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R. Weil

## MAJOR RICHARD WEIL, M.O.R.C.

Richard Weil was born in New York City in 1876, next to the youngest child in a family of seven children. His early education was directly under the supervision of his mother, who was an exceptionably able and clear-headed woman. At the age of twelve he entered the school of Dr. J. Sachs where he prepared for college, entering Columbia University, class of 1896. Already at this early age he showed a remarkably brilliant mind, which reasoned clearly and delved deeply into all subjects that presented themselves to him. At Columbia his intellectual versatility showed itself in different lines of study—prizes in English and Latin, honors in Greek, original research in science bearing testimony to his assiduous study and careful and thorough work. In recognition of his exceptional ability he was elected to the Phi Beta Kappa at the close of his junior year and he was invited by three departments, the Latin, the English and the biological to become a member of their teaching staffs. Before graduating from Columbia, his mind had been opened to the great possibilities presented by the study of medicine, and this led him to decide upon medicine as a career despite all the other attractive fields that lay open before him. Before entering the College of Physicians and Surgeons, class of 1900, he spent a summer at Wood's Hole at the biological station.

In the Medical School he was an excellent worker and found time in off hours to do experimental work in the physiological laboratory. This work led to the degree of Master of Arts. On graduating from the medical college he became an interne at the German Hospital (October, 1900, to October, 1902), where he received an excellent practical training in medicine and surgery. After the completion of this period of practical training he went to Europe for one and a half years study of medicine and its allied sciences. His clinical work was done chiefly in Vienna under Nothnagel, Neusser and Naunyn, and all three developed

boundless enthusiasm in their eager pupil. Under Marchand and v. Recklingshausen his scientific longings were further whetted and he obtained a glimpse of the wide reaches of scientific medicine. While at Strassburg he was detailed to work in a typhoid epidemic and acquitted himself most creditably.

In 1904 he returned to New York to enter upon a career as physician-investigator, and in all the years that followed he lived up to this professional ideal.

Upon taking up practice in 1904, he began his scientific investigations in the realm of medicine at Cornell Medical College and in the following year was appointed Assistant in Experimental Pathology. Four years later he became Instructor in Cornell in the Department of Experimental Therapeutics, which position he held until 1911 when he became Assistant Professor in the same department. During this period he was active in investigative work, which followed along the lines of experimental pathology and in 1915 he was given the Assistant Professorship in the Department of Experimental Pathology. The following year this department was merged with that of Experimental Medicine and Dr. Weil was given the chair which he held at the time of his death.

In 1904 he was also made Adjunct Pathologist to the German Hospital, which position he held until 1910. From 1908 until 1913 he was an Adjunct on the Visiting Staff to the Mt. Sinai Hospital, thereafter being a member of the Assistant Attending Staff. In the same year that he became an Associate Attending at Mt. Sinai Hospital he was given the positions of Assistant Director of Cancer Research and Attending Physician in the newly reorganized General Memorial Hospital. Upon his appointment to the chair of experimental medicine in Cornell University Medical College he resigned the position of Assistant Director to the Memorial Hospital but continued there as Attending Physician. During these thirteen years Dr. Weil not only performed his official duties in the positions just mentioned, but found time to conduct a fairly active private practice and to delve deeply into scientific research. Among the problems to the solution of which he bent his energies the two

to which he devoted the major portion of his time were those concerned with hemolysis and with anaphylaxis. To the former of these problems he contributed much of value, but the work by which he is known in this field is probably that concerning the variable resistance of human red blood cells to the hemolytic action of cobra venom.

In the field of anaphylaxis he stood almost alone as an exponent of the cellular theory of its mechanism. Although his work in support of this theory was vigorously criticized by many of those who supported the humoral mechanism, and although he met opposition to his views at almost every turn, he lived to see some of the leading European investigators won over to his side. His untimely death found two new studies on one of the phases of this problem in course of publication. These studies gave promise of clearing up one of the most hotly disputed points regarding the mechanism of anaphylactic reactions in the higher mammals.

All of the investigative work that came from his pen was noted for its remarkable clarity of presentation and logic. He was never content to approach any of the problems from a single point of view, but always attempted to see all sides in their true perspective and to attack the problems that presented from as many different points as possible. While the workers in the same field in this country have not yet accepted the cellular mechanism of anaphylaxis, as have some of those abroad, the evidence which Dr. Weil adduced in its support is of such a nature that we feel safe in predicting its ultimate acceptance.

It is neither necessary nor fitting that we should dwell further upon his contributions to medical science in these and other fields for his published works are known to all who are interested in the subjects with which they deal.

Dr. Weil was a member of many medical organizations including the New York Academy of Medicine, the American Medical Association, the American Society for the Control of Cancer, the American Society of Clinical Investigation, the Association of American Physicians and others of a more restricted

and narrower scientific nature. He occupied important offices in a number of scientific societies, having been vice-president of the American Association for Cancer Research and president of the Society for Serology and Hematology, and of the American Association of Immunologists. He also found time to perform the duties of an Associate Editor of the JOURNAL OF IMMUNOLOGY and of the *American Review of Tuberculosis* as well as having been the Editor of the *Journal of Cancer Research* from its foundation to the time of his death.

The range of Dr. Weil's activities was truly remarkable but it serves better than anything else to emphasize the striking features of his character. He was a man of broad interests and clear vision, and an indefatigable worker in every field that he entered. But it was not alone in medicine that he stood out above the average for he was keenly interested in the progress of the times; he was a lover of art, of history and of literature. He could talk well on almost any subject of an intellectual nature and was always ready to sharpen his wits in a friendly argument. He had a keen sense of humor and a ready wit combined with a manner of easy cordiality. It was not one but rather the combination of all of these attributes which won him the lasting friendship of all who came to know him.

Finally, he was imbued with a profound spirit of patriotism which led him to tender his services to his country at the outbreak of hostilities. Starting as a captain in the Medical Officers Reserve Corps, it was but a few months before he was made a major and detailed to Camp Wheeler as Chief of the Medical Service. There his devotion to the great task of combating pneumonia rapidly undermined his health and he himself fell a victim to the disease which he was endeavoring to conquer.

EDWIN BEER,
CARY EGGLESTON.

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# A RAPID METHOD FOR THE PRODUCTION OF PRECIPITIN ANTIGEN FROM BACTERIA: AN ATTEMPT TO APPLY IT TO THE DETERMINATION OF THE TYPE OF PNEUMOCOCCUS IN SPUTUM

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Various methods have been devised for obtaining a precipitin antigen from bacteria. Many of these require prolonged incubation of broth cultures or more or less complicated methods of extraction where growth on solid media is used. In some attempts to use the precipitin reaction to determine the presence of antigen in sputa and feces, we developed the following simple and rapid method of making a bacterial precipitin antigen which seems applicable to most, and probably to all bacteria with the exception of the acid fast types.

To a heavy suspension of bacteria in distilled water, add sufficient alkaline hypochlorite solution[1] to give a final concentration of 5 per cent; boil over the free flame or heat in a water bath for several minutes. If the bacteria have not dissolved, add more of the alkaline hypochlorite solution and reheat. Repeat this process till the bacteria are completely dissolved. This is shown by the appearance of translucency. Add several drops of an alcoholic solution of phenolphthalein. Then add $\frac{N}{1}$-hydrochloric acid till the color is just discharged To this neutralized solution, add several volumes of 95 per cent alcohol. A copious precipitate which increases on standing, should result. The precipitate is collected by centrifuging and decanting the supernatant fluid. Add normal saline solution to the sediment to give approximately a one to twenty solution by volume and

[1] For convenience we have used the solution marketed under the trade name of "antiformin."

extract at the temperature of boiling water or boil over the free flame for from three to five minutes. Then centrifuge to clear the solution of the insoluble debris. The supernatant fluid is the finished antigen. It can be prepared in a half hour or less if necessary. This antigen can be reheated for sterilization at 100°C., apparently indefinitely.

Certain points must be considered in carrying out the various steps of the process if a concentrated and economical antigen is to be obtained Although broth cultures concentrated by sedimentation in the centrifuge may be used with success, it is preferable wherever possible, to use distilled water suspensions of organisms grown over large areas of agar.

The initial suspension of organisms must be relatively heavy, in fact, as little fluid as is needed should be used in making the suspension. Furthermore, it must not be diluted unduly in adding the alkaline hypochlorite solution or in the process of neutralization. The alkaline hypochlorite solution should be added as concentrated as is feasible in measuring the desired amounts. If the neutralized extract is very concentrated several volumes of alcohol will give a prompt, copious and almost complete precipitation or so nearly complete that it would not be economical to try to precipitate the remainder by further addition of alcohol. If, however, the extract is dilute, more alcohol is needed proportionately to obtain a precipitate and this separates more slowly. Even in only moderately dilute extracts the addition of more than several volumes of alcohol may only cloud the solution and flocculation will be very slight and much delayed.

The finished antigen, even after centrifuging, may be slightly opalescent. This is due to the presence of fine particles of bacterial debris, which are difficult to throw down. As the concentrated antigen may be diluted from ten to forty or more times and as the diluted antigen is very clear this opalescence is not a factor. If for some special purpose it is necessary to use the antigen in its concentrated form, the presence of this bacterial debris must be considered as it is agglutinable and will therefore, bind antibody and constitute part of the precipitate. In one instance we collected the insoluble debris after boiling typhoid

bacilli in a 30 per cent solution of "antiformin" for one-half hour; we washed the debris till no precipitable substance was present in the washings and then suspended the debris in normal saline. With dilutions of serum that gave precipitation with a finished antigen, the bacterial debris was promptly agglutinated. The control suspension without serum flocculated to some extent and settled out. Even the severe treatment given did not destroy the agglutinability of the bacterial "rests." The presence of the fine debris can be avoided to some extent by not breaking up the sediment too vigorously in making the final extract. If for some special reason the debris is objectionable it can be removed by filtration.

The most striking point in the method outlined above is the extreme resistance of the precipitable substances to the prolonged heating in alkaline hypochlorite solutions, which allows the rapid solution of dense suspensions with subsequent concentration of antigen. This is really the only original step in the method as extraction with cold alkaline hypochlorite solution has been used before. The other steps are all well known. That which is new in the method is the combination of these steps so as to allow the rapid preparation of antigen freed from extraneous substances added in the process of preparation. We have used the method to prepare antigens from pneumococci, using the sediment from centrifugalized broth cultures, with agar cultures of *B. typhosus*, with types of *B. paratyphosus*, with *B. diphtheriæ* and with *B. mallei* and have obtained such satisfactory results from all that we see no reason why the method is not applicable to all bacteria soluble in an alkaline hypochlorite solution.

Two questions arise in relation to this method. In the process how much precipitable substance is destroyed? We attempted to determine this by preparing antigens with different concentrations of alkaline hypochlorite solutions and employing different periods of heating. By preparing these so that the concentration of the end product was as comparable as possible we could see no evidence that there was any appreciable destruction of antigen.

The other point is how far specificity is injured if at all, by the severe treatment. If the factors entering into the immune

reaction are considered; that is, standardization of antigen determined by dilution and the addition of varied dilutions of immune serum, with the subsequent employment of graded dilutions of the serum against the standard of antigen thus determined, as well as due consideration of the time factor; viz., the rapidity of the reaction, there is no evidence of loss of specificity. If any of these factors are not considered, especially if the antigen is too concentrated, cross reactions become numerous and marked, especially with closely allied organisms.

With types of bacteria that can be easily separated because

TABLE 1

*Precipitin reactions—pneumococcus sera*

| PNEUMOCOCCUS ANTIGEN | TIME | SERUM TYPES AND DILUTIONS | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Type I | | | Type II | | | Type III | | |
| | | 1-1 | 1-4 | 1-9 | 1-1 | 1-4 | 1-9 | 1-1 | 1-4 | 1-9 |
| | *hours* | | | | | | | | | |
| Type I..... | 1 | ++* | + | ± | − | − | − | − | − | − |
| | 2 | +++ | ++ | +\| | ± | − | − | − | − | − |
| Type II.... | 1 | − | − | − | ++ | ++ | + | − | − | − |
| | 2 | ± | − | − | +++ | +++ | ++ | − | − | − |
| Type III... | 1 | − | − | − | − | − | − | + | − | − |
| | 2 | − | − | − | − | − | − | +\| | ± | − |

Antigen used = 0.2 cc. Serum solution = 0.2 cc.

*+++ = profuse precipitate. ++, +|, +, ±, ± = decreasing amounts of precipitate.

of their distinctly different agglutinability even a relatively concentrated antigen gives little evidence of cross reaction; that is, the antigen so prepared loses none of its specificity. The following table illustrates this, only a slight cross being noted after long incubation. The antigens were made from broth cultures treated with antiformin and prepared according to the method already given above.

It is not the place to go into the question of the relative specificity of precipitins and of agglutinins. Because we encountered more marked cross reactions with the precipitin reactions with

very closely allied bacteria than was evident using the same serum for agglutination reactions, we feared that we were destroying some specificity in these cases To exclude this possibility we prepared antigens according to the accepted method, that is, prolonged growth in broth and subsequent removal of bacteria by filtration and compared the results obtained from the use of such antigens with the results obtained with antigens prepared according to our method. With due regard to the factors mentioned above, that is, appropriate dilutions of antigens and of sera and consideration of the time factor, both types of antigens gave comparable results. The broth antigens being very much more dilute, did not introduce the factor of concentration of the antigens so markedly. The following tables are representative of the results obtained.

No final method of standardizing the antigens has been evolved. A relatively satisfactory method, however, is to determine the volume of sediment obtained by alcoholic precipitation, using graduated centrifuge tubes, and making the final suspension by adding up to twenty volumes of saline. With comparable antigens in this way, and having obtained the appropriate dilution of one antigen against its homologous serum other antigens can be similarly diluted in testing cross reactions of this serum. This was not carried further as the practical application of the precipitin reaction for differentiation of bacteria is relatively little used. The main interest of the method, in our mind is its great value in preparing material for teaching and for demonstration purposes and for courses in immunology. It should also be of value in experimental procedures where a readily obtainable supply of concentrated antigen is needed.

We have attempted to apply the method for diagnostic purposes for the extraction of antigen from feces and from sputa. The object in attempting to extract antigen from feces was to determine whether we could detect typhoid antigen in stools and in this way determine the presence of typhoid or allied bacilli without the necessity of a prolonged bacteriological examination. Thus far such attempts have been without success. Even extracts of feces in which 95 per cent of the bacterial flora are

typhoid bacilli fail to yield a precipitable extract. Evidently we are extracting other substances from the feces which appear

TABLE 2

*Comparison of broth and of antiformin antigens*

| ORGANISM | SERUM DILUTION (ANTI-TYPHOID) | BROTH ANTIGEN (UNDILUTED) | | | ANTIFORMIN ANTIGEN DILUTED 1:10 (ANTIGEN A) | | | ANTIFORMIN ANTIGEN DILUTED 1:80† (ANTIGEN A) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | One-half hour | One hour | Ice box* | One-half hour | One hour | Ice box | One-half hour | One hour | Ice box |
| *B. typhosus* | 1:1 | ++ | ++ | ++ | ++ | ++ | ++ | + | + | + |
| | 1:6 | +\| | +\| | +\| | ++ | ++ | ++ | + | + | + |
| | 1:12 | +\| | +\| | +\| | + | +\| | +\| | + | + | + |
| *B. paratyph* "A" | 1:1 | C | ± | ± | C | ± | + | − | − | − |
| | 1:6 | − | − | ± | − | ± | + | − | − | − |
| | 1:12 | − | − | ± | − | ± | + | − | − | − |
| *B. paratyph* "B" | 1:1 | − | − | ± | ± | ± | + | − | ± | ± |
| | 1:6 | − | − | ± | − | Sl.C | ± | − | − | ± |
| | 1:12 | − | − | − | − | − | ± | − | − | ± |
| *B. pullorum* | 1:1 | + | ++ | ++ | + | +\| | +\| | − | ± | ± |
| | 1:6 | + | +\| | +\| | + | + | +\| | − | ± | ± |
| | 1:12 | + | + | + | ± | + | +\| | − | ± | ± |
| *B. sanguinarium* | 1:1 | ++ | ++ | ++ | +\| | +\| | ++ | + | + | + |
| | 1:6 | +\| | +\| | +\| | +\| | +\| | +\| | + | + | + |
| | 1:12 | + | + | +\| | ± | + | +\| | + | + | + |
| *B. abortus equi* | 1:1 | − | Sl.C. | ± | Sl.C | ± | ± | − | − | − |
| | 1:6 | − | − | − | Sl.C | ± | ± | − | − | − |
| | 1:12 | − | − | − | Sl.C | ± | ± | − | − | − |
| *B. coli* | 1:1 | − | − | − | − | − | ± | − | − | − |
| | 1:6 | − | − | − | − | − | ± | − | − | − |
| | 1:12 | − | − | − | − | − | ± | − | − | − |

Sl.C = slightly cloudy. C = cloudy. ± = slight flocculation. + = distinct flocculation. +\| = flocculation more marked. ++ = heavy precipitate. Antigen 0.2 cc. and serum dilution 0.2 cc. used.

* Overnight.

† Symbols in these columns not comparable with those of very dilute antigen in table 3. The symbols in each table are in comparison with most voluminous precipitate obtained in series represented by a table.

TABLE 3

*Comparison of broth and of antiformin antigens*

| ORGANISM | SERUM DILUTION (ANTI-TYPHOID) | BROTH ANTIGEN (1:5) | | | ANTIFORMIN ANTIGEN (1:100) (ANTIGEN B.) | | |
|---|---|---|---|---|---|---|---|
| | | One-half hour | One hour | Ice box† | One-half hour | One hour | Ice box |
| *B. typhosus* | 1–10 | + \| | ++ | ++ | + \| | ++ | ++ |
| | 1–20 | + \| | + \| | ++ | + \| | ++ | ++ |
| | 1–50 | + | + \| | + \| | ± | + \| | + \| |
| | 1–75 | C. | + | + | – | ± | + \| |
| | 1–100 | – | ± | + | – | C. | + |
| *B. paratyph.* "A" | 1–10 | – | Sl.C | Sl.C | – | ± | + \| |
| | 1–20 | – | – | – | – | Sl.C | + |
| | 1–50 | – | – | – | – | – | + |
| | 1–75 | – | – | – | – | – | – |
| | 1–100 | – | – | – | – | – | – |
| *B. paratyph.* "B" | 1–10 | C | C | ± | – | ± | + \| |
| | 1–20 | – | – | – | – | Sl.C | + |
| | 1–50 | – | – | – | – | – | – |
| | 1–75 | – | – | – | – | – | – |
| | 1–100 | – | – | – | – | – | – |
| *B. pullorum** | 1–10 | + \| | ++ | ++ | ++ | ++ | ++ |
| | 1–20 | + \| | + \| | ++ | + \| | ++ | ++ |
| | 1–50 | ± | + | + | ± | + \| | + \| |
| | 1–75 | Sl.C | ± | + | – | + | + \| |
| | 1–100 | – | C | + | – | C | + |
| *B. sanguinarium** | 1–10 | + \| | ++ | ++ | ++ | ++ | ++ |
| | 1–20 | + \| | + \| | ++ | + \| | ++ | ++ |
| | 1–50 | + | + \| | + \| | C | + \| | + \| |
| | 1–75 | C | C | + | – | + | + \| |
| | 1–100 | – | – | + | – | C. | + |
| *B. Abortus equinus* | 1–10 | C | C | C | – | – | ± |
| | 1–20 | – | – | – | – | – | ± |
| | 1–50 | – | – | – | – | – | – |
| | 1–75 | – | – | – | – | – | – |
| | 1–100 | – | – | – | – | – | – |
| *B. coli* | 1–10 | C | C | ± | – | – | – |
| | 1–20 | – | – | – | – | – | – |
| | 1–50 | – | – | – | – | – | – |
| | 1–75 | – | – | – | – | – | – |
| | 1–100 | – | – | – | – | – | – |

Sl.C = slightly cloudy. C = cloudy. ± = slight flocculation. + = distinct flocculation. + | = more marked flocculation. ++ = heavy precipitate. Antigen — 0.2 cc. and serum dilution 0.2 cc. used.

* These two paratyphoid types of fowl origin were selected because of their very close agglutinative relationship one to the other and to *B. typhosus*. The extreme cross precipitation with these types in this series is no indication of loss of specificity. Compare with similar agglutinative results of Smith and Ten Broeck. (Journ. Med. Res., 1915, xxxi, 549.)

† Overnight.

in the final antigens and interfere with the reaction. Further work is in progress to determine whether we can eliminate the inhibiting factors and to determine their character.

The application of this method in preparing antigens from sputa was attempted with the hope of evolving a rapid method of determining the type of pneumococcus present. Modifications have been found necessary and although the method at present used is far from satisfactory and has only given positive results in a relatively small percentage of the specimens examined, it is given in the hope that it will serve as a basis to which further improvements can be added. We have been handicapped by lack of satisfactory material and further work will not be possible till the next pneumonia season.[2]

The success of the method depends on several factors. One difficulty is that not infrequently the sputum submitted is salivary or pharyngeal and not a specimen raised by coughing. Naturally a positive result can only be obtained if the sputum is comparatively rich in pneumococci. The technical difficulties are first, the necessity of removing the albuminous and mucous material which otherwise passes over to the final extract, making it thick or gelatinous; secondly, the fact that the alcohol will not give a sufficiently prompt and complete precipitation where little antigen is present. The method has been modified in the attempt to overcome the first difficulty mentioned.

To the sputum add sufficient antiformin to give a concentration of from 3 to 5 per cent. If the sputum is thin, add the concentrated reagent; if it is very thick, dilute by adding one-half its volume of the appropriately diluted antiformin; that is, no more dilution should be made than is necessary for final digestion. The mixture is then heated to 100°C. and shaken till it becomes fluid. Add a few drops of phenolphthalein to the hot antiformin-sputum and neutralize with acid. Then add a sufficient excess of acid to cause coagulation of the coagulable substances in the fluid, centrifugalize and collect the supernatant fluid; neutralize and add several volumes of alcohol, making a sufficient mixture to fill a 15 cc. test tube. If a precipitate does not separate, let

[2] Three successive Type I sputa just received have been positive.

the tubes stand for a while; then if a precipitate is formed, centrifugalize and decant the supernatant fluid; add 1 cc. of saline and heat to extract the sediment and centrifuge to clear. (If too much saline is added the extract may be too dilute and beyond the reaction stage.) The supernatant fluid is then added to the tubes containing the "type" sera and the tubes are incubated in the water bath. Each tube contains 0.2 cc. of serum and 0.2 cc. of antigenic fluid The rapidity of the reaction varies with the antigenic content of this fluid With a fluid that is rich in antigen, there may be seen an immediate clouding followed by a prompt separation of the precipitate, if serum and antigen are mixed in the tube, or by a heavy ring of precipitate if the antigen is carefully "layered" on the serum. With a fluid less rich in antigen, the reaction is slower; with little antigen, it may be absent. Should the finished antigen be thick an attempt may be made to use it after dilution, but this is usually unsuccessful.

As is evident the method as far as evolved is not wholly satisfactory, but even in its present state, sputa rich in antigen can be examined and the type diagnosed in from one-half to one hour. Any increase in the number of positive reactions and in the rapidity of the reaction will depend on overcoming the technical difficulties already mentioned. Thus far no false reactions have been encountered unless the incubation was unduly prolonged. These may be found to occur occasionally because of the presence of much extraneous material in the finished antigen.

One fact has impressed us very strongly in carrying out the above work; viz., the very marked difference between bacterial and serum precipitation in regard to the dilution limits of the antigen. A serum antigen—as is well known—may be diluted as much as 1 to 50,000 times and still give a precipitate with a highly potent homologous immune serum. A bacterial antigen must be much more concentrated. The alcoholic precipitate as described when dissolved in twenty times its volume of saline cannot be diluted more than eighty or one hundred times at most. It may be that this is due to the fact that only a small fraction of the bacterial protein is precipitable. Another difference is the extraordinary resistance of the bacterial antigen to

the action of heat and of chemical agents. Acidification and boiling are without effect. In fact we have cleared antigens by adding abumin and coagulating it by heat and by acidification and neutralizing the filtrate.

## SUMMARY

A simple method of preparing a precipitin antigen from bacteria is presented, which allows of the preparation of such antigens with a rapidity and in concentration hitherto impossible. An attempt to modify the method to extract antigen from pneumonic sputum to determine rapidly the type of pneumococcus present has been partially successful. It is presented as a possible basis for further improvements.

# ON VON DUNGERN'S INDIGO TEST FOR SYPHILIS

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Von Dungern's indigo test for syphilis, which has been described in the issue of the Münchener Medizinische Wochenschrift for September, 1915 (no. 36), was recently examined by Edward P. Flood (1) and it was proved by him that its results are not constant and therefore not practically applicable to the diagnosis of syphilis.

A further study of this reaction was undertaken in our laboratory to determine more precisely its value. The reagents, which were used in our test, were prepared as follows. Though Flood stated that the preparation of the reagents was not easy and that he was obliged to introduce a modification of von Dungern's procedure in order to get a clear solution of the indigo, I was able to prepare them easily in the same manner as did von Dungern, namely:

One gram of indigo was carefully triturated and 4 cc. of concentrated sulphuric acid were added to it. After the mixture had stood for forty-eight hours (the indigo being then completely dissolved), distilled water was added up to 100 cc. A gray sediment, which was caused by unpurity of the material, was thrown down. The solution thus obtained was finally clear and indigo blue in color.

The Fehling solution no. 2 was prepared as usual; 173 grams of Rochelle salt were dissolved in 200 cc. of hot distilled water and 73 cc. of sodium hydroxide (specific weight 1.5) were added to this. Distilled water was finally added up to 500 cc.

To 1.5 cc. of the indigo-sulphuric acid there were added 10 cc. of distilled water and to 4 cc. of this solution there was added 1 cc. of Fehling no. 2. The final solution was greenish yellow

and free from any precipitate. This reagent was always used soon after the final mixture was prepared.

At first, the test was carried out exactly as described by von Dungern in order to determine whether this test is practically applicable or not. The sera to be tested were obtained from the dermatological clinic and from the medical clinic of our university. The results of these tests with syphilitic and non-syphilitic sera are shown in table 1. In our tests they were controlled with the Wassermann reaction.

TABLE 1

*Dungern's indigo test*

| REAGENT, 0.2 CC. INACTIVATED SERUM, 0.3 CC. | COAGULATION | SEMI-COAGULATION | NO COAGULATION |
|---|---|---|---|
| Wassermann ( + ) sera | 1 | 0 | 22 |
| Wassermann ( − ) sera | 1 | 2 | 31 |

Almost all of the sera did not coagulate in our tests.

Now comparing these results of the indigo test with the normal and the syphilitic sera, we see that the test is not specific, and that there is no difference between them at all. Such a non-specific reaction is evidently unavailable for the clinical diagnosis of syphilis.

In order to determine at what point coagulation of sera occurs, the reagent was used in various amounts (0.2, 0.1, 0.05 cc.). The results are as follows.

TABLE 2

*Coagulation point of sera, to which the reagent was added in various amounts*

| INACTIVATED SERUM, 0.3 CC. | REAGENT | COAGULATION | SEMI-COAGULATION | NO COAGULATION |
|---|---|---|---|---|
| | cc. | | | |
| Wassermann (+) sera | 0.2 | 1 | 0 | 22 |
| | 0.1 | 20 | 3 | 0 |
| | 0.05 | 23 | 0 | 0 |
| Wassermann (−) sera | 0.2 | 1 | 2 | 31 |
| | 0.1 | 29 | 5 | 0 |
| | 0.05 | 34 | 0 | 0 |

Almost all sera both Wassermann positive and negative coagulated with 0.1 and 0.05 cc. of this reagent. But with 0.2 cc. most of them do not coagulate; that is to say, their heat coagulation is prevented by 0.2 cc. of the reagent almost in all cases.

When 0.3 cc. of the sera was mixed with 0.2 cc. of distilled water or of physiological salt solution without any of the reagent the heat coagulation occurred soon after fifteen or twenty seconds.

In order to solve the question why the heat coagulation of the sera is prevented by the reagent, I have proceeded to the following tests.

The reagent contains indigo, sodium sulphate, sodium and potassium tartrate and sodium hydroxide. Which of these substances plays a part in preventing the heat coagulation of sera; is it the indigo or the salts or the alkali?

## I. INDIGO

The indigo sulphuric acid was neutralized with barium hydroxide, and we obtained thus a neutral solution of indigo free from sulphuric acid. This neutral solution was concentrated until it possessed the same depth of color as the original indigo sulphuric acid.

Indigo solution (0.2 cc.) was added to the syphilitic and non-syphilitic sera in order to see whether the indigo alone has the power to prevent the heat coagulation of sera. The results are shown in table 3.

TABLE 3

*Tests with pure indigo solution free from acid*

| PURE INDIGO SOLUTION (FREE FROM ACID), 0.2 CC. | COAGULATION | SEMI-COAGULATION | NO COAGULATION |
|---|---|---|---|
| Wassermann (+) sera 0.3 cc | 6 | 0 | 0 |
| Wassermann (−) sera 0.3 cc | 4 | 0 | 0 |

Coagulation occurred always rapidly, in fifteen or twenty seconds, as if only distilled water had been added, which proved that indigo does not play any part in preventing the heat coagulation of sera.

### II. COAGULATION TESTS WITH SALTS

Comparing the very small quantity of neutral sodium sulphate with that of the Rochelle salt in the reagent, we can neglect the former, because it is neutral salt and does not prevent the heat coagulation; on the contrary, it can even accelerate the coagulation in a small degree.

It was proved in test tubes that the Rochelle salt can accelerate coagulation even in the presence of the concentrated sodium hydroxide. Thus it is evident that neither indigo nor salts prevent the heat coagulation of sera.

### III. TESTS WITH ALKALI

Finally it remains to test the action of sodium hydroxide. The alkalinity of the reagent was titrated. Flood states that 1 cc. of it requires 1.21 cc. of $\frac{N}{10}$ acid for its neutralization, but I have found not only by titrating but also by mathematical calculation from the molecular weights of the ingredients of the reagent that 1 cc. of it requires 3.3 cc. to 3.4 cc. of $\frac{N}{10}$ acid for its neutralization.

Acidity of indigo sulphuric acid in the reagent:

$$37\ N \times 4 / 100 \times 1.5 / (10 + 1.5) \times 4 / (4 + 1) = 0.154\ N$$

Alkalinity of Fehling no. 2 in the reagent:

(sodium hydroxide specific weight 1.5 = 17 N)

$$17\ N \times 73 / 500 \times 1 / (4 + 1) = 0.496\ N$$

Therefore, the alkalinity of the reagent, must be

$$0.496\ N\ (\text{alkalinity}) - 0.154\ N\ (\text{acidity}) = 0.342\ N\ (\text{alkalinity})$$

To determine at just what point of alkalinity the sera coagulate, several Fehling solutions of different alkalinity were so prepared as its alkalinity in the test tubes may be each 0.12 N, 0.10 N, 0.08 N, 0.06 N, 0.04 N, 0.02 N. These solutions of different alkalinity were added (a) to the indigo and (b) to the reagent without indigo (viz., to 4 cc. of concentrated sulphuric

acid there were added 96 cc. of distilled water). The results are shown in table 4.

TABLE 4

*Coagulation tests with solutions of different alkalinities*

| SERA, 0.3 CC. REAGENT, 0.2 CC. | | ALKALINITY N | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0.12 | 0.10 | 0.08 | 0.06 | 0.04 | 0.02 |
| Syphilitic sera | | | | | | | |
| No. 1 | (a) | − | − | − | − | + | + |
| | (b) | − | − | − | − | + | + |
| No. 2 | (a) | − | − | + | + | + | + |
| | (b) | − | − | + | + | + | + |
| No. 3 | (a) | − | − | − | − | − | + |
| | (b) | − | − | − | − | − | + |
| Non syphilitic sera | | | | | | | |
| No. 4 | (a) | − | − | + | + | + | + |
| | (b) | − | − | + | + | + | + |
| No. 5 | (a) | − | − | − | − | − | + |
| | (b) | − | − | − | − | − | + |
| No. 6 | (a) | − | − | − | − | − | + |
| | (b) | − | − | − | − | − | + |

(−) = non coagulation. (+) = coagulation. (a) Reagent with indigo, (b) Reagent without indigo.

As we see in the above table, each serum has its coagulation point in its 60 per cent concentration (serum 0.3 cc., reagent 0.2 cc.), and we can not find any difference, as to this coagulation point, between syphilitic and non-syphilitic sera.

TABLE 5

| SERUM, 0.3 CC. | | ALKALINITY IN TEST TUBES | | | |
|---|---|---|---|---|---|
| | | 0.12 N | 0.10 N | 0.08 N | 0.06 N |
| Wassermann (+) sera | No. 1 | − | − | + | + |
| | No. 2 | ± | + | + | + |
| | No. 3 | − | ± | + | + |
| Wassermann (−) sera | No. 4 | − | ± | + | + |
| | No. 5 | − | − | ± | + |
| | No. 6 | − | + | + | + |

(+) = coagulation. (−) = non coagulation.

Now suspecting that not only the alkalinity but also variation in the concentration of the serum might serve to cause variation in the heat coagulation, we have tested the sera in its 75 per cent concentration instead of 60 per cent, viz., to 0.3 cc. of serum was added 0.1 cc. of the reagent. The alkalinity of the reagent used here was so prepared that the alkalinity in the test tubes was 0.12 N, 0.10 N, 0.08 N, 0.06 N respectively. The results are shown in table 5.

Comparing these results with those shown in table 4, we see that the more diluted the sera, the more easily their heat coagulation can be prevented by alkali.

## SUMMARY

1. Von Dungern's indigo test for syphilis is practically unavailable for the serum diagnosis of syphilis, because we can not find any difference as to this test between syphilitic and non-syphilitic sera.

2. Among the substances that the reagent contains neither indigo nor salts play any part in the inhibition of the coagulation, as von Dungern formerly thought.

3. The coagulation of sera in this reaction is prevented only by the alkali, as Flood proved.

4. The degree of alkalinity that can prevent the coagulation of serum, varies according to the individual, not according to the presence or absence of syphilis.

5. On the other hand, the alkalinity that prevents the coagulation of sera varies according to the concentration of the sera; that is, the more diluted the serum, the more easily may its heat coagulation be prevented by alkali.

I wish to express my thanks to Prof. Dr. K. Katayama and Prof. Dr. S. Mita for suggesting the subject and for their kind advice.

## REFERENCE

(1) Flood: Journal of Immunology, 1916, **2**, 69.

# EXTRACTS OF ANTIBODIES OBTAINED FROM SPECIFIC PRECIPITATES OF TYPHOID-ANTITYPHOID SERUM COMPLEX

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An investigation was undertaken in this laboratory with a view to precipitating the antibodies from antityphoid sera by means of a specific antigen and subsequently dissociating this antigen-antibody complex, obtaining the antibodies in a solution as free from foreign protein as possible.

As is well known, a number of antityphoid sera have been prepared and occasionally physicians have reported good results with them, but their use has never been general. In order to be successful with a bactericidal serum it seems necessary to inject large amounts. For example, Cole (1), in the treatment of pneumonia, by using very large quantities of antipneumococcus serum (from 190 to 700 cc.) has had success where others have failed. Owing to the danger of "serum sickness" following the injection of large amounts of horse serum, physicians have hesitated to treat their patients with it. Thus, the importance of eliminating as far as possible all extraneous protein and at the same time the harmful effects that it produces, can readily be seen. It was with this aim in view that the experiments described below were begun.

Within the last fifteen years great interest has been shown by investigators in regard to the fate of the antibodies in the precipitin reaction. Gay (2) was the first to demonstrate that complement was fixed by precipitates. He later (3) amplified his earlier studies and concluded that "alexin fixation by a mixture of serum and antiserum is produced by an antigen-antibody complex distinct from precipitin-

ogen-precipitin but usually brought down by the precipitate in its formation in such a way as to give the appearance that fixation is produced by the precipitate itself." Muir and Martin (4), Toyosumi (5) and Zinsser (6) likewise showed that precipitates had complement-fixing properties. In the case of agglutinins, Von Eisler and Tsuru (7) showed that normal hemagglutinins were removed from the serum through precipitation while Landsteiner and Prasek (8) proved that both hemagglutinins and bacterial agglutinins were brought down with the precipitates. Gay and Chickering (9) found that the protective bodies in an antipneumococcus serum were carried down with the precipitate.

Other investigators have shown that it is possible to break up the antigen-antibody complexes. Matsui (10) succeeded in extracting bactericidal bodies from cholera vibrios after the former had united with the latter. Muir (11) removed the hemolytic amboceptor from red blood corpuscles, while Landsteiner (12) separated agglutinins from their antigens. Chickering (13) was able to dissociate the precipitates formed by the union of pneumococcus antigen and antiserum and get the antibodies into solution. He found that his extracts contained agglutinins and precipitins besides protecting susceptible animals as efficiently as the original serum.

## EXPERIMENTS AND RESULTS

Through the kindness of Professor Park an antityphoid serum having high agglutinating and bactericidal titers was obtained. The serum was taken from a horse that had been immunized with increasing intravenous injections of dead and then living typhoid bacilli at definite intervals for a number of months.

In order to determine whether antibodies beside the precipitins were carried down with the precipitate, the original serum, the supernatant serum and the extract obtained from the precipitate were, in each experiment, tested for their agglutinin content. This antibody was chosen in preference to the others because of the ease with which the tests could be made. However, the extract that appeared to be most promising was also tested for bactericidal, complement-fixing and protective bodies. The macroscopic agglutinin test was used. 1 cc. of a twenty-four hour typhoid broth culture was added to an equal amount of the

serum or extract. The tubes were incubated at 37.5°C. for one hour and then left over-night in the ice-box. Readings were made the following morning. In the preliminary experiments extracts were prepared according to the method that Chickering had found to be best. The precipitates, which had been washed three times with normal salt solution were emulsified in normal saline to which had been added a few drops of a 1 per cent sodium carbonate solution, and were then heated for one hour at 42°C. with occasional shaking.

The method which Chickering used in preparing his antigen, namely, shaking in saline pneumococci that had been previously killed with acetone and dried in vacuo, was found to be entirely unsatisfactory in the case of the typhoid bacillus. This was undoubtedly due to the greater resistance of the typhoid bacilli to autolysis. In attempting to get a suitable antigen, the following procedures were used: (1) saline suspension of bacteria killed by heating at 54°C. for 1 hour; (2) extracts of bacteria killed with heat or acetone, dried *in vacuo* over sulphuric acid and shaken in saline or distilled water for lengths of time varying from ten minutes to twenty hours; (3) extracts of bacteria dried, ground in a mortar and then shaken in saline, (4) extracts of bacteria shaken in distilled water from seven to sixteen hours and then allowed to autolyze at temperatures ranging from 37.5°C. to 54°C. for an additional sixteen hours; (6) extracts of bacteria allowed to remain at 37.5°C. in saline or distilled water for ten days. Whenever distilled water was used the extract was rendered isotonic before adding it to the serum. None of the above antigens gave good results. Fair results were obtained with an extract of bacteria that had been killed with acetone, dried and ground eight hours a day for six days. This antigen removed approximately one-half of the agglutinins of the serum. The best results were obtained with an extract made by digesting the bacilli in a 2.5 per cent solution of antiformin. The bacterial growth on an agar slant in a quart Blake bottle was suspended in 3 cc. distilled water and was added to an equivalent amount of a 5 per cent antiformin solution. This mixture was allowed to remain at room temperature for one hour, after which it was

centrifugalized at high speed for three-quarters of an hour. The supernatant liquid was rendered neutral by the addition of normal hydrochloric acid. Equal amounts of serum and antigen solution were shaken together and put in the incubator. At the end of two hours a dense precipitate was observed. After remaining in the ice-box overnight the mixture was centrifugalized. The supernatant liquid was withdrawn, the precipitate was washed three times and then was extracted according to the method described above in alkaline saline solution equivalent to the amount of the serum used. The serum, supernatant and extract were then tested for their agglutinin content. The titer of the serum was 1:20,000; that of the supernatant was 1:4000, while that of the extract was 1:1000. It is thus seen that approximately three-fourths of the agglutinins were removed from the serum, while one-sixteenth of that amount was recovered in the extract.

By treating various dilutions of the antigen with normal horse serum as well as with immune serum, it was seen that the antigen acted specifically.

At the suggestion of Prof. Charles Krumwiede the antigen was purified in the following manner. First, the antigen solution was precipitated in three volumes of absolute alcohol. After centrifugalization, the precipitate was dried and then taken up in an amount of normal saline solution equivalent to the original volume of the antigen. After being shaken at 37°C. for a few minutes the precipitate went into solution. This purified antigen was found to be just as potent in carrying down agglutinins from the immune serum as the untreated antigen. It had the advantage of being rid of the high salt content present in the untreated antigen due to the large amount of alkali in the antiformin and its subsequent neutralization with hydrochloric acid.

### DETERMINATION OF THE AMOUNT OF ANTIGEN NECESSARY TO BRING ABOUT THE MAXIMUM PRECIPITATION OF ANTIBODIES

In order to determine the optimum amount of antigen for precipitating the antibodies from the serum, various amounts

of antigen solution and serum were mixed together. The amount of serum remained constant, while the amount of antigen varied from eight times the volume of the serum to one-eighth of its volume. Various amounts of diluted antigen were also used in order to determine whether the process took place better in weak than in concentrated solution. The tubes containing the mixtures were incubated for two hours and then left in the ice-box overnight. Abundant precipitates appeared in all of the tubes with the exception of those that contained 0.5 cc. and 0.25 cc. of antigen. After centrifugation, the supernatant liquids were pipetted off and titrated for their agglutinin content. In the last column of table 1 the actual titers of the supernatants are given, allowances having been made for the dilution of the serum by the antigen.

