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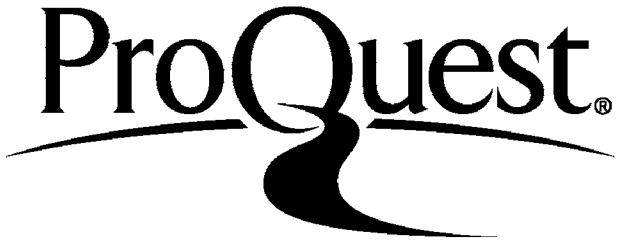

OF THE NATURE OF THE ANTIGEN.

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CONTENTS.

The study of the processes of immunity received a tremendous impetus when, in 1895, Bordet and Gengou discovered the phenomenon of complement fixation. It was apparently simplicity itself, a certain foreign substance, the antigen, injected into an animal gave rise to the formation, in the serum of that animal, of a substance, the antibody. Immune serum containing this antibody, if mixed in a test tube with the antigen which caused its formation, was capable of combining, in some way, with complement and removing it from the sphere of action; so that on the subsequent addition of heated haemolytic serum and red blood corpuscles, no haemolysis resulted. The reaction was widely applied in bacterial problems but with its extension to the Spirochaetes by Wassermann it was soon seen that the phenomenon of complement fixation was more complex since it was shown by Noguchi that syphilitic sera were capable of fixing complement in the presence not only of syphilitic liver extract but also in the presence of other liver extracts; in fact the alcoholic, or ether, extracts of most organs could furnish efficient Wassermann antigens. So it has become customary to distinguish between the "true" Bordet-Gengou reaction and other complement fixation reactions. In the former the active substance giving rise to the formation of the antibody in vivo is identical with that which reacts with the immune serum in vitro, in the latter it is different. The term "antigen" is used to cover both cases, resulting in a certain amount of confusion; "Receptor" would have served well for those substances acting only in vitro were it not for its inevitable association with the mechanical concepts of Erhlich. Some antigens are capable of producing other properties, besides those of complement fixation, in the immune serum. Haemolytic and bacteriolytic effects - precipitation - agglutination - anaphylactic reactions. The injection of foreign blood corpuscles confers on the serum the power, in the presence of complement, of liberating the haemoglobin from homologous erythrocytes, and in the absence of complement, of clumping or agglutination of the cells. The injection of dead, or living, bacteria gives rise to parallel effects with bacteria. The injection of foreign sera stimulates the production of the precipitins.

Another manifestation of antigenic action is the phenomenon of anaphylaxis. Ritchie (1903/4) found that if a guinea pig was injected with a small quantity of antigen and a certain time elapsed before a large injection of the same substance was administered intravenously; the second injection was followed by the rapid development of characteristic symptoms, frequently resulting in the death of the animal. Death

due to "Typical Anaphylactic shock" being preceded by paresis, dyspnoea and bronchial spasms, the animal ceases to breathe but the heart may beat for some time after death. The symptoms and the post mortem appearance may vary in individuals. In certain animals anaphylactic reactions are by no means constantly evoked. For example, the rabbit is a very uncertain subject and the symptoms produced differ from those in the guinea pig. In the guinea pig the reaction is delicate; and providing that the sensitising dose is not more than a very small fraction of the test dose, and that more than 10 days are allowed to elapse between the two doses, it is a reliable test for antigenic action. That the phenomenon is due to a change in the serum is shown by the production of passive anaphylaxis; for on the injection of the serum of a sensitised animal intravenously into a normal animal, the injected animal for some weeks afterwards is "sensitised", and anaphylactic reactions can be produced by the intravenous injection of a test dose. A number of skin and conjunctival reactions - such as Arthus' phenomenon and the tuberculin test - are based on this condition of hypersensibility and prove valuable tests for the antigenic properties of substances.

These results are specific. The injection of streptococci does not give rise to sera agglutinating staphylococci; here however there is an obvious morphological difference. But the injection of, or infection with, B.Paratyphosus B. does not give rise to sera agglutinating B. Paratyphosus A.; but the two bacilli are morphologically indistinguishable and biologically very similar. Passive or active immunity for the one does not, however, confer any immunity for the other. The symptoms of infection are different, the toxins are therefore different. Immune sera were found to be capable of distinguishing further than this. The clinical pictures of epidemic cerebro-spinal fever due to any of the causative cocci are identical but four strains can be distinguished by agglutinating sera (Gordon 1907). These questions then arise. Are the toxins producing the symptoms of the disease or anaphylaxis identical with the "antigen"? Is it possible to remove the antigenic principal from, the body a bacterium, or other antigenic material? Finally, to what class of substances do antigens belong?

The distinction between the antigenic products isolated from bacteria and from other parasites, from blood and other tissues, has been almost entirely based on relative solubility in different solvents. It has become customary to regard the fractions extracted by physiological saline and similar solvents as "protein"; and those isolated by organic solvents, principally ether and alcohol, as "lipoids and fats". This

division, though questionable from the chemical standpoint, gives an initial grouping of antigens. It is so far unquestioned that the proteins are antigenic. Crystallised egg albumen will give rise to antibodies, Dakin and Dale (1919) have shown that whilst by chemical and physical means it is not yet possible to distinguish between the crystallised egg albumen of the hen and the duck; antigenically they are specific in action. Further, that on racemization of these compounds different proportions were obtained of the laevao- and dextro- forms of certain of the constituent amino-acids. In this case then, protein specificity is associated with differences of stereo-chemical configuration.

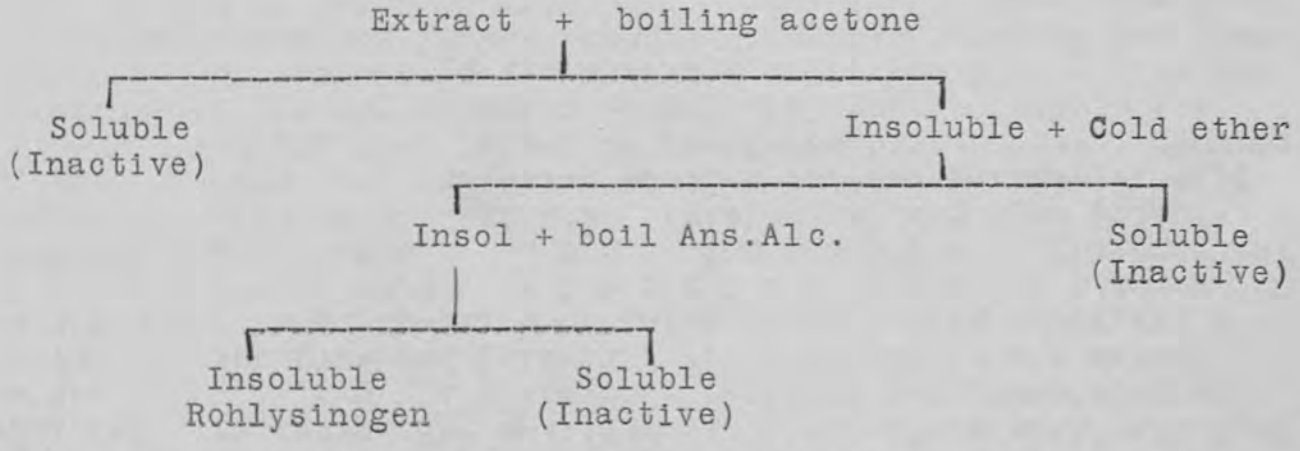
Bierry, Henri and Pettit Auguste (1904) stated that the "Nucleo-proteins" - that portion of different organs of a dog which was soluble in 0.2 % sodium carbonate and precipitated by acetic acid - when injected intraperitoneally into rabbits gave rise, in the serum of these rabbits, to products which were toxic for the organ from which they were prepared. Hence the serum of a rabbit inoculated with the nucleo-protein from dog's kidney when injected into a dog caused albuminuria.
(1916) stated that the extracts of tissues with boiling water, yielding solutions of β-nucleo-proteins gave rise to anaphylactic reactions but that the extracts from the pancreas of the pig and the ox and the spleen of the latter showed no specificity. Gideon Wells (1913) considered that the nucleic acids showed little evidence of antigenic properties; and that the so-called nucleo-proteins were mixtures of all kinds, both from the cytologist's nucleus and from the cytoplasm, probably in part denaturised and capable of giving rise to non-specific precipitins and complement fixation, even when they have lost the power of inducing anaphylactic reactions.

Apart from tissue extracts and such special antigens as Cobra venom and ricin, antigens may be placed in three classes. First, the haemolytic antigens, those which confer haemolytic properties on the serum. Secondly, bacterial antigens, those which are intimately connected with the problems of disease. Thirdly, other parasitic antigens which may be closely parallel with the processes of infection, as is hydatid fluid in tests for hydatid disease; or purely empirical, as is the extract of ox heart in the Wassermann reaction. The chemical nature of the antigenic substances in all three classes may ultimately prove the same; nevertheless it is convenient to consider them separately although the second and third classes at least are comparable in many respects.

HAEMOLYTIC ANTIGENS.

Early investigators in this field Dungern (1899) and Bordet (1900) agreed that the haemolytic antigen for blood was labile and resided neither in the haemoglobin nor in the stromata and the latter considered that it was destroyed by the action of water or ether on blood. Bierry and Pettit (1904) classified it among the nucleo-proteins. The first serious contribution to the problem was the work of Bang and Forssmann (1906), which opened up the possibility of the lipoidal nature of antigens, by showing that the lysinogenic principle of ox erythrocytes was extracted by ether and was soluble in boiling benzol. A brief account of their method of extraction is necessary as it is hoped to be able to show that the mode of preparation of the lysinogenic extracts is not without influence on their properties. The crude extract was obtained by treating wet, washed, ox corpuscles with ether; this crude extract, they stated, contained lecithin,"Amidomyelin" and several other lipoids, kephalin, sphingomyelin, cerebrosides, cholesterin, neutral fats and "organo-plastic substances in not negligible quantities". On the addition of acetone they found that the precipitate was the active fraction. This fraction was dried and repeatedly extracted with acetone, and then extracted with ether; a portion remaining undissolved containing the lysinogen. Hence they concluded that it was sphingomyelin. It was then treated with cold benzene and the lysinogen remained undissolved; but on treatment with hot benzene the active principle passed into solution, but it is to be noticed that it was not re-precipitated on cooling. They accounted for its solubility in the crude ether extract by the presence of the acetone-soluble bodies. They described the substance as having crystals resembling cholesterin. In 1907, Dautwitz and Landsteiner (1907) confirmed Bang and Forssmann's work,also extracting wet corpuscles with ether. Gottlieb and Leffmann (1907) also obtained specific haemolysins by injecting ethereal extracts obtained by the methods of Bang and Forssmann. Frouin (1907) washed dog cells and dried them with acetone and found that on injecting the corpuscles he obtained only haemogglutinins; and that by injecting the acetone-soluble portion he obtained haemolysins but no agglutinins; which was the first contradiction of Bang and Forssmann's statement. He found that the simultaneous injection of the acetone insoluble residue and the acetone extract, gave a haemolysin 5 to 6 times stronger than that obtained by the injection of the acetone extract alone. It is interesting to note here that in the same paper he stated that the inoculation of the acetone extracts of egg yolk into rabbits

gave rise to a haemolysin for dog's red cells - an early contribution to the study of heterophile haemolysin. But the solubility of the haemolysin in acetone does not appear to have received subsequent confirmation by any of the other numerous investigators. Takaki (1908) dried the corpuscles first under glass plate at 37° and extracted them with boiling benzene and treated the evaporated extract as follows:-



This active substance contained iron, sulphur and phosphorus. It gave a positive reaction with Molisch's test but no reducing substance could be split off with acid. It gave negative reactions with protein tests and elementary analysis showed that it was not of constant composition. On treating it with N/10 sodium hydroxide it became soluble in water. Landsteiner (1910) commented that its activity is not so great as would be expected if the lysinogen were soluble in the various solvents and that its properties do not agree with any known lipoid. Thiele and Embleton (1912) found that the haemolysinogenic power of sheep's red corpuscles was undiminished by "complete removal of the lipoids" by successive extraction by acetone with boiling ether and with absolute alcohol at 37°. In contradiction to the results of Frouin (1907) they did not find that the extracted cells gave rise only to haemoglutinins. They found that the extracts of the corpuscles were quite inactive thus disagreeing with the observations of the preceding investigators.

In 1908 Iscovesco (1908) contributed to the in vitro study of these substances the fact that by lysing red corpuscles with a little ether and then centrifuging and powdering the dried solid, he was able to extract a substance, insoluble in alcohol and acetone, but soluble in chloroform and ether; a fatty oil which was easily emulsified with water and which when

added to a mixture of a natural haemolytic serum and red cells prevented haemolysis.

