opsonins are also formed most probably at an early stage of cleavage of the bacterial protein molecule. The metaproteins were both capable of raising the opsonic power, but to a less extent than the unaltered and undigested bacilli./

bacilli. On the other hand, there was no rise in opsonic power to injection of proteoses and peptone, and therefore it can be said with certainty, that for opsonins to be produced the bacterial protein must be in coagulable form. Once it has been digested beyond this stage, no opsonins are formed.

The production of opsonins, therefore, is on a parallel with that of agglutinins, but with this difference, that while the protein combinations with acids and bases (metaproteins), are capable of giving rise to opsonic increase in the blood of inoculated animals, they appear to be unable to produce agglutinins. This finding emphasises the defects inherent in methods of separation of bacterial protein derivatives by chemical means, and shows how the union of a protein with a non-protein substance may modify its property as an antigen.

BACTERIOLYSINS.

A great variety of bacterial derivatives are capable of producing a rise in the bacteriolytic titre, and from the fact that bacilli digested for 4 hours with trypsin, produce a greater concentration of bacteriolysins than undigested bacilli, coupled with the finding that proteoses cause an increase in the bactericidal power, it would appear that these antibodies/

antibodies are produced in response to a further cleavage of the bacterial protein than in the case of agglutinins or opsonins. It follows as a corollary from this, that protein molecules of smaller size are capable of giving rise to bacteriolysins, but I have not determined if the increased bactericidal power in response to later fractions such as proteoses, constitutes a true specific response, and is not simply associated with the increased resistance of the body which follows the inoculation of other protein split products of a non-specific nature.

PRECIPITINS.

The formation of precipitins also would appear to follow the same lines as bacteriolysins, for they can be produced in response to cleavage of the bacterial protein beyond the coagulable stage. These precipitins formed in response to inoculation by proteoses, are found only in very low concentration and I think it probable that their detection was made possible by the employment of the bacterial digest as antigen.

There is however one feature which at first sight does not seem to be in keeping with the suggestion that precipitins are formed in response to a comparatively/

comparatively late stage in the protein cleavage. This is the fact that injections of bacterial digest do not cause so great a rise in the precipitating power as the inoculations of undigested bacilli. In my experiments, the precipitating power developed in the serum in response to inoculations of the digest, did not rise above a 1 in 8 dilution of the serum, while the controls gave a positive precipitin reaction up to 1 in 64 dilution. Moreover this latter finding was obtained when the lytic power of the sera in question was considerably diminished. This would suggest that no great cleavage of the molecule of bacterial protein was necessary for precipitin production. This point must remain at present sub judice, and further investigation is required before anything of the nature of a definite statement can be made.

There is however one conclusion that seems justified, namely that the agglutinating and precipitating properties of sera are distinct phenomena. It has been held that the phenomenon of precipitation is very closely related to that of agglutination, and in fact many authorities consider that they represent the same reaction under different condition. In other words the substances which when present in the bacterial bodies give rise to agglutination on the addition of an antiserum, produce a precipitate when free/

free in the fluid.

My investigations go to show that an antiserum may have the property of causing a precipitate in a bacterial filtrate, and yet be unable to produce agglutination when added to an emulsion of the bacilli. Again, while the entire molecule of native protein seems to be necessary for the production of agglutinins in a serum, precipitins can be produced in response to protein fractions having molecules of much smaller size.

THE EFFICACY OF THE DETOXICATED VACCINE.

In 1919, Thomson,[43] working with the gonococcus, formulated the principle that by reducing the toxicity of the germs, they could be administered for vaccine purposes in larger doses, and thus bring about a better antibody response. In order to remove the poisons which reside within the bodies of the organism, Thomson considered that the germs must be entirely disintegrated or autolysed. For this purpose, he employed alcohol, acids and alkalis, and isolated fractions corresponding to the metaproteins and proteoses. This combination of metaproteins and proteoses constituted the detoxicated vaccine and according to Thomson, in this detoxicated form the protein/

protein substances of the germs have not been so injured or changed by the chemicals as to affect their specific immunising properties. Later, he extended his principle of detoxication to organisms other than the gonococcus, so that to-day all the ordinary bacteria used in vaccine therapy, can be obtained in this form. Thomson up to the time of writing (27.2.23), has published no protocols of animal inoculations demonstrating the superiority of the detoxicated vaccine over the ordinary vaccine, and has based his conclusions mainly on the results obtained by treating patients suffering from gonococcal infection with detoxicated gonococcal vaccine - the criterion of immunity so obtained being the complement fixation reaction.

THEORETICAL CONSIDERATIONS UNDERLYING THOMSON'S WORK.

To appreciate more fully the rationale underlying the detoxication of vaccines, I have summarised below some of the main points from Thomson's paper on the subject, as I think they are worthy of comment.

Thomson determined the development of haemolysin in rabbits following injections of sheep's corpuscles, and showed that with larger doses, there was greater production of haemolysin. He states it is logical to assume from these experiments, that in order to obtain/

obtain a similarly potent antiserum in man towards bacteria, massive doses of germ substances must be injected.

Secondly he states there is a fallacy frequently preached, namely, that if one injects too much vaccine, the system becomes tired and overworked and can produce no more antibody. According to him, it is toxin that exhausts the system, and he further states that it is the bacteriolytic property one wishes to develop in an immune serum, and such a serum is much more useful than one neutralising poisons. It is to the bulk of the bacterial protein that the lysins, agglutinins, and precipitins are formed, while endotoxins are incapable of producing antibodies against themselves.

A CRITICISM OF THE DETOXICATED VACCINE BASED ON THE PRESENT WORK.

It is not contended that my series of experiments with the derivatives of the Typhoid bacillus, is sufficiently extensive to warrant any hard and fast conclusions being drawn as to the efficacy of detoxicated vaccines. My conclusions are based on work done with the Typhoid bacillus, and do not warrant generalisation to include all bacteria. Moreover/

Moreover the principle underlying the detoxicated vaccine is that it may be used in doses up to fifty times that of the ordinary untreated vaccine, while in my experiments the response to equal doses of untreated bacilli, and the various derivatives, has been determined. Allowing, however, for the want of strict parallel, certain facts emerge which throw at least some light on the problem in question.

In the case of the Typhoid bacillus, for instance, I have shown that acid and alkali metaproteins, can act as antigens, but that they constitute much weaker antigens than the untreated bacilli. In their power of producing bacteriolysins, opsonins, and precipitins they are much less effective, and agglutinins are not produced at all. Thus they act only as partial antigens.

The proteoses, also constituents of the detoxicated vaccine, are still less effective antigens, at least as regards the specific response.