TABLE 1

*Comparative agglutinating power of whole serum and sera treated with various quantities of antigen. Titer of whole serum equals 1: 20,000*

| SUPERNATANT SERUM | ACTUAL AMOUNT OF ANTIGEN USED | AMOUNT OF DILUTED ANTIGEN USED | DILUTIONS | | | | | | | | | | | | CORRECTED TITER OF THE SOLUTION |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 200 | 400 | 800 | 1000 | 1600 | 2000 | 3000 | 4000 | 5000 | 8000 | 10,000 | 15,000 | 20,000 | |
| | cc. | cc. | | | | | | | | | | | | | | |
| 1 | 8.0 | | + | + | + | + | + | − | − | − | − | − | − | − | − | 1: 8000 |
| 2 | 6.0 | | + | + | + | + | + | − | − | − | − | − | − | − | − | 1: 6400 |
| 3 | 4.0 | | + | + | + | + | + | + | − | − | − | − | − | − | − | 1: 6000 |
| 4 | 2.0 | | + | + | + | + | + | + | + | + | − | − | − | − | − | 1: 8000 |
| 5 | 1.0 | 2 (1: 2) | + | + | + | + | + | + | + | + | − | − | − | − | − | 1: 8000 |
| 6 | 0.5 | 2 (1: 4) | + | + | + | + | + | + | + | + | + | + | − | − | − | 1: 16,000 |
| 7 | 0.25 | 2 (1: 8) | + | + | + | + | + | + | + | + | + | + | + | − | − | 1: 20,000 |
| 8 | 2.0 | 4 (1: 2) | + | + | + | + | + | + | − | − | − | − | − | − | − | 1: 6000 |
| 9 | 2.0 | 16 (1: 8) | + | + | + | + | − | − | − | − | − | − | − | − | − | 1: 9000 |
| 10 | 2.0 | 20 (1: 10) | + | + | + | − | − | − | − | − | − | − | − | − | − | 1: 8800 |

The results of this experiment show that little is to be gained by the use of large quantities of antigen. An amount of antigen solution, prepared as described above, equal to that of the serum seems to give as good results as can be obtained. If too little antigen is used there is, as might be expected, little precipitation. The diluted antigens seem to work a little better than the undiluted ones; e.g., 2 cc. of a 1: 2 dilution reduces the agglutinating

titer of the serum to the same degree that 2 cc. of undiluted antigen does.

## DETERMINATION OF THE BEST METHOD OF EXTRACTING THE ANTIBODIES FROM THE PRECIPITATE

### *Effects of alkali*

The effect of weakly alkaline and strongly alkaline solutions in dissociating the precipitates was tested. Twelve tubes, each containing 2 cc. of antigen and 2 cc. of serum were incubated for two hours and then put in the ice-box overnight. The precipitates were washed. Those in the first seven tubes were suspended in 2 cc. saline with small amounts of a 1 per cent solution of sodium carbonate. The precipitates in the remaining tubes were suspended in 2 cc. of a solution of sodium carbonate or sodium hydroxide as indicated in table 2. All of the

TABLE 2

*Comparative agglutinating power of extracts treated with various amounts of alkali. Titer of whole serum equals 1: 20,000. Titer of supernatant serum equals 1: 8000*

| TUBE | ALKALINE SOLUTION | AMOUNT | TEMPERATURE | TIME | DILUTIONS | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | 20 | 200 | 400 | 800 | 1600 | 2000 | 3000 |
| | | *cc.* | *°C.* | *hour* | | | | | | | |
| 1 | 1.0 per cent $Na_2CO_3$ | 0 | 42 | 1 | + | + | + | + | − | − | − |
| 2 | 1.0 per cent $Na_2CO_3$ | 0.01 | 42 | 1 | + | + | + | + | − | − | − |
| 3 | 1.0 per cent $Na_2CO_3$ | 0.02 | 42 | 1 | + | + | + | + | − | − | − |
| 4 | 1.0 per cent $Na_2CO_3$ | 0.03 | 42 | 1 | + | + | + | + | − | − | − |
| 5 | 1.0 per cent $Na_2CO_3$ | 0.04 | 42 | 1 | + | + | + | + | − | − | − |
| 6 | 1.0 per cent $Na_2CO_3$ | 0.05 | 42 | 1 | + | + | + | + | ± | − | − |
| 7 | 1.0 per cent $Na_2CO_3$ | 0.1 | 42 | 1 | + | + | + | + | − | − | − |
| 8 | 1.0 per cent $Na_2CO_3$ | 2.0 | 42 | 1 | + | + | + | + | − | − | − |
| 9 | 0.5 per cent NaOH | 2.0 | 42 | 1 | + | + | + | + | + | − | − |
| 10 | 1.0 per cent NaOH | 2.0 | 42 | 1 | + | + | + | + | + | − | − |
| 11 | 2.5 per cent NaOH | 2.0 | 42 | 1 | + | + | − | − | − | − | − |
| 12 | 5.0 per cent NaOH | 2.0 | 42 | 1 | − | − | − | − | − | − | − |

tubes were put in a 42°C. water-bath and shaken gently for one hour. The precipitate in tube 12 (5 per cent NaOH) went into solution at once; the precipitate in tube 11 (2.5 per cent

NaOH) and half of that in tube 10 (1 per cent NaOH) were dissolved at the end of an hour.

The small amounts of alkali in the extracts evidently exert no beneficial effect, for the control tube (table 2) shows as high a titer as the others. Larger amounts of alkali are helpful. The results also clearly indicate that a strongly alkaline solution destroys the antibodies.

*Effect of time and temperature*

Ten tubes, each containing 2 cc. of antigen and 2 cc. of serum, were incubated for two hours and then put in the ice-box over-

TABLE 3

*Comparative agglutinating power of extracts of precipitates treated with weakly alkaline solutions for various lengths of time at different temperatures. Titer of whole serum equals 1: 20,000. Titer of supernatant serum equals 1: 6000*

| TUBE | ALKALINE SOLUTION | AMOUNT | TEMPERATURE | TIME | DILUTIONS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | 20 | 200 | 400 | 800 | 1600 | 2000 |
| | | *cc.* | *°C.* | *hours* | | | | | | |
| 1 | 1 per cent $Na_2CO_3$ | 0.05 | 42 | 1 | + | + | + | + | − | − |
| 2 | 1 per cent $Na_2CO_3$ | 0.05 | 42 | 2 | + | + | + | + | − | − |
| 3 | 1 per cent $Na_2CO_3$ | 0.05 | 42 | 3 | + | + | + | + | − | − |
| 4 | 1 per cent $Na_2CO_3$ | 0.05 | 42 | 4 | + | + | + | + | − | − |
| 5 | 1 per cent $Na_2CO_3$ | 0.05 | 42 | 5 | + | + | + | + | − | − |
| 6 | 1 per cent $Na_2CO_3$ | 0.05 | 42 | 6 | + | + | + | + | − | − |
| 7 | 1 per cent $Na_2CO_3$ | 0.05 | 54 | 1 | + | + | + | + | − | − |
| 8 | 1 per cent $Na_2CO_3$ | 0.05 | 54 | 2 | + | + | + | + | − | − |
| 9 | 1 per cent $Na_2CO_3$ | 0.05 | 37 | 24 | + | + | + | + | − | − |
| 10 | 1 per cent $Na_2CO_3$ | 0.05 | 20 | 24 | + | + | − | − | − | − |

night. The precipitates were washed three times with normal saline solution and then each was suspended in 2 cc. of saline with 0.5 cc. of a 1 per cent sodium carbonate solution. The tubes were left at various temperatures for varying lengths of time.

It can readily be seen from table 3 that practically nothing is gained by incubating the mixture for more than one hour at 42°C. At the end of that time a certain amount of dissociation of the precipitate has taken place. A longer time or higher temperature evidently adds nothing.

*Effect of washing the precipitate*

The next point considered was whether any of the agglutinins were lost in washing the precipitate. Accordingly, the precipitates which had formed in four tubes, through the mixture of 2 cc. of serum and 2 cc. of antigen in each, were treated as follows: No. 1 was suspended in 2 cc. of a 1 per cent sodium carbonate solution: Nos. 2, 3 and 4 were washed once, twice and three times respectively (2 cc. of normal saline solution being used

TABLE 4

*Comparative agglutinating power of extracts of washed and unwashed precipitates. Titer of whole serum equals 1: 20,000. Titer of supernatant serum equals 1: 4000*

| TUBE | PREPARATION OF SOLUTION | DILUTIONS | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 20 | 200 | 400 | 800 | 1600 | 2000 | 3000 | 4000 | 5000 |
| 1 | Precipitate treated with1 per cent sodium carbonate solution | + | + | + | + | + | + | + | − | − |
| 2 | Precipitate, washed once, treated with 1 per cent sodium carbonate solution | + | + | + | + | + | + | − | − | − |
| 3 | First washing | + | + | + | + | + | − | − | − | − |
| 4 | Precipitate, washed twice, treated with 1 per cent sodium carbonate solution | + | + | + | + | + | − | − | − | − |
| 5 | Second washing | + | + | − | − | − | − | − | − | − |
| 6 | Precipitate, washed three times, treated with 1 per cent sodium carbonate solution | + | + | + | + | + | − | − | − | − |
| 7 | Third washing | + | − | − | − | − | − | − | − | − |

for each washing) and each was then suspended in 2 cc. of 1 per cent sodium carbonate. The extracts and the washings were tested for their agglutinin content.

It will be seen from table 4 that there is a reduction in the agglutinin content of an extract of a washed precipitate. We believe this to be due to the fact that a small amount of serum, which remains on the unwashed precipitate, is carried over into the extract. The washings remove all of this serum. In other experiments it was found that when the precipitate was tightly packed in the centrifuge tube and the supernatant serum carefully removed with a capillary pipette, the titer of the first washing was not above 1: 800. It may further be seen from table

4 that the titers of the second and third washings are in no way comparable to that of the first. We may conclude that the amount of agglutinins lost through the dissociation of the precipitate by washings, when the process is carried on within a few minutes and at room temperature, is negligible.

*Tests for bactericidal bodies*

An extract was prepared in the usual way by treating the precipitate with an amount of slightly alkaline saline solution equal to the volume of the original serum. The whole serum, the supernatant and the extract were tested in vitro for their bactericidal power.

*Method.* The three solutions were inactivated by heating at 56°C. for one hour and were then diluted with normal saline. One cubic centimeter of each dilution was mixed with 1 cc. of a 1: 10 dilution of complement and 0.1 cc. of a 1: 2000 dilution of a twenty-four-hour typhoid broth culture. The complement was obtained from the pooled sera of three normal guinea-pigs. The tubes were incubated at 37°C. for two hours, at the end of which time the contents of each tube were plated in agar. The plates were incubated at 37°C. for twenty-four hours and then the colonies were counted. Controls were set up as follows: (1) culture with saline solution, in order to determine the number of bacilli used in each test; (2) culture with complement, in order to see whether the complement of itself had any bactericidal action; and (3) culture with each of the inactivated solutions, in order to see whether all of their native complement had been destroyed.

By consulting table 5 it is seen that the whole serum shows a definite bactericidal action in a dilution of 1: 40,000, the supernatant in a dilution of 1: 30,000 and the extract in a dilution of 1: 20,000. We may roughly say, therefore, that about half of the bactericidal bodies present in the whole serum are recoverable in the extract.

*Tests for complement-fixing bodies*

The whole serum, the supernatant and the extract were next tested for their complement-fixing power. The antigen pre-

TABLE 5

*Comparative bactericidal power of whole serum, supernatant and extract*

| | TYPHOID CULTURE DILUTED 1:2000 | SOLUTION | 1 CC. OF DILUTION | GUINEA-PIG COMPLEMENT DILUTED 1:10 | NUMBER OF COLONIES |
|---|---|---|---|---|---|
| | *cc.* | | | *cc.* | |
| Controls | 0.1 | | | | 3,000 |
| | 0.1 | | | | 1,200 |
| Complement controls | 0.1 | | | 1 | 1,200 |
| | 0.1 | | | 1 | 1,400 |
| Serum control | 0.1 | Whole serum | 1: 10,000 | | 3,200 |
| | 0.1 | Whole serum | 1: 10,000 | 1 | 650 |
| | 0.1 | Whole serum | 1: 20,000 | 1 | 700 |
| | 0.1 | Whole serum | 1: 30,000 | 1 | 720 |
| | 0.1 | Whole serum | 1: 40,000 | 1 | 750 |
| | 0.1 | Whole serum | 1: 60,000 | 1 | 1,120 |
| | 0.1 | Whole serum | 1: 80,000 | 1 | 1,200 |
| | 0.1 | Whole serum | 1: 100,000 | 1 | 1,280 |
| Supernatant serum control | 0.1 | Supernatant serum | 1: 5,000 | | 3060 |
| | 0.1 | Supernatant serum | 1: 5,000 | 1 | 480 |
| | 0.1 | Supernatant serum | 1: 10,000 | 1 | 680 |
| | 0.1 | Supernatant serum | 1: 20,000 | 1 | 720 |
| | 0.1 | Supernatant serum | 1: 30,000 | 1 | 900 |
| | 0.1 | Supernatant serum | 1: 40,000 | 1 | 1,100 |
| | 0.1 | Supernatant serum | 1: 60,000 | 1 | 1,300 |
| | 0.1 | Supernatant serum | 1: 80,000 | 1 | 1,100 |
| | 0.1 | Supernatant serum | 1: 100,000 | 1 | 1,200 |
| Extract control | 0.1 | Extract | 1 100 | | 3,500 |
| | 0.1 | Extract | 1: 100 | 1 | 900 |
| | 0.1 | Extract | 1: 1,000 | 1 | 600 |
| | 0.1 | Extract | 1: 5,000 | 1 | 900 |
| | 0.1 | Extract | 1: 10,000 | 1 | 780 |
| | 0.1 | Extract | 1: 20,000 | 1 | 800 |
| | 0.1 | Extract | 1: 30,000 | 1 | 1,000 |
| | 0.1 | Extract | 1: 40,000 | 1 | 1,260 |
| | 0.1 | Extract | 1: 60,000 | 1 | 1,300 |
| | 0.1 | Extract | 1: 80,000 | 1 | 1,280 |
| | 0.1 | Extract | 1: 100,000 | 1 | 1,200 |

pared with antiformin proved to be anticomplementary and therefore could not be used in making the tests. A suspension of ground bacilli was used in its place.

*Method.* In preparing the antigen,[1] typhoid bacilli that had been grown on salt-free veal agar were suspended in normal saline solution and left in flowing steam in the Arnold sterilizer for one and one-half hours. The mixture was then centrifugalized and the supernatant liquid discarded. The bacteria were washed once with 5 volumes of absolute alcohol, twice with 5 volumes of ether and then allowed to dry at room temperature for forty-eight hours. The dried bacilli were ground in a mortar for one hour. 5 cc. of this bacterial powder were taken up in 55 cc. of normal saline solution. This suspension was used as antigen. 0.1 cc. of 1: 50 dilution of the antigen, which constituted 2 units, was used in each test. For complement, the sera from ten normal guinea-pigs were pooled and 0.1 cc. of a 1: 10 dilution of this serum was used in each test. This represented $1\frac{3}{7}$ units of complement. Dilutions of the serum, supernatant and extract were made and decreasing doses of each dilution from 0.1 cc. to 0.01 cc. were put into tubes. To these, 0.1 cc. of antigen and the same amount of complement were added. The volumes of the mixtures were equalized by the addition of normal saline solution. In each rack there was one tube which contained 0.2 cc. of antigen and 0.1 cc. of complement, with no serum. This constituted the antigen control. There were also two other tubes which contained 0.04 and 0.02 cc. serum respectively and 0.1 cc. of complement with no antigen. These served as the serum controls. After incubating the tubes for thirty minutes in a 37°C. water-bath, the hemolytic system was added. This consisted of 0.1 cc. of a 5 per cent suspension of washed sheep corpuscles plus two units of hemolysin. The unit of hemolysin was found to be 0.04 cc. of a 1: 6000 dilution. The tubes were then put in the water-bath for an additional ten minutes, at the end of which time the results were read.

In titrating the various solutions, the whole serum and supernatant were used in dilutions of 1: 10 and 1: 100; the extract, undiluted and in dilutions of 1: 10 and 1: 100. In the case of the supernatant serum, the serum controls were found to be anticomplementary to a certain extent, due undoubtedly to the fact that it contained some of the

[1] This antigen was prepared in accordance with directions given by Miss M. A. Wilson of the Research Laboratories of the New York City Health Department.

"antiformin" antigen in it. This must be taken into consideration in interpreting the results.

It would seem from table 6 that the extract had about one-tenth the complement-fixing power of the serum.

*Animal experimentation*

*Historical note.* No true typhoid infection can be produced in laboratory animals, with the possible exception of the anthropoid apes. The inoculation of small amounts of stock culture has little, if any, effect. The use of large quantities, especially of a culture whose viru-

TABLE 6

*Comparative complement-fixing power of whole serum, supernatant serum and extract*

| SOLUTION | DILUTION | CONTROLS | RESULTS OF TESTS |
|---|---|---|---|
| Whole serum | 1:10 | Hemolysis | 0.001 cc. Complete fixation |
| | 1:100 | Hemolysis | 0.0008 cc. Weak fixation |
| Supernatant serum | 1:10 | 0.04 Strong fixation<br>0.02 Weak fixation | 0.005 cc. Strong fixation |
| | 1:100 | 0.04 } Trace<br>0.02 } | 0.002 cc. Weak fixation |
| Extract | Undiluted | Hemolysis | 0.01 cc. Complete fixation |
| | 1:10 | Hemolysis | 0.002 cc. Weak fixation |
| | 1:100 | Hemolysis | 0.001 cc. Weak fixation |

lence has been increased by successive passages through animals, is followed by death. That death is due to intoxication was proved by Sirotinin (14), who showed that the animals succumbed to doses of dead as well as living bacilli. Investigators have reported definite lesions in animals that have died following an injection of typhoid culture. There is usually a congestion of the abdominal organs, especially of the spleen, liver, kidney and intestinal lymph nodes. But Beumer and Peiper (15) have shown that these lesions are not specific. They were able to produce the same lesions through the injection of non-pathogenic soil and water bacteria. Metchnikoff and Besredka

(16) have shown that a true typhoid infection can be produced in the higher monkeys. In the case of fifteen chimpanzees and one gibbon, that were treated with mixtures of pure typhoid culture and the fecal material of typhoid patients, they had only one negative result. Chantemesse and Widal (17) increased the virulence of their culture by injecting it into guinea-pigs simultaneously with large doses of killed streptococci. They passed the culture from animal to animal, using decreasing doses of streptococci, until the virus acquired fixed characteristics.

*Protective power of the extract*

The object of the first experiment was to determine whether the virulence of the stock typhoid culture could be increased by following the method of Chantemesse and Widal (17), i.e., by the simultaneous injection of typhoid and killed streptococcus cultures. Accordingly three guinea-pigs were inoculated as follows:

No. 1. 4 cc. of typhoid culture subcutaneously and 8 cc. of streptococcus vaccine intraperitoneally.

No. 2. 4 cc. of typhoid culture subcutaneously.

No. 3. 8 cc. of streptococcus vaccine intraperitoneally.

No. 1 was dead within less than eighteen hours. No. 2 died after forty-eight hours. No. 3 survived. Cultures taken from the peritoneum and heart of no. 1 were positive. 1 cc. of the peritoneal exudate was mixed with 5 cc. of broth and incubated for five hours. Guinea-pig 4 was given 3 cc. of this mixture subcutaneously and at the same time 7 cc. of streptococcus vaccine intraperitoneally. The animal died within fourteen hours. Cultures from the heart and peritoneum were found to be positive. The culture obtained from the heart was inoculated into broth. Guinea-pig 5, which received 2 cc. of this broth culture intraperitoneally, became very sick within six hours after the injection. A general paralysis set in which was soon followed by death. The bacilli were obtained in pure cultures from the peritoneal exudate and heart blood. It was this virulent culture which was used in the later experiments.

Eight guinea-pigs were each given intraperitoneally 2 cc. of culture. Three of them received in addition 1, 2 and 3 cc.

respectively of serum, while three others received equivalent amounts of extract. The two control animals died within twenty-four hours after the injection. 1 cc. of the extract, as well as 1 cc. of the serum, protected against a fatal dose of culture.

In the case of mice, the minimal lethal dose was found to be 0.2 cc. injected intraperitoneally. 0.2 cc. of the extract, as well as 0.2 cc. of whole serum, was found to protect mice against the minimal lethal dose of culture.

### *Kjeldahl determinations*[2]

Determinations of the nitrogen content of the whole serum and of the extract were made. The whole serum was found to contain 1.064 grams of nitrogen per 100 cc. The content of the extract varied from 0.018 to 0.028 grams per 100 cc. In other words, the nitrogen content of the serum was reduced to from $\frac{1}{38}$ to $\frac{1}{59}$ of its original amount.

## DISCUSSION OF RESULTS

In attempting to obtain an antigen that would precipitate antibodies from an antityphoid serum, it was soon found that methods that gave good results with micrococci were of no use whatever with typhoid bacilli. Micrococci autolyze rapidly in distilled water and even in normal saline. Typhoid bacilli, on the other hand, disintegrate very slowly. The best method of preparing a typhoid antigen appeared to be the digestion of the bacteria with $2\frac{1}{2}$ per cent antiformin and the subsequent neutralization of the solution with hydrochloric acid. Such an antigen not only produced an abundant precipitate when mixed with antityphoid serum, but also carried down with the precipitate antibodies other than the precipitins. In order to free the antigen of its high salt content and of any free chlorine that might have been present, it was precipitated with three volumes

[2] These determinations were kindly carried out by R. L. Kahn of the Montifiore Home and Hospital Laboratory, New York City.

of absolute alcohol, the precipitate being subsequently redissolved in normal saline solution.

An equal amount of antigen solution and immune serum gave the best results. Larger amounts of antigen did not bring down greater quantities of antibodies from the serum. This is in keeping with the results of Dean (18) who found that the maximum precipitate was obtained by combining equivalent proportions of antigen and serum.

The mixture of antigen and serum produced the precipitation of large quantities of agglutinins, bacteriolysins, complement-fixing and protective bodies. Landsteiner and Prasek (8) found that bacterial agglutinins were carried down with the precipitate. Chickering (13) found the same thing in the case of the precipitates formed from mixtures of pneumococcus antigen and serum. That complement-fixing bodies are brought down with the precipitate has been shown by the work of Gay (3) and Zinsser (6). The removal of protective bodies from an immune serum was demonstrated by Gay and Chickering (9) and later by Chickering alone.

It was possible to dissociate the antigen-antibody complex to a certain degree and to obtain a portion of the antibodies in solution (about 5 per cent of the agglutinins, 50 per cent of the bacteriolysins and 10 per cent of the complement-fixing bodies that were present in the original serum.) Chickering had a similar experience. He found that while it was possible to remove most of the antibodies with a single extraction, a residue always remained. This residue could not be removed by repeated extractions.

Slightly alkaline solutions were found to be best for extraction. Strongly alkaline solutions would dissolve the precipitate and would also destroy the antibodies. A temperature of 42°C. was found to be necessary. Room temperature was entirely unsatisfactory. Muir (11) observed the same thing while attempting to dissociate hemolysin from red blood corpuscles.

Because a comparatively small proportion of the agglutinins are recoverable, it does not necessarily mean that the same thing is true of the other antibodies. Chickering, for example,

found that his extracts contained practically all of the protective bodies that were present in the original serum but only a small part of the agglutinins.

The work of a number of investigators shows that animal experimentation in the case of typhoid fever is a rather uncertain procedure, inasmuch as no true infection can be produced in laboratory animals, with the possible exception of the anthropoid apes. The death of animals inoculated with typhoid is most probably due to intoxication. If large amounts of the culture are given, especially if the virulence of the culture has been previously raised by passage through animals, there is an invasion of the blood stream and probably an increase in the number of bacteria. The method of obtaining a virulent culture, described by Chantemesse and Widal (17), namely, the simultaneous inoculation of typhoid bacilli and streptococcus vaccine, was found to be effective.

The enormous reduction in the protein content of the serum that is possible by extracting the antibodies, compensates for the antibodies that are lost. Such a solution as the extract, because of its low protein content, could be injected into typhoid fever patients without running the risk of having the serious reactions on the part of the patient that often follow the injection of the whole serum.

## CONCLUSIONS

1. An extract of typhoid bacilli, formed by digesting the bacteria in 2.5 per cent antiformin and neutralizing the solution with normal hydrochloric acid, produces a voluminous precipitate when mixed with antityphoid serum.

2. An equal amount of such an antigen solution and serum produces the maximum precipitate.

3. Such precipitates contain not only precipitins, but also agglutinins, complement-fixing, bactericidal and protective bodies.

4. About 5 per cent of the agglutinins, 50 per cent of the bactericidal bodies and 10 per cent of the complement-fixing bodies present in the original serum can be extracted from the precipitate in a slightly alkaline solution at 42°C.

5. The extract contains from $\frac{1}{38}$ to $\frac{1}{59}$ the amount of nitrogen that is present in the whole serum. The nitrogen content of the extracts having been reduced on the average to $\frac{1}{40}$ the amount present in the whole serum, the agglutinins were concentrated approximately twice, the bactericidal bodies twenty times[3] and the complement-fixing bodies four times.

6. By passing stock cultures of typhoid bacilli through guinea-pigs, whose resistance is lowered by the simultaneous injection of streptococcus vaccine, it is possible so to increase the virulence of the culture that small doses will kill mice and guinea-pigs in a short time.

7. 1 cc. of extract will protect guinea-pigs against 2 cc. of typhoid bouillon culture, a fatal dose. 0.2 cc. of extract will protect mice against 0.2 cc. of culture, which is likewise a fatal dose.

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[3] We do not believe that a very good method for testing bactericidal bodies has yet been devised. The plate method, used in our experiments, is not suited for accurate work.

# THE SPECIFICITY OF INTRACUTANEOUS ABSORPTION

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In previously reported experiments (1) we have shown that the absorption of antigen from the cutaneous tissues of specifically immunized animals proceeds at a rate markedly in excess of that occurring in normal animals. Horse serum, injected intracutaneously into guinea-pigs immunized against horse serum, disappeared from the site of injection much earlier than did horse serum similarly injected into normal pigs. The question was not determined, however, as to whether the heightened reactivity of the tissues, attendant upon the immune state, pertained to the specific antigen only or whether it indicated an activation of a general mechanism of elimination extending to non-specific antigens. The experiments here reported deal with this point.

In the present work guinea-pigs immunized to one antigen were tested by the intracutaneous injection of the specific and also of a heterologous antigen to demonstrate to what extent absorption depends upon a specific factor.

The technic employed was much the same as that already described. In several particulars, however, it was amplified so that a better check on the results might be obtained. Guinea-pigs immunized to normal horse serum were used with normal guinea-pigs as controls. As antigens for intracutaneous injection we employed both horse and goat serum, the former derived from a horse immunized to *B. dysenteriae* (Y-Hiss), and the latter serum secured from a goat immunized to the gonococcus. With such antigens check titrations could be made. The antidysentery horse serum could be detected by the precipitin

test with rabbit antihorse serum and by the demonstration of the dysentery agglutinins. Similarly, by the use of a rabbit anti-goat serum and the agglutination test with the gonococcus the presence of goat serum could be demonstrated. Not only were these tests made upon the tissue extracts prepared from the places of injection but titrations were also made by both tests upon the sera of the injected animals to determine the amounts of antigen absorbed into the circulation. Thus a measure was secured of both the rate of disappearance of antigen from the skin and the rate of appearance of antigen in the circulation.

The table, which follows, gives the results obtained by the use of these titration procedures in several preliminary tests upon immunized and normal guinea-pigs, all of which received for the intracutaneous injections horse serum, the antigen specific for the immunized guinea-pigs. It is evident from the data given that precipitin and agglutination tests are satisfactory means for measuring the transference of antigen. In addition, the data show the marked difference between normal animals and immunized animals.

TABLE 1

*The absorption of specific antigen*

| HOURS | IMMUNIZED PIGS | | NORMAL PIGS | |
|---|---|---|---|---|
| | Tissue | Serum | Tissue | Serum |
| | a. Precipitin tests | | | |
| 6 | 1: 25,600 | 1: 1,600 | 1: 25,600 | 1: 150 |
| 12 | 1: 6,400 | 1: 6,400 | 1: 12,800 | 1: 1,600 |
| 24 | 1: 2,400 | 1: 9,600 | 1: 6,400 | 1: 3,200 |
| 48 | 1: 400 | 1: 4,800 | 1: 2,400 | 1: 600 |
| | b. Agglutination tests | | | |
| 6 | 1: 12,800 | 1: 1,600 | 1: 12,800 | 1: 150 |
| 12 | 1: 9,600 | 1: 12,800 | 1: 12,800 | 1: 2,400 |
| 24 | 1: 2,400 | 1: 12,800 | 1: 6,400 | 1: 8,000 |
| 48 | 1: 1,200 | 1: 12,800 | 1: 1,200 | 1: 1,600 |

Examination of the figures given above shows that the agglutination titrations run parallel with the precipitin tests. Not only is this true of the tests conducted upon the tissue extracts,

but also of those in which the serum of the injected animals was used.

In addition, precipitin and agglutination tests were made upon the serum of both normal and immunized guinea-pigs, which had received no intracutaneous injections of antigen, in order to demonstrate normal agglutinins, if present, and to detect any horse serum remaining in the circulation from the immunizing treatment. Normal agglutinins were not present in the serum in amounts capable of having an appreciable bearing upon the titrations to be made upon the tissues and sera of the injected animals, nor was there a significant residue of horse serum remaining from the immunizing injections, since a positive reaction with a potent rabbit antihorse serum was never obtained in dilutions greater than 1:200. Tissue extracts from both the normal and the immunized pigs, in the dilutions tested—1:50—failed to show either normal agglutinins for *B. dysenteriae* or precipitable horse serum.

TABLE 2

*The transference of precipitable horse serum. Precipitin tests*

| HOURS | DISAPPEARANCE OF HORSE SERUM FROM THE TISSUES | APPEARANCE OF HORSE SERUM IN THE SERUM | CHANGE IN PRECIPITIN VALUE, GUINEA-PIG SERUM |
|---|---|---|---|
| 6 | 1:12,800 | 1: 1,600 | 1:3,200 |
| 12 | 1: 6,400 | 1: 6,400 | 1:1,600 |
| 24 | 1: 1,600 | 1:12,800 | 1: 600 |
| 48 | 1: 1,600 | 1: 6,400 | 1: 400 |

Tests upon the sera of the immunized pigs showed that a certain amount of precipitin had been elaborated in the course of the immunizations. In this connection it is interesting to note that the precipitin of the serum decreased as the absorption of antigen proceeded. This point is illustrated in the following table which gives the results secured in one series of titrations made upon pigs immunized to horse serum.

That the greater part of the intracutaneously injected serum passes into the circulation and is not immediately taken up by the tissues, cutaneous tissue being the index, is shown by the following. At the time of removal of the portion of skin into

which the injection was made a section of uninjected skin from the other side of the animal was removed and extracted. Titrations conducted upon these extracts in parallel with extracts from the injected tissue gave values as indicated in table 3.

TABLE 3

*The distribution of absorbed antigen in immunized pigs*

| HOURS | HORSE SERUM IN THE INJECTED TISSUE | HORSE SERUM IN THE UNINJECTED TISSUE | HORSE SERUM IN THE CIRCULATION |
|---|---|---|---|
| | a. Precipitin tests | | |
| 6 | 1: 12,800 | 1: 200 | 1: 1,600 |
| 12 | 1: 6,400 | 1: 400 | 1: 6,400 |
| 24 | 1: 2,400 | 1: 400 | 1: 12,800 |
| 48 | 1: 1,000 | 1: 400 | 1: 6,400 |
| | b. Agglutination tests | | |
| 6 | 1: 12,800 | 1: 400 | 1: 1,600 |
| 12 | 1: 12,800 | 1: 200 | 1: 6,400 |
| 24 | 1: 3,200 | 1: 1,600 | 1: 12,800 |
| 48 | 1: 800 | 1: 1,600 | 1: 12,800 |

The facts made evident in tables 1, 2, and 3 indicate clearly that the process of immunization has developed a mechanism whereby the specific antigen is removed from the cutaneous tissues to reappear in the serum at a rate far in excess of that occurring in normal animals. This conclusion is based solely upon the ability of normal and specifically immunized guinea-pigs to absorb horse serum.

Before continuing the work by introducing a second, heterologous, antigen it was necessary to determine whether or not there exists in normal animals a selective action for a particular antigen. Accordingly an experiment was conducted in which normal pigs were injected intracutaneously with the antigens previously mentioned, that is, with antidysentery horse serum on one side of the body and with antigonococcus goat serum on the other. In making the intracutaneous injections every precaution was taken to insure the retention of equal amounts of each antigen by the tissues and an effort was made to introduce the needle at the same depth into the skin. The general ap-

pearance of the elevation, which remained after injection and the removal of the needle, indicated that the procedure was carried out with considerable uniformity.

In the table which follows data are given showing the reactions secured with normal pigs.

TABLE 4

*The absorption of horse serum and goat serum in normal animals*

a. Precipitin tests

| HOURS | HORSE SERUM IN THE SKIN | GOAT SERUM IN THE SKIN |
|---|---|---|
| 6 | 1: 12,800 | 1: 12,800 |
| 12 | 1: 6,400 | 1: 6,400 |
| 24 | 1: 2,400 | 1: 3,200 |
| 48 | 1: 2,400 | 1: 2,400 |
| | HORSE SERUM IN THE CIRCULATION | GOAT SERUM IN THE CIRCULATION |
| 6 | 1: 150 | 1: 200 |
| 12 | 1: 1,600 | 1: 3,200 |
| 24 | 1: 3,200 | 1: 3,200 |
| 48 | 1: 600 | 1: 1,000 |

b. Agglutination tests

| | DYSENTERY AGGLUTININS IN THE SKIN | GONOCOCCUS AGGLUTININS IN THE SKIN |
|---|---|---|
| 6 | 1: 12,800 | 1: 12,800 |
| 12 | 1: 12,800 | 1: 12,800 |
| 24 | 1: 6,400 | 1: 4,800 |
| 48 | 1: 1,200 | 1: 1,600 |
| | DYSENTERY AGGLUTININS IN THE CIRCULATION | GONOCOCCUS AGGLUTININS IN THE CIRCULATION |
| 6 | 1: 100 | 1: 150 |
| 12 | 1: 1,600 | 1: 1,600 |
| 24 | 1: 8,000 | 1: 9,600 |
| 48 | 1: 1,600 | 1: 2,400 |

This experiment, as well as those that follow, was repeated several times, in each case duplicate animals being used upon each time interval. Minor variations occurred which could readily be ascribed to errors in technic, particularly in the intracutaneous injection, but the general type of reaction remained

constant with each of the factors used. A study of the results shows that the elimination of horse serum from the skin of normal animals is not more readily brought about than the elimination of goat serum. In fact, if curves based upon an average of the several animals are plotted for each of the several factors titrated, it is found that they coincide almost exactly throughout their entire course.

We therefore felt secure in assuming that the normal mechanism of eliminating foreign protein from the skin operates equally well with either the horse or goat serum. This gave us sufficient warranty for attempting to detect a specificity of elimination due to an alteration in this mechanism brought about through immunizing treatment.

To this end a series of guinea-pigs was immunized by repeated injections of horse serum. Titrations, similar to those indicated above, were then made upon those animals, a parallel series of normal pigs being tested as controls. The results of such a test may be tabulated as follows.

But one conclusion can be drawn from this work, namely, that the immune state so alters the process of antigen absorption from the skin that the specific antigen is removed more readily than in the normal state. Moreover, it is of especial interest that in an immunized animal the non-specific antigen is not eliminated as rapidly as is the same antigen from normal tissues, for in the animals immunized to horse serum the rate of disappearance of goat serum was delayed over that found in normal pigs. It would seem, therefore, that in immunized animals, a specific mechanism has been developed at the expense, in some measure, of the normal process. To test this point in particular many repetitions of this experiment were made and in every case such a relationship appeared.

Throughout the work with this series of animals it was repeatedly noted that in the course of the preparation of the tissue extracts the portions of tissue removed from the immunized animals differed greatly in appearance. Those sections into which horse serum, the specific antigen, had been injected were much reddened and somewhat thickened, while those from

the same pig into which goat serum had been injected remained free from thickening and inflammation. A similar condition has also been noted in connection with our work upon the absorption of antigen from the cutaneous tissues of sensitized pigs.

TABLE 5

*The absorption of horse serum and goat serum in immunized and normal animals*

a. Precipitin tests

| HOURS | IMMUNIZED PIGS | | NORMAL PIGS | |
|---|---|---|---|---|
| | Horse serum in skin | Goat serum in skin | Horse serum in skin | Goat serum in skin |
| 6 | 1: 12,800 | 1: 12,800 | 1: 12,800 | 1: 12,800 |
| 12 | 1: 6,400 | 1: 12,800 | 1: 6,400 | 1: 6,400 |
| 24 | 1: 1,600 | 1: 6,400 | 1: 3,200 | 1: 3,200 |
| 48 | 1: 400 | 1: 6,400 | 1: 2,400 | 1: 2,400 |
| | Horse serum in circulation | Goat serum in circulation | Horse serum in circulation | Goat serum in circulation |
| 6 | 1: 1,600 | 1: 50 | 1: 200 | 1: 150 |
| 12 | 1: 6,400 | 1: 100 | 1: 1,600 | 1: 2,400 |
| 24 | 1: 9,600 | 1: 100 | 1: 3,200 | 1: 2,400 |
| 48 | 1: 4,800 | 1: 200 | 1: 800 | 1: 1,600 |

b. Agglutination tests

| | Dysentery agglutinins in skin | Gonococcus agglutinins in skin | Dysentery agglutinins in skin | Gonococcus agglutinins in skin |
|---|---|---|---|---|
| 6 | 1: 12,800 | 1: 12,800 | 1: 12,800 | 1: 12,800 |
| 12 | 1: 6,400 | 1: 12,800 | 1: 12,800 | 1: 9,600 |
| 24 | 1: 1,600 | 1: 6,400 | 1: 6,400 | 1: 4,800 |
| 48 | 1: 800 | 1: 6,400 | 1: 1,600 | 1: 1,600 |
| | Dysentery agglutinins in circulation | Gonococcus agglutinins in circulation | Dysentery agglutinins in circulation | Gonococcus agglutinins in circulation |
| 6* | 1: 1,600 | None | 1: 100 | 1: 200 |
| 12 | 1: 2,400 | 1: 200 | 1: 1,600 | 1: 2,400 |
| 24 | 1: 9,600 | 1: 1,000 | 1: 6,400 | 1: 4,800 |
| 48 | 1: 12,800 | 1: 3,200 | 1: 2,400 | 1: 3,200 |

The specific antigen here, also, brought about inflammation such as never followed the injection of a non-specific antigen. Moreover, this reaction could not be due to an inherent primary toxicity, for the injection into normal pigs resulted in no reaction characteristic of either the horse or the goat serum.

It is an established fact that the process of immunization produces an altered reactivity of the body cells. Experimentation upon the reactions of the cutaneous tissues indicates that this change is specific and produces no effect upon the reacting properties of the cells for other antigens, except perhaps to lessen their activity. From the point of view of absorption of antigen, then, the immune state with the changes dependant upon it, is the result of a heightened reactivity for the specific antigen only, and does not stimulate the mechanism of elimination of heterologous antigens.

REFERENCE

(1) Smith and Cook: J. Immunol., 1917, **2**, 269.

# SPECIFIC REACTIONS OF THE BODY FLUIDS IN PNEUMOCOCCIC INFECTIONS[1]

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The problem of the reaction of the body fluids in pneumococcus infections has claimed our interest for some time. Together with the grouping of the pneumococci which was begun in our laboratory in the fall of 1916, the following studies were made to determine if possible, the extent and the way in which the body fluids reacted to the pneumococci and to antipneumococcic sera. The materials examined were obtained from patients admitted to the Allegheny General Hospital. Sputum from persons presenting clinical manifestations of pneumonia were sent to the laboratory at the earliest opportunity and the grouping made according to the method of Dochez and Gillespie (1). When the type of pneumococcus to which the infection belonged had been determined, tests were begun for the purpose of demonstrating any defensive antibody formations that might be present in the body fluids. For this purpose, examinations were made of the blood serum, spinal fluids, urine, pleural fluid and, in those instances where autopsies were held, pericardial fluids. These fluids were tested for agglutinins, precipitins, precipitable substances, etc.

The sera, urines and other materials were collected at varying intervals with reference to the time of onset of the infection and to the condition of the temperature curve. From some of the patients we obtained these materials as early as the second day of the illness and from then repeatedly until complete recovery. In some instances where death occurred early, only a single test

[1] Read before the Pittsburgh Academy of Medicine, April 24, 1917.

TABLE 1

| NUMBER OF PATIENT | PATIENTS' GROUP | GROUP I | | | | GROUP II | | | | | GROUP III | | GROUP IV | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Culture numbers | | | | | | | | | | | | | |
| | | 1 | 11 | 33 | 25 | 19 | 22 | 44 | 12 | 58 | 16a | 38 | 39 | 48 | 54 |
| 33 | I | ++++ | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 19 | II | 0 | 0 | 0 | 0 | ++++ | ++++ | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 |
| 58 | IIa | 0 | 0 | 0 | 0 | 0 | 0 | ++++ | 0 | ++++ | 0 | 0 | 0 | 0 | 0 |
| 44 | IIa | 0 | 0 | 0 | 0 | 0 | 0 | ++++ | 0 | ++++ | 0 | | 0 | 0 | 0 |
| 22 | IIx | 0 | 0 | 0 | 0 | 0 | ++++ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 39 | IV | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ++++ | 0 | 0 |
| 11 | IV and I | ++++ | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 12 and 25 | II and I | 0 | 0 | 0 | 0 | ++++ | ++++ | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 |

was made. In all instances where we could get sufficient material, the agglutination tests were made against at least four different cultures of types I, II and IV and one culture of type III. We invariably used the organism isolated from the patient whose serum was being tested. These multiple cultures were used with the hope of finding one or more cultures that the serum would agglutinate.

The method of procedure which we used is very well illustrated in table 1, wherein the reactions of a typical example of each group except group II is shown.

In this table the number of each culture corresponds to the number of the patient from whom it was isolated; i.e., "culture 33" was isolated from "patient 33," "19" from "patient 19," etc.

The patients were grouped according to the type of organism isolated from them. Patients 33 and 19 were typical group I and group II patients respectively and their sera agglutinated all cultures belonging to their respective groups and no others. All of the other patients were atypical as shown by the table. Patients 11, and 12 and 25 were especially interesting in that each had two separate and distinct attacks of pneumonia before leaving the hospital and that each recovered fully from both attacks. These two patients we will take up somewhat in detail.

Patient 11 had as his first infection a member of group IV and as his second a member of group I. He was exposed to a group I infection by having occupied a bed in the ward on two occasions of some days duration, adjacent to a patient from whom a pneumococcus, type I, had been isolated. His serum, as will be seen in the table, agglutinated all group I organisms or those corresponding to his last attack, and did not agglutinate any of the group IV organisms. Unfortunately his first culture had been lost before this phase of the work was taken up, so we do not know whether his serum would have agglutinated it or not.

Patient 12 and 25 had as his first infection a member of group II and as his second a member of group I. We have no definite history of such intimate exposure in this instance, as in the case of patient 11, but he was kept in a large ward with patients suffering from infections with the other types of pneu-

mococci. His serum showed agglutinins only for organisms belonging to group II, or those corresponding to his first infection, and none for those belonging to group I—not even for his own last culture, no. 25 of table 1. We believe that if one were fortunate enough to follow a sufficiently large number of reinfections or recurrences, much more light might be thrown upon the immunity or lack of immunity established by infections with pneumococci.