In 1911 Bang and Forssmann found that ether extracts of organs of certain animals were capable of giving rise, when injected into the rabbit, to an antibody haemolytic for sheep's red cells which was subsequently known as the Forssmann Immune Body or the Heterophile Antibody. They stated that this property was contained in the acetone insoluble fraction and that it did not fix complement in the presence of the immune serum whilst the acetone soluble fraction did so, but that it had no lysogenic properties. Schmidt (1921, 1 and 2.) extracted sheep's wet red corpuscles with ether for several days at room temperature, and then added acetone. The acetone soluble fraction was useless, the acetone insoluble ether soluble fraction he stated contained a little of the Bang and Forssmann antigen, but it was mainly active in vitro (alcohol solutions were superior). He found that extracts of dried organs were very poor in antigenic properties (1921, 2.) and also that the watery extracts of guinea pigs organs were not reliable in vitro. He did not consider (1921, 1) that the extract obtained by Bang and Forssmann was a true heterophile antigen. He found that the true isolysinogen was contained in the blood platelets. He could obtain no heterophile immune body by the injection of cells which had been extracted with various organic solvents, thus differing from the conclusions of Thiele and Embleton (1912).

Landsteiner and Prásek (1912) stated that by the extraction of wet Stromata with alcohol, the power of the stroma to fix the Forssmann Immune Body was diminished by protein coagulants. Kodama (1913) showed in a series of experiments with varying concentrated alcohol and horse flesh (the horse is a heterophile animal) that if the tissue was fresh its antigenic properties were more quickly destroyed by absolute alcohol; if dried, by 60 to 70 % alcohol. These results, he considered, concordant owing to the dilution of the alcohol caused by the water contained in the fresh tissue.

In agreement with Landsteiner and Prásek (1912). Sordelli and Fischer(1918) found that the kidneys of guinea

pigs and dogs (heterophile animals) extracted with 96% alcohol, and, subsequently with equal parts of alcohol and ether, no longer fixed complement with sera containing the Forssmann Immune Body, whilst the combined extracts did so. Further, they found that the residue was active in producing H a e m o l y s i n s i n v i v o. Whilst the s o l u b l e p o r t i o n h a d n o power of producing h a e m o l y s i n. They found a mixture of both to be inactive when injected. This is not a little remarkable in consideration of the fact that suspensions of whole kidneys of dogs and guinea pigs give rise to haemolysins and it is clear that they must both contain the residue and what can be extracted with alcohol. Sordelli (1922) was able to obtain agglutination of these lipoid suspensions by the addition of heterophile immune serum, and the agglutinant antibody was fixed by kidney of the guinea pig and by the corpuscles of sheep and goats but not those of the ox. He found that complement was fixed by agglutinated particles. Sordelli and Fischer (1919, 3) continued their researches and found that the heterophile antigen could be extracted from horses' kidneys in the following way. The tissue was extracted with boiling acetone and filtered in the cold. The residue was boiled for an hour in alcohol and filtered hot. This alchol extraction was repeated several times. A copious white precipitate was obtained on cooling. This was purified by extraction with ether in which it was insoluble: re-crystallisation from alcohol yielded a substance partly soluble in benzene. On evaporating the solution on a water bath at 60° C. a colourless, transparent glass like solid was obtained which melted at 195° C., with decomposition into a dull, red liquid. It contains nitrogen and phosphorus. The properties described closely resemble those of Sphingomyelin (Maclean 1918). This substance fixed complement vigorously with the serum of a rabbit injected with guinea pig kidney. A substance of identical appearance was obtained from the kidney of a cow but this lacked the property of fixing with the haemolysin.

Balls & Norris (1918) found that the binding properties of the stromata ~~were~~ of sheep's corpuscles were destroyed by

heating them to 80° C. for thirty minutes; and they considered that this pointed to the protein nature of the antigen. Muir (Studies on Immunity, Page 122) stated that even after heating to 100° C. they retained the power to bind the amboceptor. Balls and Korns (Loc.cit) extracted the stromata, carried down from laked blood by the addition of calcium carbonate, with alcohol and ether, and found the extract to be without binding power in vitro. The extract obtained from the residue by treatment with physiological saline had no binding properties; but on extracting the residue with 0.85% sodium chloride, containing 0.2% sodium hydroxide, the extract on neutralisation gave a precipitate which did not dissolve in excess of acetic acid : this substance had binding properties and also gave rise to isohaemolysins when injected, there being no accompanying rise in the anti-human titre. They found nucleo-protein in the extract, and also lipoids; but these latter they considered were not the active substances since the lipoidal extracts had proved inactive. In accord with Levene (1901) who had extracted dog's corpuscles with 0.5% sodium carbonate, they found that no agglutinins were formed.

Freideberger and Collier (1919) by treating urine with alcohol, in the proportion of 10 to 7 parts, obtained hetero-antigens from heterophile animals, but they could obtain no complement fixation with either iso- or heterophile urine in the presence of hetero- immune sera. After the addition of phosphotungstic acid, a small amount of antigen still remained in the filtrate, but the precipitate was without antigenic action i.e., it had been destroyed for the most part.

Landsteiner (1920) extracted 10 gms. of sheep corpuscles with 100 gms. of 80% alcohol and injected the equivalent of 2 cc. of the alcohol extract into a rabbit on two occasions and obtained no result. It is to be noted that the dose was very small and the author himself admitted this and thought that it would be desirable to repeat the experiment with larger doses. He found that a rabbit injected with a saline extract yielded a serum which fixed complement in the presence of the alcoholic extract but not with the residue. These observations confirm those of most of the more recent workers, that the antigen giving rise to the formation of haemolysins is a different substance from that which acts in the test tube phenomenon. Whilst the latter antigen appears to be lipoidal or to possess physical characters which lead to its association with these substances, the chemical nature of the antibody-producing substances is even more obscure.

BACTERIAL ANTIGENS.

The practical importance of the immunity reactions of pathogenic bacteria stimulated early inquiries into the nature of their antigenic substances. Camus and Pagniez in 1901 employing tuberculin, found it an efficient antigen in the Bordet-Gengou reaction with tubercular serum, and on account of this they considered that the antigen of the tubercle bacillus was lipoidal; Bordet (1920) criticises their conclusion by remarking that it is to be noticed that they employed crude tuberculin which contains débris of the bacilli. Calmette and Massol (1911, 1 & 2) found that aqueous extracts of the tubercle bacilli, in general, do not contain the antigen corresponding to the antibody in "Les sérums inhibitants," but that the residue of the extraction reacts with these sera. Sera "uniquement sensibilisant" fix indifferently with the antigen in the liquid or in the bacillus. They suggested that there were two antigens, the one soluble in water, and the other only in peptone water, (Calmette and Massol, 1913); but they disagreed with Camus and Pagniez who stated that the antigen was soluble in alcohol and ether; for they found (1911, 2) that whilst the "Tuberculine ancienne de Koch" contained one of the antigens, tuberculin lost much of its antigenic power when it was treated by alcohol during its purification, and that its antigenic power was independent of its toxic effect on a tuberculous animal. Here, as in the haemolytic antigens, it appears questionable whether the active antigen *in vitro* is that which acts *in vivo*. However, Thiele and Embleton (1915) found (1) that the sera of rabbits inoculated with whole tubercle bacilli reacted equally well with antigens prepared from :- (a) whole bacilli; (b) bacilli with fats and lipoids removed; (c) with the phosphatids obtained from the bacilli; (d) with Nastin.
(2) That rabbits inoculated with antigen (c) reacted well with whole or extracted bacilli, and vigorously with antigen (c). Debré and Paraf (1911) stated that whilst urine, pleural exudate, ascitic fluid, cerebro-spinal fluid, organ extracts, and serous and purulent fluids from cases of infection with the tubercle bacillus, could act as antigens in the Bordet-Gengou reaction, they did not find it advisable to use either watery or alcoholic extracts, so in this way they are at variance with Calmette and Massol and Thiele and Embleton. Blumberg (1917-1918) found that Besredka's antigen which is employed in the diagnosis of tubercle, was liable to give pseudo-positive reactions with syphilitic sera since it contained lipoids. He questioned whether true Bordet-Gengou reactions were obtainable in any infection except those which cause a polymorpho-nuclear leucocytosis; tuberculosis like syphilis gives rise to lymphocytosis, and he considered that the complement fixation obtained

with tubercular sera and Besredka's antigen was not a true specific reaction; since by employing the same technique in the preparation and substituting the egg yolk for tubercle bacilli, he was able to obtain an efficient antigen. He attributed the antigenic properties of Besredka's antigen to the presence of an emulsion of heated lipoids. Thus whilst it seems possible from the work of Thiele and Embleton that the lipoidal extracts of tubercle bacilli are antigenic in vitro, and even in vivo, their action may be non-specific; and further it seems clear that the antigenic substance is distinct from that which produces the anaphylactic phenomenon in the tuberculin reaction since it is well-known that the tuberculin reaction occurs only in animals infected with tubercle bacilli and never in animals inoculated with killed bacilli.

The solubilities of the cholera antigen were investigated by Levaditi and Mutermilch (1908) who found that the antigen was insoluble in antigen, absolute alcohol and ether; but that the residue of the bacilli, after extraction with these solvents, would yield an antigen soluble in either physiological saline or 85% alcohol. They considered that its solubility in 85% alcohol could not be attributed to a solution of protein in lipoids since these had been removed. This result is of interest in making up bacterial and helminthic antigens since one at least of these latter, as will be shown later, exhibits similar properties. They showed that the antigenic power of the solution was not lost by coagulating the proteins by heating to 100° C. for 15 minutes, and so could not be classified as a protein or a lipoid. They continued their investigations (1908, 2) and found that the same extract acted in vivo conferring active immunity with the production of an antitoxic serum which contained agglutinins and a complement fixing antibody. They found the bactericidal properties of the serum especially enhanced in a rabbit.

Ménard (1912, 1 & 2) found that whilst the lipoidal extracts of the Klebs-Loeffler bacillus had a characteristic local action, it had no immunising effect. Dumas and Pettit (1913) confirmed this local action which they said gave rise to "Une réaction fibro-plastiques" of previously unknown rapidity, beginning 20 minutes after the injection. They also found that neither passive immunity conferred by the injection of antidiphtheric serum, nor natural immunity as in the rat, gave any protection against the production of these lesions. So it seems unlikely that the antigenic substance of the diphtheria bacillus is lipoidal. This is further confirmed by Dominici and Ostrovsky (1913), who found that by extraction of the bacilli with distilled water, characteristic lesions could be produced at a distance from the seat of inoculation; but that after heating the solution to 100° C. only

local lesions were produced, which makes it probable that the toxic substance is a protein.

In the case of the typhoid bacillus, Nicolle (1898) found that a substance soluble in alcohol and ether stimulated the production of agglutinins. Pick (1902) in an intricate investigation of cultures of this bacillus found differences in the solubility of antigen for the Sachs-Georgi reaction, obtained from young and old cultures. From the former the antigen was soluble in distilled water but precipitated in 95% alcohol; from the old cultures the antigen was soluble in 95% alcohol but insoluble in ether. He also quoted Winterberg who agreed with its insolubility in ether and also found it insoluble in absolute alcohol. He considered these agglutinogens to be of unknown nature. Again it is seen that the addition of a small quantity of water causes the solution of the antigen in alcohol.

Howell and Anderson (1920) on the other hand found that the ether extract was weakly antigenic for the Bordet-Gengou reaction with immune serum. They also investigated the ethereal extract of the Meningococcus which was weakly antigenic and the Pneumococcus and Streptococcus both of which gave good antigenic extracts.

Recent work has drawn attention to bacterial fats. Stuber and Bauer considered bacterial fats to be antigenic, but Borcic did not agree with their conclusions. Jobling considered that serum lipase was increased by the injection of the sodium salts of fatty acids, and that a rise in the bacteriolitic power of the serum was thus accounted for. Thiele and Embleton (1914, 3) were unable to confirm this increase of lipase. Shaw-Mackenzie (1919) discussing Rogers' treatment of leprosy and quoting Jobling considered that lipoids might be substituted for fats since "lecithin is split by lipase and accelerates the activity of this enzyme". (If he is correct this example of the substrate or the products activating an enzyme is rather unusual.)