Secondly, a prominent feature in my immunisation experiments was, that in the case of the split products, the maximum concentration of antibodies, especially the bacteriolysins, was obtained after the third inoculation, while the fourth inoculation, of the various substances, greater in bulk than all the previous amounts put together, produced practically no/

no rise in the antibody titre. In fact, the bacteriolytic titre of the sera was lower after the last inoculation. This points to the conclusion that antibodies can be produced only up to a certain concentration, and that this maximum can often be attained after the inoculation of a comparatively small amount of the antigenic substance. Subsequent inoculation of larger doses produces no corresponding response, and it is as if a condition were established, which might be compared to 'drug fastness' in man. That this is not due to exhaustion from endotoxin such as Thomson describes is evident from the fact that the antigens used were detoxicated. Thus I do not think Thomson is justified in drawing conclusions from his experiments on the production of haemolysin in rabbits, and applying them to general antibody production following the inoculation of bacterial protein.

Lastly I have demonstrated that the protective immunity of a serum against inoculation with the living germ, may be low in spite of a high concentration of antibodies, and this would appear to be due, in the case of the bacteria under discussion, to the lack of antitoxin in the sera. Inasmuch as the deleterious effect of these germs on the body is generally held to be dependent on the liberation of endotoxin/

endotoxin following lysis, a high concentration of bacteriolysins in a serum without the necessary production of antitoxin, might reasonably be expected to yield unfavourable results where the number of living germs was at all large.

The failure to produce sera of high antitoxic value is probably associated with the difficulty of isolating endotoxin in anything approaching a pure state and not necessarily to the fact that endotoxins are incapable of producing antiendotoxins.

To sum up, the metaproteins and proteoses, which constitute a detoxicated vaccine, can act as antigens, but while the presence of antibodies to these substances may be taken as evidence of an attempt at immunisation, this does not appear to be an immunisation against the whole bacterial protoplasm. In chemically treated antigens, a change takes place yielding only a partial antigen, and the result is only partial immunisation.

This may account for the disappointing results which have been obtained clinically with detoxicated vaccines, and from my experimental findings it seems reasonable to suppose that most benefit will be derived from a vaccine as little altered as possible, either by heat or chemical agents. I am inclined to agree with Nicolle[44] when he states that a cell represents/

represents a "mosaic of antigens", and there seems to be ground for believing that a given bacterium is composed of a combination of different fractions, which when injected into the body, will call forth the formation of certain antibodies towards these fractions. There is however no reason for supposing that any fraction or combination of fractions which one can separate chemically from bacterial protein is as effective as the mosaic of antigens existing in the unaltered bacterial cell: and experimental work bears this out.

PART II.

THE TOXICITY OF THE SPLIT PRODUCTS OF THE TYPHOID BACILLUS.

INTRODUCTORY.

The study of the various toxic substances liberated by, or derived from bacteria, has stimulated a large amount of work since the discovery of the specific relations between particular microorganisms and definite pathological conditions, for it was early believed that the mere mechanical presence of bacterial bodies in the tissues of the infected individual could not be held responsible for the damage wrought. But notwithstanding the early recognition of the significance of these substances, and their continued study by many workers, there is much left undetermined in regard to the nature of bacterial toxins, and there are wide possibilities yet in the field of specific serum therapy.

Bacterial toxic substances are easily divisible into different classes. In one class we put the true soluble toxins of B. Diphtheriae and B. Tetani. These toxins are considered specific secretory products of bacterial cells, and animal organisms react to these toxins/

toxins by producing specific counteracting bodies which neutralise the toxins in definite multiple proportions. In this type of infection a wide distribution of the causal organism is not necessary for the production of systemic disturbance, since the toxic products are so easily separated from the bacterial bodies and are so readily soluble.

The toxins or toxic substances of a second class of bacteria, bear a very different relation to the bacterial bodies by which they are produced; and the reacting products of inoculated animals are different from those produced by toxins of the first class. The Typhoid bacillus belongs to this second class of bacteria.

It is hardly conceivable that Typhoid bacilli do not produce specific poisons that give rise to the symptom complex, which characterises the disease as a clinical entity. Yet it has not been definitely proved that these bacteria manufacture a secretory toxin. Toxic substances have been obtained from Typhoid bacilli, it is true, but a mechanical or chemical destruction of the bacterial bodies was necessary for their liberation. None of the various methods used to isolate toxins from B. Typhosus, has given a product of any degree of purity or one which produced an antitoxin of great value as a protective

or/

or therapeutic agent. Therefore any additional information concerning the nature and source of the toxic substances derived from this class of organism, is of first importance, since it is only through a biological and chemical knowledge of these substances that one may hope to develop methods by means of which an effective curative agent may be produced.

HISTORICAL.

Pfeiffer[45] first advanced the opinion that Typhoid toxins are liberated by a destruction of the bacterial bodies through the action of lytic substances in the body of the infected animal. He found no toxin in the filtrate of young cultures. His endotoxin produced bactericidal substances in treated animals. It was not destroyed by chloroform vapour, and was thermolabile.

Pfeiffer also believed that the endotoxin acted as an antigen in natural cases of the disease, giving rise to the antibodies in the blood of convalescents.

Buchner[46] produced poisonous bacterial substances by evaporating emulsions of bacteria to dryness, rubbing up thoroughly in hot water, boiling for an hour in a reflux condenser, filtering through kieselguhr and condensing the product thus obtained, produced pus when placed in tubes under the skin of animals,/

animals, fever and apathy in dogs and very severe local and general reaction in tuberculous animals.

Martin[47] found that bacilli killed with chloroform vapour were very toxic, more so than filtered cultures, and produced marked diarrhoea in animals. The same effects could be produced by B. Coli.

Allan MacFadyen[48] by grinding up Typhoid bacilli frozen solid by liquid air, produced a fluid whose toxic effect he attributed to the presence of intra-cellular poisons. Several other investigators, such as Besredka, Chantemesse and Castellani, using different methods, have produced toxins of varying degrees of potency.

Vaughan[49] washed bacterial bodies with alcohol, extracted them with ether and pulverised them by thoroughly rubbing up in agate mortars. Bacteria thus prepared were boiled in a reflux condenser for an hour or more with several volumes of 2% sodium hydroxide in absolute alcohol. This procedure gave a toxic and a non-toxic portion, the former soluble, the latter insoluble in absolute alcohol. The toxic portion was collected by evaporating the alcohol at low temperature. It was largely precipitated by saturation with ammonium sulphate, and gave all the protein reactions except Molischs. It was termed an alcohol soluble albumose, and the lethal dose for

guinea/

guinea pigs was 8 to 60 mg. (intraperitoneally). Animals had a slight initial rise and a subsequent greater fall in temperature, and became comatose some time before death.

Toxic substances were obtained from pathogenic and non-pathogenic bacteria, and proteins from other sources. This endeavour on the part of Vaughan to correlate intoxication from bacteria with cleavage products of the bacterial protein, stimulated a large amount of work especially in America, and certain alternatives to the original endotoxin theory of Pfeiffer were put forward to explain the primary toxicity of certain bacteria, especially the Typhoid bacillus. Briefly summarised these are:-

(1) The production of toxic split products in the animal body from the bacterial protein by proteolytic cleavage due to non-specific serum protease. (Jobling and Petersen[50])

(2) By the cooperation of antibody and complement (Friedberger[51]). In this latter sense intoxication in Typhoid infection is considered to be of the nature of an anaphylactic reaction, the combination of precipitin and complement producing an anaphylotoxin to which the pathological effects are referable.