Table 2 shows the results of the agglutination tests carried out with the sera from fifty patients.

TABLE 2

| GROUP | NUMBER | POSITIVE | PER CENT POSITIVE |
|---|---|---|---|
| I | 21 | 15 | 71.4 |
| II | 13 | 10 | 77.9 |
| III | 0 | 0 | 0 |
| IV | 10 | 5 | 50.0 |
| Unclassified (clinically pneumonia) | 6 | 0 | 0 |
| Total | 50 | 30 | 60.0 |

This differs somewhat from the report by Chickering (2) who found specific agglutinins in 100 per cent of no. I's, 53.8 per cent of no. II's and 55.5 per cent of no. IV's.

Of twenty-one patients with no. I infections six did not show specific agglutinins in their sera. Two of these died and four recovered; one of the ones who recovered had a blood stream infection and another an empyema necessitating a stay of some months in the hospital. Only two patients with fatal infections showed agglutinins in their sera and both had received anti-pneumococcic serum for group I before the test was done. Of the three patients with no. II infections who did not show specific agglutinins in their sera—one ran a very mild course; another, a small child, developed an empyema from which it recovered after many weeks; and the other one died. Of the five patients with group IV infections, showing no specific agglutinins in their sera, one had a very mild infection, two developed empyema and two died.

In the thirteen patients having infections with organisms of group II, whose sera were tested, there were three sera which showed that the infection was caused by organisms belonging to sub-group II, one to sub-group IIx, and the other two to sub-group IIa or IIb.

In regard to the appearance and duration of the agglutinins it may be said that in all instances in which the serum was obtained during the fastigium the agglutinin tests were negative, except in the two patients mentioned above who had received antipneumococcic serum therapeutically. The agglutinins appeared only during or after defervescence. As would be expected, the time during which they were demonstrable varied greatly. There was an apparent tendency for the agglutinins for organisms of group I to persist for a longer time than for the other groups (II and IV), and for those for group II to persist longer than those for group IV. We were unable to test any for group III.

As has been noted before (Chickering (2), Lister (3) ) the agglutinins in instances of human infections correspond with the experimental findings resulting from immunization of animals against the various groups of pneumococci in being specific for the organism or group to which the infecting organisms belongs. That is, a patient having an infection with a group I organism shows agglutinins only for the organisms in that group (or sub-group); a patient having an infection with an organism belonging to group IV possesses agglutinins only for his homologous organism and no other.

We have tested a number of urines from patients whose sera gave strongly positive reactions and were unable to demonstrate at any time the presence of agglutinins. Agglutinins were not demonstrable in the spinal fluid in those patients having a meningitis, except in two who had received antipneumococci serum both intravenously and intrathecally. In several of the patients with meningitis we were unable to carry out agglutination tests with their sera, so that we cannot say whether agglutinins were present or not in their blood. All of these patients died, so that as a group they were not favorable for the development of agglutinins.

In carrying out agglutinin tests on spinal fluids which contained a great number of pneumococci we noted that, upon the addition of the antipneumococcic serum, a precipitate was formed almost immediately. We did not appreciate the significance of this reaction until Blake (4) reported his observations upon the precipitin reaction on the urine. Since this precipitin reaction offered a rapid and accurate method of diagnosis, we took advantage of it and found it to be a reliable test, if a sufficient number of dilutions of both no. I and no. II sera were used. In order to explain our findings in the tests on spinal fluids and urines we made the following cross tests. Cultures were made in broth and after sufficient growth had occurred we centrifugalized them and pipetted off the supernatant, clear fluid. Serum for groups I and II were added in varying dilutions to the spinal fluid, urine and broth separately. Precipitates occurred in all three with the corresponding serum. Mixtures of spinal fluid and urine, spinal fluid and broth and urine and broth showed no precipitates. Therefore, the precipitating substance, "precipitin," was shown to be present in the serum and precipitable substance "precipitinogen" in the urine, spinal fluid and broth. We would advise, therefore, as a point of practical value—that, in cases of meningitis of indefinite etiology, a precipitin test with antipneumococcic serum be made as a rapid method for determining or excluding a pneumococcus infection with either group I or group II.

## DISCUSSION

The agglutinin tests on the sera of fifty patients showed that humans react to their acquired infections with the pneumococcus in a specific manner similar to that experimentally produced in animals; i.e., the patient produces agglutinins only for the homologous organism or group to which the homologous organisms belongs. We did not obtain, however, as high a percentage of positive results as Chickering in the instances of group I infections. The very mild infections and the fatal infections were less apt to show specific agglutinins.

The agglutinins did not appear until during or after defervescence. They remained demonstrable in the serum for variable periods of time. There seemed to be a tendency for them to persist longest in group I, next in group II and shortest in group IV.

We were unable to find any evidence of the presence of specific agglutinins in the urine, even in those patients having strong agglutination reactions in the serum. In the precipitin tests with antipneumococcic serum and urine we believe that the specific "precipitin" is in the serum and the precipitable substance, "precipitinogen," in the urine. In patients with pneumococcic meningitis, the precipitable substance may be found both in the urine and cerebro-spinal fluid.

## CONCLUSIONS

1. Specific agglutinins usually appear in the serum of patients during or just after defervescence.

2. The agglutinins in the serum seem to persist longest in group I; somewhat less in group II and for the shortest time in group IV.

3. The urine contains specific precipitable substances but does not contain agglutinins or precipitins.

4. The spinal fluid in cases of pneumococcic meningitis also contains specific precipitable substances similar to those in the urine, but neither agglutinins nor precipitins.

5. The precipitin reaction may be applied to the spinal fluid in pneumococcus meningitis as a practical means of rapid classification of the infection.

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(2) Chickering: Journal Experimental Medicine, 1914, **20**, 599.
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# STUDIES ON THE ANTITRYPSIN OF SERUM

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## INTRODUCTION

As to the nature of the antitrypsin of serum, a great deal has been written, and even at the present day various views are held in regard to it. In our laboratory several tests were performed in order to see how it can be inactivated and which part of the serum exerts the antitryptic action.

## PREPARATION AND METHOD OF OUR TESTS

In our experiments von Bergmann's casein method was modified a little to make the reaction finer. Namely:

1. Solution of casein: 0.5 gram of casein was dissolved in 50 cc. of $\frac{N}{10}$ NaOH solution and the solution was warmed a while. Then the solution was neutralized with $\frac{N}{10}$ muriatic acid and finally diluted to 500 cc. with normal salt solution. This solution of casein in our case is, therefore, less concentrated than that usually employed; i.e., it is of 0.1 per cent instead of 0.2 per cent.

2. Solution of trypsin: 0.05 gram of trypsin was dissolved in 5 cc. of physiological salt solution, to which 0.5 cc. of $\frac{N}{10}$ $Na_2CO_3$ solution was added. To make it 100 cc. (0.05 per cent) normal salt solution was then added to the mixture up to 100 cc. thus making a 0.05 per cent solution. This solution is also much more diluted than that ordinarily used (0.1 per cent).

3. Solution of acetic acid: Acetic acid, 5 cc.; alcohol, 45 cc.; aqua destillata, 50 cc.

4. The doses in each test tube were always: Solution of casein, 2 cc.; solution of trypsin in graded doses; blood serum (1 per cent), 1 cc.

The reason why all of these solutions were used in less concentrated form than ordinarily, is that the reaction may be more easily and clearly distinguished. These solutions being mixed, and after being allowed to stand half an hour in the thermostat (37°), the series were examined with the solution of acetic acid, which was poured slowly on the surface of reagents in the test tube. When the casein is not yet digested completely and a trace of it exists still in the reagents, a beautiful circle of gray precipitate is easily to be seen at the plane of contact between the acetic acid and the reagents. In our following tables antitrypsin is not expressed in units; but if it is desired to express antitrypsin content in units, it may be calculated in the same manner as in the original method, because the relation between the concentrations of casein and that of the trypsin solutions is not changed, though both solutions are less concentrated.

## I. INACTIVATING TESTS OF THE ANTITRYPSIN OF BLOOD SERUM

### *1. Inactivation by heating*

Inactivating experiments for the antitrypsin of blood serum have been performed by several investigators. Vandevelde (1) saw that the antitrypsin may be destroyed in greater or less degree by heating it at 55°, 60° and 65°C.; Achalme (2) proved that they became completely inactive by heating at 65° to 70°C. Jochmann and Kantorowicz (3) found that the antitrypsin of human serum may be completely inactivated by heating at 66°C. for half an hour. Kurt Meyer (4) has shown that the antitrypsin of serum is destroyed in but a slight degree (only one-fifth of it) by heating at 56°C. for half an hour. Kämmerer (5) compared the resistance of the antitrypsin of human serum with that of ox serum, and he saw that the former is more resistant than the latter, and that the latter is inactivated almost completely by heating at 65°C. for half an hour, but that it can scarcely be inactivated by heating at 56°C. for half an hour. Döblin (6) did not confirm these results, but he has, on the contrary, proved that the antitrypsin is thermostable and of a

colloid nature. In short, the temperature that is necessary to inactivate the serum antitrypsin has been described differently, and there is one investigator that denies its thermolability.

In order to see whether the antitrypsin of serum is thermostable or not, and if it be thermolabile, at what point of temperature it may be completely inactivated, we have begun with the following tests.

Before proceeding to the main tests, we have had to determine at what point the sera coagulate. If they begin to coagulate by heating, the factors of the reaction are changed by it and it becomes hard to judge the results correctly, so that the coagulation of the sera must be avoided as much as possible. The tests with regard to this, were performed as follows.

First, the undiluted serum was heated at various points; secondly, it was diluted with distilled water or with normal salt solution and then heated at various points. The results are shown in the following table.

TABLE 1

*Coagulation tests of rabbit serum in its different concentrations at 72°C. and 80°C.*

| CONCENTRATION OF SERUM | AT 72°C. | | AT 80°C. | |
|---|---|---|---|---|
| | Not diluted | | | |
| *per cent* 100 | + | | + | |
| | Diluted with | | | |
| | Aqua destillata | Salt solution | Aqua destillata | Salt solution |
| 50 | ± | − | + | + |
| 20 | − | − | + | − |
| 10 | − | − | − | − |
| 5 | − | − | − | − |
| 1 | − | − | − | − |

+ = Coagulation; − = no coagulation.

Thus it was decided that the coagulation of the sera can be avoided by diluting it. In our tests we have, therefore, diluted all sera to 1 per cent, and then heated them at the high temperature that is necessary for their inactivation.

*a. Serum of rabbit.* Sera of rabbits were heated at various temperatures, i.e., at 65°, 70°, 72°, 75°, 80°C. for from one minute to three hours. The results are shown in the following tables.

TABLE 2
*Rabbit serum heated at 65°C.*

| | 0.4 | 0.5 | 0.6 | 0.7 | 0.8 | 0.9 | 1.0 | 1.1 | 1.2 | 1.3 |
|---|---|---|---|---|---|---|---|---|---|---|
| No serum | + | ± | − | − | − | − | − | − | − | − |
| Serum not heated | + | + | + | + | + | + | + | + | ± | − |
| Serum heated | | | | | | | | | | |
| 3 minutes | + | + | + | + | + | + | − | − | − | − |
| 5 minutes | + | + | + | + | + | + | − | − | − | − |
| 10 minutes | + | + | + | + | ± | − | − | − | − | − |
| 30 minutes | + | + | + | + | + | − | − | − | − | − |
| 60 minutes | + | + | + | + | + | ± | − | − | − | − |
| 120 minutes | + | + | + | + | + | − | − | − | − | − |
| 180 minutes | + | + | + | + | + | − | − | − | − | − |

+ = digestion incomplete; − = digestion complete.

TABLE 3
*Rabbit serum heated at 70°C.*

| | 0.4 | 0.5 | 0.6 | 0.7 | 0.8 | 0.9 | 1.1 | 1.1 | 1.2 | 1.3 |
|---|---|---|---|---|---|---|---|---|---|---|
| No serum | + | − | − | − | − | − | − | − | − | − |
| Serum not heated | + | + | + | + | + | + | + | + | + | − |
| Serum heated | | | | | | | | | | |
| 3 minutes | + | + | + | + | + | + | − | − | − | − |
| 5 minutes | + | + | + | + | ± | − | − | − | − | − |
| 10 minutes | + | + | + | + | + | − | − | − | − | − |
| 30 minutes | + | + | + | ± | − | − | − | − | − | − |
| 60 minutes | + | + | + | ± | − | − | − | − | − | − |
| 120 minutes | + | + | + | − | − | − | − | − | − | − |

TABLE 4
*Rabbit serum heated at 72°C.*

| | 0.5 | 0.6 | 0.7 | 0.8 | 0.9 | 1.0 | 1.1 | 1.2 | 1.3 | 1.4 |
|---|---|---|---|---|---|---|---|---|---|---|
| No serum | + | ± | − | − | − | − | − | − | − | − |
| Serum not heated | + | + | + | + | + | + | + | + | + | − |
| Serum heated | | | | | | | | | | |
| 3 minutes | + | + | + | + | + | ± | − | − | − | − |
| 5 minutes | + | + | + | + | + | + | − | − | − | − |
| 10 minutes | + | + | + | + | + | − | − | − | − | − |
| 20 minutes | + | + | + | ± | − | − | − | − | − | − |
| 30 minutes | + | + | − | − | − | − | − | − | − | − |
| 60 minutes | + | + | ± | − | − | − | − | − | − | − |
| 120 minutes | + | ± | − | − | − | − | − | − | − | − |

TABLE 5

*Rabbit serum heated at 75°C.*

| | DOSES OF TRYPSIN | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0.5 | 0.6 | 0.7 | 0.8 | 0.9 | 1.0 | 1.1 | 1.2 | 1.3 |
| No serum | + | − | − | − | − | − | − | − | − |
| Serum not heated | + | + | + | + | + | + | + | + | − |
| Serum heated for | | | | | | | | | |
| 3 minutes | + | + | + | − | − | − | − | − | − |
| 5 minutes | + | + | − | − | − | − | − | − | − |
| 10 minutes | + | − | − | − | − | − | − | − | − |
| 30 minutes | + | − | − | − | − | − | − | − | − |

At 80°C. it took only seven and one-half minutes to inactivate the serum antitrypsin completely.

It may be concluded from the above tables that the sera of rabbits can be inactivated completely by heating at 75°C. for ten minutes, or at 80°C. for seven and one-half minutes, and that at temperatures lower than 72°C. the serum of rabbit cannot be inactivated completely even if it be heated for two hours.

*b. Serum of horse.* Several experiments similar to the foregoing were performed with sera of horses. The results are given in table 6.

TABLE 6

*Inactivation tests with the sera of horses*

| | DOSES OF TRYPSIN | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0.7 | 0.8 | 0.9 | 1.0 | 1.1 | 1.2 | 1.3 | 1.4 |
| No serum | + | − | − | − | − | − | − | − |
| Serum not heated | + | + | + | + | + | + | + | − |
| Serum heated at | | | | | | | | |
| 50°C. for 10 minutes | + | + | + | + | + | + | ± | − |
| 50°C. for 30 minutes | + | + | + | + | + | + | ± | − |
| 55°C. for 10 minutes | + | + | + | + | ± | − | − | − |
| 55°C. for 30 minutes | + | + | + | + | + | − | − | − |
| 60°C. for 10 minutes | + | + | + | + | ± | − | − | − |
| 60°C. for 30 minutes | + | + | + | − | − | − | − | − |
| 65°C. for 10 minutes | + | − | − | − | − | − | − | − |
| 65°C. for 30 minutes | + | − | − | − | − | − | − | − |

As we see in above table, the sera of horses can be completely inactivated by heating at a lower temperature than those of rabbits.

*c. Serum of sheep.* Further tests were undertaken in the same manner with the serum of sheep and the following results were obtained (table 7).

TABLE 7

*Inactivation tests with the serum of sheep*

| | DOSES OF TRYPSIN | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0.7 | 0.8 | 0.9 | 1.0 | 1.2 | 1.4 | 1.6 | 1.8 |
| No serum | + | − | − | − | − | − | − | − |
| Serum not heated | + | + | + | + | + | + | + | − |
| Serum heated for ten minutes at | | | | | | | | |
| 65°C. | + | + | + | + | + | − | − | − |
| 70°C. | + | + | + | − | − | − | − | − |
| 75°C. | + | − | − | − | − | − | − | − |
| 80°C. | + | − | − | − | − | − | − | − |

Though Döblin maintained that the antitrypsin of sera is thermostable, it is certain that it may be inactivated by heating; i.e., it is thermolabile. And the temperatures which must be applied for the inactivation differ in each case. Namely:

Rabbit serum at 75°C. for ten minutes
Horse serum at 65°C. for ten minutes
Sheep serum at 75°C. for ten minutes
Human serum at 65° or 66°C. for one-half hour (Jochmann and Kämmerer)
Ox serum, higher than human serum (Kämmerer)

*Conclusion.* It may be concluded that the temperatures, which must be applied for the inactivation of antitrypsin of sera, differ not according to individuality but according to species and are between 65° and 75°C. As sera never coagulate at 75°C. in their lower concentration, it would be better at first to dilute them and then heat them at 75°C. for ten to thirty minutes for their complete inactivation.

Incidentally, a brief experiment for the inactivation of the human urine was undertaken. The results obtained are shown in table 8.

TABLE 8

*Inactivation tests with human urine*

| | DOSES OF TRYPSIN | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0.6 | 0.7 | 0.8 | 0.9 | 1.0 | 1.1 | 1.2 | 1.3 | 1.4 |
| No urine | + | − | − | − | − | − | − | − | − |
| Urine not heated, 30 per cent, 1 cc. | + | + | + | + | + | + | + | + | − |
| Urine heated, 30 per cent, 1 cc. at | | | | | | | | | |
| 90°C. for ½ hour | + | + | + | + | + | + | + | ± | − |
| 90°C. for 1 hour | + | + | + | + | + | + | + | ± | − |
| 100°C. for ½ hour | + | − | − | − | − | − | − | − | − |
| 100°C. for 1 hour | + | − | − | − | − | − | − | − | − |

The antitryptic action of urine cannot be affected so easily as that of sera; urine must be heated at 100°C. for one-half hour for its complete inactivation.

### *2. Inactivation by shaking*

We can find no record of the inactivation of serum antitrypsin by shaking. It is well known that complement may be inactivated by shaking at 37°C. for one-half hour. If the antitrypsin of serum also could be inactivated by shaking as well as complement, many interesting experiments might be undertaken as to its nature. One cubic centimeter of serum was diluted to 10 cc. with normal salt solution and then shaken at various temperatures.

TABLE 9

*Inactivation tests by shaking (rabbit serum)*

| | DOSES OF TRYPSIN | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0.4 | 0.5 | 0.6 | 0.7 | 0.8 | 0.9 | 1.0 | 1.1 | 1.2 |
| No serum | + | − | − | − | − | − | − | − | − |
| Serum neither heated nor shaken | + | + | + | + | + | + | + | + | − |
| Serum shaken for two to 3 hours: at | | | | | | | | | |
| 37°C | + | + | + | + | + | + | ± | − | − |
| 49°C | + | + | + | + | + | + | + | − | − |
| Control serum (not shaken) at | | | | | | | | | |
| 37°C | + | + | + | + | + | + | + | − | − |
| 49°C | + | + | + | + | + | + | + | − | − |

Horse serum was similarly treated, the results being the same.

*Conclusions.* Antitrypsin of serum cannot be inactivated at all by shaking.

## II. EXPERIMENTS WITH SERUM GLOBULIN AND SERUM ALBUMIN

In regard to this problem, there are several different views. Landsteiner (7), Müller (8) and Opie and Parker (9) reported that antitrypsin is mostly fixed to the albumin fraction of serum. Glässner (10) stated that the antitrypsin of serum is all fixed to the euglobulin, not to albumin. But Döblin (6) and Kämmerer (5) maintained that both the globulin and the albumin fractions exert an antitryptic action, but the former less than the latter. Beside this, Kämmerer (5′) has recently made interesting experiments, in which he has proved that the globulin fraction increases after heating serum at 56°C. for one-half hour, while the albumin fraction decreases, and that the antitryptic action of the albumin fraction may be more easily inactivated than that of the globulin fraction.

In our laboratory different tests were performed bearing upon this question. To fractionate serum in globulin and albumin, we have employed several methods.

### *1. Serum treated with ammonium sulphate*

For the fractionation of 50 cc. of horse serum, there was added an equal volume of saturated solution of ammonium sulphate and the mixture was filtered. The precipitate was washed five times with a half saturated solution of the same salt.

As ammonium sulphate precipitates casein, both fractions must be dialyzed for its elimination. However, as it takes a week or more to make them completely free from the salt by dialysis, their antitryptic action might, also, be weakened by the treatment. For this reason we were satisfied with a day's dialysis, after which time the concentration of the ammonium sulphate in the globulin and albumin fractions was so little that the casein was not precipitated by it. Fortunately it was already proved by Kämmerer that ammonium sulphate does not

prevent the action of trypsin in any way; and Döblin saw that the antitrypsin was not lost at all in the first week of dialysis. Afterward the same result as Döblin's was obtained by us as to this property of trypsin (see the following chapter). It is, therefore, clear that only a day's dialysis exerts no influence upon the antitryptic action of serum.

If the doses of serum globulin (100 per cent) in the test tubes are more than 0.2 cc., the globulin may be precipitated by

TABLE 10

*Tests with serum globulin and albumin*

| | DOSES OF TRYPSIN | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0.5 | 0.6 | 0.7 | 0.8 | 0.9 | 1.0 | 1.2 | 1.4 | 1.6 |
| No serum | + | − | − | − | − | − | − | − | − |
| Serum 1 per cent. 1 cc | + | + | + | + | + | + | + | + | − |
| Serum globulin* | | | | | | | | | |
| Not heated { 20 per cent, 1 cc | + | + | + | + | − | − | − | − | − |
| Not heated { 2 per cent, 1 cc | + | − | − | − | − | − | − | − | − |
| Heated (75°, one-half hour) 20 per cent, 1 cc | + | − | − | − | − | − | − | − | − |
| Serum albumin | | | | | | | | | |
| Not heated, 2 per cent, 0.5 cc | + | + | + | ± | − | − | − | − | − |
| Heated (75°, one-half hour) 2 per cent, 0.5 cc | + | − | − | − | − | − | − | − | − |

* The serum globulin was dissolved in normal salt solution in a volume equal to that of the original serum.

acetic acid. Therefore, we must be careful in our experiments not to misjudge the reaction that is caused by the precipitation of serum globulin and not of casein. The precipitation of globulin may be easily distinguished by its coloring.

As the antitryptic action of serum is not weakened by dialysis at all, we can say that the antitryptic action of serum is considerably weakened by precipitating with ammonium sulphate, and that the antitrypsin is to be found not only in the albumin fraction but also, in a small degree, in the globulin fraction.

*2. Serum treaied with carbonic acid*

By the preceding experiment it was shown that the globulin fraction of serum also shows antitryptic action. To confirm this fact through some other experiments, we have treated the serum with carbonic acid and $\frac{N}{250}$ muriatic acid.

To 5 cc. of horse serum were added 95 cc. of aqua destillata, which was cooled in ice, and then carbonic acid was led from Kipp's apparatus into it until the precipitate no longer increased (about one-half hour). The precipitate consists solely of globulin. It was separated from the albumin portion by

TABLE 11

*Tests with serum globulin and albumin obtained by means of $CO_2$*

| | DOSES OF TRYPSIN | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0.7 | 0.8 | 0.9 | 1.0 | 1.2 | 1.4 | 1.6 | 2.6 | 2.8 |
| No serum | + | − | − | − | − | − | − | | |
| Serum 1 per cent, 1 cc | + | + | + | + | + | + | − | | |
| Serum treated with $CO_2$ | | | | | | | | | |
| Albumin part, 1 per cent, 1 cc | + | + | + | + | + | ± | − | | |
| Globulin part, 1 cc. 1.0 per cent | + | + | − | − | − | − | − | | |
| Globulin part, 1 cc. 2.5 per cent | + | ± | − | − | − | − | − | | |
| Globulin part, 1 cc. 5.0 per cent | + | + | − | − | − | − | − | | |
| Globulin part, 1 cc. 10.0 per cent | + | + | + | ± | − | − | − | | |
| Globulin part, 1 cc. 25.0 per cent | + | + | + | + | + | + | − | | |
| Globulin part, 1 cc. 50.0 per cent | + | + | + | + | + | + | + | + | − |

means of a centrifuge, and it was washed more than five times with distilled water to render it free from albumin.

Both the globulin and the albumin thus obtained were examined as usual, the results being shown in table 11.

Such an experiment was repeated with the serum of the horse and rabbit, and it was always followed by the same results. Of course, both the globulin and albumin fractions could be inactivated by heating.

Thus it has been demonstrated that the antitryptic action of serum may be weakened only a little by precipitation with carbonic acid, and that the globulin fraction undoubtedly has antitryptic action.

### *3. Serum treated with* $\frac{N}{250}$ HCl.

Sachs employed this method to separate the complement of guinea-pig serum into two components. To 0.5 cc. of horse serum, there was added 4.1 cc. of $\frac{N}{250}$ muriatic acid; after standing an hour at room temperature it was separated by means of the centrifuge into two portions. The globulin fraction (i.e., the precipitate) was washed with distilled water several times carefully. The results of the tests were mostly the same as we have seen above (table 12).

TABLE 12

*Tests with serum globulin and albumin obtained by Sach's method*

| | DOSES OF TRYPSIN | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0.5 | 0.6 | 0.7 | 0.8 | 0.9 | 1.0 | 1.1 | 1.5 | 1.7 |
| No serum | + | − | − | − | − | − | − | | |
| Serum 1 per cent, 1 cc | + | + | + | + | + | + | − | | |
| Serum treated with HCl | | | | | | | | | |
| Albumin part 1 per cent, 1 cc | + | + | + | + | + | + | − | | |
| Globulin part, 1 cc. 5 per cent | + | ± | − | − | − | − | − | | |
| Globulin part, 1 cc. 10 per cent | + | + | + | ± | − | − | − | | |
| Globulin part, 1 cc. 20 per cent | + | + | + | + | + | − | − | | |
| Globulin part, 1 cc. 50 per cent | + | + | + | + | + | + | + | + | − |

Globulin part obtained according to Sach's method shows an antitryptic action, but in less degree as in the case before.

*Conclusion.* Both globulin and albumin fractions have antitryptic action, but the latter in less degree than the former.

## III. THE NATURE OF SERUM ANTITRYPSIN

It has been shown by the above experiments that the antitrypsin of serum is to be found in both the globulin fraction and the albumin fraction. To determine whether these proteins themselves have such antitryptic action or whether they are accompanied by some specific substances which exert an antitryptic influence, further studies were undertaken.

*1. Is the antitrypsin dialysable?*

In the first place, the globulin and albumin fractions of serum were dialyzed. As we know that globulin and albumin are not dialysable, if the antitryptic action of sera be lost by dialysis, there must be some specific acting substances; if it be not lost, antitryptic acting substances in sera must be undialysable and it

TABLE 13

*Dialyzation tests*

| | DOSES OF TRYPSIN | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0.5 | 0.6 | 0.7 | 0.8 | 0.9 | 1.0 | 1.1 |
| No serum | + | − | − | − | − | − | − |
| Serum not dialyzed 1 per cent, 1 cc | + | + | + | + | + | + | − |
| Serum (1 per cent) dialyzed for | | | | | | | |
| 1 day, 1 cc | + | + | + | + | + | ± | − |
| 2 days, 1 cc | + | + | + | + | + | ± | − |
| 3 days, 1 cc | + | + | + | + | + | ± | − |
| 4 days, 1 cc | + | + | + | + | + | ± | − |
| Serum (1 per cent) not dialyzed but otherwise under the same conditions for | | | | | | | |
| 1 day, 1 cc | + | + | + | + | + | ± | − |
| 2 days, 1 cc | + | + | + | + | + | ± | − |
| 3 days, 1 cc | + | + | + | + | + | ± | − |
| 4 days, 1 cc | + | + | + | + | + | ± | − |
| Globulin (10 per cent) dialyzed for | | | | | | | |
| 2 days, 1 cc | + | + | ± | − | − | − | − |
| 4 days, 1 cc | + | + | ± | − | − | − | − |
| Globulin (10 per cent) not dialyzed, 1 cc. for 2 to 4 days | + | + | ± | − | − | − | − |
| Albumin (1 per cent) dialyzed for | | | | | | | |
| 2 days, 1 cc | + | + | + | + | + | ± | − |
| 4 days, 1 cc | + | + | + | + | + | ± | − |
| Albumin (1 per cent) not dialyzed, 1 cc. for 2 to 4 days | + | + | + | + | + | + | − |

might be said that antitryptic acting substance is nothing but protein itself. The following tests were always carefully controlled by undialyzed samples which were exposed otherwise to the same conditions.

It is seen that the antitryptic action of serum as well as that of globulin or albumin is not weakened at all after one to four

days' dialysis. We can say, therefore, that the antitryptic substance of serum at least is not dialysable.

Human urine was similarly tested but in this case the antitryptic property disappeared completely after two days' dialysis.

TABLE 14

*Dialyzation test with human urine*

| | DOSES OF TRYPSIN | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0.3 | 0.4 | 0.5 | 0.6 | 0.7 | 0.8 | 0.9 | 1.0 | 1.2 |
| No urine | + | ± | − | − | − | − | − | − | − |
| Urine 50 per cent, 0.6 cc | + | + | + | + | + | + | + | + | − |
| Urine 50 per cent, 0.6 cc. dialyzed for 2 days | + | ± | − | − | − | − | − | − | − |
| Urine 50 per cent, 0.6 cc. (control) not dialyzed (only standing for 2 days) | + | + | + | + | + | + | + | − | − |

### *2. Experiment with pepton*

A brief experiment was carried out with pepton, which is dialysable. But pepton itself was found to have no antitryptic action, and it neither prevented nor accelerated that action.

### *3. Ether extract of serum*

Serum was extracted with ether to see whether the substances that are soluble in ether, have an antitryptic action or not. O. Schwarz (11) stated that serum became inactive after the lipoids were extracted from it and that a mixture of the lipoids with protein exerts an antitryptic action. Kämmerer (5′) refuted Schwarz's theory by his experiment, in which he saw only a slight decrease of antitryptic action (only one-third) after extracting the serum five to six times with ether.

Five cubic centimeters of horse serum were treated with 50 cc. of ether in our experiment. The extract was redissolved in ether to make it pure and was made into emulsion with physiological salt solution. The results were as follows (table 15):

TABLE 15*

*Tests with ether extract of serum*

| | DOSES OF TRYPSIN | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0.5 | 0.6 | 0.7 | 0.8 | 0.9 | 1.0 | 1.1 |
| No serum | + | − | − | − | − | − | − |
| Serum 1 per cent, 1 cc | + | + | + | + | + | + | − |
| Ether extract | | | | | | | |
| 20 per cent, 1 cc | + | − | − | − | − | − | − |
| 50 per cent, 1 cc | + | ± | − | − | − | − | − |

* See the note to table 10.

It was established through the above experiments that the antitryptic substance is not dialysable and that the ether extract of serum does not contain such a substance. We have, therefore, proceeded to the following tests.

### *4. Crystalline albumin*

It seems now probable that the antitryptic substance of serum may be nothing but the protein of the serum itself. This question is not yet definitely decided, and we can not find a record of any previous experiment which was undertaken to settle it. Taking advantage of the property of serum albumin to crystallize, we have undertaken an experiment with crystalline serum albumin and, also, with crystalline ovoalbumin. As albumin that is not denatured and that is free from any admixture can be obtained by crystallization, it seemed that an experiment with crystalline albumin might bring a decision with regard to this problem. To obtain the crystalline albumin, I have employed the following method.

Fifty cubic centimeters of horse serum and the same quantity of saturated ammonium sulphate solution were mixed and then filtered. To 50 cc. of the filtrate was added more saturated ammonium sulphate solution until further precipitation began, and then 1 to 1.25 cc. of saturated ammonium sulphate solution, to which acetic acid to 10 per cent had been added. After twenty-four or forty-eight hours (in the ice box), we obtained typical crystals of serum albumin; the crystals were separated

from the fluid by centrifugation and washed five or more times with a half saturated solution of ammonium sulphate, which contains the salt and acetic acid in the same relation as above. The crystals were finally dissolved in distilled water or physiological salt solution.

TABLE 16*

*Test with solution of albumin crystal*

| | DOSES OF TRYPSIN | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0.5 | 0.6 | 0.7 | 0.8 | 0.9 | 1.0 | 1.2 | 1.4 | 1.6 |
| No serum | + | − | − | − | − | − | − | − | |
| Serum 1 per cent, 1 cc | + | + | + | + | + | + | + | + | − |
| Crystalline serum albumin, 1 cc. | | | | | | | | | |
| 5 per cent | + | + | + | ± | − | − | − | − | − |
| 10 per cent | + | + | + | + | + | + | − | − | − |
| Crystalline ovoalbumin, 1 cc. | | | | | | | | | |
| 5 per cent | + | + | + | ± | − | − | − | − | − |
| 10 per cent | + | + | + | + | + | + | − | − | − |

* See the note to table 10.

As the crystalline serum albumin is pure and free from any admixture, it must be accepted that the serum albumin itself has an antitryptic action.

*Conclusion.* There may be many kinds of substances in serum which exert an antitryptic influence, as Oppenheimer (12) has said; but there is no doubt that albumin itself exerts such action. It is still open to question, whether it is the serum protein alone that is the antitryptic agent or whether some other substances beside the protein are also antitryptic. Further quantitative experiments are necessary to solve this question.

## RESULTS

1. The temperatures that are necessary to inactivate sera, are different according to species, and are between 65° and 75°C.

2. The antitrypsin of serum can not be inactivated by shaking.

3. Both the globulin and the albumin fractions are antitryptic, but the former is less so than the latter, as Döblin and Kämmerer stated.

4. The antitrypsin of serum is not dialysable.
5. Ether extract of serum has no antitryptic action.
6. Crystalline serum albumin is antitryptic.
7. It is not yet decided whether serum contains antitryptic substances beside the serum protein or not.

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# THE CONSTANCY OF THE PROTEIN QUOTIENT DURING INTENSIVE DIGESTION AND PROLONGED STARVATION

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It is usually assumed that the fluctuations of the protein quotient under normal and pathological conditions are due directly or indirectly to variations in the general nature and especially in the rapidity of the metabolic processes.

Thus, Cervello (1) ascribes to a retardation of metabolism the rise in globulins he obtained under the administration of antipyrin. Hurwitz and Meyer (2) suppose that the increase in the serum globulins in certain actue infections is due to metabolic disturbances. Schmidt and Schmidt (3) are inclined to believe that the serum proteins have no relation to immunity and would rather attribute the increase in globulins to changes in the metabolism resulting from the toxemia and tissue waste of acute infections.

In view of the fact that alterations in the protein quotient have been so frequently attributed by various authors to disturbances in the metabolism, it was considered necessary to investigate the more precise relations of the globulin albumin ratio to difference in the rapidity of metabolism. From this standpoint it was shown in a previous communication (4) that the protein quotient of blood serum is not changed by the administration of certain drugs which are known to retard or accelerate the metabolic processes. The present work is a continuation of the former experiments, and its object is to determine the influence on the protein quotient of a disturbance of metabolism when such a disturbance is produced by periods of digestion alternating with prolonged periods of starvation.

Although many determinations of the serum proteins during starvation and digestion have been reported by various investigators; yet, the inadequate methods for estimating the serum proteins, which were then available, have so precluded accurate and thorough work, that a wide disparity in the results obtained by different workers is rather the rule than the exception. The chief disadvantage in the older methods is that large quantities of blood are required for a determination. This made frequent estimations in the smaller animals impossible, and as a result the normal variation was not known, and the intermediate effects remained undertermined.

To illustrate the marked lack of uniformity in the findings of different investigators, the following reports have been taken at random from the literature.

Salvioli (5) found that the quantity of globulins in the blood serum of two dogs starved for twenty to twenty-four hours is less than in two other dogs during a period of digestion. This change he attributed to individual variations, since he found no difference in the globulins of the same dog during fasting and digestion.

Burckhard (6), however, obtained in the serum of fasting dogs a marked increase in the globulins and a considerable decrease in the albumins. He explained these findings on the hypothesis that during starvation the globulins pass from the organs into the blood in order to cover the deficiency in the albumins.

Lewinski (7) reported only a slight increase in the serum globulins of dogs after prolonged periods of starvation.

Briggs (8) obtained a considerable decrease in the globulins of certain species of birds starved for a period of at least twenty-four hours. This work faces the criticism, however, in that the normal values were ascertained for one group of animals and the values in starvation for another; hence the comparison of the latter data with the former is not fully justified.

## METHOD AND MATERIALS

The experimental conditions were very much the same as those of the previous work (4). Robertson's micro-refracto-

TABLE 1

*Rabbit 1. Weight: June 8, 3332 grams; June 21, 2023 grams*

| DATE | TREATMENT | NON PROTEIN | ALBUMIN | GLOBULIN | TOTAL PROTEIN | GLOBULIN OF TOTAL PROTEIN | PROTEIN QUOTIENT |
|---|---|---|---|---|---|---|---|
| | | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | |
| May 7 | Normal | 1.6 | 4.4 | 1.1 | 5.5 | 20.0 | 0.25 |
| May 8 | Normal | 1.3 | 3.8 | 1.0 | 4.8 | 21.0 | 0.26 |
| May 10 | Normal | 1.6 | 3.8 | 1.1 | 4.9 | 23.0 | 0.29 |
| May 11 | Normal | 1.6 | 4.3 | 1.4 | 4.4 | 23.0 | 0.32 |
| May 12 | Normal | 1.5 | 3.8 | 1.2 | 5.0 | 24.0 | 0.32 |
| May 13 | Normal | 1.5 | 4.3 | 1.1 | 5.4 | 20.0 | 0.26 |
| May 14 | Normal | 1.5 | 4.0 | 1.1 | 5.1 | 21.0 | 0.27 |
| May 16 | Normal | 1.5 | 4.2 | 1.0 | 5.2 | 19.0 | 0.24 |
| June 8 | Normal | 1.5 | 5.2 | 0.8 | 6.0 | 13.0 | 0.16 |
| June 9 | Starved, 1 day | 1.4 | 5.1 | 1.0 | 6.1 | 16.0 | 0.20 |
| June 10 | Starved, 2 days | 1.4 | 6.0 | 0.7 | 6.7 | 10.5 | 0.10 |
| June 10 | Fed, 2 hours | 1.6 | 4.3 | 1.4 | 5.7 | 24.0 | 0.33 |
| June 12 | Starved, 1 day | 1.0 | 5.0 | 1.2 | 6.2 | 19.0 | 0.24 |
| June 14 | Starved, 3 days | 1.4 | 4.9 | 1.3 | 6.2 | 21.0 | 0.27 |
| June 14 | Fed, $4\frac{1}{2}$ hours | 1.4 | 4.9 | 1.1 | 6.0 | 18.0 | 0.22 |
| June 17 | Starved, 2 days | 1.2 | 5.5 | 1.0 | 6.5 | 17.0 | 0.18 |
| June 20 | Starved, 5 days | 1.4 | 5.5 | 1.1 | 6.6 | 17.0 | 0.20 |
| June 20 | Fed, $3\frac{1}{2}$ hours | 1.2 | 5.0 | 1.1 | 6.1 | 18.0 | 0.22 |
| June 21 | Normal | 1.4 | 4.5 | 0.75 | 5.25 | 14.0 | 0.17 |
| June 23 | Normal | 1.4 | 4.3 | 0.7 | 5.0 | 14.0 | 0.16 |

TABLE 2

*Rabbit 2. Weight: June 8, 2261 grams; June 21, 1666 grams*

| DATE | TREATMENT | NON PROTEIN | ALBUMIN | GLOBULIN | TOTAL PROTEIN | GLOBULIN OF TOTAL PROTEIN | PROTEIN QUOTIENT |
|---|---|---|---|---|---|---|---|
| | | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | |
| June 8 | Normal | 1.3 | 4.6 | 1.6 | 6.2 | 26.0 | 0.35 |
| June 9 | Starved, 1 day | 1.4 | 5.1 | 1.5 | 6.6 | 23.0 | 0.29 |
| June 10 | Starved, 2 days | 1.3 | 5.0 | 2.0 | 7.0 | 28.0 | 0.40 |
| June 10 | Fed, 2 hours | 1.4 | 5.1 | 2.9 | 8.0 | 36.0 | 0.57 |
| June 12 | Starved, 1 day | 1.4 | 5.1 | 1.7 | 6.8 | 25.0 | 0.33 |
| June 14 | Starved, 3 days | 1.2 | 5.6 | 1.8 | 7.4 | 24.0 | 0.32 |
| June 14 | Fed, 4 hours | 1.3 | 5.4 | 1.4 | 6.8 | 20.5 | 0.26 |
| June 17 | Starved, 2 days | 1.2 | 5.2 | 1.7 | 6.9 | 25.0 | 0.33 |
| June 20 | Starved, 5 days | 1.8 | 4.7 | 1.4 | 6.1 | 23.0 | 0.30 |
| June 20 | Fed, 3 hours | 1.3 | 5.0 | 1.1 | 6.1 | 23.0 | 0.22 |
| June 21 | Normal | 1.0 | 4.4 | 1.4 | 5.8 | 24.0 | 0.32 |
| June 23 | Normal | 1.1 | 4.2 | 1.15 | 5.35 | 21.5 | 0.27 |

metric method (9) was employed for the estimation of the serum proteins. Determinations were made in all cases through a fore-period of several days, to serve as a safe standard for comparison with the values to be obtained during the subsequent period of starvation and digestion. For the analysis four cubic centimeters of fresh blood drawn from a marginal ear vein was employed.

Rabbits were selected as the experimental animals.