The most remarkable contributions to the study of bacterial fats are those from Warden and his co-workers (Warden and Others, 1915-1919). They found that the fats peculiar to the gonococcus served for an antigen in complement fixation tests, and were superior to saline suspensions or to autolysates of the germs. They extended this to the bacilli of cholera, typhoid and tuberculosis. "Complexes of pure cell fats" if emulsified under certain conditions acted, Warden considered, as if they were on the surface of bacteria as they undoubtedly are in the fatty envelope of certain bacteria and in the stromata of blood corpuscles. "In other words the

action is unquestionably one directed on cell surfaces or aggregate surfaces or stromata in the sense of Bordet". (Warden and Connell, 1919, 1.) The work was extended to the production of immunity reactions in vivo. Symptoms of diphtheria were said to be produced by these specific tests, and they found that protection could be conferred by anti-diphtheric sera. These specific fats were not prepared from the bacilli but were mixtures of palmitic and stearic acids from other sources employed in the form of their sodium or potassium salts in proportion similar to those in which Warden considered they were present in the bacillus basing his figures on the mean equivalent weight of the acids and their iodine value. His theory of the mode of action is strongly reminiscent of the Lewis-Langmuir theory of catalysis and surface action in general; and if correct will furnish another striking example of the applicability of this hypothesis which is at present showing itself extraordinarily capable of interpreting many diverse phenomena.

HELMINTHIC ANTIGENS.

The Bordet-Gengou reaction has been applied to a considerable extent in the investigation of Helminthic infections and the Sachs-Georgi reaction has been used to a limited extent; but investigation of Antigenic action in vivo is largely limited by the absence, in most helminth infections, of any very definite symptoms, and of any considerable degree of active immunity; though recent work seems to point to active immunity not being so uncommon as might be supposed. Helminthic Antigens show a greater solubility in Alcohol than do many of the Bacterial Antigens; but unlike them they lack specificity in very many cases. Both Joest and Cherardini investigated serum reactions with Hydatid fluid, and in 1907 Weinberg began a series of researches on this material. He found (Weinberg, 1907) that hydatid fluid gave precipitin reactions with the sera of patients infected with echinococcus, and also with the sera of animals injected with hydatid products. Weinberg and Parvu (1908, 1) obtained positive results also with infected sheep, and negative results with Syphilitic sera. They found (Weinberg and Parvu, 1908, 2) that the duration of the reaction coincided with the persistence of eosinophilia, and so might reasonably be considered to be intimately connected with the toxic processes. Parvu and Laubry (1909, 1, 2.) employed this reaction to demonstrate that the antibody does not pass the placenta (1), and that it is present in the cerebro-spinal

fluid only when the cyst is in the central nervous system (2). Parvu (1901) showed that the antigenic substance was soluble in 85% alcohol and that there was no cross-fixation with the Wassermann antigen. The antigen was capable of filtration through collodion sacs (Parvu 1909, 2.). The Antigen does not appear to confer any lasting effect upon the serum, since Laubry and Parvu (1911) and several other observers agree that the persistence of a positive reaction beyond six months (Fairley 1922, "one year") after operation, indicates the presence of other cysts which have not been removed. Jianu (1909) found that the antigenic substance could be extracted from dessicated hydatid fluid by ether. And Israel (1910) was able to obtain antigenic alcoholic extracts both from the fluid and from the cyst wall. He used five times the bulk of alcohol to that of the material extracted. He found that syphilitic sera fixed complement with these antigens; but he considered that a specific substance was also present in that from cyst fluid but not in that from the cyst wall, which was capable of reacting with serum of cases of echinococcus infection. This was borne out by the fact that these latter sera did not give positive reactions with a Wassermann antigen, whilst fixing complement in the presence of his antigen. Brauer (1911) obtained similar results with alcoholic extracts. Fairley (1922) also obtained an alcoholic extract of cyst wall which he found inactive for echinoccal infections. He found that alcoholic extracts of dried hydatid fluid were not good; but that an extremely good antigen could be prepared from moist scolices treated with absolute alcohol, and he never found any pseudo-positive reactions when employing them. He agrees with Weinberg that the immune body appears in the serum owing to the filtration of hydatid fluid into the tissues. It is seen here that observers disagree on the question of pseudo-positive reactions with syphilitic sera and this is possibly largely attributable to the strength of the antigen they use and the consequent variation in the concentration of the lipoids. (Le Bas, 1922). They agree that the antigen is soluble in slightly diluted alcohol but, as will be shown later, this does not necessarily mean that it is either a fat or a lipoid. In regard to its specificity Servantie (1921) found that the serum of a patient infected with Fasciola hepatica gave a positive reaction with hydatid fluid. But Ghedini (1907) found that hydatid fluid did not yield a positive reaction with cases of infection with Ancylostoma duodenale or Ascaris lumbricoides. In other cestode infections, less distinctive reactions have been obtained. Weinberg and Parvu (1908) employing saline antigens of fresh or dried Taenia perfoliata and T. plicata from the horse, found that the antigens were not quite specific, but showed cross fixation with Ascaris and with Sclerostomes. Kolmer, Triste and Heist (1916) found some degree of specificity between Taenia serrata, Dipylidium caninum, Ascaris canis and Tricocephalus dispar; and injection

of these antigens into rabbits gave rise to specific sera. They found that the alcoholic extracts were rather more satisfactory. The lipoidal extracts of tapeworms have been investigated by Meyer (1904) who found that the serum of a rabbit inoculated with tapeworm, fixed complement with both crude and purified alcoholic extracts of the worm. Thiele and Embleton (1913) confirmed this. None of these observers obtained positive results with syphilitic sera. But Violle and Le Saint-Rat (1920) found that whilst tapeworm antigen prepared by Noguchi's method gave no fixation with a person infected with tapeworm it did give positive results with syphilitic sera.

Weinberg and Julien (1911) were able to demonstrate that the perienteric fluid of Ascaris megalocephala contained a toxic substance which, when injected into the nasal mucous membrane or the conjunctiva gave rise to lesions in normal animals which were marked in the case of those which were heavily infected with Ascaris. Weinberg and Séguin (1913) found that the sera of horses infected with young Ascarids were capable of neutralising small amounts of this toxin in vitro, so it is reasonable to consider that this toxin is a true antigen. Rubenstein and Julien (1913) showed that the sera of infected horses gave Abderhalden's reaction with a perienteric fluid of this parasite thus confirming the true antigenic character of the toxin. Usami (1919) found that in alcoholic extracts of hookworm gave positive results in cases of Ankylostomiasis and negative results with normal and syphilitic sera. No details of the mode of preparation of the extract are given in either the American or the English abstracts and unfortunately efforts to obtain the original Japanese paper in this country have so far failed. So there are no criteria on which to base a suggestion as to the chemical character of the nematode antigens.

More is known of Trematode antigens. Weinberg (1909) found that the saline extract of Fasciola hepatica adults filtered through a Berkefeld or a Chamberland filter gave positive results with fluke-infected sheep; and dried flukes could be used for extraction. Ando (1917) found that dogs infected with Paragonimus westermanii ground up in physiological saline and extracted in the ice chest for 24 hours with absolute alcohol, gave the following results:-

Positive	-	2 dogs infected with lung fluke.
"	-	13 men " " " "
Negative	-	2 healthy dogs.
"	-	13 " men.
Incomplete		6 syphilitics having positive Wassermanns.

Another example of the solubility of the antigen in wet alcohol and its tendency to pseudo-positive reactions with

syphilitic sera. Setsuzo Rhynji (1922) was able by similar treatment of the livers of rabbits infected with Clonorchis sinensis to extract an antigen giving the following results:-

Positive	-	10 experimentally infected rabbits.
"	-	3 " " dogs.
"	-	60% of cases of moderately or heavily infected human beings.
Negative	-	12 normal rabbits.
"	-	3 " dogs.
"	-	11 " humans.
"	-	infected patients showing no symptoms.

He obtained no pseudo-positive reactions with cases of infection with:- Ascarids, ankylostomes, Trichocephalus or Treponema pallidum: also catarrhal jaundice not due to clonorchis and cancer of the liver did not cause the serum to react positively. The complement fixation test for Bilharziasis was the first example of the use of the larval form of the trematode parasite in the intermediate host as an antigen for the detection of antibodies caused by the presence of the adult in the definitive host. Previous to this work of Fairley (1919) Rouslacroix and Payau (1911) had shown that sera from cases of Bilharziasis did not react with a Wassermann antigen if free from syphilis. Sueyasu (1917) extracted adult Schistosoma japonicum with water and with alcohol and obtained positive results with both these extracts with a serum from a horse which had recovered from Schistomiasis, and negative results with a horse which had an apparently natural immunity. (In this case also the Japanese original is not at present available and the English abstracts give no details as to the strength of the alcohol used.) Fairley (in 1919) obtained antigens by extracting the livers of Bullinus dybowski infected with S. haematobium and Planorbis boissyi infected with S. mansoni. He used both saline and alcoholic extracts but considered the latter more reliable and this was prepared by the extraction of wet snail's liver with absolute alcohol. (A more detailed account of the mode of preparation and the results obtained will be found in the accompanying published paper, Le Bas, 1922).

Murray (1920) confirmed Fairley's result using the livers of infected Physopsis africana. But Hoppli (1921) questions the specificity of the immune body since he found that the sera of patients with Bilharziasis due to S.mansoni would give positive results with alcoholic extracts of the livers of sheep infected with Fasccla hepatica.

Balls and Korns (1918) found that the binding properties of the stromata of sheep's corpuscles was destroyed by

EXPERIMENTAL.

Since it is often considered that the antigenic properties of heterophil tissues are lipoidal in character, it was thought that it might be of interest to examine the efficacy of tissue extracts, prepared in a manner calculated to obtain the various lipins in a reasonably pure state, and as little changed chemically as conditions would permit.

PREPARATION OF THE LIPOIDAL EXTRACTS OF HORSES' HEART.

The procedure adopted was based on the method described by Maclean(1918. pages 73/5), certain modifications being introduced, which were considered dedirable, to reduce the oxidation of the unstable lipins to a minimum. The bulk of the work was carried out on heart muscle from a horse. The technique in the case of the other antigenic preparations being, *mutatis mutandis*, essentially the same. The slight differences will be described separately.

The horse's heart being brought straight from the freshly killed animal to the laboratory, was washed free from blood, and as much fat and fibrous tissue as possible was cut away. The muscle tissue was then put through a mincing machine, the expressed blood drained off, and the tissue placed ina wide-necked bottle, and one volume of acetone was added. These operations were completed within 2 to 3 hours of the death of the animal, so that no appreciable amount of autolysis can have taken place. Maclean(1920)considers that the oxidation of lipins, especially of kephalin, takes place principally during the drying of the material; so to overcome this the air in the bottle was replaced by CO2. The bottle and its contents were then shaken in a mechanical shaker, at room temperature, for two to three hours; the acetone was then drained off and replaced by fresh dry acetone, the bottle again filled with CO2, and the shaking repeated for another period. This process was repeated until, as experience showed, the water had been abstracted from the tissue by the acetone. Three to four changes of acetone were found sufficient.

The acetone was drained off and the remainder was expressed by wrapping the tissue, in small quantities, in two or three thicknesses of gauze, and then pressing it in a small potatoe press. Maclean(1918)recommends

sail-cloth and a laboratory hand-press; neither were available, and the above method was found quite efficient. The small cakes so obtained, were broken up and dried in vacuo over sulphuric acid, the exhaustion of the dessicator being carried out continuously during the day. Maclean(1920) dried his tissue at this stage with fans, but, as it was desired to take no risks, in vacuo drying at room temperature, was considered preferable.

The dried tissue was then powdered in a coffee machine. The dry powder was placed in a Winchester quart bottle, and two to three volumes of absolute alcohol were added. The bottle was then filled with CO_2 and placed in a mechanical shaker for three to four hours; the alcohol was then decanted off into another bottle and maintained in an atmosphere of CO_2. The process was repeated five to six times, the alcohol extracts being collected together and stored in an atmosphere of CO_2 until all the extracts were collected. The total bulk of the extracts was about 4litres. The bulk of the extractives is contained in the first half of the alcohol extract.