(3)/

(3) By toxic split products derived from the plasma protein following the absorption by bacteria of anti-enzymes of the serum (Jobling and Petersen).[52]

(4) The presence of non-specific toxic substances in the bacterial cell body of the nature of primary and secondary albumoses, which are liberated by lysis from the bacterial cell after cell death.

This conception would differ from that of Pfeiffer in that the intracellular substances are conceived as in no sense specific toxic proteins, but rather entirely non-specific constituents representing the type of poison conceived as proteolytically produced from the antigen by Vaughan and others.

Jobling and Bull,[53] in their study of the toxic split products of the Typhoid bacillus came to the conclusion that a primary proteose derived from Typhoid bacilli by digestion with a ferment obtained from leucocytes, was the main source of the toxic properties.

In reveiwing all the literature therefore on this subject, one finds with few exceptions that the selection of the various methods used to obtain toxic substances from Typhoid bacteria, has been governed by the idea that a toxic body is stored in the inner-most parts of the bacterial bodies, and sufficient mechanical division or pressure should liberate such

a/

a body. Some workers however have believed that at least part of the toxic symptoms of natural typhoid infections are due to split products of the native bacterial protein, the hydrolysis of the protein being caused by the action of ferments in the body fluids of the infected animal.

THE AIM OF THE PRESENT WORK.

From what has been said above, it will be seen that as regards the causation of intoxication by the Typhoid bacillus, opinion is vacillating between preformed endotoxins on the one hand, and split products from the bacterial protein on the other. In Part I of this investigation, I have shown that by the aid of tryptic digestion of Typhoid bacilli, products could be isolated corresponding to progressive stages in the cleavage of bacterial protein. Having estimated their antigenic power, the question naturally arises in the light of recent work, as to what degree of primary toxicity these split products possess, and whether any derivative can be obtained of such toxicity as to warrant the assumption that to it can be attributed in large measure the intoxication produced by the Typhoid bacillus. Furthermore, are the pathological effects produced by injection of Typhoid split products comparable to what has been described/

described by previous writers as following inoculation of so called Typhoid endotoxin? On the other hand, is it that the endotoxin is liberated by digestion, so that any split products isolated chemically are practically non-toxic. Lastly, can any further light be thrown on the nature of the intoxication in Typhoid infection, and are there any grounds for associating it with the anaphylactic phenomenon?

THE PATHOGENIC EFFECTS PRODUCED IN ANIMALS BY THE TYPHOID BACILLUS.

The first difficulty encountered in an investigation of this kind is that there is no disease of animals which is identical with Typhoid fever in man, nor is there any evidence of the occurrence of the Typhoid bacillus under ordinary pathological conditions in the bodies of animals. Animals appear to be curiously immune to infection by the Typhoid bacillus, and though pathogenic effects can be produced by introducing living or dead bacilli subcutaneously or intraperitoneally, these have only a superficial resemblance to what occurs in human Typhoid. The effects produced in animals are of the nature of a short acute illness, characterised by rapid loss of weight, inability to take food, and frequently ending fatally/

fatally within forty-eight hours.

It is obvious therefore that one must not be too ready to apply to the disease in man, conclusions which have been arrived at from experiments on laboratory animals, though it appears reasonable enough to suppose that typhoid substances which prove toxic for naturally immune animals will prove relatively more toxic to man who shows the greater susceptibility to typhoid infection.

In the following experiments, mice were largely employed. The mouse is at least as susceptible to Typhoid inoculation as any other laboratory animal, and inasmuch as the split products were not available in very large quantities, the use of the smaller animals, permitted of the determination of the minimal lethal dose of any product with the least expenditure of material.

THE STRAIN OF B. TYPHOSUS EMPLOYED.

The strain of B. Typhosus used was one isolated a few days earlier from a case of Typhoid fever at the Belvidere Hospital, Glasgow.

THE TOXICITY OF TYPHOID BACILLI DIGESTED BY TRYPSIN.

It has been pointed out earlier in this work, that Typhoid bacilli when injected into man, undergo a process of solution which is attributable to the presence of Trypsin like ferments present in the body fluids. During this process, the toxic substances, whether existing as preformed endotoxins, or derived by cleavage of the protein, are set free. An attempt was therefore made to carry out digestion experiments in vitro, and to determine if the toxicity of the bacilli was affected thereby.

The technique employed was similar to that described in Part I for the preparation of digests. B. Typhosus was grown on agar, scraped off in minimal saline, precipitated with acetone, and finally extracted with acetone in a Soxhlet apparatus for 24 hours. The semi-solid mass of bacilli was then dried and weighed. 100 mg. of dried Typhoid bacilli were digested in 10 cc. of 2% Trypsin solution. Digestion was carried out for 4 hours at 37°C. after which time the solution was opalescent. 1 cc. of this digest corresponded to 10 mg. of dried bacilli.

As a control, an emulsion of dried acetone extracted bacilli was prepared containing 10 mg. to the cc. Preliminary investigation showed that the minimal/

minimal lethal dose of dried Typhoid bacilli for a mouse of 25 grams weight was about 10 mg.

A protocol of the series of inoculations, and the results obtained, is given below. All injections were made intraperitoneally.

TABLE 18./

TABLE 18. 20.12.22.

Undigested Typhoid Bacilli.				Typhoid bacilli digested with Trypsin (4 hours)			
No. of Mouse.	Weight.	Amount.	Result.	No. of Mouse.	Weight.	Amount.	Result.
A 1.	25 gr.	10 mg.	died in 48 hours.	B 1.	25 gr.	5 mg.	dead in 24 hours.
A 2.	26 "	" "	survived.	B 2.	24 "	" "	dead in 24 hours.
A 3.	29 "	7.5 "	survived.	B 3.	29 "	4 "	dead in 24 hours.
A 4.	24 "	" "	"	B 4.	27 "	" "	dead in 48 hours.
A 5.	25 "	6 "	"	B 5.	21 "	3 "	dead on 3rd day.
A 6.	23 "	" "	"	B.6.	20 "	" "	dead on 4th day.
A 7.	27 "	5 "	"	B 7.	27 "	2 "	dead on 4th day.
A 8.	29 "	" "	"	B 8.	25 "	" "	survived.
A 9.	21 "	4 "	"	B 9.	21 "	1 "	survived.
A 10.	20 "	" "	"	B 10.	26 "	" "	"
A 11.	23 "	3 "	"				
A 12.	20 "	" "	"				

From the above experiments, it is seen that while the M.L.D. for undigested Typhoid bacilli was about 10 mg., the toxicity of the digests was such that 2 mg. was a lethal dose. Thus digestion of acetone extracted bacilli with Trypsin for four hours raised their toxicity about 5 times.