During the fore-period the rabbits were fed once daily with a moderate quantity of alfalfa hay and crushed barley. Suffi-

TABLE 3

*Rabbit 3. Weight: June 8, 3332 grams; June 21, 2380 grams*

| DATE | TREATMENT | NON PROTEIN | ALBUMIN | GLOBULIN | TOTAL PROTEIN | GLOBULIN OF TOTAL PROTEIN | PROTEIN QUOTIENT |
|---|---|---|---|---|---|---|---|
| | | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | |
| May 7 | Normal | 1.4 | 4.2 | 1.0 | 5.2 | 19.0 | 0.24 |
| May 8 | Normal | 1.2 | 4.2 | 1.0 | 5.2 | 19.0 | 0.24 |
| May 10 | Normal | 1.2 | 4.4 | 1.0 | 5.4 | 18.5 | 0.23 |
| May 11 | Normal | 1.2 | 4.4 | 1.1 | 5.5 | 19.5 | 0.24 |
| May 12 | Normal | 1.5 | 4.1 | 1.0 | 5.1 | 19.5 | 0.24 |
| May 13 | Normal | 1.3 | 4.5 | 1.1 | 5.6 | 20.0 | 0.24 |
| May 14 | Normal | 1.2 | 4.2 | 1.0 | 5.2 | 19.0 | 0.24 |
| May 16 | Normal | 1.3 | 4.6 | 0.9 | 5.55 | 16.0 | 0.20 |
| May 18 | Normal | 1.4 | 3.9 | 1.4 | 5.3 | 26.0 | 0.35 |
| May 19 | Normal | 1.3 | 4.7 | 1.0 | 5.7 | 18.0 | 0.21 |
| May 20 | Normal | 1.3 | 4.4 | 1.5 | 5.95 | 25.0 | 0.34 |
| May 24 | Normal | 1.1 | 5.5 | 1.2 | 5.7 | 17.0 | 0.22 |
| May 29 | Normal | 1.3 | 4.5 | 1.2 | 5.7 | 21.0 | 0.26 |
| June 8 | Normal | 1.3 | 4.6 | 1.1 | 5.7 | 20.0 | 0.24 |
| June 9 | Starved, 1 day | 1.2 | 4.7 | 1.1 | 5.8 | 20.0 | 0.23 |
| June 10 | Starved, 2 days | 1.2 | 5.0 | 1.0 | 6.0 | 17.0 | 0.20 |
| June 10 | Fed, 2 hours | 1.8 | 4.0 | 1.1 | 5.1 | 21.0 | 0.27 |
| June 12 | Starved, 1 day | 1.1 | 4.7 | 1.2 | 5.9 | 20.0 | 0.26 |
| June 14 | Starved, 3 days | 1.3 | 5.2 | 1.1 | 6.3 | 17.5 | 0.21 |
| June 14 | Fed, 3½ hours | 1.4 | 4.7 | 1.1 | 5.8 | 20.0 | 0.23 |
| June 17 | Starved, 2 days | 1.2 | 5.0 | 1.4 | 6.4 | 22.0 | 0.28 |
| June 20 | Starved, 5 days | 1.3 | 5.0 | 1.1 | 6.1 | 18.0 | 0.22 |
| June 20 | Fed, 2½ hours | 1.3 | 4.6 | 1.0 | 5.6 | 18.0 | 0.22 |
| June 21 | Normal | 1.2 | 4.1 | 0.9 | 5.0 | 18.0 | 0.22 |
| June 23 | Normal | 1.2 | 4.1 | 1.1 | 5.2 | 21.0 | 0.27 |

TABLE 4

*Rabbit 4. Weight: June 8, 4165 grams; June 21, 3570 grams*

| DATE | TREATMENT | NON PROTEIN | ALBUMIN | GLOBULIN | TOTAL PROTEIN | GLOBULIN OF TOTAL PROTEIN | PROTEIN QUOTIENT |
|---|---|---|---|---|---|---|---|
| | | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | |
| April 11 | Normal | 1.4 | 4.8 | 1.2 | 6.0 | 20 | 0.25 |
| April 13 | Normal | 1.4 | 5.4 | 1.2 | 6.6 | 18 | 0.22 |
| April 16 | Normal | 1.4 | 5.2 | 1.5 | 6.7 | 22 | 0.29 |
| April 18 | Normal | 1.1 | 5.4 | 1.2 | 6.6 | 18 | 0.22 |
| April 20 | Normal | 1.4 | 5.1 | 1.4 | 6.5 | 22 | 0.27 |
| June 8 | Normal | 1.3 | 5.2 | 1.5 | 6.7 | 22 | 0.29 |
| June 9 | Starved, 1 day | 1.2 | 5.1 | 1.5 | 6.6 | 23 | 0.30 |
| June 10 | Starved, 2 days | 1.2 | 5.5 | 1.4 | 6.9 | 20 | 0.25 |
| June 10 | Fed, 2 hours | 1.4 | 5.1 | 1.0 | 6.1 | 16 | 0.20 |
| June 12 | Starved, 1 day | 1.3 | 4.8 | 1.6 | 6.4 | 25 | 0.33 |
| June 14 | Starved, 3 days | 1.1 | 4.8 | 1.5 | 6.3 | 25 | 0.31 |
| June 14 | Fed, 3 hours | 1.3 | 4.8 | 1.6 | 6.4 | 25 | 0.33 |
| June 17 | Starved, 2 days | 1.1 | 5.0 | 1.5 | 6.5 | 23 | 0.30 |
| June 21 | Starved, 6 days | 1.0 | 4.8 | 1.9 | 6.7 | 28 | 0.40 |
| June 21 | Fed, 4 days | 1.2 | 5.1 | 1.8 | 6.9 | 26 | 0.35 |
| June 23 | Normal | 1.2 | 4.2 | 1.7 | 5.9 | 29 | 0.40 |

TABLE 5

*Rabbit 5. Weight: June 8, 3094 grams; June 21, not weighed*

| DATE | TREATMENT | NON PROTEIN | ALBUMIN | GLOBULIN | TOTAL PROTEIN | GLOBULIN OF TOTAL PROTEIN | PROTEIN QUOTIENT |
|---|---|---|---|---|---|---|---|
| | | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | |
| April 12 | Normal | 1.4 | 3.6 | 1.8 | 5.4 | 33 | 0.50 |
| April 14 | Normal | 1.7 | 3.9 | 2.2 | 6.1 | 36 | 0.56 |
| April 17 | Normal | 1.4 | 4.3 | 1.9 | 6.2 | 31 | 0.44 |
| April 19 | Normal | 1.4 | 4.2 | 1.8 | 6.0 | 30 | 0.42 |
| June 8 | Normal | 1.2 | 4.7 | 2.2 | 6.9 | 32 | 0.47 |
| June 9 | Starved, 1 day | 1.2 | 5.3 | 2.3 | 7.6 | 30 | 0.43 |
| June 10 | Starved, 2 days | 1.2 | 4.8 | 2.2 | 7.0 | 31 | 0.46 |
| June 10 | Fed, 2 hours | 1.9 | 4.85 | 2.2 | 7.05 | 31 | 0.46 |
| June 12 | Starved, 1 day | 1.2 | 4.9 | 2.2 | 7.1 | 35 | 0.47 |
| June 14 | Starved, 3 days | | Sample lost | | | | |
| June 14 | Fed, $2\frac{3}{4}$ hours | 1.3 | 4.6 | 2.5 | 7.1 | 35 | 0.54 |
| June 17 | Starved, 2 days | 1.2 | 4.7 | 2.55 | 7.25 | 37 | 0.54 |
| June 19 | Starved, 4 days | | Died | | | | |

TABLE 6

*Rabbit 6. Weight: June 8, 2618 grams; June 21, 2023 grams*

| DATE | TREATMENT | NON PROTEIN | ALBUMIN | GLOBULIN | TOTAL PROTEIN | GLOBULIN OF TOTAL PROTEIN | PROTEIN QUOTIENT |
|---|---|---|---|---|---|---|---|
| | | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | |
| June 8 | Normal | 1.2 | 5.5 | 1.9 | 7.4 | 31.0 | 0.35 |
| June 9 | Starved, 1 day | 1.3 | 5.2 | 2.1 | 7.3 | 29.0 | 0.40 |
| June 10 | Starved, 2 days | 1.2 | 4.9 | 2.2 | 7.1 | 31.0 | 0.45 |
| June 10 | Fed, 2 hours | 1.9 | 4.1 | 2.3 | 6.4 | 36.0 | 0.56 |
| June 12 | Starved, 1 day | 1.3 | 4.5 | 2.4 | 6.9 | 35.0 | 0.53 |
| June 14 | Starved, 3 days | 1.3 | 4.8 | 2.55 | 7.35 | 35.0 | 0.53 |
| June 14 | Fed, $2\frac{1}{2}$ hours | 1.3 | 5.0 | 2.2 | 7.2 | 30.5 | 0.44 |
| June 17 | Starved, 2 days | 1.2 | 5.0 | 2.05 | 7.05 | 29.0 | 0.41 |
| June 20 | Starved, 5 days | 1.2 | 5.0 | 2.2 | 7.2 | 30.5 | 0.44 |
| June 20 | Fed, 2 hours | 1.2 | 5.0 | 2.2 | 7.2 | 30.5 | 0.44 |
| June 21 | Normal | 1.2 | 4.35 | 2.2 | 6.55 | 33.5 | 0.51 |
| June 23 | Normal | 1.3 | 4.2 | 1.9 | 6.1 | 31.0 | 0.45 |

TABLE 7

*Rabbit 7. Weight: June 8, 3094 grams; June 21, 2618 grams*

| DATE | TREATMENT | NON PROTEIN | ALBUMIN | GLOBULIN | TOTAL PROTEIN | GLOBULIN OF TOTAL PROTEIN | PROTEIN QUOTIENT |
|---|---|---|---|---|---|---|---|
| | | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | |
| May 7 | Normal | 1.3 | 4.8 | 1.9 | 6.7 | 28.0 | 0.39 |
| May 8 | Normal | 1.5 | 4.7 | 2.0 | 6.7 | 28.0 | 0.42 |
| May 9 | Normal | 1.4 | 4.8 | 2.0 | 6.8 | 29.0 | 0.41 |
| May 10 | Normal | 1.5 | 4.6 | 1.5 | 5.1 | 29.0 | 0.33 |
| May 11 | Normal | 1.5 | 4.9 | 1.7 | 5.6 | 30.0 | 0.34 |
| May 12 | Normal | 1.5 | 4.6 | 1.5 | 6.1 | 29.5 | 0.33 |
| May 13 | Normal | 1.4 | 4.0 | 2.0 | 6.0 | 33.0 | 0.50 |
| May 14 | Normal | 1.4 | 4.7 | 1.6 | 5.3 | 30.0 | 0.43 |
| May 16 | Normal | 1.4 | 4.8 | 1.7 | 6.5 | 27.0 | 0.35 |
| June 8 | Normal | 1.2 | 5.4 | 2.1 | 7.5 | 28.0 | 0.39 |
| June 9 | Starved, 1 day | 1.3 | 5.4 | 2.1 | 7.5 | 28.0 | 0.39 |
| June 10 | Starved, 2 days | 1.3 | 5.2 | 2.4 | 7.6 | 32.0 | 0.46 |
| June 10 | Fed, 2 hours | 1.4 | 4.5 | 2.4 | 6.9 | 35.0 | 0.35 |
| June 12 | Starved, 1 day | 1.3 | 5.6 | 2.75 | 8.35 | 33.5 | 0.49 |
| June 14 | Starved, 3 days | 1.1 | 5.2 | 2.5 | 7.7 | 32.0 | 0.48 |
| June 14 | Fed, $1\frac{3}{4}$ hours | 1.2 | 5.8 | 2.0 | 7.8 | 32.0 | 0.34 |
| June 17 | Starved, 2 days | 1.0 | 5.0 | 2.5 | 7.5 | 33.0 | 0.50 |
| June 21 | Starved, 6 days | 1.0 | 4.8 | 2.8 | 7.6 | 37.0 | 0.51 |
| June 21 | Fed, $\frac{3}{4}$ hour | 1.0 | 4.8 | 2.8 | 7.6 | 37.0 | 0.51 |

cient fresh water was always in the cages during the fore-period and during the period. At the beginning of the period, all the straw bedding and other possible food was removed from the cages. Where the interval of starvation exceeded two days, determinations of the serum proteins were made to show the intermediate effect of the fasting. At the termination of the starvation interval, blood was drawn for determinations. The animals were then given an excess of alfalfa hay, crushed barley, cabbage, and carrots. This interval of feeding was allowed to vary from three-quarters of an hour to four hours and a half, in order that several gradations of the intensity of digestion may be included. All the food was removed again, at the end of this feeding interval, and blood was immediately once more drawn for determinations. A longer interval of fasting then followed, and so on.

## DISCUSSION

From a perusal of the tables presented, it will be seen that the *protein quotient remains normal during digestion periods alternating with prolonged starvation periods.*

The negative data for digestion are fully in accord with the findings of Rowe (10) who showed that high protein diets have no immediate effect on the globulin-albumin ratio.

It will be recalled that the retardation of the nitrogenous metabolism as shown by the diminution in the elimination of nitrogen during starvation (11), is due to the intrinsic change, which consists of a gradual decrease in the exogenous metabolism, accompanied but not equalled by an acceleration in the endogenous metabolism. Viewed in the light of these profound metabolic disorders, the constancy of the protein quotient during prolonged starvation is not an easy matter to explain.

An analogous phenomenon is the constant and normal percentage of glucose in the blood even during an extended period of fasting (12). It is assumed that this constancy of composition is maintained chiefly by the action of enzymes elaborated in the liver cells, which convert dextrose into glycogen, or glycogen

into dextrose, depending respectively on whether the glucose concentration in the blood is in the upper or lower limits of the normal variation.

Is it not then also probable that a similar mechanism serves to adjust the constancy of the protein quotient?

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# THE IMMUNOLOGIC PROPERTIES OF UVEAL PIGMENT

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In the course of a detailed study of the anaphylactic theory of sympathetic ophthalmia, a study has been made of the antigenic properties of the uveal tract, especially the uveal pigment, of the eye. From the standpoint of the ophthalmologist the results are of little import beyond their possible correlation with clinical disease. From the standpoint of the immunologist, however, the results appear to be of more scientific interest, in so far as they indicate rather striking immunologic properties for a native body protein. It is, therefore, the purpose of this paper to report the results of this phase of our studies.

The anaphylactic theory of sympathetic ophthalmia, advanced and advocated by Elschnig, assumes that sympathetic ophthalmia is an anaphylactic uveitis brought about in the following manner. The injury to the uvea in the exciting eye, by trauma, intraocular tumor, etc., leads to a destruction or disintegration of uveal tissue. This uveal tissue is absorbed and acts as an antigen, producing a hypersensitiveness of the organism and especially of the other eye. A reaction now takes place between the sensitized cells of the uvea of the second eye and the antigen circulating in the blood or lymph. This anaphylactic reaction or intoxication is manifested clinically as a sympathetic ophthalmia.

This theory, of course, assumes that the cells of the uveal tract, or some constituent of these cells, possess antigenic properties in the homologous animal, and further assumes organ

specificity and lack of species specificity for the protein involved.

Elschnig fully realized these assumptions and in his presentation of the theory in its present form, presented experimental work to substantiate these assumed points.

## HISTORICAL

Elschnig's work (1), appearing in a series of papers, may be summarized as follows. Using as his methods the intraocular injection of sheep erythrocytes and cholera vibrio extracts, and as his indices the hemolytic titer and agglutination reactions of the blood serum, he established the fact that absorbtion from the eye could lead to immune body production. He then sought to determine three points, to give scientific support to the anaphylactic theory. These points were: (I) Does uveal tissue possess antigenic properties? If so, in the homologous animal? (II) What constituent of the uveal tract is responsible for such properties? (III) What are the antigenic properties as regards organ and species specificity? Elschnig immunized rabbits against various heterologous uveae, and against homologous uvea, and finally against so-called "chemically pure" pigment from the uveal tract of various animals. Thus he obtained hetero-immune and iso-immune uvea serum, and pigment-immune sera. He then used the complement fixation reaction for the detection of immune bodies in these sera and the study of their properties. He found that heterologous uvea produced immune bodies, as would be expected. Homologous uvea, also, acted as antigen and produced immune bodies. The sera of animals immunized to uvea fixed complement with any uvea antigen, irrespective of species—the immune bodies were organ specific. As regards species specificity, the sera of animals immunized to an emulsion of whole uvea were species specific in their reactions. The sera of animals immunized to pigment, however, were not species specific. The reason for this apparent anomaly is evident. Emulsion of whole uvea contains two elements, (1) pigment which is not species specific and (2) such

tissue as blood, smooth muscle and connective tissue which is species specific. Immunization with heterologous uveal emulsion gives an immunity not only to non-species specific pigment, but also to the other species specific elements in the uveal tract. The species specificity of uvea immune sera is due to these last elements.

Elschnig therefore concluded that uveal tissue could act as antigen in the same animal, and that the pigment was the constituent responsible for this property. The pigment was organ specific and not species specific.

The remaining work on this subject may be quickly summarized, Weichardt and Kummel (2), using the doubtfully valuable epiphanin reaction, substantiated Elschnig's findings. Likewise, with the epiphanin reaction, and complement fixation, Kummel (3), demonstrated uveal antibodies in a percentage of the sera of patients with sympathetic ophthalmia. Wissman (4), supported the anaphylactic theory by experimental work and showed uvea immune bodies in the serum of two sympathetic ophthalmia patients by the precipitin reaction, but failed to substantiate Kummel's work with complement fixation, on the sera of these patients. Rados (5), substantiated Elschnig's work only partially. Fuch and Meller (6), were unable to demonstrate uveal antibodies in the sera of patients with sympathetic ophthalmia. Von Szily (7), after lending much valuable support to the theory criticizes the antigenic properties of pigment.

## OUTLINE OF WORK

In our study of the various phases of the anaphylactic theory of sympathetic ophthalmia, we have studied primarily the immunologic properties of uveal tissue, and especially of uveal pigment. This has been done in two ways, first by a repetition of Elschnig's work with the complement fixation reaction of the sera of immunized animals, and secondly this has been confirmed by direct perfusion experiments upon the eyes of sensitized animals.

## I. THE ANTIGENIC PROPERTIES OF UVEAL TISSUE AS SHOWN BY COMPLEMENT FIXATION

This, as before mentioned, is substantially a repetition, in toto, of Elschnig's work.

Dogs were immunized, by intraperitoneal injections repeated every six days, against cow's uveal emulsion, cow's uveal pigment and dog's uveal emulsion and uveal pigment.[1] In order to avoid non-specific fixation, the sera of the dogs selected for this work were all examined, in the complement fixation reaction, with the various uveal emulsion and uveal pigment antigens, before immunization was begun. Eight dogs giving completely negative reactions with all antigens in this preliminary work, were selected for immunization. To immunize, intraperitoneal injections of uveal emulsion and uveal pigment were given to respective dogs at six day intervals. The initial injection was 5 cc. which was increased 1 cc. at each injection until 10 cc. was reached. A total of eight immunizing injections was given each animal.

At suitable intervals after the last injection, the sera of these dogs was tested again against all the antigens. The results are shown in the tables 1 to 4. In order to quantitate the strength of the reaction, three different quantities of sera were used with every antigen. A $+++$ reaction indicates complete, or practically complete, fixation of complement with all quantities of sera, a $++$ reaction similar fixation of complement in the tubes containing two largest amounts of sera, and a single $+$ reaction indicates that such fixation was observed only in the tube containing the maximum amount of serum.

The first two complement fixation reactions were done with the same antigens used in the preliminary reaction. The last reaction was done with freshly prepared antigens. All antigens were used in one-third the anticomplementary dose.

All animals showed immune sera, differing, as might be expected, in the various dogs. Dogs 16–96 and 16–98 showed the lowest immunity.

[1] For the details of technique used to prepare the uveal emulsion and uveal pigment consult references (8) and (12).

Table 1 illustrates the complement binding phenomena shown by heterologous immune sera. As would be expected these sera gave complement binding with their specific antigens. Moreover, the uvea-immune sera gave fixation of complement with pigment antigens. Pigment immune sera fixed complement with pigment antigens, showing here that the pigment itself is capable of acting as antigen.

TABLE 1

*Heterologous sensitization (cow's uvea and pigment)*

| SERA | ANTIGEN | DECEMBER 14 | Period of immunization | JANUARY 31 | FEBRUARY 14 | FEBRUARY 21, FRESH ANTIGENS |
|---|---|---|---|---|---|---|
| 16–89. Uvea immune... | Cow's uvea | Negative | | ++ | ++ | +++ |
| | Cow's pigment | Negative | | ++ | + | +++ |
| 16–90. Uvea immune... | Cow's uvea | Negative | | +++ | +++ | +++ |
| | Cow's pigment | Negative | | + | ++ | +++ |
| 16–91. Pigment immune | Cow's uvea | Negative | | +++ | ++ | +++ |
| | Cow's pigment | Negative | | ++ | ++ | +++ |
| 16–92. Pigment immune | Cow's uvea | Negative | | +++ | +++ | +++ |
| | Cow's pigment | Negative | | +++ | ++ | +++ |

Table 2 illustrates the complement binding reactions of the sera of dogs immunized to dog's uveal emulsion and dog's pigment—iso-immune sera. The iso-immune sera gave fixation of complement with their specific antigens, showing the presence of iso-immune bodies. In other words, uveal tissue possesses the power to act as a foreign protein—as antigen—in animals of the same species. This is the first, and cardinal point, to be proven in order to establish the anaphylactic theory. Furthermore, the purified pigment of the uvea possesses this power of immune body production in the homologous animal, and uvea immune sera gives fixation of complement with pigment antigens. This substantiates Elschnig's contention that it is the pigment which is the responsible factor for the peculiar antigenic properties of uveal tissue.

TABLE 2

*Homologous immunization (dog's uvea and pigment)*

| SERA | ANTIGEN | DECEMBER 14 | Period of immunization | FEBRUARY 6 | FEBRUARY 14 | FEBRUARY 21, FRESH ANTIGENS |
|---|---|---|---|---|---|---|
| 16–95. Uvea immune. | Dog's uvea | Negative | | +++ | ++ | +++ |
| | Dog's pigment | Negative | | ++ | ++ | ++ |
| 16–96. Uvea immune. | Dog's uvea | Negative | | ++ | + | +++ |
| | Dog's pigment | Negative | | + | Negative | ++ |
| 16–98. Pigment immune | Dog's uvea | Negative | | + | + | ++ |
| | Dog's pigment | Negative | | + | + | ++ |
| 16–99. Pigment immune | Dog's uvea | Negative | | ++ | + | ++ |
| | Dog's pigment | Negative | | + | ++ | ++ |

TABLE 3

*Organ specificity*

| SERA | ANTIGEN | DECEMBER 14 | Period of immunization | JANUARY 1 FEBRUARY 6 | FEBRUARY 14 | FEBRUARY 21, FRESH ANTIGENS |
|---|---|---|---|---|---|---|
| 16-89. Cow's uvea immune | Dog's uvea | Negative | | ++ | + | ++ |
| | Dog's pigment | Negative | | + | + | ++ |
| 16–90. Cow's uvea immune | Dog's uvea | Negative | | ++ | + | + |
| | Dog's pigment | Negative | | + | Negative | + |
| 16–91. Cow's pigment immune | Dog's uvea | Negative | | ++ | Negative | ++ |
| | Dog's pigment | Negative | | + | Negative | ++ |
| 16–92. Cow's pigment immune | Dog's uvea | Negative | | ++ | + | +++ |
| | Dog's pigment | Negative | | ++ | + | ++ |
| 16–95. Dog's uvea immune | Cow's uvea | Negative | | +++ | ++ | +++ |
| | Cow's pigment | Negative | | ++ | + | +++ |
| 16–96. Dog's uvea immune | Cow's uvea | Negative | | +++ | ++ | +++ |
| | Cow's pigment | Negative | | ++ | + | +++ |
| 16–98. Dog's pigment immune | Cow's uvea | Negative | | +++ | ++ | ++ |
| | Cow's pigment | Negative | | +++ | ++ | +++ |
| 16–99. Dog's pigment immune | Cow's uvea | Negative | | +++ | ++ | ++ |
| | Cow's pigment | Negative | | +++ | ++ | ++ |

Table 3 illustrates the organ specificity of the immune sera. Iso-immune sera gave positive complement binding reactions with heterologous antigens, and the heterologous immune sera gave complement binding with the iso-antigens. In other words the immune bodies were organ-specific, showing an affinity for antigens of the specific tissue, without regard to the species from which obtained. This substantiates Elschnig's second point of the organ specificity of uveal tissue. The pigment-immune sera show this property also and it is evidently to the pigment constituent that this organ specificity of uveal tissue is due

TABLE 4

*Species specificity*

| SERA | ANTIGEN | Period of immunization | JANUARY 31 AND FEBRUARY 6 | FEBRUARY 14 | FEBRUARY 21, FRESH ANTIGEN |
|---|---|---|---|---|---|
| 16–89. Cow's uvea immune | Cow's liver | | +++ | + | ++ |
| | Cow's kidney | | +++ | ++ | ++ |
| 16–90. Cow's uvea immune | Cow's liver | | +++ | +++ | +++ |
| | Cow's kidney | | +++ | +++ | +++ |
| 16–91. Cow's pigment immune | Cow's liver | | Negative | Negative | ++ |
| | Cow's kidney | | + | Negative | + |
| 16–92. Cow's pigment immune | Cow's liver | | + | + | Negative |
| | Cow's kidney | | + | ++ | ++ |

Table 4 illustrates the question of species specificity. With the sera of animals immunized to cow's uveal emulsion and cow's uveal pigment, it was determined whether or not fixation of complement occurred with antigens of cow's protein, other than uveal tissue. It will be seen by a study of this table that the uvea-immune sera give complete fixation of complement with antigens of cow's liver and kidney extract. The reason for this is that already stated—that these dogs were immunized to the whole uvea, which contained not only the uveal pigment, but also some blood, smooth muscle and connective tissue of the cow. The fixation of complement with the liver and kidney

extracts is evidently dependent upon the immunization to this last factor.

On the other hand, the sera of dogs immunized to cow's pigment alone gave either weak or negative reactions with cow's liver and kidney. The pigment was purified as much as possible, but it probably still contained traces of the other elements of the uveal tract—the blood, smooth muscle and connective tissue. The weak reactions occasionally observed are probably due to this impurity.

However, the difference in the degree of fixation of complement between the uvea immune and the pigment immune sera with the same antigens, is striking. Although in this phase of the work, our results are not so clear cut as those of Elschnig, it seems probable that the pigment acting as antigen, lacks at least in a degree, species specificity.

These observations of the complement binding reactions of uvea and pigment immune sera corroborate Elschnig's findings. Heterologous and homologous uveal tissues have the power of acting as antigens. In the case of homologous uveal tissue, the pigment is the factor responsible for its antigenic properties in animals of the same species.

Whole uveal emulsion is both organ specific and species specific, the species specificity probably being due to the blood, smooth muscle, and connective tissue in the emulsion. The pigment, however, is organ specific and probably not species specific, and in this respect is analogous to lens protein and differs from other common body protein.

These properties of uveal pigment—ability to act as foreign protein to animals of the same species, organ specificity, and lack of species specificity—are as before emphasized, fundamental properties uveal tissue must needs possess to make the anaphylactic theory a possibility.

## II. THE ANTIGENIC PROPERTIES OF UVEAL TISSUE AS SHOWN BY PERFUSION OF THE EYES OF SENSITIZED ANIMALS

### *a. The reaction of the sensitized eye to perfusion with specific antigen*

With the confirmation of Elschnig's fundamental work we next sought, both for the purpose of again confirming this work, and of more closely correlating these findings to the clinical disease, to determine whether these peculiar antigenic properties indicated by complement fixation, could be manifested in a direct anaphylactic reaction upon the eye, through general sensitization and vascular intoxication. To this end, we resorted to the perfusion of the eye. Figure 1, shows diagramatically the operation employed and the position of the inflow and outflow cannulas. The apparatus employed has already been reported (8). Fresh defibrinated dogs blood to which sufficient Ringer's solution was added to bring the red corpuscle content approximately to normal, was the perfusion fluid used.

With the technique used we were dealing with what was to all purposes a living eye, maintained on an artificial circulation with defibrinated blood, oxygenated by an artificial lung. Although the dog's heart always ceased to beat after the final ligature was placed above the heart, nevertheless the winking reflex persisted in the eyes often for an hour or more.

In this experiment the eyes were perfused for three hours, constant observations being made throughout that period.

It was found that when a sensitized dog was perfused with the defibrinated blood of a normal dog, there was no ocular reaction. When, however, the specific antigen (the antigen to which the dog was sensitized) was added to the perfusion fluid, a prompt contraction of the pupil occurred, and as the perfusion continued, small hemorrhages appeared throughout the fundus. The contraction of the pupil was marked, usually from a dilated pupil 10 to 12 mm. in diameter to a pupil from 2 to 4 mm. in diameter. This observation is in direct accord with those of Dale (9) and Schultz (10) who observed the contraction of sensitized smooth muscle in the presence of specific antigen, and

is another observation in support of the cellular theory of anaphylaxis. The hemorrhages observed cannot be so easily

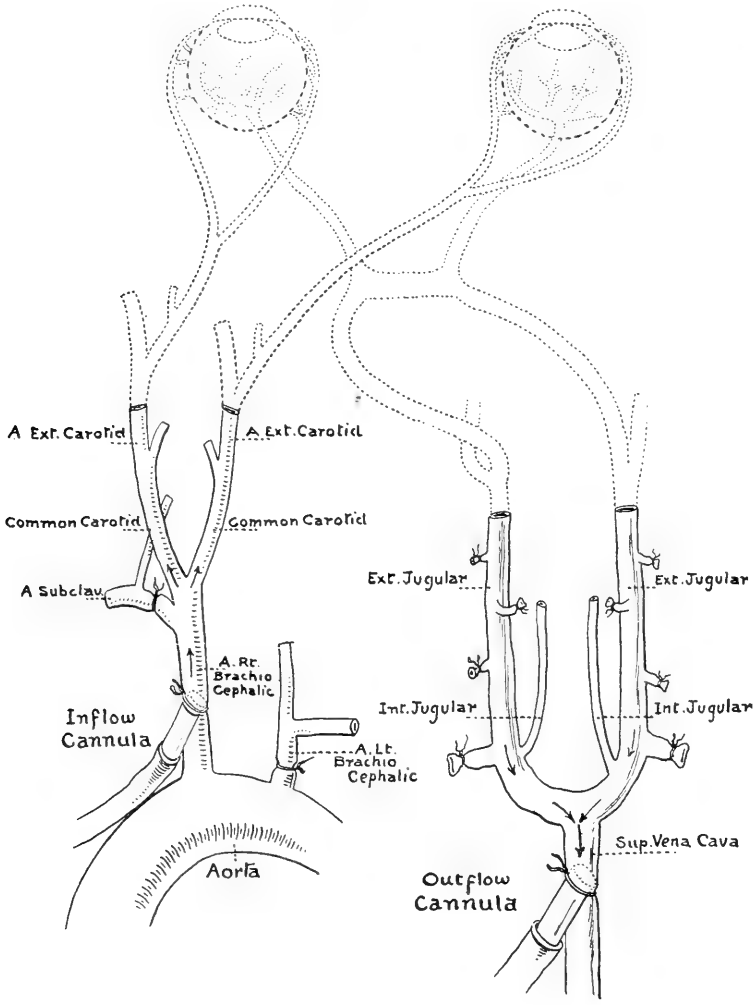


FIG. 1

explained. Petechial hemorrhages have been observed over the peritoneum of animals recovering from anaphylactic shock, and it may be that these are analogous. From their method of

formation it appeared that they were caused by an alteration of the endothelium of the capillaries, allowing a diapedesis of red cells.

In this experiment, the antigens used were horse serum and cows uveal emulsion. Before proceeding with the second phase of the perfusion work a number of perfusions were done to control this observation. These controls are illustrated in table 5. One of these controls requires a little explanation. Normal dogs perfused with horse serum showed a slight contraction of the pupil, never over 3 mm. Schultz (11) previously has shown that fresh horse serum possesses the power to produce a slight contraction of smooth muscle, and this is evidently what oc-

TABLE 5

*Controls*

| SENSITIZATION | PERFUSION FLUID | RESULTS | |
|---|---|---|---|
| | | Contraction of the pupil | Hemorrhages in fundi |
| None | D. B. | None | None |
| None | D. B. + Uveal emulsion | None | None |
| None | D. B. + Horse serum | Never over 3 mm. | None |
| Uveal emulsion | D. B. | None | None |

D. B. is defibrinated blood of normal dogs.

curred with us. This contraction of the pupil in normal dogs, perfused with horse serum, was never over 3 mm. while the contraction in the sensitized dogs was from 8 to 10 mm. Normal dogs perfused with uveal tissue showed no contraction of the pupil. In these and in all subsequent perfusion experiments here reported, each individual observation was controlled by at least two perfusions. In those experiments where for any reason (clots, thrombosis) the nature of the reaction was not clear after two perfusions had been performed, subsequent perfusions were done until the presence or absence of the anaphylactic phenomena was established beyond question.

From this it was evident, therefore, that the eyes may be sensitized as a part of general sensitization, and that anaphy-

lactic phenomena may be elicited in the eyes by means of antigen carried by the blood stream. These anaphylactic phenomena consist in a marked contraction of the pupil, and in small extravasations of blood throughout the fundus.

With the artificial condition, under which we were working, it is manifestly impossible to expect inflammatory phenomena of any kind, but the establishment of the fact that ocular anaphylaxis, however manifested, may be demonstrated by a general sensitization and vascular intoxication, gives us the second important point in the establishment of a scientific basis for the anaphylactic theory. Moreover, this observation affords us a means of studying the anaphylactic properties of uveal tissue, in vivo, by direct observation of the eye.

*b. The antigenic properties of uveal tissue in the production of the perfusion anaphylactic reaction*

Having shown that an anaphylactic reaction can be obtained through the perfusion of the eye with specific antigen, this reaction has been used to determine the antigenic properties of uveal tissue. We first sought to determine whether whole uveal tissue possessed the antigenic properties necessary to make an anaphylactic uveitis a possibility. When this point was established, we sought to determine the constituent of uveal tissue responsible for its peculiar antigenic properties. Preliminary experiments with uveal pigment showed that there was much reason to believe, with Elschnig, that the pigment was the responsible factor. A pigment solution was finally prepared which was suitable for use in perfusion (12). Experiments similar to those in which uveal emulsion was used as antigen were then performed to determine whether the pigment is the responsible factor. The reactions given by the pigment were in every way similar to those given by whole uvea, except as regards species specificity. Moreover, dogs sensitized to homologous uvea, reacted to perfusion with pigment, establishing more conclusively the fact that the pigment is responsible for the peculiar antigenic properties shown by uveal tissue.

The results of this work are shown in the following tables (6 to 9).

Table 6 represents the results obtained by sensitization and perfusion with heterologous (cow's) uveal emulsion and uveal pigment. The anaphylactic reaction was observed in all of these perfusions, establishing the power of both uveal emulsion and pigment to act as antigens.

TABLE 6

*Heterologous sensitization (cow's uvea and pigment)*

| SENSITIZATION | PERFUSION FLUID | RESULT | |
|---|---|---|---|
| | | Contraction of the pupil | Hemorrhages in fundi |
| Cow's uveal emulsion | D. B. + Cow's uveal emulsion | Marked | Marked |
| Cow's uveal pigment | D. B. + Cow's uveal pigment | Marked | Marked |

D. B. is defibrinated blood of normal dogs.

Table 7 illustrates the fundamental fact necessary in the anaphylactic theory—namely the power of homologous uveal tissue to produce an ocular anaphylactic reaction in a sensitized eye when carried there through vascular channels. It shows also that it is the pigment which is responsible for this remarkable antigenic property. Dogs sensitized to homologous uveal emulsion give an anaphylactic reaction when perfused with normal defibrinated blood to which pigment is added, and dogs sensitized to pigment give the reaction when perfused with the pigment-containing blood.

TABLE 7

*Homologous sensitization (dog's uvea and pigment)*

| SENSITIZATION | PERFUSION FLUID | RESULT | |
|---|---|---|---|
| | | Contraction of the pupil | Hemorrhages in fundi |
| Dog's uveal emulsion | D. B. + Dog's uveal emulsion | Marked | Marked |
| Dog's uveal emulsion | D. B. + Dog's pigment | Marked | Marked |
| Dog's uveal pigment | D. B. + Dog's pigment | Marked | Marked |

D. B. is defibrinated blood of normal dogs.

Table 8 illustrates the organ specific property of uveal emulsion and uveal pigment. As shown before by the complement fixation reaction, uveal tissue and pigment are organ specific. The sensitization resulting from the introduction of uveal tissue into an animal, is specific for uveal tissue, without regard for the species from which the tissue is taken. Dogs sensitized to cow's uveal emulsion react to perfusion with dog's uveal emulsion, and vice versa. Similarly dogs sensitized to cow's pigment react to perfusion with dog's pigment, and vice versa. There evidently results from sensitization with uveal tissue, a strong chemical affinity for similar tissue,—organ specificity.

TABLE 8

*Organ specificity*

| SENSITIZATION | PERFUSION FLUID | RESULT | |
|---|---|---|---|
| | | Contraction of the pupil | Hemorrhages in fundi |
| Cow's uveal emulsion | D. B. + Dog's uveal emulsion | Marked | Marked |
| Cow's uveal pigment | D. B. + Dog's pigment | Marked | Marked |
| Dog's uveal emulsion | D. B. + Cow's emulsion | Marked | Marked |
| Dog's uveal pigment | D. B. + Cow's pigment | Marked | Marked |

D. B. is defibrinated blood of normal dogs.

Table 9 shows the species specificity reaction of both uveal emulsion and pigment. The same properties are shown here as were indicated by complement fixation, the uveal emulsion is species specific, while the pigment is not species specific. Dogs sensitized to cow's uveal emulsion give an anaphylactic reaction when perfused with other cow protein, for here the sensitization is not alone with the pigment, but also with the other species specific protein contained in the whole uveal emulsion. On the other hand, dogs sensitized to the pigment alone show no reaction when perfused with other cow protein.

The last perfusion illustrated in this table is largely a control perfusion. Dogs sensitized to uveal emulsion give no reaction when perfused with other dog protein. Perfusion with the other elements of the uveal tract—blood, smooth muscle and connective tissue, evokes no anaphylactic reaction.

To sum up the work thus far, both the complement fixation reactions of the immune sera, and the perfusion reactions, have shown that uveal tissue possesses the power to act as antigen in animals of the same species, and that the pigment is the constituent of the uvea responsible for this property. In its antigenic action, uveal pigment is organ specific and not species specific. The sensitization resulting from the absorption of uveal pigment is specific. Other body protein can produce no anaphylactic reaction in animals so sensitized. Pigment alone may produce intoxication.

TABLE 9

*Species specificity*

| SENSITIZATION | PERFUSION FLUID | RESULTS | |
|---|---|---|---|
| | | Contraction of the pupil | Hemorrhages in fundi |
| Cow's uveal emulsion | D. B. + Cow's serum, liver and kidney extracts | Marked | Marked |
| Cow's uveal pigment | D. B. + Cow's serum, liver and kidney extracts | None | None |
| Dog's uveal emulsion | D. B. + Dog's liver, and kidney extracts | None | None |

*c. Ocular sensitization from antigen absorbed from the other eye*

To complete our study of the antigenic properties of uveal tissue as manifested in the ocular perfusion reaction, and as an incidental point in our study of the anaphylactic theory, we sought to determine whether uveal tissue, absorbed in one eye, could create a hypersensitiveness to uveal tissue in the second eye. To demonstrate this we sensitized dogs by the injection of uveal tissue, both heterologous and homologous, into the vitreous of one eye. After a suitable period had elapsed for sensitization to occur, the injected eyes were enucleated, in order to remove any factor which could give a possible intoxication. One week after this, the remaining eye was perfused with the specific antigen. In every case, the perfused eye gave an anaphylactic reaction, indicating that ocular sensitization had

taken place as a result of the absorption of antigen from the fellow eye. Table 10 illustrates this experiment.

TABLE 10

*Ocular sensitization*

| SENSITIZATION | | PERFUSION FLUID | RESULTS | |
|---|---|---|---|---|
| | | | Contraction of pupil | Hemorrhages in fundi |
| Vitreous injection. Left eye. Cow's uveal emulsion | Enucleation left eye | D. B. + Cow's uveal emulsion | Marked | Marked |
| Vitreous injection. Left eye. Dog's uveal emulsion | Enucleation left eye | D. B. + Dog's uveal emulsion | Marked | Marked |

## DISCUSSION

As is well known, Uhlenhuth (13) and Haendel (14) have established the fact that animals can be sensitized to their own lens protein, and Rosenau and Anderson (15) have found that guinea-pigs can be sensitized to guinea-pig's placenta. From the evidence brought out by the researches of Elschnig and this work, it seems that uveal pigment possesses these same antigenic properties. However, we realize that we have not demonstrated the actual point of auto-sensitization, the sensitization of a dog to the pigment from his own eye. The technical difficulties in the way of such a proof are enormous. One attempt to demonstrate such auto-sensitization by general anaphylactic reactions has been unsuccessful. The eye of a dog was removed, the uvea excised and macerated, and injected to sensitize. Two weeks later the second eye was removed, the uvea excised and macerated, and this again injected intravenously under ether anesthesia, and this dog was observed for a fall in blood pressure, change in the coaguability of the blood, and drop in body temperature. No such signs were observed and we have little hope of demonstrating auto-sensitization by this method. A more delicate method must be devised. For the present we must content ourselves with the demonstration that dogs uvea can produce ocular anaphylactic phenomena in the dog.

## CONCLUSIONS

The pigment of the uveal tract of the eye possesses the properties of acting as antigen in homologous animals, and in its immunologic reactions is organ specific and not species specific. These findings can be demonstrated by the complement fixation reaction with the sera of properly immunized animals, and by perfusion experiments on the eyes of sensitized animals. In the case of the perfusion experiments, the anaphylactic reaction is manifested by a marked contraction of the pupil, and the occurrence of small hemorrhages in the fundus. This reaction was used to study the antigenic properties of uveal pigment, and the results shown by complement fixation confirmed.

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# THE EXAMINATION OF THE BLOOD PRELIMINARY TO THE OPERATION OF BLOOD TRANSFUSION

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Before the operation of blood transfusion is carried out it is necessary to test the compatibility of the prospective donor's blood with that of the patient; that is, it must be determined whether the blood corpuscles of the donor will remain intact in the circulation of the patient. It is altogether probable that no two human individuals possess blood having exactly the same chemical composition; indeed, the possible different combinations of demonstrated different substances in the corpuscles alone have been estimated to be about 4000 (1).

Fortunately, however, the differences in the blood plasma and most of those in the corpuscles, although they are, perhaps, the cause of certain unpleasant symptoms, such as a chill followed by a rise of temperature, do not constitute an incompatibility such as could contraindicate the use of the blood of an individual for transfusion. Incompatibility in the latter sense is always due to the presence in the blood of the one individual of isoagglutinins or isohemolysins or both of these agencies against the corpuscles of the other individual. As the isohemolysins are never present without associated isoagglutinins and since the latter are sometimes found alone, it is necessary, for the purpose under consideration, to examine the blood of the respective individuals only for the presence of isoagglutinins.

Through the studies of Landsteiner and of von Dungern it is known that there are two different isoagglutinins in human blood.[1] These have been designated with the capital letters A

[1] A discussion of the large body of evidence supporting this assumption would lead too far from the practical purpose of this paper.

and B. The respective agglutinable elements in the corpuscles are designated with the small letters a and b. An isoagglutinin, e.g., "A" and its corresponding agglutinable substance "a" cannot be present in the same person, as this would result in the agglutination of the individual's corpuscles by his own plasma. All of the possible combinations of the substances A, B, a and b are found in human beings and individuals have been separated into four groups in accordance with the different combinations of those substances. This grouping is shown in table I.