The next step was to remove the alcohol at low temperature and without permitting oxidation. Distillation at 2 to 3cm. was attempted; owing to frothing and bumping the usual modifications of placing beans and capillary tubing in the fluid were tried, but without avail. The stream of air led in through a fine capillary tube to the bottom of the liquid was not much better and risked oxidation. The apparatus, with one small exception, was then set up in accordance with directions very kindly given by Dr. J. C. Drummond of University College, London. The alcoholic extract was placed in a Winchester bottle fitted with a rubber stopper through which was passed two glass tubes, one was short and was connected to a supply of CO_2, the other led to the bottom of the bottle. This latter was connected by a small piece of pressure tubing on which is a screw clip to another glass tube. The rubber tubing was gripped on to the glass with K-P. clips since it was essential that there should be no leak and the oily nature of the liquid passing through the tube tended to permit the slipping of the rubber on the glass of the bottle. This third tube was led through a rubber stopper fitting tightly in the neck of a 2 litre distilling flask. The end of the tube was drawn out to a hair capillary and reached to within 2 inches of the bottom of the flask. The side arm of the flask passed through a rubber stopper fitting in the neck of a litre filter flask which was

attached to an exhaust pump. The clip was screwed up the filter flask and distilling flask evacuated until the manometer measured a few millimetres pressure. The clip was then unscrewed slightly and the liquid was sucked up from the bottle and entered the distilling flask. The clip was so adjusted that as the liquid fell on the bottom of the flask it immediately distilled and the clip was constantly regulated so that more than 5 or 6 cc. of liquid never accumulated in the distilling flask. The distilling flask was gently heated on a bath which was never allowed to rise above 40°C. and was generally maintained at 32 to 35°C. It was found difficult to obtain a sufficiently steady and small stream of CO_2 to lead into the bottle from a cylinder as Dr. Drummond suggested, and so a very simple modification was made. A large paper gas balloon was charged with CO_2 and attached to the bottle. The CO_2 was then under atmospheric pressure and passed gradually into the bottle as the liquid was drawn over into the flask.

Some of the residue after distillation of the alcohol was taken up in alcohol in which most of it was easily soluble and this solution will be termed "Crude Extract." The remainder was taken up in ether and acetone was added which caused the precipitation of the lipins whilst the fats and cholesterins present in small quantities since the greater part were removed during the preliminary treatment of the tissue with acetone. The supernatant liquid was decanted off and the residual precipitate was again taken up in ether and an opalescent solution was obtained. The ether solution was centrifuged at high speed until clear, the supernatant liquid was pipetted off and the residue, which consisted of sphindomyelin and the cereberosides phrenosin and kerasin washed into a bottle by means of a small quantity of cold alcohol in which they are sparingly soluble. The acetone was again added to the ether solution and the resulting precipitate treated as before. This whole process is repeated until no precipitate is obtained on centrifuging the solution. Four or five treatments are usually sufficient. The insoluble fractions were collected together and will be termed "Sphingomyelin" antigen. The ether solution was then evaporated in vacuo until the thick viscid syrup was obtained. This was treated with absolute alcohol which dissolved the portion and precipitated another fraction. The alcohol soluble portion consisted mainly of lecithin and the alcohol insoluble fraction which was taken up in ether in which it was very soluble consisted of crude kephelin. This fraction until recently was been termed "Cuorin", being so named by

Erlandsen, who stated that it had a N.P.ratio of 1:2. Maclean (1918)cast doubts on the validity of Erlandsen's statement of Cuorin as a chemical entity. This doubt was increased by a series of analyses carried out by Levene and Komatsu(1919), and finally Maclean(1920)stated definitely that "Cuorin" was a mixture of Kephalin and the break-down and oxidation products of Kephalin. He obtained by rapid drying of the tissue by acetone a substance soluble in ether and insoluble in alcohol having a N:P. ration of 1:1, ie.the substance was Kephalin.

The four extracts of horse's heart were respectively:-

1. Crude heart.
2. Sphingomyelin(and the cerebrosides).
3. Kephalin.(crude).
4. Lecithin(mixed with dissolved Kephalin).

It was considered that in a preliminary investigation such as is the present series of experiments it was unnecessary and undesirable to attempt further purification of the lipins which could be carried out later on s should any of the crude substances exhibit striking differences from the remainder.

Rabbit(1) was inoculated with 0.1.to 0.15.gms. of "Crude Extract" on 2 occasions.
Rabbit(2) was inoculated 4 times with "Crude Extract."
Rabbit (3) was inoculated twice with "Sphingomyelin". This rabbit received about 2/3rds.of the total amounts of this fraction which was isolated from 400 to 500 gms. of horse's heart(dry weight.)
Rabbit (4) received 4 doses of "Kephalin."
Rabbit (5) received 4 doses of "Lecithin."

All inoculations were given intraperitoneally about a week elapsing between the doses. The antigens for administration were emulsified in physioligical saline and the organic solvents driven off by warming for a short time at 37°C.

The sera for the tests were obtained by bleeding the rabbit from the marginal vein of the ear on the night previous to the day on which the tests were to be carried out. The rabbits were never bled on two successive days and usually a week or more elapsed.

HAEMOLYTIC TETRE FOR SHEEP'S RED CORPUSCLES.

In these tests a 10% solution of sheep's red corpuscles were used a 100 cmm.being the unit. The cells were those which were used in the routine Wassermann tests in the laboratory. Compliment was furnished by the serum of a freshly killed guinea pig the standard unit being 20 cmm.

OH = No haemolysis

TrH = Trace of Haemolysis.

SH = Slight haemolysis.

MH = Much haemolysis.

VMH = Very much haemolysis.

ACH = Almost complete haemolysis.

CH = Complete haemolysis.

For the test graded quantities of serum were added to tubes containing 100cmm.of 10% sheep's red corpuscles and 1.cc.of physiological saline a unit of complement being added in some cases. Sera were "inactivated" by heating at 56° C.for a quarter to half an

I

	Quantities of serum in Cmm. (pure)					
	200	100	80	60	40	20
Serum active	CH	OH	OH	OH	OH	OH
" " + complement	CH	CH	CH	CH	aCH	VMH
" inactive + "	CH	CH	CH	CH	CH	CH

It was noticed that inactivated serum was more haemolytic than active serum with complement, and also that the fresh serum alone was very weakly haemolytic.

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It will be noticed that inactivated serum was more haemolytic than active serum with complement,and al so that the fresh serum alone was very weakly haemolytic.

Reference will be made to these points later. The sera of the other rabbits of this series were inactivated before the haemolytic tests were carried out as it was desired to find the smallest amount of serum that would produce haemolysis. It was not considered necessary to carry the tests above 100 cmm. of serum since this is within the range of natural sheep haemolysin which a rabbit serum may contain (Thiele and Embleton, 1912.)

II

Rabbit (2)	100 cmm inactive	1 unit Complement	= O H
" (3)	"		= O H
" (4)	"		= O H
" (5)	"		= O H

COMPLEMENT FIXATION.

In this series the Minimum Haemolytic Dose method was used 1, 3 and 5 M.H.D. of complement being employed. A Minimum Haemolytic Dose being that amount of complement which will just produce complete haemolysis in in 100 cmm. of a 10% suspension of sheep's red cells to which has been added 5 units of sheep amboceptor. 1 unit of amboceptor being that amount of amboceptor which will just produce complete haemolysis of 100 cmm. of a 10% suspension of sheep's red cells in 1cc. of physiological saline in the presence of 20 cmm. of pure guinea pig serum. (For further particulars of the test reference may be made to the accompanying paper Le Bas, 1922). The tests were set up according to the table given below. Each tube containing 100cmm. of

III

tion to

Serum	1 MHD	3 MHD	5 MHD	Control
200 cmm	1)			
100 "	2)			
50 "	3)			

Reference will be made to these points later. The sera of the other rabbits of this series were inactivated before the haemolytic tests were carried out as it was desired to find the smallest amount of serum that would produce haemolysis. It was not considered necessary to carry the tests above 100 cmm.of serum since this is within the range of natural sheep haemolysin which a rabbit serum may contain (Thiele and Embleton,1912.)

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The three control tubes contained no antigen and served as serum anti-complementary controls. Rabbit(1) which had received only two doses was examined with another series and those results will be given later.

Courin antigen was repeatedly made using different ways of emulsification adding saline slowly to the ether solution--adding the ether solution to a bulk of saline--adding the alcohol to emulsify first; but whatever method was used the anti-complementary factor of this antigen was so high as to make it totally unsatisfactory and unfit for use in complement fixation tests.

IV

Antigen	MHD	200	400	600
I Crude	1	OH	OH	OH
	3	CH	ACH	MH
	5	CH	CH	aCH
II Sphingomyelin	1	OH	OH	OH
	3	CH	CH	CH
	5	CH	CH	CH
III Kephalin	1	OH	OH	OH
	3	SH	OH	OH
	5	ACH	OH	OH
IV Lecithin	1	ACH	SH	SH
	3	CH	VMH	VMH
	5	CH	CH	CH

It was thought from these results that working with 1, 3, and 5 M.H.D. and using 100 cmm. of antigen, any trace of fixation would be detected and controls would be provided by the tubes containing 1 M.H.D. The slightest fixation should have shown with the tubes having 3 M.H.D. and any considerable amount with the tubes having 5 M.H.D.

The sera were tested in the quantities employed and all the injected rabbits were slightly anti-complementary in quantities of 200 cmm. of serum; the normal rabbit was not anti-complementary. This being the case it was expected that a great deal of pseudo-fixation would result owing to the operation of the anti-complementary adjuvenant phenomenon. (Thiele and Embleton, 1914, 1). But the actual results obtained were unexpected. At the time of the test controls were put up of the quantities actually used in the test, both of antigens and sera and it was a fairly constant feature that more haemolysis were shown in the tubes containing slightly anti-complementary quantities of serum together with slightly anti-complementary quantities of antigen than were shown by either the antigen or the serum control tube. No explanation of this "anti-complementary neutralising phenomenon" as it may be termed, has been found in the literature examined, nor has it apparently been noted before. But almost certainly, it was not due to faulty technique which was the first and obvious suggestion as was shown by its frequent occurrence with different sera in spite of great care being taken.

Although the quantities of complement and the conditions were such as to show up strongly even a very slight tendency to fixation yet not more than occasional traces of inhibition of Haemolysis were obtained and these were well within the possibilities of additive phenomena.

PREPARATION OF ANTIGENS FROM OTHER HETEROPHILEE ORGANS.

a. Acetone insoluble fraction of the ether extract of dried red corpuscles of the sheep.

Sheep's red cells which had been dried in vacuo over sulphuric acid were extracted with ether at room temperature the procedure being similar to that for the preparation of crude horse's heart extract. The ether extract was then treated with acetone the precipatate taken up in ether and re-precipatated by acetone and this precipatate was injected into rabbit (6). Only enough for one dose was obtained; but since some authors state that the development of the heterophile haemolysin is greater after one dose than after several

successive doses, it was thought desirable to include this experiment. The haemolytic titre of the active serum with added guinea pig complement about one week after innoculation was eighty cubic millimetres of pure serum causing complete haemolysis of one unit of sheep's red corpuscles. And since the rabbit received the equivalent of 2 to 3 hundred c c. of blood, there seems little evidence that the cold ether extracted from the dry cells yields a lysinogen.

(b) "Sphingomyelin" from sheep's red corpuscles.

This antigen was prepared in an exactly similar way to the same fraction from horse's heart. Only traces of kephalin and lecithin fractions were obtained. 0.0047 gm. of Sphingomyelin was obtained from 100cc.of blood and this was suspended in alcohol and divided into three doses given at weekly intervals to Rabbit (7). The initial titre of this rabbit was 400cmm.pure serum producing complete haemolysis in 1 unit of sheep's red corpuscles.

	Cum of Serum				
Serum	100	80	60	40	20
Active alone.	O H	O H	O H	O H	O H
Active + complement	A H	C H	V M H	M H	O H
Inactive + complement.	C H	C H	C H	C H	V M H

Again it will be noticed that the inactivated serum with complement is more haemolytic than the active serum with complement and that the active serum alone shows very little haemolytic power.

(c) Complement fixation.

The sera of these two rabbits together with Rabbit (1) and Rabbit (3)of the horse's heart series, were tested against antigens I, II, and IV on two occasions. First about a week after their last inoculation and again after several weeks. The first time the unit system of complement and simultaneous titration of amboceptor and complement(Thiele and Embleton, 1914, 2.)was used, and the second time the M.H.D.

system was substituted for the former. The tests were set up according to Table VI. on the first occasion each tube contained 100 cmm. of antigen and 1cc. of physiological saline. After fixation 200 cmm. of a mixture of amboceptor and sheep's red corpuscles was added.

VI

	units of Complement		
Serum	3/4	1	1 1/2
200 cmm	"	"	"
100 "	"	"	"
80 "	"	"	"
60 "	"	"	"
40 "	"	"	"
20 "	"	"	"

The results with 1 unit of complement are shown in Table VII

VII

Antigen	Rabbit (1)	Rabbit (3)	Rabbit (6)	Rabbit (Y)	normal
I	+ + + +				
II	+ +				
IV	+ + + +	+ + +	+ + +		+
					+

VIII

	Rabbits					
Antigen	(1)	(3)	(6)	(Y)	normal	Lecitho-vitellin
I	+ +	+ +	+ + +	+ +		+
II	+	+ +	+ +	+		
IV	±		+	+	+	±

Complement = 1. M H D.

system was substituted for the former. The tests were set up according to Table VI.on the first occasion each tube contained 100 cmm.of antigen and 1cc.of physiological saline. After fixation 200 cmm.of a mixture of amboceptor and sheep's red corpuscles was added.