A second series of experiments was carried out, using Typhoid bacilli which had been killed at 56°C., and which had not been extracted with acetone. A thick emulsion of Typhoid bacilli was made in saline, and the bacilli killed by heating to 56°C. for 30 minutes in a water bath. To 4.5 cc. of this emulsion was added 0.5 cc. of Injectio Trypsini Co. giving a final concentration of 10% Trypsin. Another 4.5 cc. of the emulsion was diluted with 0.5 cc. of saline to get the same strength as the digest, and was used as a control. I have stated previously in this work that bacilli simply killed by heating are difficult to digest with proteolytic ferments, and it is for this reason that I employed 10% Trypsin, and carried out digestion for 8 hours at 37°C.

The diluted emulsion contained about 10,000 million bacilli per cc. and 0.5 cc. was the approximate minimal lethal dose of the undigested emulsion. A record of the inoculations carried out is given below, all injections as before were made intraperitoneally.

TABLE 19. 21.12.22.

Typhoid Emulsion (undigested)				Typhoid Emulsion (digested 8 hours at 37°C. with 10% Trypsin.)			
No. of Mouse.	Weight	Amount.	Result.	No. of Mouse.	Weight.	Amount.	Result.
C 1.	25 gr.	0.5 cc.	dead in 24 hours.	D 1.	25 gr.	0.5 cc.	Dead in 12 hours.
C 2.	26 gr.	"	" "	D 2.	25 "	"	"
C 3.	23 "	0.4 cc.	Survived.	D 3.	22 "	0.4 cc.	"
C 4.	29 "	"	"	D 4.	26 "	"	"
C 5.	26 "	0.3 cc.	"	D 5.	23 "	0.3 cc.	Dead in 24 hours.
C 6.	26 "	"	"	D 6.	27 "	"	"
C 7.	25 "	0.2 cc.	"	D 7.	24 "	0.2 cc.	Very ill but survived.
C 8.	28 "	"	"	D 8.	21 "	"	"
C 9.	25 "	0.1 cc.	"	D 9.	28 "	0.1 cc.	Survived - no ill effects.
C 10.	26 "	"	"	D 10.	27 "	"	" "

From the second protocol, though the differences were not so striking as in the experiments where acetone extracted bacilli were used, it is seen that the digests were about twice as toxic as the control emulsions of undigested bacilli. The difference between this series of results, and the preceding, is probably dependent on the fact that in the second case, where the bacilli were not subjected to acetone extraction as a preliminary, digestion even with the stronger solution of Trypsin did not take place to anything like the same extent as when acetone extracted bacteria were employed.

The mice which died as a result of inoculation of digested bacilli, were examined post-mortem, partly to exclude any liver condition having been present previously which might have lowered the threshold of their resistance to toxins, and partly to compare the pathological effects produced by injections of digested bacilli with those produced by a lethal dose of undigested bacilli. It was found that the pathological appearances in the two series of animals, were identical, and consisted mainly in great congestion of the abdominal viscera, especially the ileum of the small intestine which showed subserosal haemorrhages here and there. The mesenteric vessels were much/

much congested, and the spleen was enlarged and congested.

The mode of death in the two cases was therefore identical, except that the animals which received toxic doses of the digests, suffered from a more fulminant type of illness with more rapid death. It would appear therefore as if previous digestion of the bacilli with Trypsin, presented the toxic substances preformed, with a proportionately quicker fatal result on injection. On the other hand, in the case of undigested bacilli, the organisms had to be gradually broken down to the toxic phase inside the animal body so that the liberation of toxin was slower, and in amounts such as the animal might for a time be able to deal with.

The effect of proteolytic digestion on the toxicity of certain bacteria, has been determined by other observers. Sluyts[54] for instance, as early as 1894, demonstrated that cultures of vibrios submitted to pancreatic digestion in a solution of carbonate of soda, were not modified in their toxic power towards rabbits, and this is confirmed by Sanarelli[55]. Douglas,[56] working with B. Dysenteriae (Shiga), an organism which produces a very powerful toxin, found that tryptic digestion when carried out for an optimum period of time, was capable of increasing the toxicity of/

of these organisms.

In the case of B. Typhosus, I think it has been shown fairly conclusively that tryptic digestion by breaking down the bacterial bodies, liberates toxic substances, so that such digests become much more toxic than a corresponding amount of the undigested bacilli.

That these toxic substances produced in vitro are the same as those produced in vivo by the proteolytic action of the body fluids seems most probable, but the similarity of the pathological appearances produced in animals cannot be accepted as a criterion of this for reasons which will be discussed later.

THE TOXICITY OF THE PROTEIN SPLIT PRODUCTS OF THE TYPHOID BACILLUS.

2 grams of dried powdered Typhoid Bacilli were digested with 2% Trypsin for about 6 hours at 37°C. An almost completely clarified opalescent solution was obtained, from which the cleavage products were separated by the method previously described in Part I. The test animals used were white mice, and the tables below give the results of the inoculations made to determine the minimal lethal dose of the various derivatives. Injections were given intraperitoneally in all cases.

TABLE 20.

Determination of the M.L.D. of Alkali Metaprotein.

No. of Mouse.	Weight.	Date.	Amount.	Result.
E 1.	23 grams.	18.12.22.	10 mg.	Survived.
E 2.	26 "	"	"	"
E 3.	21 "	20.12.22.	20 mg.	"
E 4.	27 "	"	"	"
E 5.	25 "	"	30 mg.	Dead in 36 hrs.
E 6.	25 "	"	"	" 48 "

As far as inoculations were carried out, acid metaprotein behaved similarly to alkali metaprotein, and 30 mg. was a lethal dose. On account however of the small amount of acid metaprotein available, a complete series of controlled experiments was not made.

TABLE 21.

Determination of the M.L.D. of Primary Proteose.

No. of Mouse.	Weight	Date.	Amount.	Result.
G 1.	25 grams	13.12.22.	10 mg.	Survived.
G 2.	20 "	"	"	"
G 3.	27 "	15.12.22.	15 mg.	Lost weight and died after 1 wk.
G 4.	25 "	"	"	Survived.
G 5.	24 "	"	20 mg.	Died in 48 hrs.
G 6.	21 "	"	"	Died on 3rd day.

TABLE 22.

Determination of M.L.D. of Secondary Proteose.

No. of Mouse.	Weight.	Date.	Amount.	Result.
H 1.	25 grams	13.12.22.	20 mg.	Survived.
H 2.	25 "	"	"	"
H 3.	26 "	15.12.22.	30 mg.	"
H 4.	24 "	"	"	"
H 5.	22 "	18.12.22.	50 mg.	"
H 6.	25 "	"	"	"

No further injections were made on account of lack of material.

ANALYSIS OF THE ABOVE RESULTS.

For mice, the minimal lethal dose of dried undigested Typhoid bacilli was 10 mg., so it is seen that the various derivatives isolated are relatively much less toxic. Alkali and acid metaprotein were fatal to mice in doses of 30 mg., but secondary proteose was practically non-toxic, the mice withstanding doses of 50 mg. without even signs of illness.