TABLE 1

| | | | |
|---|---|---|---|
| Group I | Plasma.....A and B<br>Corpuscles.....0 | Group III | Plasma.....A<br>Corpuscles.....b |
| Group II | Plasma.....B<br>Corpuscles.....a | Group IV | Plasma.....0<br>Corpuscles.....a and b |

The grouping is schematically shown in figure 1, which consists of four test tubes containing blood of the four groups of individuals.

Since the plasma of individuals of group I contains both A and B isoagglutinins it is able to agglutinate the corpuscles of the individuals of all of the other groups all of which contain one or both of the agglutinable substances a and b. Similarly it is clear that a group II plasma will agglutinate group III or group IV corpuscles but not those of group I. Group III plasma will agglutinate group II or group IV corpuscles but not those of group I. Finally, group IV plasma is not able to agglutinate any corpuscles since it lacks both isoagglutinins.

The proportional (percentage) representation of the four groups among human individuals has been estimated in three different studies as follows:

| | VON DUNGERN AND HIRSCHFELD (2) | W. L. MOSS (3) | MIRIAM OLMSTEAD (4) |
|---|---|---|---|
| Group I | 36.0 | 43 | 46 |
| Group II | 47.0 | 40 | 39 |
| Group III | 11.0 | 7 | 13 |
| Group IV | 5.7 | 10 | 2 |

These results show that by far the greater number (about

83 per cent) of human individuals are nearly equally divided between groups I and II.

It is evident that donors that are of the same group as the patient should be preferred for the transfusion, but if such a donor is not available then one of another group must be selected whose corpuscles are not agglutinated by the patient's plasma. In such a case the donor's plasma will usually contain agglutinins for the patient's corpuscles but this fact does not constitute a contraindication to the use of the donor's blood for the trans-

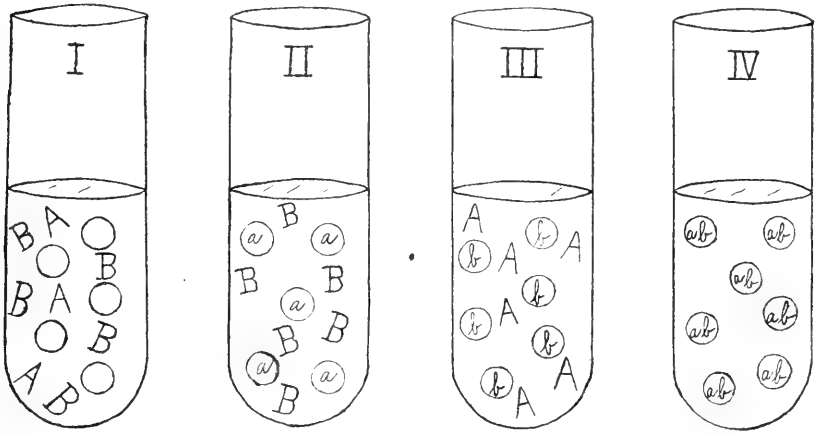


FIG. 1. BLOOD GROUPING OF HUMAN INDIVIDUALS (SCHEMATIC)

The corpuscles are represented by the circles, the plasma by the space surrounding the circles.

fusion because the donor's plasma is so diluted in the patient's circulation that its agglutinating or hemolytic power is reduced below the point of injury to the patient's corpuscles.

The testing of patient's and donor's blood preliminary to transfusion may be done directly or indirectly.

### DIRECT TEST

For carrying out the direct test it was formerly customary to obtain several cubic centimeters of blood by venepuncture from both patient and donor a small part of which was mixed with

sodium citrate solution the remainder being allowed to clot. The citrated blood was washed with normal saline solution and in a dilute (1–10) suspension it was mixed with the clear serum of the other individual. After an incubation of one or two hours at 37°C. the presence of agglutination and hemolysis was determined macroscopically.

Weil (5) took the first step in the simplification of this time consuming procedure by mixing the citrated blood of the two individuals in three proportions: namely, one part of A with nine parts of B; one of A with one of B; and nine of A with one of B. He found that the phenomenon of agglutination was not interfered with by the presence of the even more numerous corpuscles not taking part in that reaction, and that the reaction could be observed macroscopically after the usual incubation period. Hemolysis, also, could be detected by the presence of hemoglobin in the supernatant fluid of the mixtures after centrifugation.

A further modification in the interest of economy of blood was contributed by Rous and Turner (6). These authors using the ordinary white blood cell mixing pipet drew up first, 10 per cent sodium citrate to the mark 1 and then blood from the finger to the mark 11 and blew out the citrated blood into a narrow test tube. The two bloods were then mixed with the use of the Wright capillary pipet in the proportions suggested by Weil, and the mixtures sealed in the capillary pipet by fusing the tip. After fifteen minutes the tips of the sealed pipets were broken and a drop of the mixtures examined microscopically in normal saline solution for agglutination.

The following method, which is based on a suggestion of Dr. James Ewing, permits the mutual tests to be made with practically a single drop of blood from each individual and with the use of little more than the usual apparatus employed for a white blood cell count.

The patient and the donor or donors being in the same or adjoining rooms, a finger of each individual is prepared as for an ordinary blood count, for which purpose soap and water meet every requirement. Three ordinary glass slides are placed

in order as shown in figure 2. For convenience the right and left ends of all of the slides or of only slide 3 may be marked *A* and *B*, the first letter referring to the patient, the second one referring to a donor. The stem of a white blood cell mixing pipet is filled with normal saline solution up to the ninth mark and the fluid thus measured (nine divisions) is deposited on slide 1 at the right (position *B*). The pipet is then washed out with a 10 per cent solution of sodium citrate, enough of this solution being left in the stem to fill the terminal division. The

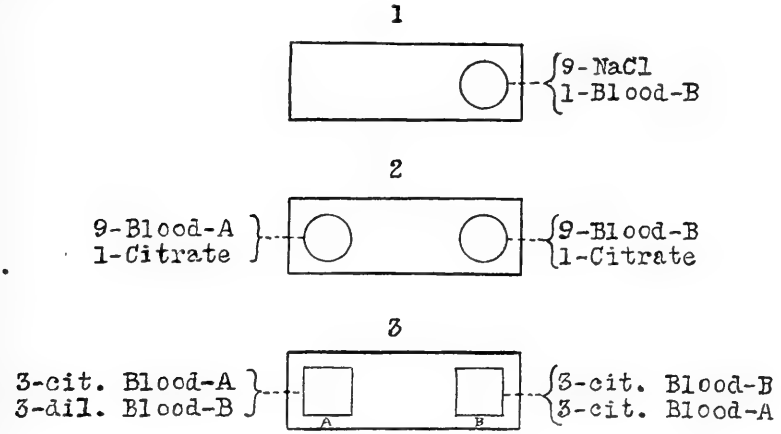


FIG. 2. SHOWING ARRANGEMENT OF SLIDES IN THE BLOOD TEST.

patient's finger is punctured as for a blood-count and the blood is drawn up into the stem of the pipet to the mark 1. This mixture of blood and citrate solution is deposited on slide 2 at the left and a similar mixture of the donor's blood with citrate solution is deposited at the right of the same slide, the pipet being thoroughly washed, between the two operations, first with saline solution and then with the citrate solution. One division of the donor's citrated blood (*B*) is well mixed with the nine divisions of saline solution at the right of slide 1, the resulting mixture thus representing a 1 to 10 dilution of the donor's blood *B*. If equal parts of this diluted blood and of the undiluted citrated blood of the patient *A* are mixed, the resulting mixture

will represent 9 parts of blood *A* and 1 part of blood *B* in 10 parts of saline diluent. Such a mixture is made by drawing up with the white blood cell mixing pipet, first, 3 divisions of citrated blood-*A* at the left of slide 2 and then 3 divisions of diluted blood-*B*, the two portions being then deposited together at the left of slide 3, stirred once with the tip of the pipet and covered with a $\frac{7}{8}$ inch cover-glass. At the right of slide 3 is placed a mixture of 3 divisions each of the two undiluted citrated bloods at right and left of slide 2, this mixture, likewise, being covered with a cover-glass. In making this second mixture, which, in a sense, is intended as the reverse of the first mixture, it is not necessary to dilute the patient's citrated blood —*A*— ; first, because, in most instances, the corpuscular content of the patient's blood is reduced sufficiently to meet the purpose of the dilution and secondly, because the quantitative relation in the mixture, as far as the ratio of donor's plasma to patient's corpuscles is concerned, is nearer that at the actual transfusion than if the patient's blood were used in a 1 to 10 dilution. If, however, mutual tests are being made in two normal individuals in order, for example, to determine their group relationship, then blood-*A* must be diluted 1 to 10 before being mixed with blood-*B* in the second mixture.

Agglutination usually begins at once and can easily be detected with the microscope as it has been described by previous authors. If agglutination does not appear the observation should be continued for fifteen minutes, at the end of which time only the borders of the covered blood films will have begun to dry.

To recapitulate: three ordinary glass slides are placed in order as shown in figure 1 and designated 1, 2 and 3, the third slide being marked left and right for identification with the letters *A* and *B*. With a white blood cell mixing pipet a mixture of 9 divisions of the patient's blood and 1 division of 10 per cent sodium citrate is placed at the left on slide 2 and a similar mixture of the donor's blood and the citrate solution is placed at the right of the same slide. A mixture of 1 division of the citrated donor's blood and 9 divisions of normal saline solution is placed at the right end of slide 1. Three divisions of the citrated

blood-*A* and 3 divisions of the diluted blood-*B* are drawn up into the pipet deposited at the left of slide 3 and covered with a cover-glass. Three divisions each of the two undiluted citrated bloods are similarly deposited at the right of slide 3 and covered with a cover-glass. These two latter mixtures are immediately examined microscopically for agglutination.

If more than one donor is to be tested the series of slides must be duplicated or triplicated et cetera according to the number of prospective donors. In such case two divisions of the citrate solution are left in the end of the pipet before obtaining the patient's blood the latter being then taken twice up to the mark 1. This amount suffices for the examination of three donors.

### INDIRECT TEST

If the blood-grouping of patient and donors has been determined it is unnecessary to carry out the direct test because the availability of the donor's blood for the transfusion can be learned by referring to the constitution of the blood of the different groups as shown in table 1 or in figure 1. A patient of group I, for example, requires a donor of group I, the blood of all other groups being incompatible. The indirect test is based on the foregoing principle and for its performance, as Moss pointed out, it requires the storing of sera of the two groups II and III. If the corpuscles of an individual are not clumped by either of these sera, that individual belongs to group I; if they are clumped by both sera they must be group IV corpuscles; if they are clumped only by the group II serum or only by the group III serum then they belong respectively to a group III individual or to a group II individual.

The technical procedure of the direct test is applicable also to the indirect test. If the patient and a single donor are to be examined the mixtures on slides 1 and 2 (figure 1) are prepared exactly as for the direct test. Two slides—3a and 3b—must take the place of slide 3 in figure 1 since each blood is to be mixed with both stock sera. Three divisions each of the patient's undiluted citrated blood are mixed respectively with three divisions of the two sera and placed under cover-glasses at the

two ends of slide 3a and similar mixtures of the diluted donor's blood with the two sera are placed on slide 3b. Clumping, if present, can be seen at once with the microscope and in a few minutes it becomes apparent to the naked eye.

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# EXPERIMENTS UPON THE PASSIVE TRANSFER OF ANTIBODIES FROM THE BLOOD TO THE CEREBROSPINAL FLUID

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Since the cerebrospinal fluid under normal conditions is generally free of certain normal or natural antibodies or other constituents which may be present in the blood, as, for example, hemolysins for the erythrocytes of various animals, agglutinins for various micro-organisms, diphtheria antitoxin and complement, the mechanism concerned in the production of the cerebrospinal fluid under normal conditions is regarded as an effectual barrier against the entrance of antigens, antibodies and various chemical substances into the cerebrospinal fluid. For this reason the presence of antibodies in the cerebrospinal fluid during disease is generally interpreted as indicating infection of the tissues of the central nervous organs with the production of antibodies by tissues in direct communication with the cerebrospinal fluid or, by an injurious effect upon the choroid plexus and subarachnoid villi, facilitating the passage of antibodies from the blood to the cerebrospinal fluid. As shown by Wile and Stokes (1, 2 and 3) and Hauptmann (4) the cerebrospinal fluid may show the presence of syphilis reagin (the antibody concerned in the Wassermann reaction) and present abnormal chemical and cytological changes in the late primary or early secondary periods of that infection, with or without demonstrable clinical evidences of infection of the nervous system. For this reason Wile and Stokes very properly emphasize the importance of careful clinical examinations of the nervous system during the early stages of syphilis, coupled with a thorough examination of the

cerebrospinal fluid and regard the presence of syphilis reagin in the fluid as an indication of spirochaetic invasion of the tissues of the central nervous organs.

As agglutinin for *B. typhosus* may be found in the cerebrospinal fluid during typhoid fever without clinical manifestations of involvement of the central nervous organs, it would appear possible for *T. pallida* or other micro-parasites lodged in tissues other than the nervous tissues to stimulate the production of antibodies to such extent as to reach a high degree of concentration in the blood with a passive or forcible transfer of these antibodies into the cerebrospinal fluid in a manner analogous to the transfer or elimination of the syphilis reagin in the secretions of the mammary glands and kidneys. The object of our experiments was to determine whether antibodies introduced into the venous blood of normal experimental animals passed into the cerebrospinal fluid and more particularly if this occurred after the introduction of the syphilis antibody or reagin, in order to study by experimental means whether the presence of antibodies in the cerebrospinal fluid is to be accepted as indicating the presence and activity of the respective antigen or antigens in the tissues of the central nervous organs, or, whether antibody in high concentration in the blood may be passed into the cerebrospinal fluid by the uninjured mechanism governing the production of cerebrospinal fluid.

## EXPERIMENTAL

Dogs were employed in all experiments because of the ease with which amounts of blood-free cerebrospinal fluid up to 2 cc. may be secured by spinal puncture. In experiments concerning the passive transfer of the syphilis reagin from the blood to the cerebrospinal fluid, normal dogs were bled from the carotid artery under ether anesthesia and an equal amount of human serum from active cases of syphilis was injected intravenously. Wassermann reactions were conducted with the blood and cerebrospinal fluid of each animal prior to injection and at varying intervals afterward; in conducting these tests, the sera were used in amounts of 0.1 cc. and the cerebrospinal fluid in amounts of

0.5 cc. with each of three antigens; namely, an alcoholic extract of beef heart re-enforced with cholesterin; an alcoholic extract of syphilitic liver and an extract of acetone insoluble lipoids. The following protocols of several experiments express the results observed.

*a. The passive transfer of human syphilis reagin in the blood of a normal dog to the cerebrospinal fluid.* Weight 6250 grams; preliminary Wassermann reactions with serum and cerebrospinal fluid negative with all antigens. Under ether anesthesia 150 cc. of blood was removed from the carotid artery and 210 cc. of human syphilitic serum yielding ++++ Wassermann reactions with all antigens, were injected into the jugular vein.

Wassermann tests with cerebrospinal fluid removed twenty-two hours later were weakly positive.

Wassermann tests with blood removed two and four and one-half hours later were moderately positive, twenty-two hours after, the tests were weakly positive and seventy hours later, completely negative.

The tests with urine secured four and one-half and twenty-two hours after the injection of syphilitic serum were negative in doses of 0.5 cc. urine with each antigen.

*b.* A second dog weighing 5420 grams was bled 150 cc. under ether anesthesia and 250 cc. of human syphilitic serum yielding ++++ Wassermann reactions with all antigens, were injected into a femoral vein. Preliminary Wassermann tests with blood and cerebrospinal fluid yielded negative reactions with all antigens.

Cerebrospinal fluid removed four hours later yielded doubtfully positive reactions with each of the three antigens; fluid removed twenty-four hours after transfusion yielded negative reactions.

Wassermann tests with blood serum secured two and four hours after injection yielded moderately positive reactions, while tests with serum secured twenty-four and seventy-two hours after injection were negative with all antigens.

*c. The passive transfer of human syphilis reagin in the blood of a dog to the cerebrospinal fluid after the preliminary injection of sterile horse serum into the spinal canal.* Since the experiments of Flexner and Amoss (5) with the virus of acute anterior poliomyelitis have indicated that the injection of sterile fluids into the spinal canal of monkeys facilitates the passage of the virus from the blood to the central nervous organs, due presumably to injury to the mechanism governing the pro-

duction of cerebrospinal fluid, we have conducted experiments similar to those already summarized except that the animals received an intraspinal injection of 1 cc. of sterile horse serum twenty-four hours before transfusion with human syphilis serum, followed by tests of the cerebrospinal fluid and blood for the syphilis reagin.

Dog, weighing 9150 grams, was given an intraspinal injection of 1 cc. sterile horse serum. Wassermann tests with blood and cerebrospinal fluid were negative with all antigens. Twenty-four hours later 150 cc. of blood were removed from the carotid artery under ether anesthesia and 150 cc. of human syphilitic serum yielding ++++ Wassermann reactions with all antigens, were injected into a femoral vein.

Cerebrospinal fluid removed four hours later yielded moderately positive Wassermann reactions with all antigens; cerebrospinal fluid removed twenty-two hours after the injection of serum yielded completely negative reactions with all antigens.

Blood serum secured four hours after transfusion yielded moderately positive reactions and twenty-two hours after transfusion weakly positive reactions. Tests made with the serum forty-eight hours after transfusion were negative with all antigens.

While in this experiment the amount of human syphilitic serum injected was less per kilogram of body weight than employed in the former experiments, the amount of reagin in the cerebrospinal fluid appeared to be somewhat greater as judged by the degree of complement fixation with each of the three antigens; a similar result was observed in the following experiment.

*d.* A dog weighing 7050 grams was given an intraspinal injection of 1 cc. of sterile horse serum. Preliminary Wassermann tests with cerebrospinal fluid and blood serum were negative with all antigens. Twenty-four hours later 150 cc. of blood were removed from the carotid artery under ether anesthesia and 250 cc. of human syphilitic serum yielding ++++ Wassermann reactions with each of the three antigens, were injected into a femoral vein.

Cerebrospinal fluid removed three hours later yielded moderately positive reactions with each of the three antigens; fluid secured twenty-two hours after the transfusion yielded doubtfully positive reactions, after forty-eight hours the fluid yielded completely negative reactions.

Blood serum secured three hours after transfusion yielded strongly positive reactions; serum secured twenty-two hours after yielded weakly positive reactions while with serum secured seventy hours after the reactions were completely negative.

*e. The passive transfer of typhoid agglutinin (dog) from the blood of a normal dog to the cerebrospinal fluid.* As in the experiments already mentioned a heterologous immune serum (human) was employed, it was not surprising that elimination of the syphilis reagin from the blood stream of the dogs was rapid and usually completed within seventy-two hours to such extent that the reagin could not be demonstrated in the blood serum by the Wassermann tests. We have immunized a dog with typhoid bacilli until the amount of agglutinin in the blood reached a high degree of concentration. During the process of immunization the blood serum and cerebrospinal fluid were examined at intervals for the presence of agglutinin. At the completion of the period of immunization the animal was bled to death under ether anesthesia and the serum was injected into a second normal dog followed by examinations of the cerebrospinal fluid for the presence of agglutinins.

A dog weighing 9450 grams yielded negative agglutination reactions with an emulsion of typhoid bacilli in final dilutions of 1: 2 with blood serum and cerebrospinal fluid. After a series of fourteen intravenous injections with typhoid vaccine at intervals of four and five days, the serum agglutinated in final dilution of 1: 5120 and the cerebrospinal fluid in final dilution of 1: 8 (microscopic technic). The animal was now bled and 150 cc. of serum secured.

From a second dog weighing 5000 grams 100 cc. of blood were taken from a carotid artery under ether anesthesia and 150 cc. of the typhoid-immune serum were injected into a femoral vein. Preliminary agglutination tests with the serum and cerebrospinal fluid of this animal yielded negative agglutination tests in final dilutions of 1: 2 with an emulsion of typhoid bacilli.

Cerebrospinal fluid removed three hours after transfusion yielded complete agglutination of *B. typhosus* in dilutions up to 1: 5; fluid removed twenty-four hours later agglutinated 1: 4 while forty-eight hours after transfusion agglutination was not in evidence with the lowest dilution; namely, 1: 2.

The blood serum removed three and again twenty-four hours after transfusion yielded complete agglutination in final dilutions up to 1: 160; forty-eight hours after transfusion the titer was 1: 40 and ninety-six hours later only partial agglutination occurred in final dilutions of 1: 4 and 1: 8.

## DISCUSSION

These experiments have, in our opinion, demonstrated two facts with special reference to syphilis reagin, namely, that large amounts of this antibody in the blood may result in the passage of small amounts into the cerebrospinal fluid with a normal condition of the central nervous organs and of the mechanism governing the production of the cerebrospinal fluid; secondly, that subarachnoid injection of sterile serum appears to facilitate the passage of antibody by injury to the mechanism governing the production of the fluid or by the production of localized congestion with increased transudation of blood constituents into the cerebrospinal fluid. Accordingly it would appear possible that in syphilis or other acute infections accompanied by a high concentration of antibody in the blood, small amounts of antibody may be found in the cerebrospinal fluid and that this finding does not of itself necessarily indicate infection of the central nervous organs. These observations, however, should have no further significance and do not by any means lessen the value of the studies of Wile and Stokes in syphilis, because they have found the reagin in the cerebrospinal fluid during the preroseolar period when the concentration of reagin in the blood cannot be regarded as having reached a point of high concentration and, furthermore, found in some cases an increase of protein and cells in the cerebrospinal fluid, which cannot be explained at present on any other basis than actual infection of the nervous tissues with *T. pallida*.

## SUMMARY

1. The removal of blood from normal dogs followed by the intravenous injection of human syphilitic serum in amounts varying from 30 to 50 cc. per kilogram of body weight was followed by the presence of small amounts of syphilis reagin (the antibody concerned in the Wassermann reaction) in the cerebrospinal fluid.

2. The reagin was found in the cerebrospinal fluid as early as three hours after transfusion with syphilitic serum; tests at shorter intervals were not made. The amount of reagin found in 0.5 cc. of cerebrospinal fluid was small in all experiments as based upon the degree of complement fixation with all antigens.

3. After irritation of the spinal meninges by the preliminary injection of sterile horse serum the amount of reagin gaining access to the cerebrospinal fluid after transfusion of syphilitic serum, appeared to be somewhat greater.

4. All traces of syphilis reagin in the cerebrospinal fluid of dogs following transfusion of human syphilitic serum apparently disappeared after 22 to 48 hours as determined by completely negative Wassermann reactions.

5. The intravenous injection of dog-typhoid immune serum into a normal dog in amount of about 30 cc. per kilogram of body weight, was followed by the appearance of traces of agglutinin in the cerebrospinal fluid within three hours after transfusion; fluid removed forty-eight hours later was free of agglutinin.

6. These experiments demonstrate the possibility of the passage of antibody from the blood into the cerebrospinal fluid without primary involvement of the central nervous organs or injury to the mechanism concerned in the production of cerebrospinal fluid, when the amount of antibody in the blood has reached a point of high concentration. While it is possible that in human syphilis the presence of traces of reagin in the cerebrospinal fluid may be due to the passive transfer of this substance from the blood, as shown by Wile and Stokes the presence of the reagin with or without other changes in the fluid, as an increase of protein and cells usually indicates the presence and activity of *T. pallida* in the tissues of the central nervous organs.

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# THE ISOLATION, PURIFICATION AND CONCENTRATION OF IMMUNE BODIES: A STUDY OF IMMUNE HEMOLYSIN

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The numerous attempts to isolate immune bodies from their original sera and to concentrate them have, up to this time, not given satisfactory results. To obtain pure immune substance free from serum protein is, on the one hand, a most important condition for the solution of problems as to the biophysical and biochemical properties of immune bodies and it has, on the other hand, an even more important relation to the therapeutic use of antibodies, as, for example, in the possibility that by such means the uncomfortable results of serum disease, which are said to be engendered by serum protein, may be avoided.

Many workers have inquired into this problem with various methods, which will be classified according to their purpose in two groups. One group of methods endeavors to eliminate all of the serum protein except the immune substance from the immune serum, while the other seeks to extract only the immune bodies from the respective sera.

### *1. The elimination of serum protein*

With the purpose of facilitating the practical use of immune bodies the attempt to eliminate the serum protein has been chiefly performed with respect to diphtheria or tetanus antitoxin.

*a. Fractionating method.* By saturation with magnesium sulphate or half saturation with ammonium sulphate the antitoxin in serum is precipitated with some kinds of globulin. Thus, by treating certain immune sera with ammonium sulphate, Pick (1) found that from diphtheria antiserum produced in the horse the antitoxin is precipitated with the pseudoglobulin whereas from the antiserum of the goat it is precipitated with the euglobulin. By this means the total amount of serum protein can be remarkably diminished and the concentration

of antitoxin presumably can be expected. Pick states that by the isolation of those fractions it is possible to concentrate the protective power ten to fifteen times. This method of Pick was proved thereafter by many workers not to be practicable.

Brieger and Krause (2) state that they succeeded in eliminating 75 per cent of total nitrogen from diphtheria serum, while preserving the original antitoxic power. The antitoxic serum is diluted with an equal volume of distilled water and it is then treated with ammonium sulphate. The resulting precipitate is placed in a 10 per cent watery solution of glycerin and treated with sodium chloride. The precipitate produced by the sodium chloride contains no antitoxin, while the fluid portion has protective power.

Gibson (3) prepared a refined and concentrated diphtheria antitoxin in a similar way. By means of half saturation of ammonium sulphate, and treatment of the precipitate with a saturated solution of sodium chloride containing acetic acid, he could concentrate the number of units of antitoxin per cubic centimeter from 200 to 500. But in carrying out this process there was a loss of about 30 per cent of antitoxin units. Gibson applied this method of concentration not only to diphtheria antitoxin, but also with his co-worker Collins (4) to the concentration of agglutinin.

Frouin's (5) method is more widely known. He added 2 to 5 per cent watery solution of glycerin to diphtheria immune serum and then saturated it with sodium chloride. The filtrate was placed on a water bath at 75° to 80°C. for ten to fifteen minutes. The coagulated serum was placed in 2 to 3 volumes of a half saturated solution of sodium chloride and macerated in the ice-box for twelve to thirteen hours. The maceration was repeated at least three times, each time with a fresh saline solution. After this manipulation the total decanted fluid was dialyzed and concentrated *in vacuo* at about 35°C. to the original volume.

All of the above mentioned or other so-called fractionating methods, however, do not give constant and satisfactory results, though they have been more frequently employed in the practical preparation of antitoxin than the other methods of concentration that are immediately to be described.

*b.* Evaporation and freezing also have been employed for the concentration of immune bodies as the works of Bujwid (6), Ernst, Coolidge and Cook (7) and Hata (8) show, but the use of these methods has not been continued, because by these methods not only the immune substance, but also all the colloidal substances in serum are concentrated.

*c.* Reasoning from the assumption that antitoxin and other immune bodies are of non-proteid nature, many workers have attempted to eliminate the serum protein by trypsin digestion. Thus Pröscher (9) treated diphtheria serum with extract of pancreas at 32°C. and expected to peptonize all of the protein substance associated with the immune bodies. Then by half saturation with ammonium sulphate he precipitated the antitoxin out of the solution of pepton. The last trace of pepton was eliminated by dialysis. Similar attempts, which were made by Belfanti and Carbonne (10), Pick and Brieger have not given satisfactory results, because the antibodies, also, appear to be attacked by trypsin, though their destruction is very slow.

All of these methods designed to eliminate serum protein from immune serum are too incomplete to be used in general, because there are difficulties in the technique of handling the blood proteins and our knowledge of serum proteins is still in confusion. Whether immune substances are of proteid nature or not is quite unknown. Though an increase in the globulin content of the blood as the result of immunization was proved by Atkinson (11), Moll (12) and recently by Hurwitz and Meyer (13), which may be indicative of the serum-globulin nature of immune body, Joachim (14) has observed that the increase is manifested in the non-protective fraction. Glässner (15), also, states that immunization can be accomplished without any essential globulin change.

These methods have, therefore, only a practical interest their use having resulted, to some degree, in a concentration of the immune bodies, but they have no biological interest, because the purification of the immune bodies with these methods is not possible. It seems likely, then, that it will not be possible to solve this great problem by any process of elimination of the serum protein.

*2. Extraction of immune bodies from their original sera*

*a. By employing their antigens.* The question whether antigen and antibody once combined are again separable or not has been treated by many workers. When antigen has been mixed with antibody, the two unite at once if the conditions are favorable, but this union differs from a neutralization in a chemical sense inasmuch as it is not so difficult to separate the former combination again. Thus Calmette (16) made a neutral mixture of snake venom and its antitoxin again toxic, by heating it at 68°C. v. Wassermann (17) also obtained the same result with respect to a neutral mixture of pyocyaneus toxin and its anti-

toxin. Morgenroth (18) extracted the toxic substance from the toxin-antitoxin mixture of both cobra venom and diphtheria toxin by means of weak hydrochloric acid.

Hahn and Trommsdorf (19) treated agglutinated bacteria with 1/100 normal sulphuric acid and succeeded in separating the active agglutinin again.

Landsteiner (20), and Landsteiner and Jagic (21) proved that agglutinated red blood corpuscles washed with physiological salt solution gave off some of the agglutinin again, when they were brought into physiological salt solution and that the quantity of liberated agglutinin is proportional to the temperature applied to the medium. They also ascertained that the same relation could be established with respect to precipitin.

Bail and Tsuda (22), and Spaet (23) proved that immune bodies which combined with cholera vibrios can easily be separated from the latter in physiological salt solution at 40° to 42°C. and also that serum of various animals can separate that combination. Tsuda (24) succeeded also in separating the so-called normal immune substances in normal sera from their union with the respective antigen, but he was not able so well to separate the immune bodies in immune sera.

The preceding group of experiments demonstrate that a union of antigen and antibody is separable without the loss of their respective functions; however, they are far from effecting the purification and concentration of immune bodies.

*b. By employing inorganic surfaces.* From the standpoint that the antigen-antibody combination may be explained as an adsorption in a merely physical sense, many workers have studied the phenomena of adsorption of immune bodies by inorganic surfaces. Thus Biltz, Much and Siebert (25); Andrejew (26); Landsteiner and Reich (27) have found that, in common with other colloidal substances, immune bodies also could be adsorbed by surfaces such as caolin and animal charcoal. These surfaces act selectively and there is some distinction with respect to the adsorbing power between electropositive and electronegative surfaces.

Then, in logical sequence, the effort has been made to separate the adsorbed immune substances from the inorganic surfaces. But these experiments have always ended in negative or unsatisfactory results. Thus, the well known work of Jacqué and Zunz (28) has shown that after the antitoxin or the toxin has been adsorbed by such surfaces they are by no means easily separable *in vitro*, although *in vivo* a separation, in

greater or less degree, is observed. According to Zunz, however, if antitoxic serum is subjected to adsorption by animal charcoal, after a great deal of the protein substance in the serum has been diminished by the above mentioned method of Frouin, only a small quantity of antitoxin can be separated again into physiological salt solution.

Nicholas Ssbolew (29) states that when the immune serum of typhus or cholera is treated with iron hydroxide, the precipitate that forms contains all of the immune bodies, but attempts to separate the immune bodies from that precipitate by means of various physical or chemical processes succeeded only *in vivo*, never *in vitro*.

The use of these inorganic surfaces for the concentration of immune body can not give satisfactory results, because such surfaces adsorb not only the immune substances, but also the serum protein. Those agents have not a selective power to adsorb only the immune substance such as the specific antigens have. Therefore, even if a separation has succeeded, the purification must be quite questionable.

### *Studies on hemolysin*

To solve the problem of the isolation, purification and concentration of immune bodies, it is most convenient to use hemolysin, because its biological reaction is more easily recognized *in vitro* than that of other immune bodies such as precipitin, agglutinin or bacteriolysin. And since the antigen for hemolysin is red blood cells, we can use them as ideal surfaces for our purpose, because they can take up their amboceptors without adsorbing any blood protein. Moreover since this antigen-amboceptor union can exist without hemolysis taking place so long as complement is absent, the manipulation must be accordingly easy. But on the other hand, as red blood cells are very susceptible to slight physical or chemical changes in their environment, there are, in this respect some technical difficulties connected with their use.

*1. Studies on normal hemolysin.* Normal blood serum of many animals causes hemolysis to a greater or less degree when mixed with the red corpuscles of another species of animal. We call this hemolytic substance "normal hemolysin." Bail and Rotky (30) showed that the blood cells of the horse or guinea pig, that were sensitized with normal active human serum could render hemolytic the physiological salt solution, in which they were afterward digested; that is to say, the normal hemolysin of human serum which was combined with the red blood cells can be separated again into physiological salt solution. Bail (31)

also reported a similar experiment with the blood corpuscles of sheep that had been sensitized with inactive pig serum. Similar attempts, however, with immune hemolysin have never been successful, though the biological property of immune hemolysin appears to be quite analogous with that of normal hemolysin.

*2. Studies on immune hemolysin.* Von Liebermann and Fennyvessy (32) have succeeded in the extracting immune hemolysin against pig blood cells from its union with the latter by means of 1/100 normal hydrochloric acid. They state that the pure immune hemolysin that is isolated by this method shows no protein reaction with most sensitive protein tests and moreover it does not go through animal membrane. But this isolation of immune hemolysin, as was pointed out by Pietro Rondoni (33), cannot be said in the strict sense of the word to be a true separation of immune body and antigen union, for hydrochloric acid destroys not only red blood cells but also the immune body, especially when the acid is of high concentration.

Pietro Rondoni extracted active immune hemolysin from sensitized red blood cells by means of diluted NaOH solution, which has been added to physiological salt solution. He says that in that case there is no alteration of blood cells and immune hemolysin and that the extraction of combined amboceptor is accomplished in a short time either at 0°C. or at 37°C. But this method has one and the same failing as that of von Liebermann, for alkali also effects hemolysis, though in a different degree from that of acid, and Rondoni was not able to purify the isolated hemolysin.

With the encouragement of Professor Mita I have taken up the study of this problem and I have employed, as the most convenient material, the immune hemolytic serum of the rabbit against sheep's blood. The first question to be settled was whether the union of amboceptor and antigen is completely separable or not. The method of Liebermann and Fennyvessy has its value only with respect to the isolation and concentration of immune hemolysin. That of Rondoni has also the disadvantage that alkali cannot be used in the higher concentrations on account of its hemolytic action. Furthermore, the experiment of Bail and his co-workers proves that the same manipulation employed for normal hemolysin cannot be applied to immune hemolysin.

It occurred to me that in order to separate the immune hemoly-

sin (hemolytic amboceptor) and red corpuscle combination, it must be necessary to use some other agent beside sodium chloride in the medium, because it seemed very unlikely that antigen and amboceptor combined in ClNa solution could be separable again in the same solution, even if a little alkali or acid be added or if the temperature be modified.

From this point of view I examined various salts and non-electrolytes and I found, after long investigation, that some kinds of *sugar* have a surprising property of separating the united blood cells and hemolysin.

*Technique*

By means of the usual repeated administration of the red blood cells of the sheep, I obtained from rabbits an immune hemolysin for my experiments the hemolytic power of which was 1: 10,000; which means that 1 cc. of a 1 to 10,000 dilution of the inactivated immune serum with 0.1 cc. of fresh guinea-pig's serum could lake all of the blood cells in 1 cc. of a 5 per cent emulsion of washed sheep's blood cells in two hours at 37°C.

I employed this serum for my study, diluting it to 100 volumes with physiological salt solution. When 4 cc. of washed sheep's blood is mixed with 5 cc. of this diluted inactive immune serum and left at room temperature for fifteen to twenty minutes, the red blood cells will unite with all the amboceptors in it. This fact is in accordance with the results of the work of Morgenroth (34), who found that red corpuscles can combine with far more amboceptor than the minimal quantity by which the former will be completely laked.

The antigen and amboceptor union thus obtained was repeatedly washed with physiologic salt solution, till the last trace of blood protein was removed. If, under this condition, it is possible to separate the combination, the amboceptor thus isolated must be pure. The union was digested in solutions of various chemical substances and the extracted fluid after centrifugalization was tested as to its hemolytic power. The results that were negative will be omitted from this report.

EXPERIMENTAL RESULTS

With the above mentioned technique, two test tubes of pure antigen-amboceptor union were made and then digested at 37°C.

A. In 5 cc. of physiological salt solution.

B. In 5 cc. of 10 per cent watery solution of saccharose.

After two hours, during which the tubes were shaken several times, both were centrifugalized and red tinged extracts were thus obtained. The hemolytic test was performed in the usual way, 1 cc. of a 5 per cent emulsion of washed sheep's blood cells and 0.1 cc. of fresh guinea-pig's serum being used in each tube.

The results of this experiment are shown in table 1.

TABLE 1

| HOURS | EXTRACT A (WITH SALT SOLUTION UP TO 1 CC.) | | | EXTRACT B (WITH SALT SOLUTION UP TO 1.0 CC.) | | | ORIGINAL DILUTED SERUM CONTROL (WITH SALT SOLUTION UP TO 1.0 CC.) | |
|---|---|---|---|---|---|---|---|---|
| | 0.5 cc. | 0.3 cc. | 0.1 cc. | 0.5 cc. | 0.3 cc. | 0.1 cc. | 0.03 cc. | 0.01 cc. |
| 0.5 | – | – | – | Compl. | Weak | Weak | Weak | Weak |
| 1.0 | – | – | – | Compl. | Compl. | Very strong | Almost compl. | Very strong |
| 1.5 | – | – | – | Compl. | Compl. | Almost compl. | Compl. | Almost compl. |
| 2.0 | – | – | – | Compl. | Compl. | Compl. | Compl. | Compl. |

It is seen that the hemolytic amboceptor that had been united with the red blood cells was easily separated into the solution of saccharose, while the physiological solution was quite indifferent.

Beside the saccharose I used also glucose and lactose, and found that these sugars also have the property of separating the amboceptor-corpuscle union. I should like to note here that these sugars must be pure and especially that the glucose must be that of Merck. The following experiments were performed with saccharose.

It was important to determine whether the separation of antigen-amboceptor union by sugar could be made complete and it was necessary, therefore, to examine the various physical factors that favor the separating power of the sugar.

Since the volume of the sugar solution used in our first experiment (table 1) for extracting the hemolysin from the sensitized corpuscles is the same (5.0 cc.) as that of the original hemolytic serum used for the sensitization of the cells, we can estimate the proportion of the absorbed hemolysin recovered by the digestion

in the sugar solution by directly comparing the hemolytic titer of the original serum and of the extraction fluid B. This comparison (0.1 cc.: 0.01 cc.) shows that 10 per cent of the absorbed amboceptor was separated from the sensitized cells during the digestion in the sugar solution. This calculation does not take into account the inhibitory action of sugar on the hemolysis (see under "Discussion") but as the amount of sugar present in the test mixtures was relatively small, as compared with the amount of salt present, its inhibitory influence may be disregarded.

## FACTORS THAT INFLUENCE THE SEPARATING POWER OF SUGAR

The separation of amboceptor from its antigen combination with sugar depends upon various physical factors.

### *1. Influence of temperature*

Pietro Rondoni states that the separation of the antigen-amboceptor union by alkali is the same either at 0°C. or at 37°C. But as Landsteiner showed that the quantity of the separated

TABLE 2

*Showing the effect of temperature on the extracting power of sugar solution.*

| HOURS | EXTRACT A | | | EXTRACT B | | |
|---|---|---|---|---|---|---|
| | 0.5 cc. | 0.3 cc. | 0.1 cc. | 0.5 cc. | 0.3 cc. | 0.1 cc. |
| 0.5 | Compl. | Weak | Weak | — | — | — |
| 1.0 | Compl. | Almost compl. | Strong | — | — | — |
| 1.5 | Compl. | Compl. | Almost compl. | Weak | Trace | — |
| 2.0 | Compl. | Compl. | Compl. | Almost compl. | Strong | Weak |

agglutinin from agglutinated bacteria is proportional to the temperature of the medium, it is most probable that the separation of hemolytic amboceptor from the sensitized blood cells is also proportional to the temperature applied.

With the usual technique two test tubes of pure antigen amboceptor union were made and digested:

A. In 5 cc. of 10 per cent saccharose, at 37°C.

B. In 5 cc. of 10 per cent saccharose, at 5°C.

After two and one-half hours, during which the tubes were shaken several times, both tubes were centrifugated and the hemolytic power of the extracts was tested in the usual way.

The results of this experiment are shown in table 2.

The experiment makes it evident that the higher temperature

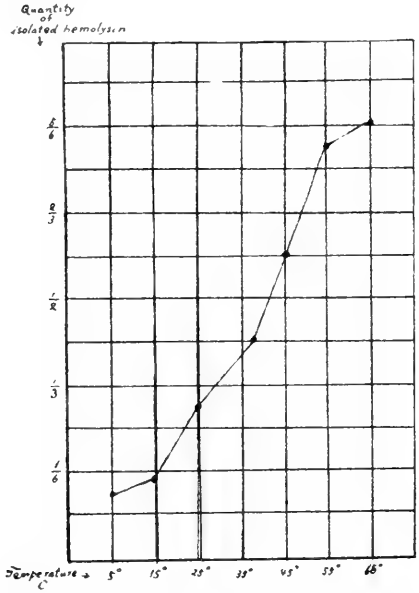


CHART I. SHOWING THE INFLUENCE OF TEMPERATURE ON THE SEPARATION OF HEMOLYSIN FROM SENSITIZED CORPUSCLES IN SUGAR SOLUTION

Concentration of saccharose, 10 per cent; quantity of solution of saccharose, 20 cc.; time, fifteen minutes.

favors the separation of the hemolysin. It is known that the blood cells are laked in physiological solution when certain high temperatures are applied; we call this phenomenon heat-hemolysis. According to Gros's study, red blood cells are destroyed at 56°C. after eighteen minutes in 0.9 per cent ClNa solution. But the heat hemolysis, as he states, depends upon the concentration of the emulsion of blood cells. A concentrated emulsion of blood

cells is dissolved more slowly than a diluted one. In my second experiment temperatures up to 65°C. were examined, a 25 per cent emulsion (in this case, for only fifteen minutes) and the macroscopic appearance of the extract after centrifugation was about the same as that obtained at lower temperature. The experiment makes it seem probable that the heat hemolysis is inhibited by sugar. The results of this experiment are shown in Chart 1.