VI

	units of Complement		
Serum	3/4	1	1 ½
200 cmm	"	"	"
100 "	"	"	"
80 "	"	"	"
60 "	"	"	"
40 "	"	"	"
20 "	"	"	"

The results with 1 unit of complement are shown in Table VII

VII

Antigen	Rabbit (1)	Rabbit (3)	Rabbit (6)	Rabbit (Y)	normal
I	+ + + +			Anticomplementary	
II	+ +				
IV	+ + + +	+ + +	+ + +		+
Wasserm	+ + +	+ + + +	not done		±

Some weeks later the M.H.C. system carried out as in Table III.yielded the results shown in Table VIII.

In this case 1 M.H.D. was used yet as in the case of the horse's heart test the fixation was minimum and i in no case amounted to even 1 M.H.D. which is a very small degree of fixation when it is taken into consideration that the sera were slightly entiocomplementary. In the case of the first tests the crude extract injected Rabbit No.(1) gave fixation of one unit with antigens I. and IV. but it also gave a considerable degree of fixation with the Noguchi antigen prepared from Ox heart as did also Rabbit (3).

In order to test whether these antigens could act in vitro although they did not exhibit any marked antigenic power in vivo, they were tested with the serum of a rabbit which had a high induced antifheep titre. Its amboceptor titre being 60 omm. of 1:100. The method adopted was to take 1:10 serum and use graded quantities of serum with a standard amount of antigen. The tests were set up according to Table XII.

XII

Serum	antigen	Complement	
100	200	3/4 unit	1 unit
100	100	" "	"
80	100	" "	"
60	100	" "	"
40	100	" "	"
20	100	" "	"

After half an hour at room temperature and half an hour at 37°C. in a Wassermann bath, ordinary sheep's red cells were added and after another half hour at 37° the readings were taken. It was found that there was complete haemolysis in all tubes except those containing antigen III. However, in this case there was very powerful agglutination due no doubt to the large amount of amboceptor; this coupled with the apparently anti-complementary tendency of this antigen exhibited in previous

experiments makes it probable that the lack of haemolysis in these tubes was due to an interference with the complement and not to a true re-action between antigen serum and complement. It might be thought that such a great excess of amboceptor would reduce the complement required for haemolysis below the amount which would be left unfixed by the antigen&serum. So a control was set up using quantities of complement from one-half down to a twentieth of a unit with the largest amount of serum used. It was found that with half a unit of complement Haemolysis was far from complete. So that the necessary quantity of complement lay between three-quarters and half a unit, since the tubes containing three-quarters of a unit were completely haemolysed there could have been no fixation of complement as great as a quarter of a unit.

PREPARATION OF A FAT-AND LIPOID-FREE ANTIGEN.

Cat's corpuscles were obtained and washed thoroughly in physiological saline and defatted by Hardy's method. (Hardy and Gardiner, 1910) The cells from 100 cc. of cat's blood were cooled to 0°C. and several volumes of alcohol cooled to 8°C. were added and the mixture allowed to stand for some time in a freezing mixture at -14° C. and then filtered at 0°C. The cells were washed on the filter paper with ether cooled to below 0°C. until all the alcohol was removed. They were then abstracted in a Suxhlet apparatus for two days with ether. The residue so obtained was innoculated into a rabbit at weekly intervals intraapertoneally. The animal received three inoculations; and then after several weeks a final "whip-up" dose. The haemolytic titre was then found for cat's corpuscles Table IX. (isolysins) and for sheep's corpuscles Table X. (heterolysins).

IX

Quantities Serum	100	80	60	40	20	10	8	6	4	2	c.mm.
Active Serum	C H	A C H	M H	S H	T. H	0	0	0	0	0	
" + Complement	C H	C H	C H	A C H	T. H	0	0	0	0	0	
Inactive + " "	C H	A C H	V M H	M H	T. H	0	0	0	0	0	

Cats corpuscles = 100 c.mm 10%.

It will be noticed that there is no anti-complementary effect the active serum evidently containing nearly 1 unit of complement in 80 cmm.

X

Serum reactivn + complement	Quantities of Serum in cmm (pure).									
	100	80	60	40	20	10	8	6	4	2
	CH	CH	CH	ACH	ACH	MH	MH	SH	SH	T.H

Besides producing a haemolysin for cat's red cells which are not present in the normal rabbit an augmentation of the normal antisheep haemolysin is exhibited.

The agglutinating power of the serum for cat and sheep corpuscles is shown in Table XI.

XI

	Quantities of Serum in cmm (pure).									
	100	80	60	40	20	10	8	6	4	2
Cat's Corpuscles	a_{qq}	a_{qq}	a_{qq}	—	—	—	—	—	—	—
Sheep's " "	a_{qq}	a_{qq}	a_{qq}	—	—	—	—	—	—	—

The agglutination was slight for cat cells and was faint for sheep's cells.

ANAPHYLACTIC REACTION.

In order to test whether the rabbits had developed a heterophile antigen which was not detectable by the preceding methods the anaphylactic reaction was performed by injecting the sera of the inoculated rabbits intravenously into guinea pigs. The presence of a heterophile antibody makes the serum toxic for guinea pigs.

The Three rabbits which were selected as a preliminary were those two which had exhibited some transient haemolytic power for sheep's red cells, the third rabbit being that one which was inoculated with the defatted cat's red cells. Three c.c. were given; Tanigouchi(1920) states that as little as one-tenth of this amount will sometimes prove fatal.

Guinea Pig(1) injected with 3cc.of serum from a rabbit inoculated with the Sphingomyelin fraction of sheep's red corpuscles.

Result: NO EFFECT.

Guinea Pig(2) injected with 3cc.of serum from rabbit injected with crude horse's heart.

Result: Slight and transient paralysis of the hind limbs lasting only a few minutes.

Guinea Pig (3) injected with 3 ccc.of serum from rabbit injected with cat's defatted red cells.

Result: NO EFFECT.

BILHARZIA ANTIGEN.

Opportunity occurred to examine the solubility of the antigenic principle of the snail's liver employed by Fairley (1919--21)in the complement fixation test for Bilharziasis. This antigen can be extracted either by treating the liver of the snail with physiological saline or with absolute alcohol. Its solubility resembles in this respect the Bang and Forssmann immune body--the heterophile antigen. So that it was considered of interest to examine the various extracts treating the livers as the horse's heart and sheep's red cells had been treated. Only the crude alcoholic extract was employed, the material available being quite insufficient for further fractionation. When it was found that the absolute alcohol soluble fraction was not antigenic for Bilharziasis part of the insoluble material was extracted with 50% alcohol, and this extract was found to be antigenic proving that the antigenic substance was not destroyed by the treatment to which the material

XIII

TABLE V.—SUMMARY.

Serum	Saline I.	Absolute Alcohol II.			50% Alcohol.	50% Saline III.	Absolute Alcohol IV.	Echinostoma	
100 c.mm.	100 cm.	100 c.mm.	200 c.mm.	300 c.mm.	100 c.mm.	200 c.mm.	100 c.mm.	200 c.mm.	300 c.mm.
H—d	+ + + +	— — — —	— — — ±	— — — +	— — + +	— + + +	— — — —	— — — —	...
Normal ...	— — — —	...	...	— — — +	— — — —	— — — ±	— — — —	— — — —	...
Syphilitic ...	— — — —	...	...	— — + +	— — — —	— — — ±	— — — —	— — + +	+ + + +
Normal + Syphilitic	— — — —	— — — ±	— — — +	...	...	...	...	...	...

+ + + + = complete inhibition of hæmolysis. XIII
— + + + = almost complete ,, ,, ,,
— — + + = partial ,, ,, ,,
— — — + = slight ,, ,, ,,
— — — ± = trace of ,, ,, ,,
— — — — = complete hæmolysis.

Guinea Pig(1) injected with 3cc.of serum from a rabbit inoculated with the Sphingomyelin fraction of sheep's red corpuscles.

Result: NO EFFECT.

Guinea Pig(2) injected with 3cc.of serum from rabbit injected with crude horse's heart.

Result: Slight and transient paralysis of the hind limbs lasting only a few minutes.

Guinea Pig (3) injected with 3 ccc.of serum from rabbit injected with cat's defatted red cells.

Result: NO EFFECT.

BILHARZIA ANTIGEN.

Opportunity occurred to examine the solubility of the antigenic principle of the snail's liver employed by Fairley (1919--21)in the complement fixation test for Bilharziasis. This antigen can be extracted either by treating the liver of the snail with physiological saline or with absolute alcohol. Its solubility resembles in this respect the Bang and Forssmann immune body--the heterophile antigen. So that it was considered of interest to examine the various extracts treating the livers as the horse's heart and sheep's red cells had been treated. Only the crude alcoholic extract was employed, the material available being quite insufficient for further fractionation. When it was found that the absolute alcohol soluble fraction was not antigenic for Bilharziasis part of the insoluble material was extracted with 50% alcohol, and this extract was found to be antigenic proving that the antigenic substance was not destroyed by the treatment to which the material been submitted in the preparation of the absolute alcohol ract. Full particulars of the technique and results will found in the accompanying paper(Le Bas,1922).Table XIII. narises the results.

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+ + + + = complete inhibition of hæmolysis. XIII
− + + + = almost complete ,, ,, ,,
− − + + = partial ,, ,, ,,
− − − + = slight ,, ,, ,,
− − − ± = trace of ,, ,, ,,
− − − − = complete hæmolysis.

It will be noticed that the absolute alcohol extract of the livers of snails suffering from infections of both Schistosomes and Echinostomes was antigenic for syphilitic sera; and since the publication of this paper the behaviour of a similar extract of normal snail's liver has been investigated. The snails were of the same genus--Planorbis--the species employed being P.corneus, a common form found in ponds on Hampstead Heath. Table XIV. showing also the results obtained with the ordinary Noguchi and Cholesterin antigens, both of which were in constant routine use; shows that the extracts from snail's livers runs closely parallel in its antigenic action to the Cholesterin antigen.

XIV

Serum	Snail liver	Noguchi	Cholesterin
1	++++	=	++++
2	++++	=	=
3	++++	=	++++
4	++++	++++	++++
5	+	=	=
6	++++	=	++++
7	=	=	++++
8	=	=	++
9	+	=	++++
10	=	=	=
11	=	=	++++
12	+ + + + + +	+ + + + + +	+ + + + + +
14	++++	=	++++

++++ = Complete haemolysis in "Control" tube & no haemolysis in the tube containing serum + antigen.

++++ ++++ = Anti-complementary serum i.e. no haemolysis in Control.

= = Complete haemolysis in "Control" and in "Test" tube

The two sera with which positives were obtained with the snail antigen and not with cholesterin were both from cases of secondary syphilis which had been treated. Varying results with different antigens is not surprising in such cases. Of the two speciments which gave positives with the cholesterin antigens and negatives with the snail liver antigen, one was cerebro-spinal fluid from a case of disseminated sclerosis in which a positive result has not been anticipated and the other serum was from a case of heart trouble the Wassermann reaction being examined to confirm the clinical diagnosis.

EXAMINATION OF DATA.

Those authors who found the alcoholic or ether extracts active antigens when injected into animals made their initial extract with moist solvents in nearly every case. And there is general agreement that the extracts of dried organs are very deficient in antigenic properties. Especially is this noticeable in the case of antigens employed for the production of antibodies. Little attention has been directed to this point although the fact is constantly noted and the reason for this neglect seems to be based on one or other of two assumptions.

It is taken for granted that drying in some way destroys the antigenic properties. If that is so then the lipins will be affected by oxidation (Maclean 1922). Hitherto no precautions have been taken to prevent this. It was with the intention of finding an answer to these questions that the experiments described in this paper have been carried out. If the antigen were an unstable lipin it would be largely destroyed in the drying of the tissue as generally carried out, i.e. by spreading out in thin layers and drying with warm air. If it is the stable lipin, sphingomyelin, or a cerebroside it would be unaffected in the process of drying and could be equally well extracted from dried tissues and no difference would then be found between the extracts of dried or fresh tissues. In the first case, it would be expected that the care taken to prevent oxidation in these experiments would result in an extremely active extract being obtained. This is far from the case and it is justifiable to conclude that the antigenic substance - the heterolysinogen of horse's heart is not lipoidal.