The primary proteose fraction was relatively the most toxic, doses of 20 mg. causing death in 48 to 56 hours, while doses of 15 mg. set up a rapid wasting which/

which terminated fatally in about a week.

When it is remembered that digested Typhoid bacilli were fatal to mice in doses equivalent to 2 mg., it is obvious that the chemical methods associated with the separation of the bacterial split products, have resulted in a very marked detoxication, and none of the protein bodies isolated is of a sufficient degree of primary toxicity for animals, as to warrant its being identified with the toxin present in the digests or even in the undigested bacilli. It may be that toxic substances present in the digests are of a very simple protein nature, for instance amino-acids, and as such are not capable of being isolated by the precipitation methods employed.

In respect of the primary proteose fraction being the most toxic of the Typhoid split products isolated, I can confirm the findings of Jobling and Bull,[57] but that it is mainly responsible for the injurious effects wrought on the animal organism, appears at least from my experimental findings to be unlikely. Albumoses have long been recognised as possessing primary toxicity for animals, but in the light of present knowledge, this toxicity must be classed apart from that of the true intracellular bacterial toxins.

THE MODE OF DEATH FOLLOWING INOCULATION WITH THE TYPHOID SPLIT PRODUCTS.

The relative immunity of laboratory animals both to the Typhoid bacillus and its derivatives, necessitates the inoculation of comparatively large amounts to obtain definite pathological results, and insofar as the inoculation of massive quantities of germs or germ substances into animals, has no parallel as regards the aetiology of the disease in the human subject, it is questionable how far the experimental findings obtained can be applied to man. Moreover, by any form of treatment with Typhoid bacilli it appears impossible to reproduce the symptom-complex of Typhoid fever, but instead a rapidly fatal issue, often with acute cachexia, is brought about.

At the same time, a review of the literature discloses a strange divergence of opinion as regards the actual mode of death and the pathological findings obtained with the Typhoid bacillus and its derivatives. Some observers have isolated toxic substances from Typhoid bacilli, which they claimed produced specific lesions in animals very analogous to what occurs in the course of the disease in man, and have deduced that there is a special affinity between Typhoid toxins and certain cells and organs of the body. (Arima[58]).

On/

On the other hand, Friedberger,[59] Neufeld and Dold,[60] Rosenow,[61] and Weichardt,[62] found that bacteria or substances derived from bacteria, produced acute symptoms and death in animals, apparently identical with the symptoms and manner of death occurring in anaphylaxis due to a second or toxic infection of egg albumen or serum. Friedberger formulated the opinion that infection was a mild protracted form of anaphylaxis, and anaphylaxis an extreme acute form of infection. Thus it was not necessary to assume any specific bacterial toxin in infection, but according to him, symptoms were produced by the same poisonous substances irrespective of the microorganism. Similar views were held by Vaughan and Wheeler.[63]

In the succeeding work to be described, I have carried out a short investigation into the mode of death following the inoculation of Typhoid bacilli and their split products, with special reference to the morbid histology, the chief end in view being a comparison between the results obtained with the proteose fraction and the intact Typhoid bacilli. Mice have been used in many of the experiments inasmuch as the fractions were not obtained in sufficient quantity, or of such a degree of toxicity, as to cause death in the larger animals. Some experiments have also/

also been carried out with rabbits and guinea pigs, to study especially the pathological results obtained with egg globulin and killed Typhoid bacilli.

PATHOLOGICAL FINDINGS IN RABBITS RECEIVING LETHAL DOSES OF TYPHOID BACILLI.

Four rabbits were inoculated intravenously with a lethal dose of an emulsion of B. Typhosus - the growth from eight agar slants emulsified in 4 cc. of 0.85% saline constituting the fatal dose. In two rabbits, the bacilli were killed by heat before injection, in the remaining two, living bacilli were inoculated.

After inoculation, all the rabbits showed marked tremor, probably associated with the shock from the bulk of the fluid injected. Following this, the animals became quiet, and sat crouched in the corner of the cage. Severe diarrhoea set in, and dyspnoea was marked. Weakness of the hind extremities developed, and the temperature remained subnormal. Three of the animals died in 12 to 18 hours, while the fourth died after two days.

The final picture in each case was one of extreme weakness and paralysis of the extremities.

At the autopsy, the naked eye appearances were the/

the same in all the rabbits, and the more prominent findings are given below:-

In the Abdomen, there was congestion of the mesenteric blood vessels and small intestine, most marked in the lower end of the ileum. Small petechial haemorrhages were present in the wall of the small intestine, and to a lesser degree in the wall of the stomach. The liver was congested, with some pallor of the periportal areas. No changes could be made out naked eye in the spleen, kidney, and suprarenal gland.

In the Thorax, there was excess of fluid in the pleural sacs. The lungs were moderately distended and markedly congested. They showed numerous focal haemorrhages scattered throughout their structure. The Heart showed congestion of the epicardial vessels with one or two small haemorrhages. In the case of rabbits receiving living bacilli, organisms could not be cultivated from the heart blood.

MICROSCOPIC APPEARANCES.

Intestine:- Congestion and focal haemorrhages in the submucous coat. Some congestion and swelling of the Peyer's patches.

Liver:-/

Liver:- Slight congestion. In scattered areas here and there the liver cells are indistinct in outline, and the nuclei are undergoing fragmentation and disappearance, suggestive of early parenchymatous necrosis. The sinusoids contain clumped masses of Typhoid bacilli with some degree of leucocyte aggregation in the neighbourhood of the Clumps.

Kidney:- Marked congestion in the cortex especially in the glomerular tufts. Focal haemorrhages scattered throughout the cortex. Parenchyma cells of the collecting tubules show commencing degeneration, and many of the lumina contain hyaline casts. The medulla shows practically no change.

Spleen:- Clumps of Typhoid bacilli made out in the sinuses, but no general change in the organ.

Suprarenal:- Slight congestion and a few small haemorrhages in the intermediate zone between cortex and medulla.

Lungs:- Marked congestion of the whole organ with extravasations into the alveoli. Some of the haemorrhages are of considerable size.

Heart:-/

Heart:- No change in the muscle fibres, but small focal haemorrhages from the superficial vessels.

It is interesting to note that the same pathological picture was presented by the animals irrespective of whether the Typhoid bacilli injected were living or dead. Moreover where living bacilli were employed, the organisms apparently did not multiply to any extent in the blood stream, and cultures could not be obtained from the heart blood after 18 hours. It appears, therefore, that death in those cases is due to a pure intoxication and not to an actual infection, with progressive septicaemia.

The appearances of the animals prior to death, coupled with the congestion of the abdominal organs, and the focal haemorrhages in the intestine, liver, and lungs, constitute a picture which is almost identical with what has been described as occurring in late anaphylactic death.

By previously sensitising rabbits with egg globulin (20 mg.), and after 14 days injecting a toxic dose of the same substance, I have produced death in rabbits within 12 hours, and the appearances at autopsy are very similar to what I have just described as following first injections of Typhoid bacilli.