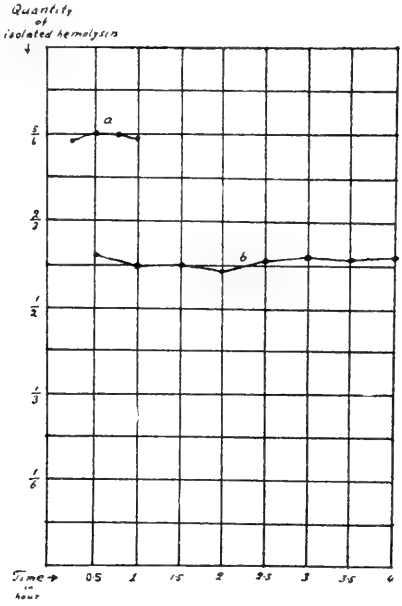


CHART II. SHOWING THE INFLUENCE OF TIME ON THE DISSOCIATION OF CORPUSCLE AND AMBOCEPTOR IN SUGAR SOLUTION

Curve a: Concentration of saccharose, 10 per cent; quantity of solution of saccharose, 20 cc.; temperature, 56°C.

Curve b: Concentration of saccharose, 10 per cent; quantity of solution of saccharose, 20 cc.; temperature, 45°C.

If temperatures above 70°C. are applied, the red corpuscles will be altered in few minutes. If the same experiment is performed with agglutinin or bacteriolysin it may be possible to apply the higher temperature. After all, it is quite apparent that higher temperatures greatly favor the dissociation of the

corpuscle-hemolysin combination and it also appears that the separation can be made complete, if the conditions are favorable, though in my experiment the separation has been carried out only up to 84 per cent.

*2. Influence of time*

It might be thought that at the lower temperatures a longer period of digestion would result in the same degree of separation

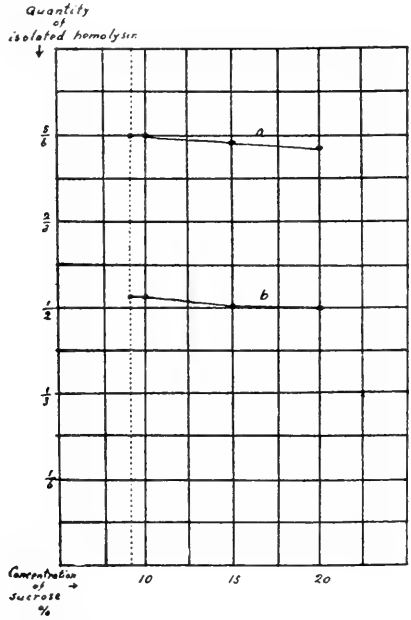


CHART III. SHOWING THE INFLUENCE OF THE CONCENTRATION OF THE SUGAR ON THE DISSOCIATION OF THE CORPUSCLE-AMBOCEPTOR COMBINATION

Curve a: Temperature, 56°C.; time, thirty minutes; quantity of solution of saccharose, 20 cc.

Curve b: Temperature, 40°C.; time, thirty minutes; quantity of solution of saccharose, 20 cc.

of the hemolysin as would a shorter period at a higher temperature. But within the limit of four hours the length of time, according to my experience, does not exercise any influence upon the separation of the immune body. In this fact the dissociation of the hemolysin differs from other biological reactions, such as hemol-

ysis by heat. Whether, as seems improbable, any different effect would be obtained by a still longer digestion I am unable to say. A more prolonged digestion of the cells causes considerable hemolysis which, moreover, interferes with the further manipulation of the extract.

*3. The influence of the concentration of saccharose*

It is, of course impossible to use a hypotonic solution, on account of its hemolytic action.

The isotonic solution of saccharose is theoretically a 7.8 per cent watery solution. I examined the effect of different concentrations from the isotonic solution up to a 25 per cent solution, but I found that concentrations above 20 per cent are also unavailable, because in such concentrated solutions there appears also partial destruction of the red corpuscles at higher temperatures.

The result of this experiment, graphically presented in chart III shows that the concentration of the sugar does not exert any considerable influence upon its separating power, but it seems as though the isotonic or slightly hypertonic solution is the optimum for the action of the sugar.

*4. Influence of the quantity of sugar solution*

It would be most convenient for subsequent study if all the combined hemolysin could be separated in a small quantity of sugar, but when the quantity of sugar solution is little, the separated hemolysin also is relatively small in amount; it seems as though the hemolysin becomes saturated, to a certain extent, in the solution. In such case a new solution should act effectively to separate more of the combined hemolysin, and the following experiment shows that this is true.

With the usual technique one test tube of pure antigen-amboceptor union is prepared. This is digested for two hours at 37°C. in 5 cc. of a 10 per cent watery solution of saccharose; after which it is centrifugated and a red tinged extract is obtained. (Extract A.) To the sediment of sensitized red blood cells a second por-

tion of 5 cc. of 10 per cent saccharose is added and after 1 hour incubation also at 37°C., it is centrifugated. The second digestion caused considerable hemolysis. (Extract B.)

The hemolytic power of the two extracts was tested in the usual way. The results of this test are shown in table 3.

TABLE 3

*Showing the effect of a second treatment of the corpuscle-amboceptor combination with sugar solution*

| HOURS | EXTRACT A | | | EXTRACT B | | |
|---|---|---|---|---|---|---|
| | 0.5 cc. | 0.3 cc. | 0.1 cc. | 0.5 cc. | 0.3 cc. | 0.1 cc. |
| 0.5 | Compl. | Weak | Weak | Compl. | Almost compl. | Weak |
| 1.0 | Compl. | Almost compl. | Strong | Compl. | Compl. | Almost compl. |
| 1.5 | Compl. | Compl. | Compl. | Compl. | Compl. | Compl. |
| 2.0 | Compl. | Compl. | Compl. | Compl. | Compl. | Compl. |

It is seen to be possible to extract a considerable quantity of hemolysin by a second digestion and it may also be possible by a third or more treatments to dissociate all of the combined hemolysin. But as even the second extraction caused more or less destruction of red blood cells, it was necessary to employ such a quantity of the sugar solution that by first treatment most of the united immune hemolysin will be separated. Experiments were carried out to determine what that quantity is and the result of this study is shown in chart IV.

The experiment shows that it is necessary to use at least 20 cc. of sugar solution in order to extract almost all of the amboceptor that was combined with 4 cc. of red blood cells.

To summarize the foregoing experimental results: when 20–30 cc. of a 10 per cent watery solution of cane sugar are mixed with 4 cc. of sheep's red blood cells that have taken up the amboceptor in 0.05 cc. of immune hemolytic rabbit's serum and this mixture is incubated at 60°C. (1: 10,000), after fifteen to twenty minutes almost all of the immune substance, at least five-sixths of it, will be transferred from the corpuscles into the sugar solution.

Thus the separation of antigen-amboceptor union is practically

complete and in that separating action not only the quality of medium, but also its quantity and the temperature play important rôles.

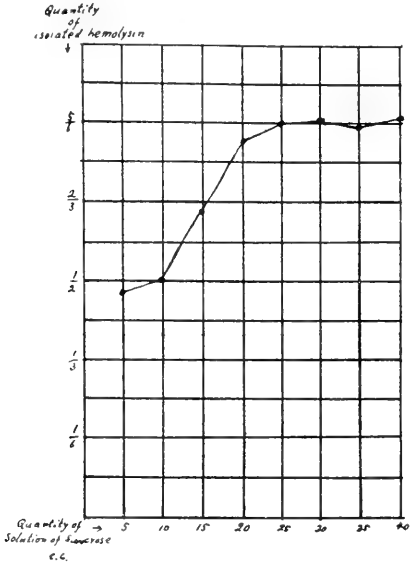


CHART IV. SHOWING THE INFLUENCE OF VOLUME ON THE DISSOCIATING POWER OF THE SUGAR SOLUTION ON THE AMBOCEPTOR-CORPUSCLE COMBINATION

Concentration of saccharose, 10 per cent; temperature, 56°C.; time, thirty minutes.

## PURIFICATION AND CONCENTRATION OF THE ISOLATED IMMUNE HEMOLYSIN

As shown above, I was able to isolate immune hemolytic amboceptor from the union with its antigen. It was, however, very important to purify and concentrate the isolated hemolysin in order to investigate its biological properties.

### *a. Elimination of corpusclar substances including hemoglobin*

It is true that the sugar extract of the sensitized corpuscles does not contain, beside the immune hemolysin, any serum protein, but it does contain a small quantity of destroyed red blood

cells. Therefore the extract has always a slight red nuance of hemoglobin. To purify the hemolysin this trace of corpuscular substance must be excluded. For the solution of this second difficulty I examined various means.

1. Dialysis. Though the hemolysin does not pass through parchment, hemoglobin, also, is not dialyzable; therefore we can not use dialysis in order to exclude hemoglobin.

2. Adsorption. By inorganic surfaces, such as caolin, hemoglobin is completely adsorbed but at the same time the hemolysin is also adsorbed.

3. All other methods except the following have ended in a negative result.

4. Successful method. It is well known that the hemolysin in immune serum can not be extracted with fat solvents such as ether, petroleum ether, aceton and chloroform and it is by no means susceptible to them, while complement is easily attacked by them. *By shaking the sugar extract with ether in the manner to be described I have succeeded in removing from the extract all traces of the corpuscular substance thus obtaining the hemolysin in a pure condition.*

If, to the sugar extract, 5 to 10 volumes of pure ether are added, and the mixture is shaken in a separatory funnel for one to two hours, there appear three layers in it. The upper layer is of ether; the middle layer consists of a coagulated mass of corpuscular substance, which is colored red and looks gelatinous; the lowest layer is the sugar solution, which is now quite colorless and contains the same quantity of the extracted hemolysin as before the treatment with ether.

When, instead of ether only, ether and hydrochloric acid are used together and likewise shaken in a separatory funnel, the exclusion of the hemoglobin is far easier, but in that case some of the hemolysin is destroyed, especially when the acid is concentrated.

If the exclusion of the hemoglobin by shaking with ether is not accomplished by one treatment then the lowest layer is separated into another funnel and treated again with fresh ether. After repeating this manipulation twice or thrice the last trace of hemoglobin will be excluded.

*b. Elimination of sugar and the trace of salt*

The solution thus purified with ether contains still a certain quantity of sugar and a trace of ClNa. These substances are quite easily eliminated by dialysis; i.e., the purified solution is poured into parchment and kept in running water for about two days.

*c. Concentration of purified hemolysin*

Finally the watery solution of the hemolysin is brought into an exsiccator and concentrated in vacuum to the required volume.

*d. The nature of pure immune hemolysin*

A further study is necessary to determine this. I can only report here that the isolated hemolysin preparation does not react so sensitively as true protein with protein tests such as that with sulphosalicylic acid or with ClNa + acetic acid.

## DISCUSSION

*1. The influence of the medium upon the action of hemolytic serum*

The action of compound hemolysin depends upon the reaction of its environment or the quantity of ions in it. It is well known that the action of hemolysin is prevented by a higher concentration of salts and von Liebermann (35), Michaelis and Skwirsky (36), and Hecker (37) proved that both acid and alkali prevent the hemolytic action of hemolysin. Eisler (38) found that in a non-electrolyte medium the complex immune hemolysin cannot produce its characteristic effect. But as regards the explanation of these phenomena, all of the authors are agreed that alkali and acid and the absence of electrolytes act in such a way as to prevent the interaction of amboceptor and complement upon the corpuscles.

On the basis of his observation, however, that the combined antigen and amboceptor are separable in alkaline reaction, Rondoni explains the above mentioned inhibitory phenomena as follows: those agents which prevent the hemolytic action of

hemolysin act upon the combination of antigen and amboceptor instead of the combination of the latter and complement. This explanation appears to be correct, in the light of my own observation, that the sugar solution or more correctly the non-electrolyte medium can separate the union of hemolytic amboceptor and its antigen.

But Rondoni's statement that the preventive power of alkali is the same at 0°C. as at 37°C., cannot hold for other agents, because my experiments show that at the higher temperatures the antigen-amboceptor union is better separated than at lower temperatures. Indeed it seems likely, that the other inhibitory agents also will act more effectively at higher temperatures and this belief is supported by the observation of Landsteiner, that the quantity of agglutinin separated from agglutinated bacteria is proportional to the temperature applied.

The general conclusion appears justified that the combination of antigen and antibody of any kind is more or less influenced not only by the quality of the medium, but also by the degree of temperature applied.

### *2. Reversibility of antigen and amboceptor union*

Though it had been proved that the union of antigen and antibody is reversible, as I have already stated, it had not yet been determined whether that reversibility is complete or partial. The experiments of von Liebermann and Fennyvessy have no bearing on the question of reversibility and those of Pietro Rondoni tell us nothing as to the degree of the reversibility. (See, also, the work of Morgenroth (39). )

My own experiments demonstrate that the reversibility of antigen and amboceptor is almost or quite complete, so far as immune hemolysin is concerned.

Those antigen antibody unions, of which the reversibility was proved only *in vivo*, would probably be found separable also *in vitro*, if the quality of medium and its temperature were suitably modified.

*3. Indispensability of salt in the production of immune reactions*

It is quite evident that salt is indispensable to the carrying out of the biological reaction between immune hemolysin and red corpuscles. If this fact is taken together with the observation of Bordet (40), that the agglutination of bacteria with specific agglutinin does not occur in a medium free from salt, it may be said in general that an immune body can not exert its characteristic effect without salt. As to the reason for this, I should like to suggest that salt mediates the combination of antigen and antibody.

*4. Difference between normal and immune antibodies*

This question is an old one and it has been often discussed and the difference between the two sorts of antibodies has been shown by several workers; for example, by Shibayama (41) by means of dialysis of hemolytic serum, by Eisenberg and Volk (42) from the standpoint of absorption of agglutinin and by Landsteiner and Reich (43) from the standpoint of separability of hemagglutinin and blood cells.

To this evidence may be added the experiments of Bail that I have already referred to dealing with the separability of the hemolysin and blood cell combination. In view of all of these demonstrated differences between the normal and the immune antibodies, it must appear doubtful that the normal hemolysins are merely increased by immunization with red blood cells.

*5. Concentration of the immune hemolysin*

Any method of isolation and concentration of immune bodies must be, in its principle, simple and certain and also there must not be any loss of immune body during the manipulation. The method of Rondoni is not applicable to a concentration because, with that method, a complete reversibility of the antigen and amboceptor union was not shown to be attainable. Though the method of von Liebermann and Fennyvessy was heretofore the most convenient, these authors succeeded only with the hemolysin against pig's blood and there was necessarily some loss of hemolysin, because they employed a very strong hydrochloric

acid, which is able to destroy hemolysin even in its weaker concentration.

As for my own method, since the reversibility is complete, there is no loss of immune body and though the method has hitherto been applied only to the immune hemolysin of rabbit against sheep's blood, there can be no doubt that it would produce the same results with other hemolysins.

*6. The biological properties of pure immune hemolysin*

It has been shown that hemolysin does not pass through animal membranes and that it is not susceptible to ether; but as to its chemical nature or other physical properties further experiments are required. As, however, von Liebermann and his co-worker say, it is certain that hemolysin does not react so sensitively to the protein reagents such as sulphosalicylic acid or NaCl + acetic acid as is the case with the known protein. Nevertheless I hesitate to assert now that immune hemolysin or immune substance generally are of non-proteid nature.

*7. The pure isolation and concentration of other immune bodies*

As to the isolation of agglutinin or bacteriolysin a similar method will probably be available, though such attempts have succeeded hitherto chiefly *in vivo*. If the antigen is not formed; e.g., toxin, the purification of the corresponding antibody cannot be accomplished by the method under consideration.

If antitoxin is of non-proteid nature, then, with the fractionating method when the serum globulin is precipitated, it is possible that the immune bodies are adsorbed by the surfaces of smallest particles of globulin by a mere physical process so that a separation of the antitoxin might be expected, though this was attempted by Pick with the use of alcohol and other chemical agents with negative result.

As the works of Zunz and others show, it is difficult to separate immune bodies only, after they have been adsorbed by inorganic surfaces, because if the active substance is separated again, some serum protein is separated with it. The study of the separability of immune substance from inorganic surfaces has, there-

fore, a different biological interest from the one that engaged our attention in this paper.

## SUMMARY

1. The reversibility of the antigen and amboceptor union is proved to be practically complete, so far as the immune hemolysin of the rabbit against sheep's blood is concerned.

2. The isolation of hemolytic amboceptor from its antigen union is accomplished by a simple method: When the hemolytic power of the original immune serum is 1: 10,000, it is diluted to 100 times its volume with physiological salt solution; 5 cc. of this diluted serum is poured into 4 cc. blood cells, which are washed free from serum protein with physiological salt solution. After 15 to 20 minutes at room temperature all of the hemolytic amboceptor has been adsorbed by blood cells; and the antigen-amboceptor union is thus obtained. After the sensitized corpuscular sediment is washed with physiological salt solution several times, till the last trace of serum protein has been removed, this pure antigen-amboceptor combination is mixed with an isotonic or slightly hypertonic watery solution of saccharose, glucose or lactose and left at 55°C. for fifteen to thirty minutes, during which time the vessel is shaken several times. The sugar extract, which contains nearly all of the hemolysin used to sensitize the cells, is obtained by centrifugation.

3. In order to purify this sugar extract, which contains substances from destroyed blood cells, it is placed in a separatory funnel and shaken for one to two hours with 5 to 10 volumes of ether, this treatment, if necessary, being repeated twice or thrice, till at last the solution becomes quite colorless. This colorless solution is dialysed in parchment against running water in order to eliminate the sugar and traces of ClNa.

4. The solution thus obtained is concentrated *in vacuo* to the required volume.

I wish to express my indebtness to Professor S. Mita for the facilities and encouragement, which he has extended to me in carrying out these experiments.

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# A NEW METHOD OF ESTIMATING THE ANTITRYPTIC INDEX OF BLOOD SERUM

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## I. INTRODUCTION

The methods that are at present employed for the estimation of the antitryptic indices of blood sera yield only an arbitrary measure of antitryptic power, which bears no necessary relationship to the actual quantity or proportion of trypsin that is inactivated by the serum. Thus in the Gross-Fuld casein method (1) the "antitryptic index" is expressed by the concentration of trypsin that is requisite to overcome the inhibiting action of a given quantity of serum to the extent of permitting the complete hydrolysis (removal of substances precipitable by acetic acid) of a given quantity of sodium caseinate in a given time. In the Loeffler plate method (2) the "antitryptic index" is estimated in terms of the concentration of trypsin that is required to overcome the inhibiting action of a unit volume of serum to the extent of permitting some digestion of heat-coagulated blood to occur in a given interval of time. In the method advocated by Mintz the antitryptic index is similarly estimated as the per cent of a given trypsin solution that is just able to overcome the "paralyzing" action of an arbitrary unit of blood serum (3).

While each of these methods yields results of qualitative value, they are not comparable with one another, nor do they yield any quantitative measure of the relative trypsin-inactivating powers of different sera. They would do so were (a) the quantity of protein digested in a given time always directly proportional to the concentration of trypsin acting upon it, and (b) the quantity

of trypsin bound by serum directly proportional to the quantity of antitrypsin that it contains. Both of these assumptions are, however, demonstrably invalid.

In the first place the relationship between time of digestion and the quantity of protein digested is not one of simple proportionality, and in fact in the case of sodium caseinate (employed in the Gross-Fuld method of estimating the antitryptic index) the relationship between time and degree of hydrolysis is very accurately expressed by the logarithmic equation

$$\log \frac{a}{a - x} = Kt$$

which is that which usually obtains in monomolecular chemical reactions (4).

In the second place, the quantity of trypsin bound by different samples or quantities of serum is not directly proportional to their antitrypsin content, for in that event double the amount of serum which suffices to halve the activity of a given concentration of trypsin should reduce its action to zero. Now the experimental fact is that although it is an easy matter to halve the activity of trypsin by the addition of serum it requires a relatively enormous excess of serum to abolish its proteolytic activity altogether. Thus in rabbit 1 (table 1) the addition of 0.15 unit of serum neutralized one-half the specified quantity of trypsin employed, but the addition of 0.33 unit of serum only reduced the tryptic activity to one-third. Evidently in the interaction of trypsin and antitrypsin we have, as in so many reactions involving biological antibodies (5) a "balanced" or incomplete reaction in which the station of equilibrium is determined by the relative masses of all of the reacting components.

Since the velocity-constant ($K$) of the hydrolysis of sodium caseinate is, as usual in catalyzed reactions, directly proportional to the concentration of trypsin (4) we have in the value of this constant a quantitative measure of the concentration of free trypsin in the digest. We have accordingly measured the value of $K$ in various mixtures of sodium caseinate, trypsin and antitryptic sera with the object of ascertaining the actual quantita-

tive relationships which obtain between the trypsin and antitrypsin, on the one hand, and the quantity of trypsin inactivated on the other. The following was the procedure employed.

### II. PROCEDURE OF THE ESTIMATION

A number of glass tubes 12 to 14 cm. in length, having an inside diameter of 4 to 5 mm. and walls about 1 mm. thick are prepared and sealed at one end. The open ends are sealed by means of short lengths of sealed glass tubing of the same diameter as the tubes and inserted into short lengths of rubber tubing.

The following are the reagents required:

| | |
|---|---|
| Sodium hydroxide | 0.048 N |
| Acetic acid | 0.600 N |
| Sodium chloride | 6.0 per cent |
| Sodium carbonate | 0.01 per cent |
| Casein | 6.0 per cent |
| Trypsin (Gruebler's) | 1.0 per cent |
| Serum diluted with 6.0 per cent sodium chloride | |

The acetic acid is prepared with sufficient accuracy by diluting 35 cc. of glacial acetic acid to one litre. This is kept as a stock solution.

The casein solution is prepared from commercial casein (Eimer and Amend's or Merck's C. P. "Nach Hammarsten") specially purified by washing in water, alcohol and ether (6). One and a half grams of the casein powder is introduced into a 50 cc. flask and 25 cc. of the 0.048 N sodium hydroxide solution is added. The casein gradually dissolves with the aid of frequent shaking and gentle warming. When clear and homogeneous it is filtered to remove any particles which may not have completely dissolved. The solution thus prepared is neutral to phenolphthalein; it must be freshly made up on the day on which it is to be used.

The trypsin solution was made by dissolving Gruebler's trypsin in 0.01 per cent sodium carbonate.

In preparing the diluted serum, fresh clear and only very slightly haemolyzed serum was employed. The serum and the requisite amount of 6 per cent sodium chloride to dilute it to the desired concentration are introduced into a glass tube, a glass bead dropped in and the mixture shaken thoroughly.

These various solutions having been prepared, the estimation of the antitryptic power of a given dilution of serum is carried out as follows:

Three of the glass tubes are labelled A, B and C. A glass bead is dropped into each of these. Into A is introduced 0.125 cc. of the diluted serum and into B and C 0.125 cc. of 6 per cent sodium chloride solution. One cubic centimeter of the 6 per cent casein solution is then added to each of the tubes and 0.125 cc. of the 1 per cent trypsin solution. The tube labelled C is immediately acidified by the addition of 0.125 cc. of the 0.6 N acetic acid which stops all tryptic action and precipitates the casein. The contents of all the tubes are mixed thoroughly by affixing a stopper and shaking. The tubes A and B are now incubated at 36°C. for one hour. After removal from the incubator these tubes are acidified in the same way as C and again stoppered and shaken. The liquid and precipitate in each tube are separated by centrifugalization and the refractive index of each of the fluids is determined at the same time (i.e., at the same temperature) in a Pulfrich Refractometer, the angle of total reflection being read to within one minute, a sodium flame being the source of light.

It has been previously shown that the refractive index of a solution of the mixed products of the tryptic hydrolysis of casein is identical with that of the original solution of casein from which the products were derived (7). Hence the difference between the refractivities of the fluids derived from the tubes B and C is proportional to the amount of casein digested in B by the action of the trypsin during the period of incubation. One gram of casein or its hydrolysis products dissolved in 100 cc. of dilute alkali changes the refractive index of the solvent by 0.00152. Hence, dividing the difference between the refractive indices of the fluids derived from B and C by the factor 0.0152 we obtain the percentage of casein digested by the trypsin (7).

The difference between the refractivities of the fluids derived from A and C similarly yields the percentage of casein digested by the trypsin which has not been bound by the antitrypsin of the serum. It is necessary to recollect, however, that the glob-

ulins of the serum are precipitated along with the casein by the addition of the acetic acid.

It is for this reason that 6 per cent sodium chloride is employed as the diluent of the serum, for it is found that on the average, and within the experimental error of the determination, the refractive index of serum from which the globulins have been precipitated by acetic acid is equal to that of a 6 per cent solution of sodium chloride. Hence by using this concentration of sodium chloride as diluent the refractivities of the fluids derived from the tube containing serum and from those containing 6 per cent sodium chloride in the place of serum are equally affected by the respective additions.

The difference between the refractivities of the fluids from the tubes B and C, therefore is a measure of the hydrolysis attributable to the total amount of trypsin added, acting for one hour at 36°C., while the difference between the refractivities of the fluids from the tubes A and C is a measure of the hydrolysis due to the proportion of the trypsin that remains unbound by the antitrypsin of the serum, acting for the same period and at the same temperature.

The order followed in mixing the reagents in the tube should not be changed. If the trypsin and serum are mixed before the casein is added, irregular and untrustworthy results are obtained.

We have thus measured the relative hydrolysis in the presence and in the absence of the antitrypsin of the serum. We proceed to ascertain from these measurements the actual proportion of trypsin bound by the serum by computing the velocity constant of hydrolysis in the presence and absence of serum by applying the monomolecular formula

$$\log_{10} \frac{a}{a - x} = Kt$$

where $t$ is the time in hours, $a$ is the initial percentage of casein and $x$ is the percentage of casein that has been digested. The value of $K$ thus computed is directly proportional to the concentration of active (unbound) trypsin in the digest.

### III. EXPERIMENTAL RESULTS

Illustrative results are shown in the accompanying tables in which $A$ denotes the dilution of serum employed, the undiluted serum being taken as unity, and $T$ denotes the proportion of the trypsin computed to have been neutralized by the serum.

TABLE 1

*Rabbit 1 Concentration of casein solution 4.36 per cent*

| $X$ | $K \times 10^3$ | $A$ | $T$ | $\frac{T}{A(1-T)} = C$ |
|---|---|---|---|---|
| 1.26 | 148 | 0.33 | 0.68 | 4.00 |
| 1.52 | 186 | 0.25 | 0.60 | 6.00 |
| 1.68 | 211 | 0.20 | 0.54 | 5.87 |
| 1.78 | 228 | 0.15 | 0.51 | 6.93 |
| 2.04 | 274 | 0.10 | 0.41 | 6.95 |
| 2.55 | 383 | 0.05 | 0.17 | 5.00 |
| 2.79 | 444 | 0.03 | 0.04 | 4.83 |
| 2.86 | 463 | 0.00 | 0.00 | |

Considering the number of factors involved in the determination, there is a very evident tendency towards constancy of the ratio $\frac{T}{A(1-T)}$. The significance of this fact will be clear from the following considerations:

If we consider the neutralization of trypsin by antitrypsin to be due to the formation of a proteolytically inactive compound, the simplest conception we can form of the process is that one molecule of trypsin combines with one molecule of antitrypsin to form the inactive compound. Applying the mass-law, therefore, we should expect the following relationship to hold good:

$$k\alpha\,(A-\beta T)\,(1-T) = T$$

where $\alpha$ is the number of molecules of antitrypsin contained in the specified proportion of *undiluted* serum, $\beta$ is the number of molecules of trypsin-antitrypsin compound which would be formed by the neutralization of the total quantity of trypsin employed, and $k$ is the equilibrium constant of the reaction.

Rearranging, this equation may be written:

$$\frac{T}{(A - \beta T)\ (1 - T)} = C$$

where $C$ is a constant which is proportional to the molecular concentration of antitrypsin in the sample of serum employed.

The experimental fact being that $\frac{T}{A\,(1-T)}$ is constant, it is evident that $\beta T$ is negligibly small in comparison with $A$, or in other words the number of molecules of trypsin-antitrypsin compound which would be formed by the neutralization of all of the trypsin present would be negligible in proportion to the total number of molecules of antitrypsin contained in the serum.

TABLE 2

*Rabbit 1. Serum taken twenty-four hours subsequently (this animal was receiving injections of trypsin)*

| $A$ | $C$ |
|---|---|
| 0.40 | 8.84 |
| 0.33 | 9.50 |
| 0.25 | 9.00 |
| 0.20 | 8.50 |

TABLE 3

*Rabbit 2*

| $A$ | $C$ |
|---|---|
| 0.50 | 3.13 |
| 0.33 | 3.32 |
| 0.17 | 3.23 |

TABLE 4

*Rabbit 3*

| $A$ | $C$ |
|---|---|
| 0.50 | 3.55 |
| 0.33 | 3.25 |
| 0.25 | 3.14 |
| 0.13 | 2.10 |
| 0.06 | 2.59 |

Evidently only a small fraction of the antitrypsin in serum is actually engaged in binding the quantities of trypsin employed and a very great excess of antitrypsin must be present in the serum. We should thus be inclined to identify the antitryptic fraction of serum with some quantitatively important fraction, for example, as several authors have suggested, the serum albumins (8). In the succeeding article, however, it is shown by one of us (Hanson) that the antitryptic index of serum, as measured by the constant $C$, may be increased by immunization no less than 300 per cent without any definite increase in the percentage of albumins in the serum. It is evident, therefore, that if the binding of trypsin is indeed accomplished by the albumin

fraction the process of immunization against trypsin accomplishes, not an increase in the quantity of anti-trypsin, but an alteration in its physical or chemical condition of such a character as to enhance its affinity for trypsin. On the other hand it is possible that the neutralization of trypsin is in reality accomplished by some other and hitherto unidentified constituent of the serum, which must, however, be present therein in disproportionate quantity to the actual molecular concentration (which is of course very small) of trypsin employed in these experiments.

## SUMMARY

1. A simple and accurate method of measuring the antitryptic indices of blood-sera is described.

2. It is shown that for varying proportions of antitrypsin ($= A$) added to a specified amount of trypsin (regarded as unity) the relation:

$$\frac{T}{A\,(1-T)} = C$$

holds good for any given serum, $T$ being the proportion of the trypsin neutralized by the serum and $C$ a constant which is a direct measure of the number of molecules of antitrypsin contained in a specified volume of the serum.

3. The molecular concentration of antitrypsin in blood serum is in great excess of the molecular concentration of proteolytically active material in a 1 per cent solution of Gruebler's trypsin.

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# THE NON-INFLUENCE OF INJECTIONS OF TRYPSIN UPON THE PROTEIN QUOTIENT IN BLOOD SERUM

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## INTRODUCTION

The relation of the serum proteins to infection and immunity has engaged the attention of many investigators. The earlier experimental evidence indicated that the production of antibodies is always accompanied by an increase in the globulins. Later work, however, has shown that immunity could be attained without any change in the quantity of globulins.

Among the earlier workers may be mentioned, Hiss and Atkinson (1), and Ledingham (2), who obtained a marked increase in the globulins in the serum of horses immunized against diphtheria toxin.

A great part of the recent work has been done by Hurwitz and Meyer (3), and Schmidt and Schmidt (4). These investigators found by extensive and varied experimentation that with small doses of antigen carefully administered, a high degree of immunity can be produced without a decided rise in the globulins. The antigens employed by Hurwitz and Meyer (3), were certain living bacteria, dead bacteria and fowl typhoid endotoxin. The antibodies to all of these antigens are carried down with the globulins on fractionating the immune sera. An antigen, however, whose antibody is known to be in the albumin fraction has not been employed by Hurwitz and Meyer, Schmidt and Schmidt, nor, to the writer's knowledge, by any previous investigators. According to Kämmer and Mogulesko (5), pan-

creas trypsin and yeast trypsin are antigens of this sort. It was of interest to find whether or not a carefully produced immunity to this rather unique type of antigens is associated with a change in the protein quotient. This is the problem that incited the undertaking of the present work.

## METHODS AND MATERIALS

The experimental conditions were in general similar to those described in a previous communication (6). Seven normal rabbits were selected as the experimental animals. Determinations of the serum proteins and of the antitryptic index were made through a prolonged fore-period. At the end of the fore-period the immunization of six of the animals was begun, while one served as a control.

Solutions of Grübler's pancreas trypsin *puriss. sicc.*, freshly made up and filtered was the particular trypsin employed.

The strength of the sample of this trypsin used was such, that the velocity constant of digestion of an 0.38 per cent solution freshly made up and filtered, acting in an equal volume of a 4.40 per cent sodium caseinate solution at a temperature of 37°C., was found to be $36 \times 10^{-2}$ by Robertson's refractometric method (7). The same sample of trypsin unfiltered showed only a slightly higher potency.

This enzyme was administered subcutaneously and intravenously in order that the slower and more rapid effects, respectively, may be elicited. The quantities and frequency of injection were also varied in the different rabbits so that the optimum rapidity of immunization may be at least more or less closely approached. In no case were the doses used so large as to cause severe reactions or even a loss of weight in the animals.

The determinations of the serum proteins were made by Robertson's micro-refractometric method (8).

The estimations of the antitryptic index were carried out by the method described in the preceding article (9). The principles on which this method is based are briefly as follows:

Since it is a fact that the refractive index of a sodium caseinate

solution is practically not altered by tryptic digestion, it is possible to estimate the extent of hydrolysis in such a solution containing trypsin by precipitating the undigested casein with a definite volume and strength of acetic acid, centrifuging down the precipitate and determining the refractive index of the supernatant fluid, which is a solution of the products of hydrolysis (6).

The velocity constant ($k$) of the trypsin in question is then calculated from the monomolecular formula,

$$\log \frac{a}{a-x} = Kt$$

where $a$ is the initial concentration of the substrate and $x$ the amount digested during a period of time $t$.

The velocity constant ($k_1$) of the rate of hydrolysis is then obtained in a similar manner for a casein-trypsin solution to which a known quantity of serum has been added.

Since $K$ is the velocity constant of the rate of normal hydrolysis and $K_1$ is the velocity constant of the rapidity of digestion after a part of the trypsin was neutralized by the antitrypsin of the serum,

$$\therefore \frac{k_1}{K} = \text{fraction of trypsin unneutralized}$$

It has been observed (9), that the trypsin-antitrypsin reaction is reversible; hence it is practicable to calculate the antitryptic index ($C$) from the equilibrium equation,

$$\frac{x}{S\ (1-x)} = C$$

where $x$ = the fraction of trypsin neutralized and $s$ the amount of serum employed.

## DISCUSSION

The experimental results presented indicate that the normal variation in the antitryptic index is on the average approximately 50 per cent in any particular rabbit and is considerably greater in different animals. Rabbits 1 and 3, and to a lesser extent

TABLE 1

*Rabbit 1. Weight; July 17, 2862 grams; July 28, 2798 grams*

| TREATMENT | DATE | NON-PROTEIN | ALBUMIN | GLOBULIN | TOTAL PROTEIN | GLOBULIN OF TOTAL PROTEIN | PROTEIN QUOTIENT | C |
|---|---|---|---|---|---|---|---|---|
| | | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | | |
| Normal | 7– 3 | 1.2 | 4.0 | 1.5 | 5.5 | 27 | 0.37 | |
| Normal | 7– 5 | 1.3 | 4.2 | 1.8 | 6.0 | 30 | 0.43 | 2.78 |
| Normal | 7– 7 | 1.0 | 4.1 | 1.8 | 5.9 | 30 | 0.44 | 3.00 |
| Normal | 7– 9 | 1.2 | 3.5 | 1.7 | 5.2 | 33 | 0.48 | |
| Normal | 7–15 | 1.5 | 4.0 | 1.2 | 5.2 | 23 | 0.30 | 3.25 |
| 0.2 gram trypsin subcutaneously on the 16th | 7–17 | 1.3 | 3.8 | 1.4 | 5.2 | 27 | 0.37 | 1.21 |
| 0.3 gram trypsin subcutaneously on the 19th | 7–18 | 1.4 | 4.1 | 1.5 | 5.6 | 23 | 0.36 | 3.62 |
| 0.4 gram trypsin subcutaneously on the 22d | 7–20 | 1.6 | 3.95 | 1.35 | 5.3 | 26 | 0.34 | 8.11 |
| 0.6 gram trypsin subcutaneously on the 25th | 7–23 | 1.4 | 3.5 | 2.8 | 6.3 | 44 | 0.80 | 5.42 |
| | 7–26 | 1.3 | 3.2 | 1.7 | 4.9 | 35 | 0.50 | 2.70 |
| | 8– 2 | 1.3 | 4.0 | 2.2 | 6.2 | 35 | 0.55 | 2.35 |

TABLE 2

*Rabbit 2. Weight: July 17, 2894 grams; July 28, 2724 grams*

| TREATMENT | DATE | NON PROTEIN | ALBUMIN | GLOBULIN | TOTAL PROTEIN | GLOBULIN OF TOTAL PROTEIN | PROTEIN QUOTIENT | C |
|---|---|---|---|---|---|---|---|---|
| | | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | | |
| Normal | 7.5 | 1.3 | 4.2 | 1.8 | 6.0 | 30.0 | 0.43 | 2.78 |
| Normal | 7–7 | 1.3 | 4.0 | 1.5 | 5.5 | 27.0 | 0.37 | 2.22 |
| Normal | 7–9 | 1.4 | 4.1 | 1.8 | 5.9 | 30.5 | 0.44 | |
| Normal | 7–15 | 1.4 | 4.5 | 1.4 | 5.9 | 24.0 | 0.31 | 2.55 |
| 0.2 gram trypsin on the 16th subcutaneously | 7–17 | 1.4 | 4.7 | 1.2 | 5.9 | 20.0 | 0.26 | 1.90 |
| | 7–18 | 1.6 | 4.1 | 1.3 | 5.4 | 24.0 | 0.31 | 2.14 |
| 0.3 gram trypsin on the 19th (subcutaneously) | 7–20 | 1.4 | 3.4 | 1.5 | 4.9 | 31.0 | 0.44 | 3.99 |
| 0.5 gram trypsin on the 22d (subcutaneously) | 7–23 | 1.5 | 4.0 | 1.4 | 5.4 | 26.0 | 0.35 | 5.00 |
| 0.6 gram trypsin on the 25th (subcutaneously) | 7–26 | 1.3 | 3.6 | 1.7 | 5.3 | 32.0 | 0.47 | 3.06 |
| | 8–2 | 1.2 | 4.8 | 1.2 | 6.0 | 20.0 | 0.25 | 1.85 |

TABLE 3

*Rabbit 3. Weight: July 17, 2610 grams; July 28, 2383 grams*

| TREATMENT | DATE | NON PROTEIN | ALBUMIN | GLOBULIN | TOTAL PROTEIN | GLOBULIN OF TOTAL PROTEIN | PROTEIN QUOTIENT | C |
|---|---|---|---|---|---|---|---|---|
| | | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | | |
| Normal | 7–3 | 1.2 | 4.1 | 1.9 | 6.0 | 32.0 | 0.46 | |
| Normal | 7–5 | 1.3 | 4.8 | 1.85 | 6.65 | 28.0 | 0.38 | 2.78 |
| Normal | 7–7 | 1.3 | 4.4 | 1.8 | 6.2 | 29.0 | 0.41 | 3.00 |
| Normal | 7–9 | 1.4 | 4.3 | 1.8 | 6.1 | 29.5 | 0.42 | |
| Normal | 7–15 | 1 4 | 4.5 | 1.25 | 5.75 | 22.0 | 0.27 | 3.25 |
| 0.1 gram trypsin on the 16th (subcutaneously | 7–17 | 1.2 | 4.3 | 1.3 | 5.6 | 23.0 | 0.32 | 2.35 |
| Same dose on the 17th and 18th | 7–18 | 1.4 | 4.1 | 1.7 | 5.8 | 29.0 | 0.41 | 3.12 |
| Daily on the 19th to 25th inclusive 0.2 gram subcutaneously | 7–20 | 1.4 | 4.3 | 2.0 | 6.3 | 32.0 | 0.46 | 9.00 |
| | 7–23 | 1.2 | 3.9 | 1.9 | 5.8 | 33.0 | 0.49 | 8.61 |
| | 7–26 | 1.1 | 4.0 | 2.2 | 6.2 | 35.0 | 0.55 | 4.80 |
| | 8–2 | 1.2 | 4.8 | 1.6 | 6.4 | 25.0 | 0.33 | 2.81 |

TABLE 4

*Rabbit 4. Weight: July 17, 2270 grams; July 28, 2383 grams*

| TREATMENT | DATE | NON PROTEIN | ALBUMIN | GLOBULIN | TOTAL PROTEIN | GLOBULIN OF TOTAL PROTEIN | PROTEIN QUOTIENT | C |
|---|---|---|---|---|---|---|---|---|
| | | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | | |
| Normal | 7–3 | 1.3 | 4.25 | 1.25 | 5.5 | 24.0 | 0.29 | |
| Normal | 7–5 | 1.3 | 4.4 | 1.3 | 5.7 | 23.0 | 0.23 | 2.50 |
| Normal | 7–7 | 1.3 | 4.0 | 2.1 | 6.1 | 34.0 | 0.52 | 2.22 |
| Normal | 7–9 | 1.05 | 3.9 | 1.4 | 5.3 | 26.5 | 0.36 | |
| Normal | 7–15 | 1.4 | 3.9 | 1.0 | 4.9 | 20.0 | 0.26 | 3.25 |
| 0.1 gram trypsin on the 16th (intravenously) | 7–17 | 1.4 | 3.7 | 1.3 | 5.0 | 26.0 | 0.35 | 3.52 |
| Same dose also on the 17th and 18th | 7–18 | 1.3 | 3.8 | 1.2 | 5.0 | 24.0 | 0.32 | 2.93 |
| Daily 19th to 23d inclusive 0.2 gram | 7–20 | 1.5 | 4.3 | 1.1 | 5.4 | 20.0 | 0.26 | 4.25 |
| On 24th and 25th 0.4 gram (intravenously) | 7–23 | 1.3 | 4.0 | 1.7 | 5.7 | 30.0 | 0.42 | 4.43 |
| | 7–26 | 1.2 | 4.3 | 1.8 | 6.1 | 30.0 | 0.42 | 3.06 |
| | 8–2 | 1.2 | 4.6 | 1.3 | 5.9 | 22.0 | 0.28 | 1.85 |

TABLE 5

*Rabbit 5. Weight: July 17, 2100 grams; July 28, 2270 grams*

| TREATMENT | DATE | NON PROTEIN | ALBUMIN | GLOBULIN | TOTAL PROTEIN | GLOBULIN OF TOTAL PROTEIN | PROTEIN QUOTIENT | C |
|---|---|---|---|---|---|---|---|---|
| | | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | | |
| Normal | 7–3 | 1.3 | 3.9 | 1.6 | 5.5 | 29 | 0.41 | |
| Normal | 7–5 | 1.3 | 4.4 | 1.7 | 6.1 | 28 | 0.39 | |
| Normal | 7–7 | 1.3 | 4.0 | 1.6 | 5.6 | 29 | 0.40 | 3.99 |
| Normal | 7–9 | 1.0 | 4.5 | 1.3 | 5.8 | 22 | 0.29 | |
| Normal | 7–15 | 1.3 | 4.4 | 1.2 | 5.6 | 21 | 0.27 | 4.88 |
| Normal | 7–17 | 1.4 | 4.1 | 1.2 | 5.3 | 23 | 0.29 | 3.52 |
| Normal | 7–18 | 1.5 | 3.8 | 1.3 | 5.1 | 25 | 0.34 | 2.93 |
| Normal | 7–20 | 1.5 | 4.0 | 1.0 | 5.0 | 25 | 0.25 | 4.69 |
| Normal | 7–23 | 1.3 | 4.0 | 1.7 | 5.7 | 30 | 0.42 | 5.00 |
| Normal | 7–26 | 1.2 | 4.3 | 2.5 | 6.8 | 37 | 0.58 | 2.58 |
| Normal | 8–2 | 1.2 | 4.6 | 1.1 | 5.7 | 20 | 0.26 | 1.85 |

TABLE 6

*Rabbit 6. Weight: July 17, 2383 grams; July 28, 2440 grams*

| TREATMENT | DATE | NON PROTEIN | ALBUMIN | GLOBULIN | TOTAL PROTEIN | GLOBULIN OF TOTAL PROTEIN | PROTEIN QUOTIENT | C |
|---|---|---|---|---|---|---|---|---|
| | | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | | |
| Normal | 7–3 | 1.2 | 4.7 | 1.8 | 6.5 | 28 | 0.38 | |
| Normal | 7–5 | 1.2 | 5.1 | 2.0 | 7.1 | 28 | 0.39 | 3.99 |
| Normal | 7–7 | 1.2 | 4.3 | 2.1 | 6.5 | 34 | 0.49 | 3.32 |
| Normal | 7–9 | 1.4 | 4.3 | 2.4 | 6.7 | 36 | 0.56 | |
| Normal | 7–15 | 1.3 | 4.8 | 2.0 | 6.8 | 29 | 0.42 | 3.82 |
| 0.1 gram trypsin on the 16th (intravenously) | 7–17 | 1.2 | 4.3 | 1.5 | 5.8 | 26 | 0.35 | 1.58 |
| Also same dose daily to the 23d inclusive | 8–18 | 1.3 | 3.8 | 1.7 | 5.5 | 31 | 0.45 | 2.26 |
| | 7–20 | Lost | | | | | | 5.86 |
| On the 24th and 25th 0.2 gram | 7–23 | 1.3 | 4.4 | 2.2 | 6.6 | 33 | 0.50 | 4.43 |
| | 7–26 | 1.3 | 4.6 | 2.2 | 6.8 | 32 | 0.48 | 2.70 |
| | 8–2 | 1.3 | 4.7 | 1.6 | 6.3 | 25 | 0.34 | 0.95 |

TABLE 7

*Rabbit 7. Weight: July 17, 2610 grams; July 28, 2472 grams*

| TREATMENT | DATE | NON PROTEIN | ALBUMIN | GLOBULIN | TOTAL PROTEIN | GLOBULIN OF TOTAL PROTEIN | PROTEIN QUOTIENT | C |
|---|---|---|---|---|---|---|---|---|
| | | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | | |
| Normal | 7–3 | 1.1 | 4.2 | 1.3 | 5.9 | 29 | 0.31 | |
| Normal | 7–5 | 1.2 | 4.3 | 1.7 | 6.0 | 28 | 0.40 | 2.35 |
| Normal | 7–7 | ..... Lost ..... | | | | | | 2.22 |
| Normal | 7–9 | 1.0 | 3.9 | 1.7 | 5.6 | 30 | 0.44 | |
| Normal | 7–15 | 1.3 | 4.2 | 1.6 | 5.8 | 28 | 0.40 | 1.80 |
| Daily 0.1 gram trypsin subcutaneously from 16th to 23d inclusive | 7–17 | 1.4 | 3.7 | 2.1 | 5.8 | 36 | 0.57 | 2.35 |
| | 7–18 | 1.6 | 4.0 | 2.0 | 6.0 | 33 | 0.50 | 1.50 |
| On the 24th and 25th 0.2 gram... | 7–20 | 1.4 | 3.7 | 1.9 | 5.6 | 34 | 0.51 | 2.55 |
| | 7–23 | 1.3 | 4.0 | 2.0 | 6.0 | 33 | 0.50 | 2.93 |
| | 7–26 | 1.3 | 3.9 | 2.4 | 6.3 | 38 | 0.61 | 2.05 |
| | 8–2 | 1.2 | 4.3 | 2.1 | 6.4 | 33 | 0.49 | 0.95 |

rabbits 2, 4 and 6, developed a certain degree of immunity to trypsin as shown by the rise in the antitryptic index. Rabbit 5 (the control) showed also a rise in the antitryptic index. Although this increse is considerably less than that shown by rabbits 1 and 3, it is yet beyond the limits of normal variation. Rabbit 7 failed to produce any immunity.