If the antigen is protein in character drying and the treatment with the various organic solvents may denaturise it. So the "defatting" of the cat's red cells was carried out by the process devised by Hardy (q.v.) for obtaining protein free from fats and lipoids but with its characters unchanged. In this case it was found that the protein residue was active so that the lysinogen for cats red cells does not seem to be lipoidal in character. But Thiele and Embleton and others whose results have been described found that the protein residues were active after vigorous treatment. Ritchie and Miller (1913) found that the residue was inactive only after treatment with chloroform which evidently destroyed the antigen since it was not found in chloroform extracts hence it does not seem probable that the antigenic power of dried organs is due to the destruction of the antigen in the process of drying.

Another assumption that is made is that a substance which is subsequently found in solution in a pure solvent is necessarily soluble in that solvent in its original state. That this conclusion is wholly unjustified was shown by the work of Mayer and Terroine (1907) on the solubility and mode of formation of the complexes known as the "Lécithalbumines" or the "Lipo-proteins". Lierbermann, Hoppe-Scyler, Osborne and Campbell agree that these substances are soluble in - solutions of neutral salts - in dilute alkalis - in alcohol and in ether; whilst they are insoluble in distilled water and in acids. Mayer and Terroine, in agreement with most other investigators, doubt their existance in natural conditions. Although they are insoluble in acids yet their formation only takes place in acid media by the interaction of proteins with lipoid solutions or emulsions. It is clear that the tissues in which bacterial action is taking place will furnish acid conditions for the formation of these complexes. The sheep red cells dried in vacuo, or by acetone, may owe their small degree of activity to the fact that it was not possible to obtain them immediately from the freshly killed animal as in the case of the horse's heart and so a certain amount of bacterial action may have produced a media suitable for the formation of the lipoid protein complexes resulting in the subsequent extracts containing soluble protein. This is supported by the fact that the extraction of the fats and lipoids from the cat's corpuscles did not remove their antigenic power for these cells were obtained in as sterile a manner as conditions would permit and were used almost immediately whereas the sheep's blood was collected at the slaughter house many hours elapsing before its treatment. The solubility of these complexes in organic solvents would lead to their confusion with thelipins; they are extremely soluble in xylol and chloroform, very soluble in benzene and carbon disulphide and soluble in alcohol and in ether, but insoluble in acetone. Another point noticed by Mayer and Terroine was that whilst lecithin moves towards the positive pole lecithalbumen moves to the negative pole when placed in an electric field. It might be possible by this means to separate the constituents of these antigenic "lipoidal"solutions.

Bang and Forssmann, Takaki and others who found the heterolysin in the ether extract and then subsequently treated it with various organic solvents so removing the lipoids had an active residue. Now this solid was not soluble in cold benzene but was soluble in hot; as are sphingomyelin and the cerebrosides. But unlike them, it is not reprecipitated on coolingthefore either it is not insoluble in cold benzene in which case it does not correspond to sphingomyelin or the cerebrosides or it does not form a true solution but is emulsfied, or it is changed by boiling in benzene. In neither of the last two cases does its properties correspond with that of sphingomyelin or cerebrosides.

Further Wernicke and others found that a similar substance prepared in a like manner was not capable of stimulating the production of heterolysin but could fix complement in the presence of heterophile immune bodies. In this they are in accord with most of the more recent results (Taniguchi 1921/22) (Schmidt 1921, 2) (Césari 1922). It is difficult to account for the fact that Bang and Forssmann found the complement fixing antigen in the acetone solution. The solubility of this antigen in acetone is not found by others. But it is easy to see that the "organo-plastic" substances in their crude ether extract may never have been subsequently removed.

In some cases it has actually been shown that by preliminary drying followed by extraction with absolutely dry solvents, the lipoids can be removed without the antigen (Levaditi and Mutermilch - Cholera Bacillus; Le Bas - Bilharzia Antigen; q.v.) and that subsequently the antigen could be extracted with moist alcohol. Against this must be placed the observation of Ritchie and Miller (1913) that after extracting sheep's serum dried <u>in vacuo</u> with alcohol and ether, and then extracting with 85% alcohol no complement fixing antibody was evoked on injection of the extract and in the case of wet ox cells no haemolysin was obtained by the same procedure. But it is to be noted in this case that the ether and alcohol extracts were also without action and the residue of the ox cells was active; so in this case the antigen appeared not to be soluble in a high concentration of alcohol.

Supposing that the antigen is contained in the initial extract owing to its solubility in diluted organic solvents and that the dried extract is extracted with the pure solvent the antigen will then be in the presence of a very high concentration of lipoidal substances. And in these conditions emulsification may take place and the antigen pass into apparent solution in a solvent in which it is not in the true sense soluble, by accompanying the lipoids the activity of the extracts will be attributed erroneously to these lipoids.

Krumwiede and Noble (1921) carrying out researches on the lipoidal nature of antibodies found it almost impossible to rid the lipoid solutions of proteins. It is difficult to put forward any suggestion as to the nature of those antigens which can be obtained after the removal of all fats and lipoids by extraction with slightly diluted alcohol. They cannot be placed in any of the recognised classes of animal proteins. The alcohol-soluble protein - the Prolamines which are insoluble in absolute alcohol and in water but soluble in 50% to 85% alcohol have so far only been recognised among the vegetable proteins (Osborne and others 1891-1903). Whilst perhaps the bacterial antigens may be placed among them the animal antigens cannot be so classified at present. α-proteo-proteose and α-deutero-

proteose are not precipitated from a 2% solution of Witte's peptone by the addition of an equal volume of alcohol (Schryver 1909). Serum albumen is soluble in dilute neutral salt solutions containing as much as 85% of alcohol only after dialysis (Mayer 1907). The jecorins which are probably artificial products (Maclean 1918), and which can be prepared artificially (Drechsel) were found by Victor (1907) to be soluble in aqueous alcohol but precipitated by 99% of alcohol.

The fact also that by slight variations of conditions some antigenic substances are extracted with either the lipoid or the protein fraction suggests that they may not belong to any recognised class or either the proteins or the lipins.

The very divergent opinions on the character of the heterolysinogen are so surprising that they raise some doubt of the true antigenic nature of these lysogins. It is significant that sheep haemolysin in small and varying amounts is present in normal rabbit serum. And this haemolysin can be increased by the injection of substances which cannot be considered as true antigens. Arken (1915) by injecting "sodium oxybenzoate" was able to increase the haemolysin and agglutinins in an immune rabbit. And Teale (unpublished results) found that the intravenous injection of colloidal salicylic acid into rabbits previously prepared with Hirudin raised both the antisheep haemolytic titre and the complement titre enormously; the former being increased several hundreds of times by a few injections. Frouin (1920) injected ceric salts into rabbits and increased their haemolytic titre for sheeps red cells. In this connection it may be noted that Césari (1922) found that the haemolysin for ox corpuscles which is normally present in the guinea pig to a small extent, can be increased by injections of sheeps red corpuscles.

The anticomplementary character of the injected rabbits is in accord with the results of Kolmer (1916, 1 and 2) which showed that the parenteral or enteral administration of lipoids increased the anticomplementic power of rabbits serum. He found that many normal rabbits' sera gave non-specific fixation with fat extracts which was least marked with the acetone-insoluble fraction. Bergel (1913) accounted for the Wassermann reaction in a similar way by assuming the existence of a lipoidal antigen "Leuslipoid" and he had found (Bergel, 1912) that the administration of lecithin had increased the fixation with lipoids.

Table VII. shows similar results, but the effect in these cases was transient as will be seen by comparison with Table VIII. of Experiments performed some weeks later. The nature of the anticomplementary condition of the serum is not clear. Noguchi (1906) stated that it was removable from rabbits serum by treatment with lipoid solvents. Zinsser and Johnson (1911) denied this. Marminger (1920) found that the anticomplementary condition in horse, mule and rabbit sera was a function of the globulin and depended not on the total amount of the globulin but on the proportion of globulin to albumen in the serum ("Eiweiss-quotient"). Moore (1920) investigated the effect of the complement titre on immunization by using guinea pigs naturally deficient in complement. He found a low complement titre did not in the least affect the formation of amboceptor but that it did affect the opsonins. So that it cannot be objected that the complement deficiency shown by the injected rabbits was the cause of the lack of antibody formation.

The experiments on the reaction between the antigens and the sheep Amboceptor (Table XII) were undertaken also with a view to the possible explanation of the "anticomplementary neutralising phenomenon." If it could have been shown that there was combination between the antigen and the sheep amboceptor with or without the combination of complement this would have accounted for any anticomplementary tendency in the antigen anticomplementary controls. And it might be supposed that in those tubes in which the anticomplementary rabbit serum was also present combination might take place between antigen and true antibody in preference to the combination of the antigen with the sheep amboceptor and the fixation perhaps of some complement as in the first case, but the amboceptor would be free and the inhibition of haemolysis would be diminished. But there seems no justification for supposing that there was any interaction between the antigen and the sheep amboceptor. (See Table XII) and so this phenomenon cannot be accounted for at present.

CONCLUSIONS.

1. That the Forssmann Immune Body is not generated in rabbits by the injection of lipins.

2. That the true antigenic character of heterolysinogens is doubtful.

3. That the anticomplementary power of sera may be stimulated by the injection of lipoidal extracts.

4. That anticomplementary effects are not necessarily additive.

5. That it is not justifiable to base the chemical classification of antigens on their solubility in various solvents.

6. That some antigens probably belong to a class of compounds not yet recognised at any rate in connection with them.

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I

Of the Nature of the Antigen in the Complement-Fixation Test for Bilharziosis.

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THE application of the Bordet-Gengou Complement-fixation test to the investigation of Helminthic infections during the last fifteen years has produced a number of results. In some cases they are not concordant and also appear discordant with the majority of the results obtained by this reaction in other immunity problems, notably those of bacterial infection and of antibodies produced against tissue extracts. The antigens employed, from the point of view of their mode of preparation, fall into two classes: those obtained by the extraction of the material with physiological saline, and those obtained by extraction with alcohol or, rarely, ether.

(The term "Antigen" will be employed in this paper to cover the term "Receptor," which has fallen into disuse owing to its association with the mechanical concepts of the Ehrlich side-chain theory. It must not be taken to imply that the so-called Antigen is necessarily capable of stimulating the production of specific antibodies by injection into an animal; but that in the presence of certain sera complement fixation can be obtained.)

Antigens extracted with physiological saline—to be referred to in this paper as saline antigens—have been found to yield positive results of a varying degree of specificity for individual helminthic infections. Ghedini (1907) tried sera from cases of infection with *Ancylostomum duodenale* against antigens prepared from *A. duodenale*, *Trichocephalus dispar* and against hydatid fluid: and also sera from cases of infection with *Ascaris lumbricoides* against an antigen from *A. lumbricoides* and against hydatid fluid. He obtained complement fixation when he employed the homologous antigen and negative results with the other antigens. Weinberg and Parvu (1908), investigating helminthic infections of the horse (*Sclerostoma*, *Ascaris*, *Tænia perfoliata* and *T. plicata*), and employing saline antigens prepared either from fresh material or from parasites dried in alcohol, found that they generally obtained consistent results with sera from heavily infected horses, but that the fixation was not quite specific. Weinberg (1909) obtained good results in a large number of experiments with saline solutions of desiccated hydatid fluid from cysts in man or sheep, the sera being obtained from cases of echinococcus infection in man. Kolmer, Trist and Heist (1916), carrying out complement fixation tests with the sera of dogs infected with *Tænia serrata*, *Dipylidium caninum*, *Ascaris canis* and *Trichocephalus dispar*, considered that their results showed some degree of specificity with the saline antigens; although the dogs' sera frequently yielded positive results with antigens prepared from parasites "the ova of which could not be

found in the fæces" (this may be attributable to the previous and unknown infections of the dogs employed in the experiments). But they found that if rabbits were inoculated with their saline antigens, specific complement fixation could be obtained with their sera. Fairley (1919) obtained good and consistent results employing saline antigens prepared from the livers of snails—*Planorbis boissyi* infected with *Schistosomum mansoni* and *Bullinus dybowski* infected with *S. hæmatobium*. The sera of patients having Bilharziosis due to either species reacted equally well with antigens from either species of snail. He found, however, that he obtained more satisfactory results with an alcoholic extract.