Yet/

Yet although there is great similarity between the results obtained with Typhoid bacilli, and with a toxic dose of foreign protein in a previously sensitised animal, it is hardly justifiable to regard death in the former case as being of the nature of true anaphylaxis.

In the first instance, there is an absence of the phenomenon of sensitisation which is the very essence of anaphylaxis, the illness is not acute but subacute, and the lungs post-mortem have not the dry voluminous appearance which is typical of true anaphylaxis. The subacute nature of the illness, as compared with typical acute anaphylactic death, points therefore to a solution or partial disintegration of the bacterial protein being necessary before the toxic substances, whatever they may be, are liberated.

Of the actual bacterial protein derivatives isolated from a digest of Typhoid bacilli, I have shown previously that the most toxic is the primary proteose fraction. I have therefore investigated the mode of death, and the post-mortem appearances in animals receiving lethal doses of this product.

THE EFFECTS PRODUCED BY THE INOCULATION OF PRIMARY PROTEOSE.

At a very early stage in my investigation of the properties of Typhoid split products, and in an endeavour to get some idea of the toxicity of these products for the purposes of dosage in the immunisation experiments, I injected about 50 mg. of Typhoid primary proteose intravenously into a rabbit. Although far from constituting a lethal dose, very striking effects were witnessed.

A few minutes after the injection, the animal showed signs of peripheral irritation, uneasiness, and scratching. This stage of stimulation passed into a stage of depression with rapid breathing, and subnormal temperature. Within half an hour, diarrhoea developed, and exhaustion, and partial paralysis became prominent - the hind extremities being pushed out. The animal began to recover in less than two hours and ultimately became completely well, though it refused food for two days following the injection.

The effect resembled that produced in rabbits by injections of large doses of the unaltered Typhoid bacilli, but the condition in the former instance developed more quickly, and was a more typical shock picture./

picture. I have unfortunately not been able to secure this proteose fraction in sufficient quantity to produce death in rabbits, and so I have not had the opportunity of comparing post-mortem findings. In this direction, however, investigations have been carried out on mice.

PATHOLOGICAL FINDINGS IN MICE FOLLOWING THE INOCULATION OF LETHAL DOSES OF (1) KILLED TYPHOID BACILLI. (2) DIGESTED TYPHOID BACILLI. (3) TYPHOID PRIMARY PROTEOSE.

Killed Typhoid Bacilli:-

The inoculation of 8 to 10 mg. of dried Typhoid bacilli produced death in mice in from 24 to 48 hours. The most prominent features at autopsy were, the congestion of the small intestine especially in its lower half, the appearance of miliary pale areas in the liver, and a very congested and haemorrhagic condition of the lungs.

On section, the intestine, liver, and lungs, showed well marked change microscopically.

Intestine:- Showed congestion and mild catarrhal change in the mucosa. There were petechial haemorrhages in the submucous and subserous coats. The Peyer's patches were congested.

Liver:- Some general congestion, and cloudy swelling of the liver cells. There were present also areas of commencing focal necrosis with disappearance of liver cells, fragmentation of cell nuclei, and collections of leucocytes, among which were some larger endothelial cells. There were one or two small haemorrhages into the necrosing areas.

Lung:- Generalised and very intense congestion, with a small extravasation here and there. Commencing proliferative changes in the walls of the alveoli.

Kidneys:- Showed appearance of toxic necrosis affecting chiefly the epithelium of the collecting tubules. Glomerular tufts were swollen, and some general congestion of the cortex was present.

Spleen:- Slight congestion, and a few aggregations of endothelial phagocytes lying apart from the lymph nodes.

Digested Typhoid Bacilli.

When amounts of bacterial digest, corresponding to 5 mg. of Typhoid bacilli were inoculated into mice, death took place in 12 to 18 hours, with changes in the/

the intestine and lungs, similar to what have been described as occurring with whole Typhoid bacilli. The liver changes however were not so marked. When smaller doses of 2 - 3 mg. were inoculated, and death took place after 48 hours, the pathological picture, both naked eye and microscopic was almost identical with that obtained with undigested bacilli. Changes in the liver were more generalised, however, and focal distribution was not a feature.

Typhoid Primary Proteose:-

The inoculation of 20 mg. of primary proteose isolated from digests of Typhoid bacilli, was followed by death in 48 - 56 hours. At autopsy, the intestines, liver, and lungs, showed definite changes which resembled naked eye those present in death from undigested or digested Typhoid bacilli. Liver and lungs were the organs most affected.

MICROSCOPIC APPEARANCES:-

Liver:- Cloudy swelling of the whole organ. There were areas of focal necrosis, varying in size and degree, scattered throughout the liver substance. They resembled miliary tubercles, and contained phagocytic endothelial cells. The areas where/

where the change was most advanced showed central necrosis and surrounding leucocyte proliferation. The changes resembled in some measure those met with in the liver in cases of human Typhoid fever. Congestion of the liver was not a marked feature.

Intestine:- Showed some general congestion and swelling of the Peyer's patches, but the changes were not so marked in the mice receiving proteose injections as in those killed with Typhoid bacilli either digested or undigested.

Lungs:- Showed very intense congestion all over, with a few haemorrhages. Proliferative changes in the walls of the alveoli were marked, and there were many phagocytic endothelial cells present. Some detached masses of endothelial cells lay free in the alveoli. The condition was similar to what is seen in the human lung in cases of severe toxemia.

Spleen:- Congested and showed a few focal lesions similar to those met with in the liver, and these lesions contained large endothelial cells.

Kidney:- Showed a toxic necrosis of the epithelial cells of the collecting tubules. There was a slight degree of general cortical/

cortical congestion, and the glomeruli were swollen.

An analysis of these post-mortem findings on mice brings out this fact that the pathological picture in death from inoculation of the proteose fraction, is practically the same as in death following inoculation of the whole bacilli or bacilli digested with Trypsin. The only differences to be made out were firstly, that the intestinal changes were less marked in mice dying from proteose inoculations than in those dying from inoculations of whole Typhoid bacilli or Trypsin digests. Secondly, where suspensions containing solid particles were inoculated, as in the case of the mice receiving undigested bacilli and proteose, focal lesions in the liver were a prominent feature. On the other hand, when the toxic substances were administered in the more soluble form of digests, the liver changes were more generalised. Again variations naturally occurred according to the period elapsing between inoculation and death of the animal. When death took place in less than 24 hours, congestion and haemorrhages were the prominent features, but in animals dying after 48 hours degenerative and necrotic changes became more manifest, and the picture was one of typical toxic action.

HAS PRIMARY PROTEOSE ANY POWER OF SENSITISATION.

In my experiments on rabbits, I found that the appearances met with, when lethal doses of Typhoid bacilli were inoculated, were comparable to those produced when sensitised animals were killed with egg globulin, and where death occurred late. I next endeavoured to find out if a primary proteose fraction isolated from digested egg albumin was capable of sensitising an animal in the same manner as the intact native protein.