Very interesting is the phenomenon that soon after the first one or two injections there is a marked fall in the antitryptic index. This decrease in the antitrypsin was possibly due to a neutralization of the antitrypsin by the trypsin injected, without an accompanying equal regeneration of the former for a time.

The injections of trypsin in the doses used is not followed by any decided change in the protein quotient. This fact is in harmony with the negative results of immunization against other antigens, cited at the beginning of this article.

## SUMMARY

1. The normal variation in the antitryptic index is very marked in any particular rabbit, and is even greater in different animals.

2. An unmistakable rise in the antitryptic index has been produced in at least two of the six rabbits immunized against trypsin.

3. The progress of immune body production against trypsin is very peculiar, in that at first there is a marked fall in the antitrypsin, followed soon, however, by rather a sudden rise which is at best only 300 per cent above the normal variation. At this level the antibody content remains approximately stationary for a comparatively brief period and is then supervened by a rapid return to the normal, in spite of continued periodical injections of trypsin.

4. *Immunity to pancreas trypsin appears to cause no change in the protein quotient. This fact may serve as additional evidence in favor of the recently emphasized view that immunity is non-dependent on the concentration of the serum proteins.*

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# EFFECTS OF INTRAVENOUS INJECTIONS OF A COLLOID (GELATIN), UPON RABBIT SERA

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The general purpose of the following experiments was to study the effects of intravenously injected gelatin upon the non-proteins and proteins in normal rabbit sera. The special phases considered were:

1. The effects of the injected gelatin upon the ratio of the serum albumins and globulins and upon the total amounts of proteins and non-proteins.

2. The time required for the gelatin to disappear from the blood stream.

3. The effects due to the gelatin attracting fluids from the body tissues, resulting in an excessive dilution of the blood (hydremic plethora).

Four medium sized female rabbits (2.0–2.5 kilo), were used as experimental animals. For several days before and throughout the experimental period these animals were under uniform conditions as to feed, water, etc.

The micro-refractometric method of Robertson (1) was used for the determination of the albumins, globulins and non-proteins. This method makes it possible to obtain accurate results with small amounts of serum, which is necessary when using the smaller experimental animals. The blood was removed from the vein of one ear and the injections made into a vein of the opposite ear. The analysis of the serum was made on alternate days, injections being given every day during the experimental period. The analytical work was carried out under

uniform conditions, about six hours being the average time for completing an entire set of determinations.

Gelatin was selected as a colloid suitable for injection because it is non-toxic and is not precipitated by N/50 acetic acid and subsequent boiling. It therefore appears quantitatively among the non-protein constituents. The chief disadvantage in using gelatin arises from the fact that the term "gelatin" has only a relative meaning. Nelson's "Gold Label" gelatin was selected for this work but before making up the solutions it was placed in a desiccator over $CaCl_2$ and allowed to dry several days. The gelatin then contained 7.5 per cent water and 1.5 per cent ash. From this product two solutions were made; a 10 per cent and a 20 per cent (10 grams of gelatin to 90 grams of water and 20 grams of gelatin to 80 grams of water, respectively). Not all of the water was added as such, since the solutions were acid and it was necessary to add several cubic centimeters of N/10 alkali to make the solutions neutral to phenolphthalein. After neutralizing and diluting to weight the solutions were sterilized in an Arnold steam sterilizer on three successive days to insure killing any spore-forming organisms. For injection the solutions were warmed on a water bath to 37 to 40°C.

The accompany tables on pages 149 to 152, show that the serum proteins of an individual rabbit vary from day to day through quite a wide range and that this variability applies to a greater extent between different rabbits. The variability of the serum proteins has been observed by Schmidt (2) and others employing the same methods of experimentation. From the same tables it is also evident that the injected gelatin showed no marked effect in altering either the ratio or the total amount of the serum albumins and globulins, nor the amount of non-proteins.

Moll (3), in the course of investigations on the effects of immunization upon the globulin content of their blood sera, injected gelatin into rabbits and obtained a very marked increase of the globulins in every case, in one instance his figures indicate an increase of 81 per cent. His method (4) for estimating the globulins depended upon a separation by salting out, followed

by successive washings with water, alcohol, ether, then drying at 110°C. and weighing. This method could hardly be expected to give quantitative results since the salt used to coagulate the proteins would be carried down in considerable amounts with

TABLE 1

*Ratio of globulins to total proteins*

| ANIMAL NO. | FORE-PERIOD | PERIOD | AFTER-PERIOD |
|---|---|---|---|
| | *per cent* | *per cent* | *per cent* |
| I | 18.4 | 17.1 | 22.0 |
| | 19.8 | 29.7 | 22.1 |
| | 18.1 | 14.6 | |
| | 14.3 | 24.1 | |
| | 18.2 | 22.2 | |
| | | 21.4 | |
| Average | 17.8 | 21.5 | 22.0 |
| II | 18.0 | 19.9 | 20.3 |
| | 20.9 | 22.8 | |
| | 24.7 | 28.0 | |
| | 19.7 | | |
| | 19.5 | | |
| | 17.4 | | |
| | 17.8 | | |
| | 19.3 | | |
| Average | 19.6 | 23.6 | 20.3 |
| III | 18.6 | 28.7 | 28.3 |
| | 24.8 | 18.6 | 28.5 |
| | 26.8 | 24.1 | |
| | 22.1 | 27.3 | |
| | 17.3 | | |
| Average | 21.9 | 24.7 | 28.4 |

the precipitate and would not be readily removed by the washings.

Hurwitz and Meyer (5) suggest that inflammatory irritants may give rise to an increase in serum globulins. A glance at table 1, above, for rabbit 1 on the second day of the period shows there has been a very marked increase in the globulins, rising from 17 to 30 per cent of the total proteins. This value

is considerably higher than any obtained with this animal. On the previous day this animal received, through accident, quite a large extravascular injection of gelatin which collected in a hard lump at the base of the ear. The animal did not exhibit any out-

TABLE 2

*Total proteins*

| ANIMAL NO. | FORE-PERIOD | PERIOD | AFTER-PERIOD |
|---|---|---|---|
| | *per cent* | *per cent* | *per cent* |
| I | 6.46 | 6.27 | 5.68 |
| | 5.95 | 6.13 | 6.07 |
| | 6.07 | 5.74 | |
| | 5.96 | 5.81 | |
| | 6.00 | 6.27 | |
| | | 6.44 | |
| Average | 6.08 | 6.11 | 5.88 |
| II | 6.63 | 6.08 | 6.41 |
| | 6.07 | 6.12 | |
| | 5.83 | 6.03 | |
| | 6.23 | | |
| | 6.32 | | |
| | 6.38 | | |
| | 6.29 | | |
| | 6.20 | | |
| Average | 6.24 | 6.08 | 6.41 |
| III | 6.14 | 5.88 | 5.73 |
| | 5.88 | 6.24 | 5.86 |
| | 5.74 | 5.81 | |
| | 6.05 | 5.41 | |
| | 6.75 | | |
| Average | 6.11 | 5.84 | 5.80 |

ward signs of disturbance except that the ear was feverish for thirty-six hours, by this time the gelatin lump was very much smaller.

To determine the time the gelatin remained in the blood it was only necessary to make determinations of the non-proteins before and after the injection of the gelatin. The analysis of the blood just before the injection gives the normal non-protein

content, the increased amounts obtained after injection are due to the normal non-protein plus varying amounts of gelatin. The question also arose as to whether the elimination of gelatin from an animal having previously had several injections would

TABLE 3

*Non-proteins*

| ANIMAL NO. | FORE-PERIOD | PERIOD | AFTER-PERIOD |
|---|---|---|---|
| | *per cent* | *per cent* | *per cent* |
| I | 1.21 | 1.33 | 1.50 |
| | 1.13 | 1.23 | 1.40 |
| | 1.13 | 1.41 | |
| | 1.43 | 1.33 | |
| | 1.41 | 1.43 | |
| | | 1.23 | |
| Average | 1.26 | 1.33 | 1.45 |
| II | 1.23 | 1.24 | 1.31 |
| | 1.31 | 1.41 | 1.40 |
| | 1.13 | 1.31 | |
| | 1.33 | | |
| | 1.23 | | |
| | 1.24 | | |
| | 1.13 | | |
| | 1.33 | | |
| Average | 1.25 | 1.32 | 1.36 |
| III | 1.31 | 1.14 | 1.43 |
| | 1.31 | 1.23 | 1.31 |
| | 1.13 | 1.33 | |
| | 1.23 | 1.30 | |
| | 1.33 | | |
| Average | 1.26 | 1.25 | 1.37 |

be more or less rapid than from an animal which had had no other injections. From the tables and curves on pages 152, 154 and 155 it will be seen that the animal having had several injections of gelatin disposed of it more rapidly than the animal which had received gelatin for the first time. It is also evident that the elimination of gelatin commenced immediately after injection

and continued at a fairly uniform rate. The table on this page shows that the analyses made approximately twenty-four hours after the injection of gelatin give a very constant value for the non-proteins, the variations during the period of gelatin injection being no greater than those preceding and following this

TABLE 4

| DATE | RABBIT NO. | NON-PROTEINS | TOTAL PROTEINS | GELATIN SOLUTION INJECTED | | CALCULATED $V$ | $V + v$ |
|---|---|---|---|---|---|---|---|
| *1917* | | *per cent* | *per cent* | *cc.* | *per cent* | *cc.* | *cc.* |
| May 27 | Ia[1] | 1.23 | 6.13 | 20 | 10 | 194.1 | |
| | Ib[2] | 2.10 | 5.23 | | | | 227.1 |
| | IIIa | 1.23 | 6.24 | 20 | 10 | 280.0 | |
| | IIIb | 1.81 | 5.20 | | | | 336.0 |
| May 28 | IIa | 1.33 | 6.20 | 20 | 10 | 256.7 | |
| | IIb | 2.00 | 5.61 | | | | 285.3 |
| | IIIa | 1.33 | 5.81 | 20 | 10 | 212.1 | |
| | IIIb | 2.10 | 4.73 | | | | 240.1 |
| May 29 | Ia | 1.33 | 5.81 | 10 | 20 | 457.6 | |
| | Ib | 1.71 | 5.05 | | | | 526.2 |
| | IIa | 1.24 | 6.08 | 10 | 20 | 318.5 | |
| | IIb | 1.81 | 5.44 | | | | 356.7 |
| | IIIa | 1.30 | 5.41 | 10 | 20 | 247.2 | |
| | IIIb | 2.00 | 4.68 | | | | 286.8 |
| May 31 | Ia | 1.43 | 6.27 | 10 | 20 | 455.6 | |
| | Ib | 1.79 | 5.14 | | | | 555.8 |
| | IIa | 1.41 | 6.12 | 10 | 20 | 330.0 | |
| | IIb | 1.99 | 5.25 | | | | 344.5 |
| June 1 | IVa | 1.23 | 6.44 | 10 | 20 | 319.0 | |
| | IVb | 1.70 | 4.82 | | | | 425.9 |

1, "a" indicates that blood samples were taken just before the gelatin was injected.

2, "b" indicates blood samples taken 4 hours after the injection of the gelatin.

period. Having followed the gelatin in the blood, at one hour intervals for six hours, beginning immediately after injection and finding none at the end of twenty-four hours, it is evident that the gelatin disappears from the blood within these time limits. On extending the curves, page 154, assuming the rate of elimination

to be uniform, a value of approximately twelve hours is obtained as the time in which the gelatin should be completely eliminated.

Buglia (6) conducted some experiments upon dogs to determine the manner in which intravenously injected gelatin was eliminated and in addition followed the amount of gelatin in the blood. Using the viscosity (determined by the Ostwald Viscosimeter at 37°C.), and the conductivity (Kohlrausch conductivity cell at 37°C.), as a means of determining the presence of gelatin, he found that a portion of it remained in the blood as long as forty hours. The more direct refractometric method of estimation described above would appear, as has been pointed out, to indicate complete disappearance of the gelatin at a much earlier period.

In order to obtain an idea of the volume of the blood in the rabbits and the dilution resulting from the attraction of tissue fluids by the injected gelatin, the following method of estimation was used;

If we let

$V$ = original volume of blood in the rabbit,

$v$ = increase in above volume, $V$, due to dilution by attracted tissue fluids,

$p_0$ = per cent of gelatin in the blood immediately after injection,

$p_1$ = per cent of gelatin in the blood after $V$ has become $V + v$,

$T_0$ = per cent of total proteins in the blood before the injection of gelatin,

$T_1$ = per cent of total proteins in the blood after $V$ has become $V + v$,

$n_0$ = per cent of non-proteins in the blood before the injection of gelatin,

$n_1$ = per cent of non-proteins after $V$ has become $V + v$.

Then,

$$\frac{T_0 - T_1}{T_0} = \frac{v}{V + v} = s, \text{ whence } v\,(1 - s) = sV \text{ and } v = \frac{s}{1 - s}\,V. \quad (1)$$

$$p_0 V = p_1\,(V + v) \quad (2)$$

As an example take the following:

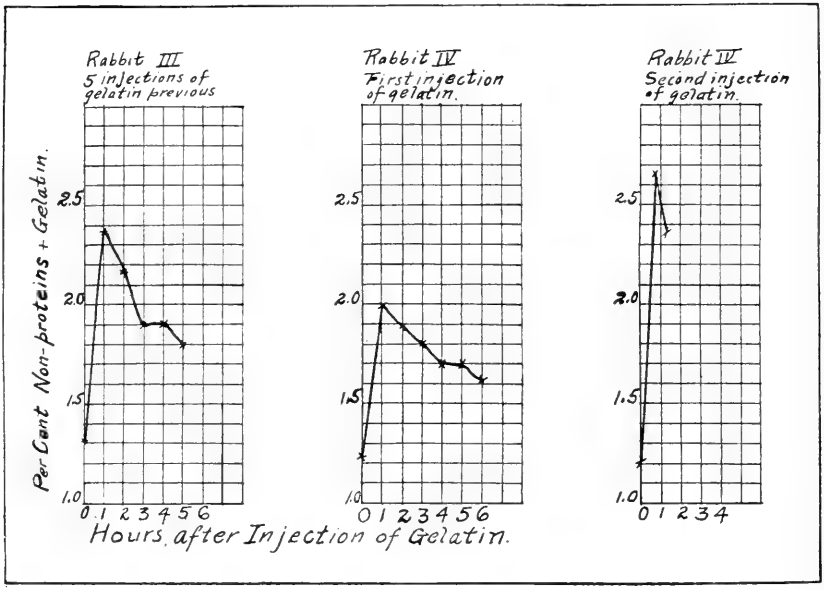
$T_0$ = 6.44 per cent, after four hours $T_1$ = 4.82 per cent,

$n_0$ = 1.23 per cent, after four hours $n_1$ = 1.70 per cent,

hence,

$$\frac{6.44 - 4.82}{6.44} = 0.251, \; v = \frac{0.251}{0.749} V = 0.335 V$$

Rate at which gelatin disappears from the blood




and

1.70 − 1.23 = 0.47 per cent, the difference in non-protein due to gelatin remaining in the blood,

hence,

$p_0$ = 0.0047 × 1.335 = 0.00627 per cent of gelatin in the original volume of blood when 2 grams of gelatin are injected,

therefore,

$V = \frac{2.0}{0.00627}$ = 319.0 cc., and $V + v$ 1.335 × 319.0 = 425.9 cc., hence the apparent increase in the volume of the blood, due to the attraction of water from the tissues, after four hours was 33.5 per cent.

In a similar way the results of other determinations are calculated, table 4, on page 152, presents a brief summary of these calculations. The observed dilutions are both apparent and real for while the gelatin as a whole is drawing tissue fluids into the blood stream, the gelatin is also disappearing from the blood at a rapid rate. A glance at the table on this page and at the curves on the opposite page shows clearly how constant and how rapid this elimination of gelatin becomes. Since the different

TABLE 5

| DATE | RABBIT NO. | NON-PROTEINS + GELATIN | REMARKS |
|---|---|---|---|
| *1917* | | *per cent* | |
| June 1..... | IV | 1.23 | Sample taken before injection |
| | | 1.99 | Sample taken 1 hour after injection |
| | | 1.89 | Sample taken 2 hours after injection |
| | | 1.80 | Sample taken 3 hours after injection |
| | | 1.70 | Sample taken 4 hours after injection |
| | | 1.70 | Sample taken 5 hours after injection |
| | | 1.61 | Sample taken 6 hours after injection |
| June 2..... | III | 1.31 | Sample taken before injection |
| | | 2.37 | Sample taken 1 hour after injection |
| | | 2.17 | Sample taken 2 hours after injection |
| | | 1.90 | Sample taken 3 hours after injection |
| | | 1.90 | Sample taken 4 hours after injection |
| | | 1.80 | Sample taken 5 hours after injection |
| June 4..... | IV | 1.21 | Sample taken before injection |
| | | 2.66 | Sample taken 45 minutes after injection |
| | | 2.36 | Sample taken 75 minutes after injection |

body fluids are isotonic the substances carried into the blood by the tissue fluids would not materially affect the refractive index of the blood serum. If proteins are brought into the blood stream there must also be some elimination, for the results presented in table 1, page 149, show that the ratio of the globulins to the total proteins does not change to any marked extent.

That actual dilution of the blood did occur was evident to the eye by the distended appearance of the blood vessels. Two hours after the injection of the gelatin, the visible blood vessels,

especially the ear veins, were much enlarged and samples of blood taken at this time and for several hours later presented a very watery appearance. Previous to this two hour period it was extremely difficult to bleed the rabbits from the ear veins as the blood clotted very rapidly.

## SUMMARY

Experimental results are presented which show that:

1. The serum proteins of individual rabbits vary through a wide range and that the variation is even greater between different rabbits.

2. Intravenous injections of large amounts of gelatin does not produce any marked change in the ratio of the globulins to the total proteins.

3. The gelatin begins to disappear from the blood immediately after injection and continues to do so at a fairly constant rate, being completely eliminated at some time between six and twenty-four hours.

4. The injection of gelatin causes tissue fluids to be drawn into the blood stream, resulting in hydremic plethora.

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# THE INFLUENCE OF ACTIVE NORMAL SERUM (COMPLEMENT) UPON MENINGOCOCCI[1]

## I. THE OPSONIC ACTIVITY OF FRESH NORMAL SERUM ALONE AND IN COMBINATION WITH ANTIMENINGITIS SERUM FOR MENINGOCOCCI

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### INTRODUCTION

It is generally true that the antibacterial properties of both normal and immune sera are greater when perfectly fresh than after being heated or allowed to stand for weeks or months with the addition of a preservative as shown in chart 7 with the opsonins in antimeningitis serum. This is probably due not only to deterioration of specific antibodies in immune serum but also to the loss of certain labile substances in normal and immune sera alike and particularly those substances generally designated as complements and normal opsonins. Antibacterial sera are especially likely to show differences in activity in their fresh state as compared with the same sera after being heated or after standing, and particularly when examined by test tube methods, whereas the antitoxic sera are usually more stabile; these differences in the case of the antibacterial sera may be ascribed in part to the loss of the labile complementary substances regarded as essential or important in the mechanism of their antibacterial activity.

The curative and protective properties of antimeningitis serum have been ascribed to the presence and activities of opsonins,

[1] Aided by a grant from the Pediatric Society of Philadelphia.

bactericidins and an antitoxin capable of neutralizing the endotoxin believed to result from the disintegration of virulent meningococci. Jochmann (1) first drew attention to the high phagocytic powers of antimeningitis serum and this property of the antiserum has been emphasized by the intensive researches of Flexner (2, 3), Flexner and Jobling (4) and others, not only in test tube experiments but also in experimental infections in monkeys and in persons suffering with meningococcus meningitis. Neufeld (5) proposed a technique for measuring the opsonin content of antimeningitis serum which was adopted by Jobling (6) as a means of standardizing the serum. At the present time this method is less widely used owing to technical difficulties and likelihood of error, and it is not regarded as yielding an accurate measure or index of the antibody content of an antiserum; furthermore it does not measure the antitoxic and bactericidal activity and Flexner (2) particularly has shown that the latter properties exert an important part in the therapeutic activities of antimeningitis serum. One may accept as an established fact however, that the phagocytic activity of a *potent* antimeningitis serum is higher than that of normal serum; that resistance to and recovery from meningococcus meningitis is to be ascribed in part to the phenomenon of phagocytosis and that the intraspinal injection of a potent antimeningitis serum is frequently followed by an increased ingestion and diminution in the numbers and viability of meningococci and finally their complete disappearance, and that these curative effects may be ascribed in part to the influence of opsonin contained in the antiserum. Normal human serum has been found by Davis (7) on the basis of test tube experiments, to contain opsonin for meningococci as, likewise, bactericidal substances, and Flexner (3) has shown that normal monkey serum is capable of reducing appreciably the toxic effect of given doses of virulent meningococci. McKenzie and Martin (8) have also observed that fresh human serum contained thermolabile substances having an inhibitory effect upon the growth of meningococci, and have used fresh human serum in treatment by intraspinal injection, with encouraging results. It does not appear however, that anyone has studied the influence

upon meningococci of a combination of normal and antimeningitis serum, as the latter is secured in the market ready for administration.[2]

Normally the cerebrospinal fluid is free of complement as likewise of certain antibodies which may occur normally or naturally in the blood, as agglutinins, hemolysins and diphtheria antitoxin; investigations upon the presence of complement in the cerebrospinal fluid during epidemic meningitis have not been

TABLE 1

*Results of tests for hemolytic complement in the fresh cerebrospinal fluids of suppurative meningitis*

| TYPE OF INFECTION | RESULTS WITH INCREASING AMOUNTS OF CEREBRO SPINAL FLUID* | | | | | |
|---|---|---|---|---|---|---|
| | 0.4 cc. | 0.8 cc. | 1 cc. | 1.5 cc. | 2 cc. | 3 cc. |
| Meningococcus | NH† | NH | NH | NH | NH | NH |
| Meningococcus | NH | NH | NH | NH | NH | NH |
| Meningococcus | NH | NH | NH | SH | SH | SH |
| Meningococcus | NH | NH | NH | NH | NH | NH |
| Meningococcus | NH | NH | SH | SH | SH | MH |
| Meningococcus | NH | NH | NH | NH | NH | NH |
| Pneumococcus | NH | NH | NH | NH | NH | SH |
| Pneumococcus | NH | NH | NH | SH | SH | SH |
| Sterile; acute congestion | NH | NH | NH | NH | NH | NH |
| Sterile; acute congestion | NH | NH | NH | NH | NH | NH |

* All fluids were tested within a few hours after removal by spinal puncture. The tests were conducted by placing increasing amounts of cerebrospinal fluid in a series of test tubes with two units of antisheep hemolysin and 1 cc. of 2.5 per cent suspension of washed sheep cells; the results were read after one hour in a water bath at 38°C.

† NH = no hemolysis; SH = slight hemolysis; MH = marked hemolysis.

sufficiently numerous or extensive to warrant a statement on this point; Fairley and Stewart found but one case in an extended series in which the cerebrospinal fluid in 1.5 cc. contained a trace of complement, probably due to admixed blood. Our own studies of the fresh cerebrospinal fluid from six cases of epidemic meningococcus meningitis during the acute stages of the disease

[2] Fairley and Stewart (9) have however, recommended the thorough trial of the complement reinforcement of commercial sera, in the treatment of meningitis.

showed the presence of traces of hemolytic complement in but two; consequent to the acute congestion of the vessels of the meninges in this disease together with the inflammatory exudation of serum and cellular elements from the blood, it is reasonable to expect the presence of traces complement and natural anti-sheep hemolysins during the early stages of acute meningitis as found in acute poliomyelitis by Kolmer, Freese, Matsunami and Meine (10), although owing to the fact of high dilution in the cerebrospinal fluid the amount of complement in a given volume of fluid is likely to be small and may escape detection (table 1).

While the serum treatment of epidemic meningitis by the subdural method of injection, has been an acknowledged success when potent serum was employed, as shown by the reduction of a gross mortality of 70 to 90 per cent among cases treated without serum compared with 30 per cent or less among serum treated cases (Flexner), yet continued efforts are necessary in order to lower the 20 to 30 per cent mortality remaining among the serum treated cases; naturally most can be expected in this direction from efforts toward increasing the potency and polyvalency of the immune serum and from efforts toward early diagnosis of the disease, in the bacteremic stage if possible, with prompt use of the serum intravenously and intraspinally. Inasmuch as the curative properties of antimeningitis sera are in most part ascribed to the presence and activity of opsonins, bactericidins and an antitoxin and, as the first two antibodies are most active when the serum is fresh, due, presumably, to the presence of labile opsonin and complement in fresh serum, and since the various antimeningitis sera are several weeks or months old when employed and lack the presence of these substances, which are to be found only in fresh serum and, since the cerebrospinal fluid in meningitis likely contains but traces of complement and normal opsonin or none at all, our efforts towards increasing the efficiency of the serum treatment of meningitis have been confined to a laboratory study of the influence of fresh normal human and guinea-pig sera alone upon meningococci and more particularly in combination with various antimeningitis sera, with special

attention to their influence upon the opsonic and bactericidal activities of antimeningitis serum in vitro and upon the curative value of the serum as tested with mice infected with lethal doses of virulent meningococci.

It is the purpose of this communication to present a summary of the results of our experiments bearing upon the opsonic activities of fresh normal human and guinea-pig sera alone for virulent meningococci and in various dilutions with antimeningitis sera.

## EXPERIMENTAL

*Technic.* Several strains of meningococci and various polyvalent antimeningitis sera prepared for the market and ready for administration, were employed.[3] In some experiments fresh sterile and preservative free antisera were used, but the results given in this paper are based upon experiments conducted with the former unless otherwise stated. As our purpose was to determine the opsonic activity of the antisera plus fresh normal serum, we have not determined the limits of opsonic activity of each immune serum but adopted one dilution or a series of dilutions as low as possible to avoid the agglutinating properties of each serum. The human and guinea-pig sera were used immediately after collection or kept at a low temperature and used within twenty-four hours after collection. In all experiments the various sera were used unheated; all dilutions were prepared with 0.85 per cent sodium chlorid in distilled water.

Various strains of meningococci cultivated upon plain dextrose agar and suspended in isotonic salt solution were employed; the majority of experiments were conducted with strain No. 124 furnished by Doctors Hitchens and Robinson, which grew fairly well on plain dextrose agar and proved highly virulent for white mice.

Human leucocytes obtained from the blood of healthy persons were employed routinely; comparative tests were also made with

[3] *Acknowledgment.* We are indebed to Dr. A. P. Hitchens, Dr. C. H. Robinson and Dr. H. L. Amoss, for several strains of meningococci, parameningococci and polyvalent immune sera and to Dr. J. F. Anderson for immune sera, to all of whom we express our thanks.

exudative leucocytes of guinea-pigs obtained by aleuronat irritation, but the results given in this paper are those observed with the leucocytes of normal healthy persons.

The pipet method of Wright was employed throughout, the mixtures of equal parts of sera, meningococci and leucocytes being incubated at 37°C. for fifteen minutes when smears were prepared. With this technic the following studies were made with various normal and immune sera and strains of meningococci:

1. The opsonic activity displayed in equal parts of fresh sterile human serum, human leucocytes and suspension of meningococci.

2. The opsonic activity displayed in equal parts of fresh sterile guinea-pig serum, human leucocytes and meningococci.

3. The opsonic activity of a 1:100 dilution of antiserum alone and in combination with equal parts of varying dilutions of fresh sterile human or guinea-pig serum, human leucocytes and meningococci.

4. The opsonic activity displayed in mixtures of equal parts of varying dilutions of antisera, undiluted human or guinea-pig serum, human leucocytes and meningococci.

Two values were determined with each smear:

*a*. The phagocytic index or percentage of polymorphonuclear leucocytes found to engulf diplococci.

*b*. The opsonic index or average number of cocci per phagocyte. In computing this index each pair of cocci was counted as two.

As Kite and Wherry (11) have shown that the amount of mechanical agitation to which mixtures of leucocytes and bacteria or leucocytes, bacteria and serum are subjected influence the degree of phagocytosis, we have endeavored to determine the influence of agitation due to the mixing of the various substances in conducting our tests, but comparative experiments consisting in mixing serum, leucocytes and meningococci by expelling and drawing in the pipet six times, yielded phagocytic and opsonic indices entirely similar to those observed when the various components were agitated by mixing thirty times in the same manner; although phagocytic tests are well known to yield irregular results due to errors both within and beyond the control of the manipulation, we have endeavored to control this one factor by adhering to a uniform technic.

While leucocytes from different sources have been found to vary in phagocytic activity, we have used uniformly the leucocytes obtained from the peripheral blood of healthy persons after washing three times, as serving the simple purposes of our study,

TABLE 2

*Phagocytic and opsonic indices with varying dilutions of fresh normal human and guinea-pig serum and different strains of normal meningococci*

| FINAL DILUTION OF HUMAN AND PIG SERUM | WITH NORMAL COCCI STRAIN I | | WITH NORMAL COCCI STRAIN II | | WITH NORMAL COCCI STRAIN III | | WITH NORMAL COCCI STRAIN 124 | | WITH NORMAL COCCI STRAIN IV | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Human serum | Pig serum | Human serum | Pig serum | Human serum | Pig serum | Human serum | Pig serum | Human serum | Pig serum |
| 1:2 | 40.0* | 32.0 | 67.0 | 40.0 | 30.0 | 36.0 | 14.0 | 14.0 | 32.0 | 10.0 |
| | 1.7† | 1.2 | 2.1 | 1.7 | 1.0 | 1.3 | 1.0 | 1.7 | 1.2 | 1.1 |
| 1:4 | 36.0 | 36.0 | 56.0 | 44.0 | 22.0 | 20.0 | 20.0 | 14.0 | 22.0 | 8.0 |
| | 1.6 | 1.3 | 1.5 | 1.7 | 1.0 | 1.2 | 1.1 | 1.0 | 1.1 | 1.0 |
| 1:8 | 36.0 | 16.0 | 48.0 | 34.0 | 22.0 | 22.0 | 10.0 | 16.0 | 8.0 | 6.0 |
| | 1.4 | 1.4 | 1.6 | 1.5 | 1.0 | 1.0 | 1.0 | 1.2 | 1.0 | 1.0 |
| 1:16 | 20.0 | 20.0 | 44.0 | 26.0 | 14.0 | 10.0 | 10.0 | 10.0 | 4.0 | 6.0 |
| | 1.1 | 1.1 | 1.2 | 1.9 | 1.3 | 1.4 | 1.0 | 1.2 | 1.0 | 1.0 |
| 1:32 | 14.0 | 6.0 | 36.0 | 24.0 | 12.0 | 10.0 | 8.0 | 10.0 | 4.0 | 10.0 |
| | 1.0 | 1.0 | 1.3 | 1.5 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| 1:64 | 18.0 | 12.0 | 34.0 | 22.0 | 14.0 | 14.0 | 6.0 | 16.0 | 4.0 | 8.0 |
| | 1.0 | 1.0 | 1.5 | 1.7 | 1.0 | 1.0 | 1.8 | 1.0 | 1.0 | 1.2 |
| 1:128 | 14.0 | 14.0 | 30.0 | 28.0 | 14.0 | 14.0 | 6.0 | 8.0 | 4.0 | 4.0 |
| | 1.0 | 1.1 | 1.7 | 1.8 | 1.0 | 1.0 | 1.0 | 1.2 | 1.0 | 1.0 |
| 1:256 | 14.0 | 16.0 | 38.0 | 28.0 | 12.0 | 10.0 | 4.0 | 6.0 | 2.0 | 8.0 |
| | 1.0 | 1.0 | 1.5 | 1.1 | 1.0 | 1.0 | 1.0 | 1.3 | 1.0 | 1.0 |
| 1:512 | 18.0 | 12.0 | 30.0 | 18.0 | 10.0 | 8.0 | 6.0 | 8.0 | 2.0 | 10.0 |
| | 1.1 | 1.0 | 1.3 | 1.0 | 1.4 | 1.0 | 1.0 | 1.0 | 1.0 | 1.2 |
| No serum.. | 14.0 | 11.0 | 28.0 | 14.0 | 12.0 | 10.0 | 5.0 | 10.0 | 2.0 | 7.0 |
| | 0.8 | 1.0 | 1.2 | 0.6 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |

* Upper figure in each column gives the phagocytic index (percentage of leucocytes found to engulf meningococci).

† Lower figure in each column gives the opsonic index (average number of meningococci per phagocyte).

namely, the influence of fresh sera upon the phenomenon of phagocytosis of meningococci in vitro. Human leucocytes were found to possess somewhat greater phagocytic activities than guinea-pig leucocytes and the leucocytes from the peripheral blood of guinea-pigs were found to be more active than those secured from the peritoneal cavity sixteen hours after the intraperitoneal injection of a sterile emulsion of aleuronat.

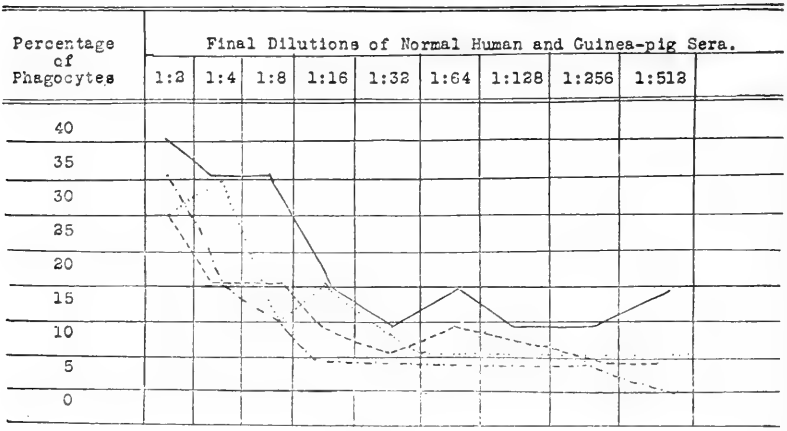


CHART 1

——— = results with human serum and normal meningococci, no. 1, phagocytosis without serum was 14 per cent.

..... = results with guinea-pig serum and normal meningococci, no. 1, phagocytosis without serum was 11 per cent.

- - - - - = results observed with human serum and normal meningococci no. 124; phagocytosis without serum was 12 per cent.

- · - · - · = results observed with guinea-pig serum and normal meningococci no. 124; phagocytosis without serum was 10 per cent.

## RESULTS

*a. Opsonic activity of normal human and guinea-pig serum for meningococci.* The phagocytic and opsonic indices observed with five human and five guinea-pig sera and three different strains of meningococci are summarized in table 2; the phagocytic indices observed with two human and two guinea-pig sera and two strains of meningococci are shown in chart 1.

The results may be summarized as follows:

1. The fresh serum of persons and guinea-pigs possess well marked amounts of opsonin for meningococci.

2. As a general rule human serum contains more opsonin for meningococci than guinea-pig serum.

3. Dilutions of human and guinea-pig serum greater than 1:16 or 1:32 generally failed to influence the phagocytic and opsonic indices.

TABLE 3

*The opsonic activity of human serum alone in final dilution of 1:3 and in conjunction with various antimeningitis sera in final dilution of 1:400, for strains of normal meningococci*

| ANTIMENINGITIS SERUM | STRAIN OF MENINGOCOCCUS | NO ANTISERUM; NO HUMAN SERUM | | ANTISERUM ALONE (1:400) | | HUMAN SERUM ALONE (1:3) | | ANTISERUM (1:400) AND HUMAN SERUM (1:3) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Phagocytic index | Opsonic index | Phagocytic index | Opsonic index | Phagocytic index | Opsonic index | Phagocytic index | Opsonic index |
| Serum 1..... | 124 | 12 | 0.8 | 16 | 1.2 | 30 | 1.1 | 36 | 1.6 |
| Serum 2..... | 124 | 10 | 0.8 | 15 | 1.2 | 26 | 1.1 | 35 | 1.4 |
| Serum 3..... | 124 | 8 | 0.4 | 14 | 1.8 | 15 | 1.1 | 50 | 1.6 |
| Serum 4..... | 124 | 8 | 0.6 | 18 | 2.2 | 40 | 1.7 | 45 | 1.7 |
| Serum 3..... | Normal 1 | 6 | 0.4 | 12 | 1.0 | 32 | 1.2 | 55 | 2.6 |
| Serum 4..... | Normal 1 | 2 | 0.5 | 11 | 1.1 | 34 | 1.1 | 43 | 2.2 |
| Serum 1..... | Normal 1 | 15 | 1.2 | 25 | 1.2 | 40 | 1.7 | 73 | 1.8 |
| Serum 2..... | Normal 1 | 20 | 1.2 | 26 | 1.4 | 40 | 1.6 | 67 | 1.8 |
| Serum 3..... | Normal 11 | 12 | 1.0 | 16 | 1.2 | 15 | 1.2 | 50 | 1.6 |
| Serum 4..... | Normal 11 | 8 | 1.0 | 10 | 1.1 | 15 | 1.2 | 20 | 2.0 |

4. The majority of phagocytes were found to contain less than an average of three cocci and the normal sera were found to increase the percentage of leucocytes engulfing cocci rather than increasing the number of cocci per leucocyte.

5. Different strains of meningococci were found to exhibit varying degrees of resistance to phagocytosis.

6. The sera of different persons and guinea-pigs were found to vary in a slight degree in opsonic activity for the same strains of meningococci.