The results recorded with alcoholic antigen extracts of helminth material vary greatly; both in regard to their specificity for individual helminthic infections, and in regard to their capability for serving as antigens in the Wassermann reaction for Syphilis. As in the results recorded by Kolmer, Trist and Heist (1916) (see above) a marked difference in specificity is noticeable between results obtained from injections of parasite tissue, and results obtained by infection. Meyer (1904) found that the serum of a rabbit inoculated with tapeworm fixed complement in the presence of either crude or purified alcoholic extract of tapeworm. These results were confirmed by Thiele and Embleton (1913). These observers found that the serum from a rabbit inoculated with killed *Tænia crassicollis* gave complement fixation with both the "free" and the "bound" "Lecithin" (Erlandsen's classification), extracted from homologous tapeworms. On the other hand Violle and Le Saint-Rat (1920) state that an extract of tapeworm prepared by Noguchi's method gave no fixation with the sera of patients *infected* with tapeworm. Further, these authors found that whilst their extract was negative with normal serum, complement was fixed in the presence of syphilitic serum; whereas both Meyer (1904) and Thiele and Embleton (1913) obtained negative results with the sera from cases of syphilis. Jianu (1909) obtained positive results with cases of echinococcus infection by employing an antigen prepared from desiccated hydatid fluid, the extraction being carried out with ether, according to the method used by Lesser (1909) in preparing a Wassermann antigen from normal heart tissue. Israel (1910) prepared alcoholic antigens from hydatid fluid and from the cyst wall; using five times the bulk of alcohol to that of material for extraction. He found that syphilitic sera were capable of fixing complement in the presence of these antigens, but he considers that a specific substance capable of reacting with sera from echinococcic infections is also present in the antigens prepared from cyst fluid, but not from the cyst wall; this is borne out by the fact that these latter sera will fix complement with his antigens but not with lipoidal extracts from other sources, which are capable of acting in the Wassermann reaction. Brauer (1911) obtained similar results with alcoholic antigens prepared from hydatid fluid. Usami (1919) found that an alcoholic extract of hookworm gave positive results with cases of hookworm, and negative results with normal and syphilitic sera. Details of his mode of preparation of this extract are not available in either the English or the American abstract.

Regarding the relative value of saline and alcoholic antigens, Kolmer, Trist and Hirst (1916) considered that they obtained more specific results with their alcoholic extracts than with their saline antigens (results described above). Fairley (1919), as is stated above, found that the alcoholic antigen was more stable and more sensitive. He obtained (*a*) in cases of infection of less than two years' standing with the saline antigen 72 per cent., and with the alcoholic 88·8 per cent. of positives, (*b*) in cases of over two years'

standing with the saline antigen 67·4 per cent., and with the alcoholic antigen 74·2 per cent. of positives. In no case did he obtain a positive reaction with cases infected with syphilis but free from bilharzia.

The fact that alcoholic extracts seem efficacious antigens in helminthic infection is in contrast with that of most bacterial infections. Tubercle bacilli and possibly *B. lepræ* seem exceptional; for Camus and Pagniez in 1901 found that sera of tuberculous patients would fix complement with tuberculin. But Bordet (1920) remarks on this, that it is to be noted that the substance employed was crude tuberculin containing debris of the bacilli, and he says that Calmette and Massol found the active principle termed "tuberculin" inactive as an antigen. Thiele and Embleton (1915) found, however, (1) That the sera of rabbits inoculated with whole *B. tuberculosis* reacted equally well with antigens prepared from (*a*) whole bacilli, (*b*) bacilli with fats and lipoids removed, (*c*) with phosphatids obtained from the bacilli, (*d*) with Nastin. (2) That rabbits inoculated with antigen (*c*) reacted well with whole and extracted bacilli, and vigorously with antigen (*c*). With these exceptions the Bordet-Gengou reaction has been found to be applicable only to saline extracts, or saline suspensions of bacteria or infected organs. In the case of tissue extracts there has been some divergence of opinion, but in general it is found that saline extracts are efficacious and purified alcoholic extracts are not. An exception must be made in the case of the alcoholic extracts of organs which exhibit heterophilic antigenic properties (Taniguchi (1921), Forssmann and many others). It was on this account and because Fairley (1919) obtained better results with alcoholic extracts, that it was considered of interest to examine more closely the behaviour of alcoholic extracts of infected snails with Bilharziosis serum.

In the preparation of the alcoholic extract Fairley (1919) used 1 c.c. of absolute alcohol for the extraction of each snail's liver. Now this entailed considerable dilution of the alcohol employed; both by reason of the water content of the snail's liver and by reason of the amount of water which invariably surrounds the liver in the dissection. The result will be that the alcoholic extract will be liable to contain a considerable amount of protein, which may account for its antigenic properties. On this account an antigen (Antigen II) has been prepared by extraction of *dried* snails' livers with absolute alcohol in order to obtain an antigen as far as possible free from protein. The next step has been the preparation of an antigen (Antigen III) by the extraction of the residue left from the absolute alcohol extraction, with diluted alcohol; thus giving an antigen, corresponding to Fairley's alcoholic antigen, with the fats, lipoids cholesterin and other allied substances so far as possible removed.

Experimental.

Technique of Complement-Fixation Test.

Complement:—1½ units are employed, that is 30 c.mm. of normal guinea-pig serum. The standardization of the complement being carried out by the method advised by Thiele and Embleton (1914 (2)) for the simultaneous standardization for the amboceptor and complement.

Hæmolytic System.—Washed normal sheep red cells 10 per cent. in normal saline, and an equal quantity of standardized solution of amboceptor (Burroughs Wellcome and Co.).

Hæmolytic Control for the Antigen is put up with ordinary sheep red cells

10 per cent. and no amboceptor; and is placed in the rack at the beginning of the experiment and left the whole time.

Test.—The antigen anticomplementary titre, and the serum tests are set up with the complement; and the fixation was carried out for half-an-hour at room temperature and for half-an-hour at 37° in a Wassermann bath. Red cells and amboceptor are then added, and the rack of tubes replaced in the bath for another half-hour; after which the reading is taken. In all doubtful cases the tubes were centrifuged. All sera were inactivated, by heating to 56°C. for a quarter-of-an-hour before use in order to destroy any natural complement, and to remove any thermolabile anti-complementary tendency which they may have possessed. The known normal and syphilitic sera were all pooled sera; which had been tested the same day in the laboratory with the Wassermann reactions: using both Noguchi and Cholesterin antigens. All sera employed were those which had proved either fully negative or fully positive.

The serum from a patient, H—d, was tested by the Wassermann reaction with both antigens and was found completely negative.

The Bilharziosis serum was tested each time with the saline antigen, prepared, according to Fairley (1919), by extracting the livers of *P. boissyi* infected with *S. mansoni*. Two snails' livers were shaken vigorously for half-an-hour with 2 c.c. physiological saline, and the mixture was then incubated at 37° C. for 24 hours; it was then filtered and the filtrate was used as the antigen. (Table I.)

II.—Antigen II extracted with Absolute Alcohol.

Preparation :—About thirty livers of snails infected with *S. mansoni* were placed in dry acetone, immediately after their dissection and diagnosis; the bottle containing the mixture being filled with CO_2 and kept stoppered and shaken at intervals. After some hours the acetone was poured off, and fresh dry acetone added, the bottle was again filled with CO_2, and stoppered as before.

This was repeated, making three treatments with dry acetone. The dried livers were then placed in a test-tube and about 5 c.c. of absolute alcohol was added, the tube was filled with CO_2, and stoppered and shaken in a vaccine shaker 2 to 3 hours. The alcohol was then decanted off, a fine suspension of liver tissue in alcohol was obtained which was collected in a bottle which was filled with CO_2 and kept stoppered. This process was repeated 5 to 6 times. The collected extracts were then evaporated at room temperature (Egypt, December, 18°C. to 20°C.) by bubbling CO_2 through the mixture. A fine buff coloured powder consisting of the alcoholic extract and suspended liver tissue was obtained. The bottle was filled with CO_2 and sealed with paraffin wax. (This part of the process was completed on the 31st December.)

The dry powder so obtained was extracted with about 10 to 15 c.c. of absolute alcohol for 24 hours. The mixture was placed in the incubator at 37°C., for one hour of the 24 hours, to facilitate extraction, the bottle was frequently shaken. After extraction the mixture was filtered, and the clear alcoholic filtrate was collected in a test-tube of known weight (the residue was kept, see experiment III). The alcohol was removed under reduced pressure (10 to 30 mm.) at 40°C. The tube was re-weighed.

Test-tube	11·6855 grm.
Residue + Test-tube ...	11·7046 ,,
(Antigen)	0·0161

TABLE I.—SALINE ANTIGEN I. PREPARED ACCORDING TO FAIRLEY (1919).

1 (*a*) Tests performed on 21st January 1 (*b*) Tests performed on 11th February.

Nature of Test— 1 (*a*)	Sera 100 c.mm.	Antigen in c.mm.	Complement in Units	Normal Saline in cc.	Sheep Red Cells. 10 % in c.mm.	Amboceptor in c.mm.	Result
Antigen Anticomplementary	...	300	1½	1	100	100	− − − −
,, ,,	...	400	1½	1	100	100	− − − −
Antigen Hæmolytic	...	200	...	1	100	...	+ + + +
H—d for Bilharzia	H—d	100	1½	1	100	100	+ + + +
H—d Anticomplementary Control ...	,,	...	1½	1	100	100	− − − −
Pooled Serum for Normal Control ...	Pooled serum	100	1½	1	100	100	− − − −
Pooled Serum Anticomplementary ...	,, ,,	...	1½	1	100	100	− − − −
Nature of Test—1 (*b*).							
Antigen Anticomplementary	...	300	1½	1	100	100	− − − −
Antigen Hæmolytic	...	200	...	1	100	100	+ + + +
H—d for Bilharzia	H—d	100	1½	1	100	100	+ + + +
H—d Anticomplementary	,,	...	1½	1	100	100	− − − −
Pooled Serum for Normal Control ...	Pooled Normal	100	1½	1	100	100	− − − −
Pooled Serum Anticomplementary ...	,, ,,	...	1½	1	100	100	− − − −
Syphilitic Serum for Control	PooledSyphilitic	100	1½	1	100	100	− − − −
Syphilitic Serum Anticomplementary	,, ,,	...	1½	1	100	100	− − − −

Saline Antigen prepared according to Fairley (1919) is effective in that (1) it gives negative results both with known normal and known syphilitic sera: (2) it gives positive results with a suspected case of Bilharzia.

The serum of the case submitted (H—d) showed a fully positive reaction on both occasions. This man was in S. Africa in 1902 ; and contracted vesical Bilharziosis due to infection with S. hæmotobium. Since this date he has not been exposed to re-infection. Although under observation no ova have been found in the urine during the past year, and no ova could be found even after a provocative treatment with 2 gr. of sodium antimonyl tartrate given between the dates of the taking of the blood for the two complement-fixation tests. A differential leucocyte count made on 6th February gave the following result:

Polymorphs	48 per cent.	+ + + + =	complete inhibition of hæmolysis.
Lymphocytes	36 ,,	− + + + =	almost complete ,, ,, ,,
Large Mononuclears	4 ,,	− − + + =	partial ,, ,, ,,
Eosinophiles	12 ,,	− − − + =	slight ,, ,, ,,
Basophiles	— (500 counted)	− − − ± =	trace of ,, ,, ,,
		− − − − =	complete hæmolysis.

TABLE II.—ANTIGEN EXTRACTED WITH ABSOLUTE ALCOHOL FROM LIVERS OF SNAILS INFECTED WITH BILHARZIA.

(21.1.22.)

Nature of Test.	Sera 100 c.mm.	Antigen in c.mm.	Complement in units.	Normal Saline in c.c.	Sheep Red Cells. 10% in c.mm.	Amboceptor in c mm.	Result.
Antigen Anticomplementary	...	100	1½	1	100	100	− − − −
,, ,, ...	...	200	1½	1	100	100	− − − −
,, ,, ...	...	300	1½	1	100	100	− − − −
,, ,, ...	...	400	1½	1	100	100	− − − −
,, ,, ...	...	500	1½	1	100	100	− − − −
,, ,, ...	...	600	1½	1	100	100	− − − −
Antigen Hæmolytic	...	200	...	1	100	...	+ + + +
,, ,, ...	...	400	...	1	100	...	+ + + +
,, ,, ...	...	600	...	1	100	...	+ + + +
H—d to test Antigenic properties for Bilharzia	H—d	25	1½	1	100	100	− − − −
,, ,, ,,	,,	50	1½	1	100	100	− − − −
,, ,, ,,	,,	100	1½	1	100	100	− − − −
,, ,, ,,	,,	150	1½	1	100	100	− − − −
,, ,, ,,	,,	200	1½	1	100	100	− − − ±
,, ,, ,,	,,	300	1½	1	100	100	− − − +
H—d Anticomplementary	,,	...	1½	1	100	100	− − − −
Pooled Serum Control	Pooled	100	1½	1	100	100	− − − ±
,, ,, ,, ...	,,	200	1½	1	100	100	− − − +
Pooled Serum Anticomplementary ...	,,	...	1½	1	100	100	− − − −
Normal Serum Control	Normal	300	1½	1	100	100	− − − +
Normal Serum Anticomplementary ...	,,	...	1½	1	100	100	− − − −
Syphilitic Serum Control	Syphilitic	300	1½	1	100	100	− − + +
Syphilitic Serum Anticomplementary	,,	...	1½	1	100	100	− − − −

The resulting solid was emulsified with normal saline by vigorous shaking for half-an-hour at ordinary temperature. The proportion used 0·5 grm. solid : 20 c.c. saline (Fairley, 1919). The resulting emulsion was then used as Antigen II. This second part of the preparation was carried out on January 17 to 21. The antigen, prepared in this way, should contain no protein ; only lipoids, fats, cholesterin and similar alcohol soluble compounds. (Table II.)