Egg albumen was digested with trypsin, and the primary proteose separated in the same manner as described for bacterial digests. Guinea pigs were used as being the most susceptible animals, and four were inoculated subcutaneously with 20 mg. of egg proteose. After 14 days 200 mg. of proteose were injected. The animals showed some degree of excitement after the inoculations, but there was no evidence of anaphylaxis. Controls inoculated with the same sensitising and toxic doses of egg albumen died with typical anaphylaxis within an hour.

Further experiments showed that animals inoculated with 20 mg. of egg proteose were not sensitised towards egg albumen, and 200 mg. produced no shock. Again, animals sensitised with egg albumen showed no anaphylaxis/

anaphylaxis after a second inoculation of primary proteose.

In the short series of experiments carried out in this connection, my findings agreed with those of Wells,[64] namely that proteose fractions possess no anaphylactogenic power.

DISCUSSION AND CONCLUSIONS.

The regularity of the intestinal lesions produced by injections of Typhoid bacteria or toxic products derived from them, have inclined workers to believe that a special affinity exists between these substances and the intestinal tissues. In fact many observers have considered these lesions to be absolutely specific. In a publication by Arima,[65] the symptoms and pathology produced by toxic products of Typhoid bacteria injected into rabbits, are described. He concluded that there is a special affinity between the toxin, and the tissues affected. The main features described are diarrhoea, paralysis of the hind extremities, haemorrhages and hyperemia of the small intestine, swelling of the Peyer's patches, parenchymatous degeneration of the kidneys, congestion and cloudy swelling of the liver, and congestion and haemorrhages in the lungs.

In/

In comparing the pathological findings described by Arima, with those obtaining in my own series of rabbits, there were very few points of difference. I have never been able to obtain intestinal lesions so intense as those illustrated in Arima's publication, nor were changes in the heart muscle a marked feature, but otherwise the lesions were very similar. On the other hand, I found that egg albumin in sensitised rabbits was capable of producing changes which were to my mind very similar to those produced by injections of Typhoid bacilli, and certainly intestinal lesions were present. The picture resembled very closely what is described as occurring in late anaphylactic death, and it would appear as if the pathological lesions were of a non-specific nature, dependent on similar toxic protein groups in bacterial protein, and protein from other sources. Certain it is, that the action of any specific toxin that may be present in Typhoid bacilli is largely overshadowed by the action of non-specific toxic protein. There is confirmation of this in the literature. Pearce and Eisenbrey[66] have described the symptoms and pathology associated with anaphylactic shock in dogs. They used foreign serum to produce the anaphylactic state, and the pathology has many features in common with what I have described as occurring in rabbits. They lay emphasis on the intestinal/

intestinal lesions. Again, Schittenhelm and Weichardt[67] found that bacterial emulsions and peptone produced in dogs pathological changes which were similar to those obtained by injecting egg albumin into previously sensitised animals. They called the condition produced 'enteritis anaphylactica'.

Rosenow and Atkin[68] using pneumococcal extract obtained similar results. In their cases the lungs were especially affected, being hyperemic and haemorrhagic. Vaughan[69] found that all proteins could be split up so as to yield poisonous and non-poisonous fractions. The toxic fraction could be derived from bacterial, animal, or vegetable proteid, and produced similar symptoms in animals. Furthermore he concluded that all bacteria contained similar poisonous protein groups and that toxic fractions derived from B. Coli or B. Typhosus were in no way specific for these organisms. This is confirmed by Schittenhelm and Weichardt, and by Jobling and Bull, who obtained a swollen and hyperemic condition of the Peyer's patches and mesenteric lymph glands with inoculations of staphylococcus aureus and meningococcus, as well as with Typhoid and Colon bacilli.

Single doses of egg albumin and casein do not produce symptoms such as have been described, and Abderhalden[70] has shown that sera of animals sensitised with/

with native proteins possess proteoclastic ferments not found in the sera of normal animals. If, as would appear, sensitisation by egg albumin is associated with the calling forth of a ferment capable of attacking native protein, then the proteins of bacteria are attacked by ferments incapable of acting upon the proteins ordinarily used in animal experimentation, since a sensitising dose is not required. There is thus a biological difference between bacterial and other proteins; bacterial protein has a high degree of primary toxicity, but other proteins are only toxic after sensitisation.

The classification of the toxic substances derived from B. Typhosus and the particular part each plays in Typhoid infection, must at present remain unsettled questions. The great toxicity of digests of Typhoid bacilli, as compared with whole Typhoid bacilli, and with the proteose fraction, suggests that preformed endotoxins do exist in the bodies of the bacilli and that they are liberated by cleavage of the protein molecule. These endotoxins are apparently not represented by the proteose or any other protein fraction that can be isolated from the bacilli, and the possibilities are either that the endotoxins are thermolabile, like Pfeiffer's endotoxin, or that they are simple protein bodies like the amino-acids, and cannot/

cannot be isolated by methods such as have been employed. The toxic effects of histamine - an amino-acid product - and the shock pictures produced by its experimental inoculation, lend some support to the theory that bacterial endotoxins may be substances of similar structure.

At the same time, however, my experiments have lead me to believe that primary proteose is capable of producing very much the same shock picture as the original Typhoid bacilli which contained all the endotoxins, and the ultimate pathological lesions (at least in mice) were the same in each case. The primary proteose fraction, however, even though the most toxic of all the protein split products, could not be said to possess a very high degree of primary toxicity, as comparatively large amounts had to be inoculated to obtain the results described above.

Again, those symptoms which have been described as following the inoculation of Typhoid primary proteose into rabbits, are practically the same as those obtained by other observers using commercial peptone, and called by them "Peptone Shock".

Peptone shock has been supposed to be due to the combined effect of histamine and proteose, but Auld[71] has shown that typical shock can be produced by peptone freed from histamine, and that this effect is entirely due to the proteoses - especially primary proteose.

During later years, Witte's peptone and albumoses have been largely used instead of vaccines in the treatment of disease both chronic and acute, and good results have been claimed for this distinctive protein therapy. The actual mode of action of these substances in bringing about either increased resistance against disease, or defervescence of already existing disease, is still largely a matter of conjecture. According to Weichardt[72] it is due to mild shock to the whole cellular mechanism of the body - an omnicellular plasma activation'.

In reviewing my experimental work with Typhoid proteose, it will be recalled that in Part I, I found that the specific antibody response was not marked. There was a slight increase in the bactericidal power following proteose injections, but as bactericidal substances are present in the serum of normal rabbits, there is the possibility that this might be of the nature of a non-specific response, for several observers[73] have obtained an increase in antibody titre following non-specific injections. The latter apparently are incapable of actually generating antibodies, but can mobilise them where they have previously existed. It may be therefore that the proteose fraction from Typhoid bacilli has a definite rôle in immunisation. Though its power of producing specific antibodies/

antibodies is slight, it may bring about a mild general reaction, which on the principle of non-specific protein therapy, may be associated with increased resistance against disease.