Antigen II. (1) shows no difference between the known positive Bilharzia serum and the known normal serum. (2) In the higher concentration it shows definitely more fixation of complement, both with a mixed normal and syphilitic serum and with a known pooled syphilitic serum. The addition of cholesterin made the antigen anticomplementary ; as was shown by the complete lack of hæmolysis in all tubes, to which were added 50 c.mm. 1 per cent. alcoholic solution of cholesterin.

III.—Antigen III extracted with 50 per cent. Alcohol.

The residue from II was placed in a bottle and about 4 c.c. of absolute alcohol + 4 c.c. of saline and placed in incubator at 37° C. for 24 hours, and was frequently shaken. The extract so obtained was filtered and the filtrate was collected in a test-tube of known weight. The solution was then evaporated by placing the test-tube in a water bath and bubbling a stream of air through the solution. Owing to an accident the top of the tube was broken, and so the weight of the residue could not be obtained. The residue was then emulsified with about 3 to 4 c.c. of normal saline and filtered. The filtrate was used as Antigen III. This extract should contain various substances which are soluble in 50 per cent. alcohol, diluted with physiological saline ; but it will not contain those substances which are precipitated by 50 per cent. alcohol. It will not contain more than minute traces of fats, cholesterin and lipoids.

Since the fats and cholesterin are removed by the preliminary treatment with acetone and the lipoids are removed by the alcohol (the possible exception is cuorin, which is insoluble in alcohol, being precipitated from its solution in ether by this reagent (Maclean, 1918, p. 75), it forms an emulsion in water, but no statement as to its solubility in 50 per cent. alcohol diluted with physiological saline can be traced. Maclean (1918) (pp. 54 and 74) states that this compound is of doubtful existence. It is unlikely that it will contain any lipo-protein since these substances, if indeed they exist at all, are considered to be decomposed by alcohol at ordinary temperatures and even at 8° C. (Hardy (1910)). (Table III.)

The antigen is anticomplementary in large quantities, but not in 300 c.mm., and there is no anticomplementary adjuvant phenomenon (Thiele and Embleton (1914 (1)) to an appreciable extent with serum in the concentration used as is shown by the two controlled pooled sera. This antigen is quite a definite one for bilharzia ; the readings were quite clear, and the haemolysis in the case of the known bilharzia serum, with 200 c.mm. of antigen, was only demonstrable by centrifuging.

IV.—Antigen IV extracted with Absolute Alcohol.

In order to test whether the positive result obtained with Antigen II with the syphilitic serum could be attributable to the infection of the snail with *S. mansoni*, a similar antigen was prepared from the livers of *P. boissyi* infected

TABLE III.—RESIDUE FROM II EXTRACTED WITH EQUAL PARTS OF ABSOLUTE ALCOHOL AND NORMAL SALINE, *i.e.*, 50% ALCOHOL.

Nature of Test.—III.	Serum 100 c.mm.	Antigen III in c.mm.	Complement in Units.	Normal Saline in c.c.	Sheep Red Cells 10% in c.mm.	Amboceptor in c.mm.	Result.
Antigen Anticomplementary	...	300	1½	1	100	100	− − − −
,, ,, ...	...	600	1½	1	100	100	+ + + +
Antigen Hæmolytic	...	200	...	1	100	...	+ + + +
H—d. To test Antigenic properties for Bilharzia	H—d.	100	1½	1	100	100	− − + +
	H—d.	200	1½	1	100	100	− + + +
H—d. Anticomplementary	H—d.	...	1½	1	100	100	− − − −
Normal Control for Antigen	Normal.	100	1½	1	100	100	− − − −
,, ,, ...	,,	200	1½	1	100	100	− − − ±
Normal Anticomplementary	,,	..	1½	1	100	100	− − − −
Syphilitic Control for Antigen ...	Syphilitic	100	1½	1	100	100	− − − −
,, ,, ...	,,	200	1½	1	100	100	− − − ±
Syphilitic Anticomplementary ...	,,	...	1½	1	100	100	− − − −

with echinostoma employing the same mode of preparation as that employed for Antigen II. (Table IV.)

This antigen appears to be comparable with Antigen II, but rather clearer readings are obtained. It shows no antigenic power for either normal serum or for the serum of the patient infected with Bilharzia. It shows increasing antigenic properties, proportional to its concentration for a pooled serum which was known to have given a positive Wassermann reaction. (Table V.)

Conclusions.

The fact that Fairley (1919) found the alcoholic antigen more efficient may be due to the greater solubility of the active principle in alcohol diluted with physiological saline than in physiological saline alone. Or it may be due to the increase of the number of positives by the presence of some slightly anti-complementary substance such as cholesterin (Thiele and Embleton (1914 (1)). The relatively poor results obtained with Antigen III may be due to the partial coagulation of the substance by the prolonged alcohol treatment resulting in a very dilute solution being obtained; or it may be due to its destruction by keeping. Fairley (1919) advises that the antigens should be prepared as fresh as possible, and that they lose their antigenic power on keeping.

In regard to the positive results obtained with syphilitic sera, antigens employed in the Wassermann reaction are alcoholic or ethereal extracts of various organs, and the alcohol-soluble, acetone-insoluble fraction is employed. So that, whilst snails' livers have not been previously used as a source for Wassermann antigens (mammalian livers are largely used) and efficient Wassermann antigens can be prepared from invertebrates (cf. Brauer (1911), Violle et Le Saint-Rat (1920)), the positive results obtained are not surprising. The concentration of the alcoholic extract employed was less than that used in the preparation of Standard Wassermann antigen (in the proportion of 25-30) which may account for better results being obtained with larger quantities of antigen. Good results were shown by the extract obtained from the Echinostoma infected snail. The fact that Fairley (1919) found his antigen inactive with syphilitic sera may have been due to the very much smaller concentration of lipoids which his antigen would contain—(the percentage of solids employed in both cases being the same)—and his antigen probably containing in addition some emulsified fats and cholesterin as well as the active antigen.

From the results obtained it would appear that the antigenic substance, acting in the test for Bilharziosis, is insoluble in absolute alcohol and acetone; since the treatment of the material by these two reagents did not remove it; and it was not present in the alcohol solution fraction. Further, the antigenic substance cannot have been destroyed by prolonged treatment with acetone and absolute alcohol, nor irreversibly precipitated by them; since it would not then have been found in solution in the subsequent treatment with 50 per cent. alcohol.

It must be soluble to some extent in physiological saline, since extracts with this solvent yield an efficient antigen. Further, it cannot be insoluble in a considerable concentration of alcohol, for Fairley (1919) found that his alcoholic antigen, extracted by absolute alcohol which was diluted by the tissue water-content and by the water necessarily accompanying the liver, was a very efficient and specific antigen. The substance is soluble in 50 per cent. alcohol diluted with physiological saline; as is shown by the reactivity of Antigen III, which was prepared from the material after the previous removal of fats, cholesterin and lipoids, by successive treatment with alcohol and

TABLE IV.—ANTIGEN EXTRACTED WITH ABSOLUTE ALCOHOL FROM LIVERS OF PLANORBIS BOISSYI INFECTED WITH ECHINOSTOMA.

Nature of Test. IV.	Serum 100 c.mm.	IV. Antigen in c.mm.	Complement in units.	Normal Saline in c.c.	Sheep Red Cells 10% in c.mm.	Amboceptor in c.mm.	Result.
Antigen Anticomplementary	...	300	1½	1	100	100	− − − −
,, ,, ...	...	600	1½	1	100	100	− − − +
Antigen Hæmolytic	...	200	...	1	100	...	+ + + +
H—d for Antigenic props. for Bilharzia	H—d	100	1½	1	100	100	− − − −
	,,	200	1½	1	100	100	− − − −
H—d-Anticomplementary	,,	...	1½	1	100	100	− − − −
Normal Antigen Control	Normal	100	1½	1	100	100	− − − −
,, ,, ,, ...	,,	200	1½	1	100	100	− − − −
Normal Anticomplementary	,,	...	1½	1	100	100	− − − −
Syphilitic Antigen Control	Syphilitic	100	1½	1	100	100	− − − −
,, ,, ,, ...	,,	200	1½	1	100	100	− − + +
,, ,, ,, ...	,,	300	1½	1	100	100	+ + + +
Syphilitic Anticomplementary ...	,,	...	1½	1	100	100	− − − −

TABLE V.—SUMMARY.

Serum	Saline I.	Absolute Alcohol II.			50% Alcohol.	50% Saline III.	Absolute Alcohol IV. Echinostoma		
100 c.mm.	100 cm.	100 c.mm.	200 c.mm.	300 c.mm.	100 c.mm.	200 c.mm.	100 c.mm.	200 c.mm.	300 c.mm.
H—d	+ + + +	− − − −	− − − ±	− − − +	− − + +	− + + +	− − − −	− − − −	...
Normal ...	− − − −	...	...	− − − +	− − − −	− − − ±	− − − −	− − − −	...
Syphilitic ...	− − − −	...	...	− − + +	− − − −	− − − ±	− − − −	− − + +	+ + + +
Normal + Syphilitic	− − − −	− − − ±	− − − +	...	...	...	...	...	...

acetone. (The possible exception being cuorin, see Antigen III above). It is to be noted that the solid remaining after the extraction of Antigen II was only a small quantity of the total bulk of the livers; consisting of the fine suspension in the alcoholic extract made in Egypt which was not filtered but decanted, before desiccation for bringing back to England. Since only a very small proportion of this was soluble in 50 per cent. alcohol the antigenic substance must be extremely active.

There seems some difficulty in classifying it by its solubilities in the recognized classes of proteins. The solubility character of the class of alcohol-soluble proteins—the Prolamines—which are insoluble in absolute alcohol and insoluble in water, but are soluble in 50 per cent. to 85 per cent. alcohol, would appear to be coincident with that of the antigen. But so far these have only been recognized among the vegetable proteins. (Osborne and Voorhees, Osborne and Chittenden, Osborne and Harris and others (1891-1893)).

Schryver (1909) states that on the addition of an equal volume of alcohol to a 2 per cent. solution of Witte's peptone, α-proto-proteose and α-deutero-proteose remain in solution.

Mayer (1907) found that some proteins, e.g., serum albumen, *if dialysed*, were soluble in very dilute neutral salt solutions, containing as much as 80 to 85 per cent. of alcohol. If they were *not dialysed* they could not be dissolved by dilute alcohol except in the presence of strong alkali.

Victor (1907) found that by extraction with 99 per cent. alcohol, concentration, and subsequent solution in aqueous alcohol, jecorins were obtained as a precipitate by the addition of 99 per cent. alcohol. These artificial jecorins, like the "natural" jecorins, are soluble in aqueous alcohol, but are precipitated by pure alcohol. It will be noticed that in this mode of preparation, which was devised by Drechsel (1886), they are obtained from the alcoholic extract. Maclean (1918), p. 156, considers them an artificial product. They are generally obtained from mammalian liver tissue.

In spite of the difficulty of its classification with the proteins, it seems unlikely the antigen is lipoidal in character, since it is not present in the alcoholic extract. It is improbable that cuorin should be a specific antigen. It is unlikely to be a lipo-protein as was shown above. (Antigen III, above.) So far, only proteins, or possibly lipoids, or substances accompanying them, have been shown to be antigenic. Therefore, it seems that the activity of this antigen is to be attributed either to a substance of a protein class, or some active principle associated with protein as yet unidentified and unclassified.

My best thanks are due to Professor Leiper, for enabling me to carry out the work: not only by providing the material and the opportunity to begin the preparation of the antigens in Cairo in conjunction with his Mission to Egypt. But also for the interest which he has so kindly taken in it, and the assistance which he has given me throughout. Owing to the kindness of Dr. F. H. Teale, I have been able to continue the work in London in the Bacteriological Laboratories of University College Hospital Medical School; and I also owe much to his advice and supervision. I am indebted to the Department of Helminthology of the London School of Tropical Medicine, for providing the snails for the saline antigen; and to Dr. J. B. Christopherson, C.B.E., for obtaining the bilharziosis serum.

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