Lastly, though from my experiments I am in no position to dogmatise, it would seem that several factors enter into the intoxication which is a feature of Typhoid infection. These are probably:-

(1) Preformed toxins of a protein nature stored up in the bodies of the bacilli, and liberated by lysis - endotoxins.

(2) Protein split products of the bacterial cell, e.g. primary proteose.

(3) In man, where the disease has a definite incubation period, native coagulable proteins from the bacteria may be associated with the intoxication produced, the system having become gradually sensitised to bacterial protein during the incubation period.

S U M M A R Y.

(1) Typhoid bacilli are naturally resistant to digestion by proteolytic ferments such as Pepsin or Trypsin, but when extracted with acetone for 24 hours, they are almost completely dissolved by 1 - 2% solutions of Trypsin in 4 - 6 hours.

(2) From such digests of B. Typhosus, by appropriate methods, bacterial protein derivatives, corresponding to progressive phases of digestion, can be isolated.

(3) These derivatives are, alkali and acid metaprotein, coagulable protein, primary proteose, secondary proteose and peptone.

(4) Equal amounts of these bacterial products have been inoculated into rabbits, and the antibody response compared with that obtained by inoculating equivalent amounts of dried undigested Typhoid bacilli.

(5) Metaproteins are definitely antigenic, but compared with unaltered Typhoid bacilli, they act only as partial antigens. Agglutinins are not produced in response to inoculation of Typhoid metaprotein, and the other antibodies are present in/

in much less concentration than in the controls. There is no difference to be made out between acid and alkali metaprotein. Though these are the earliest recognisable derivatives of the unchanged bacterial protein, their combination with acid or basic radicals brings about a marked deterioration in their antigenic power.

(6) Coagulated Protein produced all the recognisable antibodies against the Typhoid bacillus, but in lesser concentration than the untreated bacilli. In spite of the alteration in molecular grouping associated with coagulation, this antigen proved more efficient than acid or alkali metaprotein.

(7) Proteoses act as very weak antigens. No agglutinins or opsonins were formed, and complement fixation antibodies could not be demonstrated. Their inoculation caused a slight rise in the bacteriolytic power of the serum, and a positive precipitin response, demonstrable only in the lowest dilutions of the serum.

(8) No demonstrable antibodies against the Typhoid bacillus were formed as a result of inoculations of bacterial peptone.

(9) Typhoid bacilli digested for 4 hours with 1% Trypsin were tested as antigens. Inoculation of these/

these filtered digests produced a rise in the bacteriolytic power of the serum greater than that found in the controls receiving undigested bacilli. Agglutinins, precipitins, opsonins and complement fixation antibodies were produced, but to a lesser degree than in the controls.

(10) Typhoid bacilli, extracted with acetone, are not thereby appreciably weakened in their antigenic power.

(11) The lipoid material, removed from bacilli by extraction with acetone, is completely devoid of antigenic properties.

(12) In experimental animals, the titre of antibodies in the serum cannot be accepted as an accurate criterion of the protective power against the living bacillus.

(13) Evidence is brought forward to show that antibodies are not all produced together, but in response to different phases of the digestion of bacteria. In this respect Douglas' findings are confirmed.

(14) It is suggested that agglutinins are formed in response to a very early phase in the cleavage of the bacterial protein, and that unaltered native protein is necessary for their production.

(15) There is also evidence that opsonins are produced in response to an early stage of cleavage of the protein molecule, though not so early as is required for the production of agglutinins. For the production of opsonins, the protein must not have passed the coagulable stage.

(16) It is further suggested that bacteriolytic antibodies are produced in association with a later period of digestion of the bacterial protein than either agglutinins or opsonins.

(17) The production of precipitins seems to run on a parallel with that of bacteriolysins, but the evidence on this point is inconclusive.

(18) It is concluded that agglutinating and precipitating properties of sera are not very closely related, but are really distinct phenomena.

(19) Judging from the experiments carried out, it would appear as if bacteriolysins in a serum are only capable of being stimulated up to a certain titre, and this can sometimes be reached after two or three inoculations. Subsequently in spite of the inoculation of larger amounts of antigen, there is a tendency for the bacteriolytic power to decline. This is especially apt to occur where undigested bacilli are employed.

(20) The efficacy of so called detoxicated vaccines, which consist of metaproteins and proteoses, is discussed. From experience with Typhoid derivatives, it is concluded that while the constituents of detoxicated vaccines can act as antigens and produce antibodies, the immunisation is only partial. This appears to be due to the fact that chemically treated antigens undergo some alteration, as a result of which they become less effective.

(21) Typhoid bacilli digested for 4 hours with 1 to 2% Trypsin are much more toxic than undigested bacilli.

(22) The split products which can be isolated chemically from digests of Typhoid bacilli are less toxic than the original organisms.

(23) The primary proteose fraction is the most toxic of the protein split products derived from the Typhoid bacillus. The metaproteins are less toxic than primary proteose, and secondary proteose is the least toxic of all.

(24) The symptoms and pathological findings in rabbits, which have received lethal doses of both living and dead Typhoid bacilli, are described.

(25) The mode of death, and the post-mortem appearances including the congestion of the abdominal organs, the hyperemia and focal haemorrhages in the intestine, and the congested and haemorrhagic condition of the lungs, are very similar to what is found in sensitised rabbits which have received a toxic dose of egg globulin and in which death has occurred comparatively late.

(26) The subacute nature of the illness as compared with acute anaphylactic death, points to a solution or partial disintegration of the bacterial protein being necessary before the toxic substances are liberated.

(27) The pathological picture in death from inoculation of Typhoid primary proteose is practically the same as in death following inoculation of the whole bacilli or of bacilli which have been digested with Trypsin.

(28) Primary proteose, derived from egg albumen, has no power of sensitising animals either towards itself or towards the native protein from which it was derived.

(29)/

(29) The pathological picture following inoculation of animals with Typhoid bacilli is a non-specific one, and can be produced not only by colon bacilli, but by non bacterial protein.

(30) The symptoms produced in animals by Typhoid primary proteose are the same as those described following inoculation with "Peptone", and peptone shock seems to be dependent on the presence of proteoses - especially primary proteoses.

(31) From a study of the toxic substances derived from Typhoid bacilli, it is probable that the intoxication produced by these bacteria is dependent upon.-

(a) Preformed toxins of a simple protein nature, stored up in the bacterial body and liberated by lysis - endotoxins.

(b) Protein split products of the bacterial cell - e.g. primary proteose.

(32) It is suggested that in man, the native bacterial proteins may be associated with the intoxication produced, the system having become sensitised to these proteins during the incubation period.

In/

In closing, I would here express the debt I owe to the late Professor James Ritchie. He it was who suggested this line of research, and his help and kindly criticism during the earlier period of the investigation, were invaluable. His sad and untimely death was a sore blow to all, and to no one more than his pupils in the laboratory who had benefited from the inspiration of his teaching, and to whom he was a real friend.

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