*b. Opsonic activity of various antimeningitis sera and in combination with normal human and guinea-pig sera.* The results of ex-

periments showing the degree of phagocytosis in mixtures of leucocytes and meningococci (to show degree of spontaneous phagocytosis); in mixtures of antiserum in one final dilution of 1:400 with leucocytes and meningococci; in mixtures of varying dilutions of normal human and guinea-pig sera with leucocytes and meningococci and in mixtures of antiserum, normal serum,

TABLE 4

*The opsonic activity of guinea-pig serum alone in final dilutions of 1:3 and in conjunction with various antimeningitis sera in final dilutions of 1:400 for different strains of normal meningococci*

| ANTIMENINGITIS SERUM | STRAIN OF MENINGOCOCCI | NO ANTISERUM; NO PIG SERUM | | ANTISERUM ALONE (1:400) | | PIG SERUM ALONE (1:3) | | ANTISERUM (1:400) AND PIG SERUM (1:3) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Phagocytic index | Opsonic index | Phagocytic index | Opsonic index | Phagocytic index | Opsonic index | Phagocytic index | Opsonic index |
| Serum 3..... | 124 | 12 | 0.2 | 14 | 1.2 | 12 | 1.3 | 30 | 1.4 |
| Serum 4..... | 124 | 11 | 0.4 | 20 | 1.2 | 24 | 1.4 | 46 | 1.3 |
| Serum 3..... | Normal 1 | 6 | 0.4 | 7 | 1.2 | 10 | 1.2 | 20 | 1.3 |
| Serum 5..... | Normal 1 | 6 | 0.3 | 10 | 1.4 | 8 | 1.1 | 16 | 1.2 |
| Serum 1..... | 124 | 9 | 0.2 | 15 | 1.1 | 35 | 1.3 | 40 | 1.4 |
| Serum 1..... | 124 | 8 | 0.3 | 16 | 1.1 | 36 | 1.3 | 46 | 1.5 |
| Serum 1..... | Normal 11 | 14 | 1.1 | 30 | 1.4 | 40 | 1.7 | 67 | 2.0 |
| Serum 2..... | Normal 11 | 12 | 0.6 | 30 | 1.2 | 36 | 1.6 | 48 | 1.8 |
| Serum 1..... | Normal 1 | 15 | 1.1 | 28 | 1.4 | 32 | 1.4 | 64 | 1.7 |
| Serum 2..... | Normal 1 | 15 | 0.8 | 40 | 1.6 | 30 | 1.2 | 58 | 1.8 |
| Serum 4..... | Normal 1 | 10 | 1.0 | 12 | 1.1 | 15 | 1.6 | 30 | 1.8 |

leucocytes and meningococci, are shown in tables 3, 4, and 5; charts 2 and 3 show the phagocytic curves observed in two experiments and charts 4 and 5, the phagocytic and opsonic curves shown in two other experiments.

In table 6 are given the phagocytic and opsonic indices of another set of experiments with dilutions of different antimeningitis sera varying from 1:300 to 1:2560 with a constant dilution of human nd guinea-pig serum (1:4); in chart 6 are shown the phagocytic curves observed in one of these experiments with varying dilutions of an antimeningitis serum alone and of similar dilutions of antiserum with 1:4 dilutions of fresh normal human and guinea-pig sera.

TABLE 5

*Phagocytic and opsonic indices with final 1:400 dilutions of various antimeningitis sera, different strains of normal meningococci and varying dilutions of fresh normal human and guinea-pig sera*

| FINAL DILUTIONS OF HUMAN AND PIG SERUM | ANTISERUM 1, (1:400) NORMAL COCCI 1 PLUS: | | ANTISERUM 2, (1:400) NORMAL COCCI 1 PLUS: | | ANTISERUM 1, (1:400) COCCI 124 PLUS: | | ANTISERUM 3, (1:400) COCCI 124 PLUS: | | ANTISERUM 3, (1:400) NORMAL COCCI 1 PLUS: | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Human serum | Pig serum | Human serum | Pig serum | Human serum | Pig serum | Human serum | Pig serum | Human serum | Pig serum |
| 1:4 | 72.0* | 64.0 | 50.0 | 68.0 | 36.0 | 41.0 | 50.0 | 30.0 | 56.0 | 20.0 |
| | 1.8† | 1.7 | 2.2 | 2.0 | 1.6 | 1.4 | 1.6 | 1.2 | 2.6 | 1.2 |
| 1:8 | 52.0 | 44.0 | 60.0 | 74.0 | 24.0 | 24.0 | 42.0 | 22.0 | 54.0 | 12.0 |
| | 1.7 | 1.7 | 2.2 | 2.2 | 1.1 | 1.1 | 1.5 | 1.8 | 1.7 | 1.1 |
| 1:16 | 50.0 | 48.0 | 50.0 | 48.0 | 20.0 | 16.0 | 28.0 | 26.0 | 34.0 | 12.0 |
| | 1.5 | 1.7 | 2.2 | 2.3 | 1.3 | 1.0 | 1.0 | 1.8 | 1.2 | 1.0 |
| 1:32 | 30.0 | 40.0 | 44.0 | 48.0 | 16.0 | 16.0 | 24.0 | 26.0 | 24.0 | 14.0 |
| | 1.1 | 1.5 | 3.0 | 2.2 | 1.0 | 1.0 | 1.1 | 1.3 | 1.1 | 1.4 |
| 1:64 | 28.0 | 36.0 | 36.0 | 30.0 | 18.0 | 8.0 | 22.0 | 24.0 | 6.0 | 8.0 |
| | 1.1 | 1.3 | 1.8 | 1.9 | 1.0 | 1.0 | 1.4 | 1.1 | 1.1 | 1.0 |
| 1:128 | 22.0 | 20.0 | 40.0 | 42.0 | 16.0 | 6.0 | 22.0 | 18.0 | 6.0 | 8.0 |
| | 1.1 | 1.2 | 1.5 | 1.5 | 1.1 | 1.0 | 1.1 | 1.0 | 1.0 | 1.2 |
| 1:256 | 16.0 | 16.0 | 50.0 | 30.0 | 12.0 | 12.0 | 14.0 | 12.0 | 6.0 | 10.0 |
| | 1.1 | 1.1 | 1.4 | 1.8 | 1.0 | 1.5 | 1.0 | 1.0 | 1.0 | 1.0 |
| 1:512 | 16.0 | 12.0 | 42.0 | 24.0 | 16.0 | 10.0 | 18.0 | 12.0 | 2.0 | 10.0 |
| | 1.1 | 1.3 | 1.5 | 2.4 | 1.1 | 1.2 | 1.1 | 1.5 | 1.0 | 1.0 |
| 1:1024 | 12.0 | 22.0 | 38.0 | 30.0 | 16.0 | 10.0 | 18.0 | 12.0 | 2.0 | 8.0 |
| | 1.4 | 1.0 | 1.8 | 1.7 | 1.1 | 1.0 | 1.1 | 1.0 | 1.0 | 1.0 |
| Antiserum (1:400) alone | 16.0 | 22.0 | 24.0 | 25.0 | 16.0 | 11.0 | 10.0 | 10.0 | 2.0 | 7.0 |
| | 1.1 | 1.2 | 1.1 | 1.2 | 1.1 | 1.1 | 1.1 | 1.1 | 1.0 | 1.6 |
| No antiserum; no normal serum | 12.0 | 12.0 | 14.0 | 13.0 | 12.0 | 10.0 | 8.0 | 10.0 | 2.0 | 7.0 |
| | 1.0 | 1.0 | 1.0 | 1.1 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |

* Upper figure in each column gives the phagocytic index (parcentage of leucocytes found to engulf meningococci).

† Lower figure in each column gives the opsonic index (average number of meningococci per phagocyte).

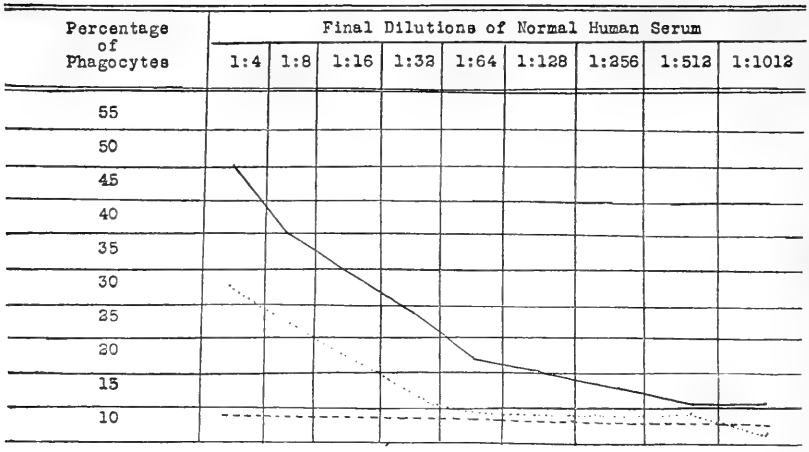


CHART 2

——— = results with an antimeningitis serum in final dilution of 1:400 with varying final dilutions of normal human serum.

..... = results with normal human serum alone.

- - - - - = results with antimeningitis serum alone diluted 1:300.

* The average phagocytic index without antiserum or normal serum was 12 per cent (spontaneous phagocytosis).

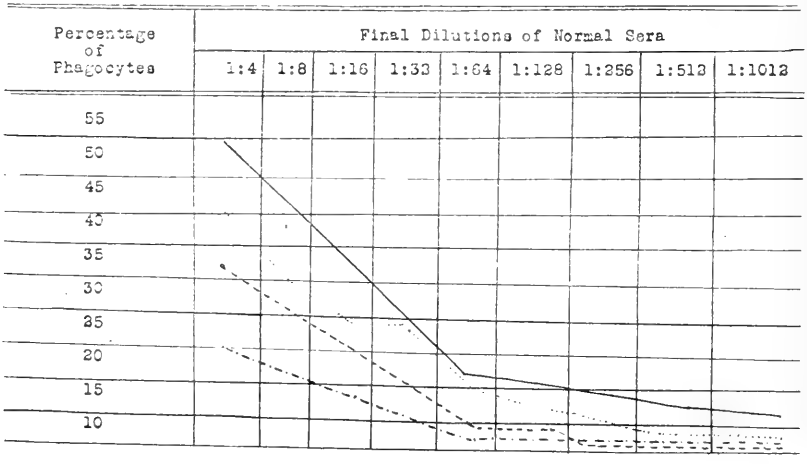


CHART 3

——— = results with varying dilutions of normal human serum with a final 1:400 dilution of antimeningitis serum.

..... = results with varying dilutions of normal guinea-pig serum with a final 1:400 dilution of antimeningitis serum.

- - - - - = results with varying dilutions of human serum alone.

-.-.-.- = results with varying dilutions of guinea-pig serum alone.

* The average phagocytic index without antiserum or normal serum was 13 per cent (spontaneous phagocytosis). The average phagocytic index of the 1:300 dilution of antiserum alone was 17 per cent.

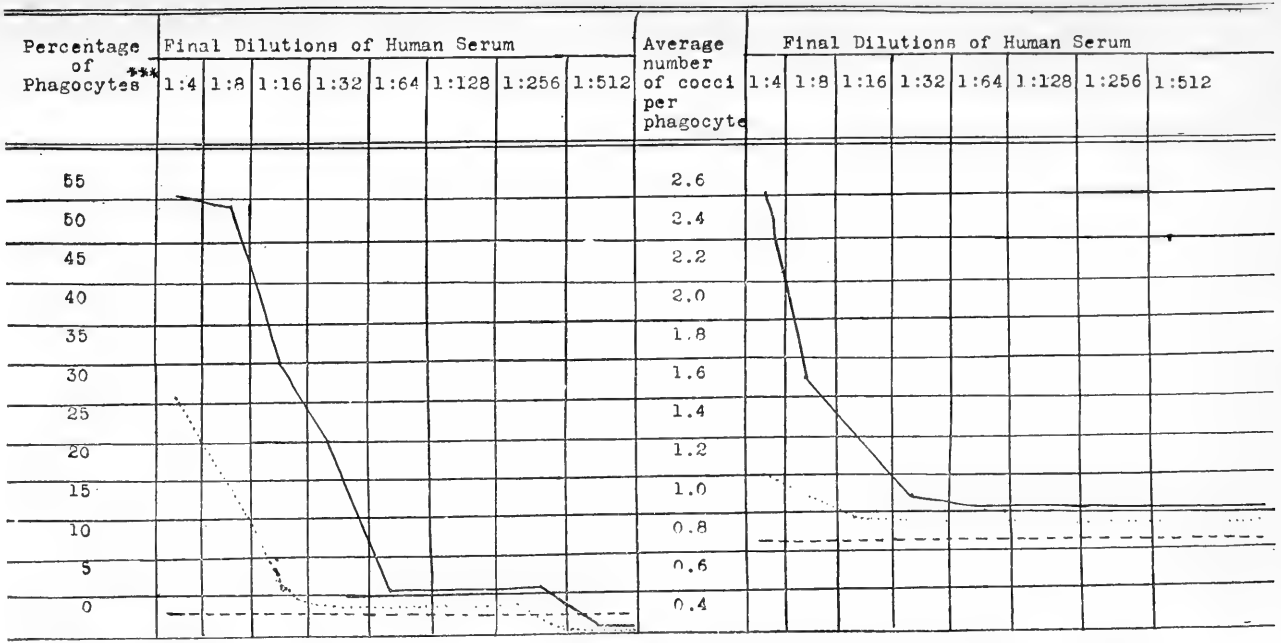


CHART 4

——— = results with varying dilutions of normal human serum with a final 1:400 dilution of antimeningitis serum.

..... = results with varying dilutions of normal human serum alone.

- - - - - - = results with 1:400 dilution of antimeningitis serum alone.

* Antimeningitis serum No. 3.

** Normal meningococci No. 1.

*** The average phagocytic index without antiserum or normal serum was 2 per cent (spontaneous phagocytosis); the average number of cocci per phagocyte was 0.4.

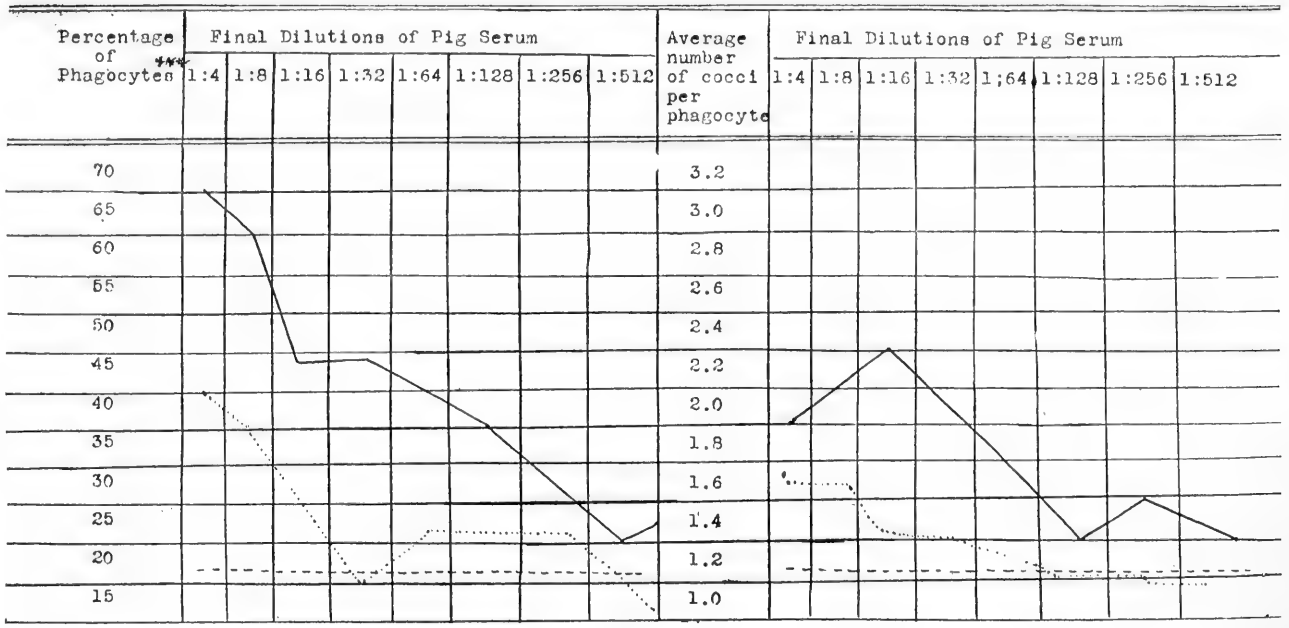


CHART 5

——— = results with varying dilutions of normal guinea-pig serum with a final 1:400 dilution of antimeningitis serum.

..... = results with varying dilutions of normal guinea-pig serum alone.

- - - - - = results with 1:400 dilution of antimeningitis serum alone.

* Antimeningitis serum No. 2.

** Normal meningococci No. 2.

*** The average phagocytic index without antiserum or normal serum was 25 per cent; the average number of cocci per phagocyte was 1.1.

TABLE 6

*Phagocytic and opsonic indices with varying dilutions of antimeningitis sera, different strains of normal meningococci and fresh normal human and guinea-pig sera in final dilution of 1:4*

| FINAL DILUTIONS OF ANTISERA | ANTISERUM 1, NORMAL COCCI 1 PLUS: | | ANTISERUM 2, NORMAL COCCI 1 PLUS: | | ANTISERUM 1, NORMAL COCCI 124 PLUS: | | ANTISERUM 3, NORMAL COCCI 124 PLUS: | | ANTISERUM 3, NORMAL COCCI 1 PLUS: | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Human serum | Pig serum | Human serum | Pig serum | Human serum | Pig serum | Human serum | Pig serum | Human serum | Pig serum |
| 1:200 | 76.0* | 58.0 | 86.0 | 48.0 | 40.0 | 50.0 | 66.0 | 40.0 | 48.0 | 24.0 |
| | 1.9† | 1.6 | 3.3 | 2.1 | 1.2 | 1.9 | 2.0 | 1.5 | 2.3 | 1.2 |
| 1:320 | 60.0 | 48.0 | 92.0 | 58.0 | 38.0 | 46.0 | 62.0 | 36.0 | 42.0 | 24.0 |
| | 1.6 | 1.7 | 3.8 | 1.8 | 1.4 | 1.5 | 1.6 | 1.5 | 2.3 | 1.1 |
| 1:400 | 62.0 | 54.0 | 66.0 | 48.0 | 36.0 | 46.0 | 44.0 | 48.0 | 42.0 | 16.0 |
| | 1.9 | 1.4 | 2.3 | 1.8 | 1.4 | 1.5 | 1.7 | 1.3 | 2.2 | 1.0 |
| 1:640 | 64.0 | 44.0 | 62.0 | 36.0 | 30.0 | 46.0 | 46.0 | 22.0 | 38.0 | 14.0 |
| | 1.8 | 1.5 | 2.3 | 1.7 | 1.1 | 1.5 | 1.7 | 1.2 | 1.4 | 1.0 |
| 1:800 | 54.0 | 44.0 | 70.0 | 44.0 | 32.0 | 48.0 | 56.0 | 22.0 | 40.0 | 10.0 |
| | 1.5 | 1.1 | 2.6 | 1.5 | 1.1 | 1.6 | 1.5 | 1.5 | 1.7 | 1.0 |
| 1:1280 | 48.0 | 36.0 | 68.0 | 44.0 | 30.0 | 38.0 | 72.0 | 39.0 | 32.0 | 10.0 |
| | 1.5 | 1.3 | 2.4 | 1.8 | 1.3 | 1.4 | 2.2 | 1.4 | 1.3 | 1.0 |
| 1:1600 | 42.0 | 40.0 | 72.0 | 38.0 | 24.0 | 42.0 | 76.0 | 28.0 | 24.0 | 8.0 |
| | 1.3 | 1.2 | 2.4 | 2.4 | 1.0 | 1.6 | 2.1 | 2.0 | 1.5 | 1.0 |
| 1:2560 | 48.0 | 26.0 | 52.0 | 32.0 | 26.0 | 34.0 | 68.0 | 18.0 | 34.0 | 6.0 |
| | 1.5 | 1.2 | 1.5 | 1.3 | 1.1 | 1.5 | 2.2 | 1.1 | 1.2 | 1.0 |
| Normal sera 1:4 alone | 40.0 | 30.0 | 64.0 | 34.0 | 27.0 | 38.0 | 49.0 | 23.0 | 34.0 | 8.0 |
| | 1.4 | 1.1 | 2.4 | 1.5 | 1.0 | 1.4 | 1.7 | 1.4 | 1.1 | 1.0 |
| No antisera; no normal sera..... | 14.0 | 13.0 | 37.0 | 22.0 | 12.0 | 7.0 | 8.0 | 12.0 | 1.0 | 6.0 |
| | 1.0 | 1.0 | 1.6 | 1.1 | 1.0 | 1.0 | 1.2 | 1.0 | 0.5 | 1.0 |

* Upper figure in each column gives the phagychtic index (percentage of leucocytes found to engulf meningococci).

† Lower figure in each column gives the opsonic index (average number of meningococci per phagocyte).

A study of the results of these experiments have shown:

1. In mixtures of equal parts of human leucocytes and emulsions of various strains of meningococci, from 2 to 14 per cent of leucocytes were found to engulf the diplococci (spontaneous phagocytosis); the average number of cocci (not diplococci) per phagocyte varied from 0.4 to 1.2. The degree of spontaneous phagocytosis varied considerably among the different strains of meningococci employed.

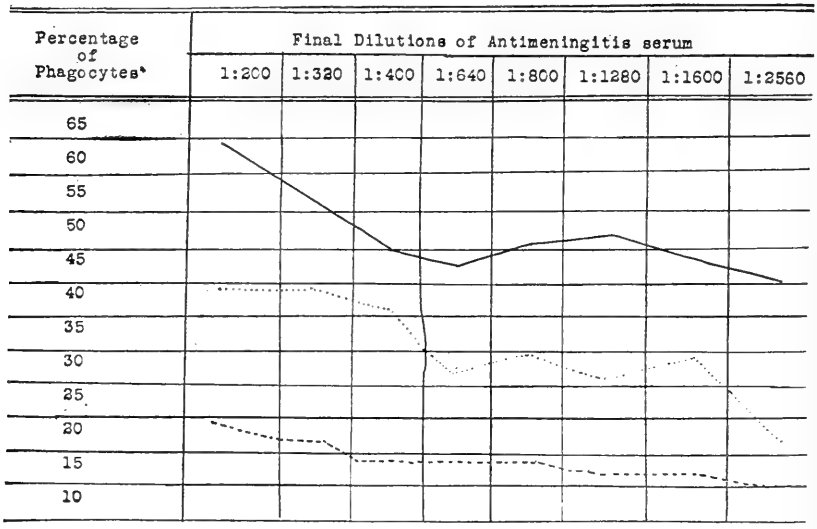


CHART 6

——— = results with diluted human serum and antiserum.

..... = results with diluted guinea-pig serum and antiserum.

- - - - - = results with antiserum alone.

* Average phagocytic index with diluted human serum alone was 43 per cent; average opsonic index 1.5.

Average phagocytic index with diluted guinea-pig serum alone was 27 per cent; average opsonic index 1.3.

Average phagocytic index without normal serum or antiserum was 14 per cent; average opsonic index 1.1.

2. As previously stated and shown in table 2 and chart 1, both human and guinea-pig serum in fresh and normal conditions showed unmistakable opsonic activity in final dilutions varying from 1:2 to 1:32 or 1:64. As a general rule human serum dis-

played greater activity than guinea-pig serum, as will be appreciated by comparing tables 2 and 3.

3. The opsonic activity of the different antimeningitis sera varied considerably and with at least two of them in final dilution of 1:400, the phagocytic and opsonic indices were generally lower than observed with normal human and guinea-pig sera alone in final dilutions of 1:3.

4. The addition of either fresh normal human or guinea-pig serum and particularly the former to antimeningitis serum always

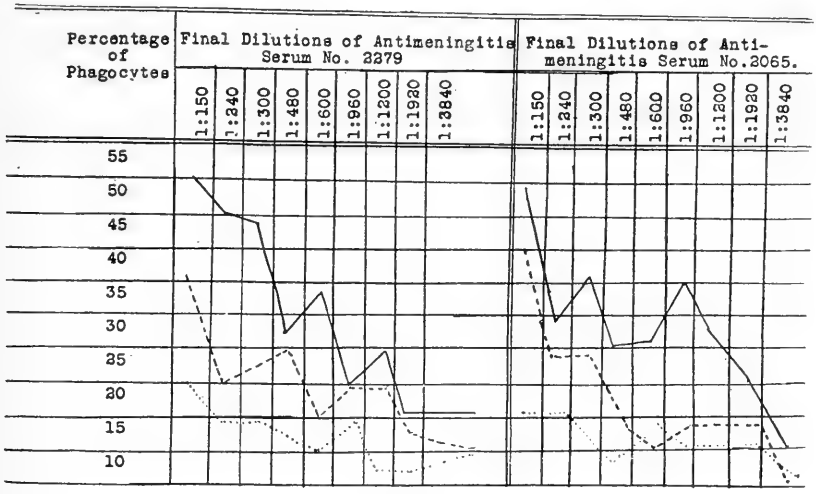


CHART 7

——— = results with fresh (about six hours after bleeding) sterile antimeningitis sera.

- - - - - = results with same sera after heating at 60°C. for thirty minutes.

..... = results with same sera after the addition of tricresol to 0.2 per cent and standing at room temperature for four days.

The average phagocytic index without antiserum (spontaneous phagocytosis) was 12 to 20 per cent.

yielded the higher phagocytic and opsonic indices with all strains of meningococci. The highest values were found when the final dilutions of normal human and guinea-pig serum were less than 1:32.

*c. The opsonic activity of sterile antimeningitis sera alone in* (1) *a fresh or active condition;* (2) *after heating or inactivation and* (3)

*after the addition of tricresol to 0.2 per cent and standing at room temperature for four days.* As previously stated and shown in chart 7, the opsonic activity of antimeningitis serum has been found most marked while the serum was fresh and active; after heating at 60°C. for thirty minutes the phagocytic activity is diminished to a considerable degree as is likewise true after the addition of tricresol and standing at room temperature for four days or longer.

## CONCLUSIONS

1. The phagocytic activity of antimeningitis serum was found most marked while the serum was fresh and active; after heating or after the addition of 0.2 per cent tricresol to a serum followed by standing at room temperature for four days or longer, the phagocytic activity was diminished to a considerable degree due, presumably, to the loss of labile opsonin or complement.

2. Fresh normal human and guinea-pig sera possess well defined amounts of opsonin for virulent meningococci in final dilutions as high as 1:16 or 1:32; as a general rule human serum has a higher opsonin content than guinea-pig serum. The sera of different persons and guinea-pigs were found to vary to a slight extent in opsonic activity.

3. The addition of fresh normal human or guinea-pig serum to various anti-meningitis sera as prepared and marketed for administration, was found to definitely and uniformly increase the opsonic activity for various strains of meningococci.

4. The cerebrospinal fluid in meningococcus meningitis is generally free of complement or contains but traces; normal cerebrospinal fluid is always free of this serum constituent as, likewise, of opsonin for meningococci (Davis).

5. As based upon accepting the opsonic activity of antimeningitis serum in vitro as an index of its curative power, it would appear justifiable to conclude that fresh normal human and guinea-pig sera alone may possess curative properties when brought into direct relation with meningococci and that the addition of either these normal sera and particularly fresh human serum to antimeningitis serum as secured in the market, increases opsonic activity.

6. It would appear worthy of clinical test to add 1 cc. of fresh sterile human or guinea-pig serum to each 9 cc. of antimeningitis serum, before intraspinal injection and particularly in the treatment of severe and serum-resistant cases of meningococcus meningitis, for the purpose of increasing the phagocytosis of the microörganisms.

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# THE INFLUENCE OF ACTIVE NORMAL SERUM (COMPLEMENT) UPON MENINGOCOCCI[1]

## II. THE BACTERICIDAL AND PROTECTIVE VALUE OF FRESH NORMAL SERUM ALONE AND IN COMBINATION WITH ANTIMENINGITIS SERUM FOR MENINGOCOCCI

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In addition to specific opsonins and toxin neutralizing antibodies, potent antimeningococcus sera are generally regarded as possessing a distinct meningococcidal activity; the latter is ascribed to the presence of specific bacteriolysin requiring the presence of complement for lytic activity although this does not explain the total bactericidal activity inasmuch as the early experiments of Flexner (1) demonstrated that heated serum possesses bactericidal activity and advanced the hypothesis that it is only necessary that the fresh or heated serum should injure the cocci in order that their intracellular enzymes should be rendered active and destructive for the microörganisms.

The defibrinated bloods and sera of normal persons have been found by Davis (2), Flexner, (1), McKenzie and Martin (3) and others to possess meningococcidal properties; this activity was found most marked with fresh active sera and diminished but not totally removed by heating at 60°C. for thirty minutes. Inasmuch as the antimeningococcus sera ordinarily administered are free of complement and the cerebrospinal fluid in meningococcus meningitis apt to be free or contain but traces of this constituent of serum, we have sought to determine the bactericidal activity of the fresh serum of persons and guinea-pigs alone and in conjunction with anti-meningococcus serum for virulent men-

[1] Aided by a grant from the Pediatric Society of Philadelphia.

ingococci, in an experimental study of the probable value of complemental bacteriolysis in resistance to meningococci.

In our first communication concerning the influence of active normal serum (complement) upon meningococci (4) we have shown that the opsonic activity of fresh active antimeningitis serum is greater than the opsonic activity of the same serum after heating or the addition of tricresol, followed by exposure to room temperature for several days; also that the addition of active normal serum to commercial antimeningitis serum increases opsonic activity. It would appear, therefore, that the opsonic activity of antimeningitis serum is maximal when the serum is fresh and active, that is, contains the labile opsonin or complement, and that this constituent may be restored and the commercial immune serum reactivated to a certain degree, by the addition of fresh human or guinea-pig serum.

In this investigation we have followed three main lines of study:

1. To determine the bactericidal activity of normal human and guinea-pig sera and various anti-meningococcus sera alone, and in combination, for virulent meningococci, by in vitro, plating and pipet methods.

2. A study of the agglutination of meningococci by normal and immune sera in relation to the bactericidal activity of these sera.

3. A study of the mouse test of Hitchens and Robinson (5) for determining the protective value of antimeningitis serum and the influence of normal sera alone and in conjunction with antimeningitis serum upon virulent meningococci, as determined with this technic.

The results of a large number of experiments are briefly summarized in this communication; with plating methods the bactericidal activity of various antimeningococcus sera alone as well as the sera of normal persons and guinea-pigs alone and in combination with antisera, has been found surprisingly low although these sera and particularly the whole blood of certain persons and guinea-pigs, have been found definitely bactericidal for meningococci with a delicate technic. The protection test of Hitchens and Robinson has, we believe, a definite and practical value al-

though not sufficiently delicate for the purposes of our experiments, namely, to determine the influence of complemental bacteriolysis alone as a factor in protection against virulent meningococci. Inasmuch as any animal protective test calls into consideration the influence of immunity principles in the body fluids of the experimental animal and thereby masking the possible influence of the addition of fresh normal serum (complement) upon the bactericidal activity of the immune serum, we have given most attention to test tube experiments.

### THE BACTERICIDAL ACTIVITY OF FRESH NORMAL SERUM ALONE AND IN COMBINATION WITH ANTIMENINGITIS SERUM

*a. The bactericidal activity of anti-meningococcus and normal sera alone and in combination, as determined by plating methods.* All experiments were conducted with strain 124 of normal meningococci[2] kindly furnished by Dr. George Robinson. Antimeningococcus sera were obtained from various laboratories; normal human sera were obtained from ourselves and others of the laboratory staff by vein puncture, and from normal adult guinea-pigs by heart puncture.

Bactericidal tests in vitro, and particularly with plating methods, are generally unsatisfactory; strict attention to the reaction of the culture medium and numerous preliminary tests to determine the proper dose of culture to employ in order to avoid too few or too many colonies in a plate with frequent repetitions of the main experiments, were necessary in this study. The protocols of several experiments are given in tables 1, 2, 3 and 4 and the results may be summarized as follows:

1. As shown in table 1, active or fresh antimeningitis sera are more bactericidal than the same sera after inactivation by heating at 60°C. for thirty minutes. The addition of 0.3 per cent tricresol to the sera increased bactericidal activity in dilutions up to about 1:20, but this preservative did not appear to appreciably injure or reduce the bactericidal activity of the serum itself as tested within one week after the addition of tricresol, although it reduces to some degree the opsonic activity of the serum.

[2] This strain was isolated on June 2, 1917, on the hospital ship *Solace*.

2. Complement free antimeningitis sera possess bactericidal activity as previously shown by Flexner and it is highly probable that complemental bacteriolysis exerts but a minor rôle in the total bactericidal activity of immune serum.

TABLE 1

*The influence of heat and tricresol upon the bactericidal activity of antimeningococcus serum*

| ANTIMENINGITIS SERUM (NO. 2279) | FINAL DILUTIONS OF ANTISERUM | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1:2 | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1280 | 1:2560 |
| Fresh active..... | 1500* | 900 | 400 | 200 | 180 | 30 | Sterile | Sterile | Sterile | Few |
| Five days after addition of 0.25 per cent tricresol. | Sterile | Sterile | Sterile | 20 | 40 | 240 | 20 | Sterile | Sterile | Few |
| Fresh serum after heating at 60°C. for one-half hour | 1400 | 1600 | 1800 | 300 | 180 | 20 | 30 | 10 | Sterile | Few |
| Heated serum + active human serum 1:5..... | 1800 | 1600 | 1200 | 1900 | 1400 | 1500 | 1800 | 1400 | 1200 | 1800 |

* Colonies per plate.

In these experiments the culture (no. 124) was used in amounts of 0.5 cc. of a 1:500,000 emulsion of a twenty-four hour serum agar culture; 0.5 cc. of this emulsion mixed wih 0.5 cc. sheep serum broth and plated in amounts of 0.5 cc. after two hours incubation, showed 600 colonies per plate.

Dilutions of serum in amounts of 0.5 cc. of culture were incubated at 37°C. (water bath) for two hours and plates prepared with 0.5 cc. from each tube in 10 cc. of sheep serum dextrose agar.

0.5 cc. of human serum (1:5) plus 0.5 cc. emulsion of cocci and plated in amounts of 0.5 cc. after two hours incubation, showed 900 colonies per plate.

3. The bactericidal activity of practically all the antisera tested was low according to the results observed with the technic employed although some bactericidal activity was generally apparent as compared with the controls; not infrequently the higher dilutions of serum were more bactericidal than the lower, these results being similar to those published by Jochmann (6).

4. As shown in table 4 active human and guinea-pig sera gen-

erally possess some bactericidal activity for meningococci; the bactericidal activity of these sera vary in different persons and animals and, as a general rule, human sera are more bactericidal than pig sera.

5. In practically all experiments however, the addition of equal parts of 1:5 or 1:10 dilutions of fresh active human or guinea-pig sera to varying dilutions of different anti-meningococcus sera, was without appreciable increase of bactericidal activity; indeed

TABLE 2

*Bactericidal action of anti-meningococcus serum alone and in combination with equal parts of a 1:5 dilution of active guinea-pig serum*

| SUBSTANCES | FINAL DILUTIONS OF ANTISERUM | | | | | | | | | | PIG SERUM AND CULTURE | CULTURE ALONE |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1:2 | 1:4 | 1:8 | 1:12 | 1:16 | 1:24 | 1:32 | 1:48 | 1:64 | 1:96 | | |
| Antiserum (commercial) alone.... | Sterile | Sterile | 540* | 2160 | Unc. | Unc. | Unc. | Unc. | Unc. | Unc. | Unc. | Unc. |
| Antiserum + pig serum.... | Unc. | Unc. | 6480 | 9720 | 4300 | 8000 | Unc. | Unc. | Unc. | Unc. | | |

* Colonies per plate; Unc. = too many colonies for counting.

In this experiment a series of dilutions of the immune serum was prepared in sterile test tubes with sheep serum broth, in amounts of 1 cc.; each tube was seeded with 0.1 cc. of a twenty-four hour serum dextrose broth culture of meningococci and incubated in a water bath at 37°C. for two hours, shaken and 0.5 cc. from each tube plated in 10 cc. of sheep serum dextrose agar. In the second series a 1:5 dilution of fresh sterile pig serum was used instead of sheep serum broth in making the dilutions, thereby rendering the final dilutions of immune serum the same in both series. Controls on the sterility of the immune and normal sera, culture, etc., were included. The plates were counted after forty-eight hours incubation.

the colonies of meningococci were generally more numerous than with the antiserum alone, suggesting that the addition of normal serum enriched the culture medium and thereby favored the more rapid multiplication of the surviving cocci.

TABLE 3

*Summary of bactericidal tests with antisera alone and in combination with normal human and guinea-pig serum*

| EXPERIMENT | SUBSTANCES | FINAL DILUTIONS OF ANTISERUM | | | | | | NORMAL SERUM ALONE | CULTURE ALONE | NORMAL HORSE SERUM ALONE |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | | | |
| 1 | Antiserum alone | 2100 | Unc. | Unc. | Unc. | Unc. | Unc. | Unc. | Unc. | Unc. |
| | Antiserum + human serum | 5700 | Unc. | Unc. | Unc. | Unc. | Unc. | | | |
| 2 | Antiserum (commercial) alone | Sterile | Sterile | Sterile | Few | 40 | Contam. | 360 | 2000 | 4500 |
| | Antiserum + human serum | Sterile | 2560 | 2200 | 1420 | 1080 | 340 | | | |
| 3 | Antiserum alone | 5400 | 6840 | 10800 | Unc. | Unc. | Unc. | 6000 | 10800 | Unc. |
| | Antiserum + human serum | Unc. | Unc. | Unc. | Unc. | Unc. | Unc. | | | |
| 4 | Antiserum alone | 300 | 300 | 300 | 480 | 540 | 240 | 7000 | 6400 | 8000 |
| | Antiserum + human serum | 9180 | 9720 | 7020 | 9180 | 9000 | 8000 | | | |
| 5 | Antiserum alone | 200 | 900 | 1000 | 1200 | 1380 | 1200 | 3200 | 7000 | 0 |
| | Antiserum + human serum | 300 | 900 | 1400 | 1500 | 1200 | 2000 | | | |
| 6 | Antiserum alone | 200 | 900 | 1000 | 1200 | 1380 | 1200 | 4800 | 7000 | 0 |
| | Antiserum + guinea-pig serum | 400 | 2500 | 2100 | 2200 | 2700 | 2200 | | | |

Experiment 1. Culture used in 0.5 cc. of 1:1000 serum broth culture; human serum 0.5 cc. of 1:10; antimeningitis serum 1.

Experiment 2. Culture used in 0.5 cc. of 1:2500 serum broth culture; human serum 0.5 cc. of 1:5; antimeningitis serum 2.

Experiment 3. Culture used in 0.5 cc. of 1:1000 serum broth culture; human serum 0.5 cc. of 1:5; antimeningitis serum 3.

Experiment 4. Culture used in 0.5 cc. of 1:1500 serum broth culture, human serum 0.5 cc. of 1:5; antimeningitis serum 4.

Experiment 5. Culture used in 0.5 cc. of 1:500,000 suspension of serum agar growth human serum 0.5 cc. of 1:5; antimeningitis serum 2.

Experiment 6. Culture used in 0.5 cc. of 1:500,000 suspension of serum agar growth; pig serum 0.5 cc. of 1:5; antimeningitis serum 2.

In these experiments the antisera were previously heated in a water bath at 56°C. for thirty minutes and diluted with equal parts of sheep serum broth of with 1:5 or 1:10 dilutions of fresh sterile human serum. The tubes were seeded with 0.5 cc. of varying dilutions of twenty-four hour serum dexrose broth cultures of meningococci; the final dilutions of immune serum were always the same in each series with immune serum alone or in combination with the normal human serum.

All tubes were incubated at 37°C. for three hours, shaken and 0.5 cc. from each plated with 10 cc. of sheep serum dextrose agar; counts were made after forty-eight hours incubation.

Numerous controls in culture and sera were included in all experiments.

*b. The bactericidal activity of anti-meningococcus and normal sera alone and in combination, as determined with a pipet method.* In this technic which was modified after that of Wright (7) by Dr. George D. Heist and Prof. Lacey, the number of microörganisms exposed to the action of the serum for many hours was reduced to a minimum thereby rendering the test for bactericidal activity quite delicate.

From five to eight dilutions of a culture of normal meningococci (strain 124) prepared with sheep serum broth in a series of tubules, were employed with a many stemmed pipet; the various dilutions of culture were taken up in the respective sterile pipets and then expelled, leaving meningococci clinging to the interior

TABLE 4

*Bactericidal activity of active human and guinea-pig sera for meningococci**

| SERA | FINAL DILUTIONS OF SERA | | | | | |
|---|---|---|---|---|---|---|
| | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 |
| Dr. M. | 1200 | 1200 | 1400 | 1600 | 1800 | 3000 |
| Dr. K. | 2700 | 900 | 1000 | 3700 | 3300 | 5000 |
| Pig 4 | 2700 | 7500 | 4500 | 3700 | 2700 | 5500 |
| Pig 5 | 7500 | 9000 | 9000 | 8000 | 5000 | 8000 |

* In these experiments 1 cc. of each dilution of serum was mixed with 1 cc. of culture and incubated for two hours on a water bath when plates were prepared.

The culture alone showed 1200 colonies per plate before incubation for two hours in the water bath and 7000 per plate after this period of incubation.

of the pipets. Each pipet was now filled with serum, sealed and incubated for twenty-four hours when smears were prepared, stained and examined for meningococci. Numerous controls of each dilution of culture were included in which sheep serum water broth was substituted for serum. In these pipets in which all meningococci were killed, microörganisms were not found in the smears, whereas in those in which killing was incomplete or entirely absent, few or many cocci were to be found in the stained smears. After practice in the manipulation of the pipets, the results become fairly consistent and the method is well adapted as a ready and simple test for measuring the bactericidal activity of whole blood inasmuch as but a drop or two is required and this amount is easily obtained from a finger.

With this technic fresh normal human and guinea-pig blood were found distinctly meningococcidal, as shown in the protocols given in table 5; the blood of different persons and guinea-pigs were found to vary in bactericidal activity. As a rule human

TABLE 5

*The bactericidal activity of normal human and guinea-pig blood for meningococci measured with a pipet method*

| FRESH WHOLE BLOOD | DILUTIONS OF CULTURE* | | | | |
|---|---|---|---|---|---|
| | Undiluted | 1:5 | 1:25 | 1:125 | 1:625 |
| Human 1 | +† | − | − | − | − |
| Human 2 | + | + | + | + | + |
| Human 3 | − | − | − | − | − |
| Human 4 | − | − | − | − | − |
| Human 5 | − | − | − | − | − |
| Human 6 | + | + | + | − | − |
| Human 7 | − | − | − | − | − |
| Guinea-pig 1 | + | + | + | + | + |
| Guinea-pig 2 | + | + | − | − | − |
| Controls | + | + | + | + | + |

* A twenty-four hour serum broth culture of normal meningococci (strain 124).

† + = growth; − = no growth (sterile).

TABLE 6

*Whole blood more bactericidal for meningococci than serum*

| SOURCE | RESULTS WITH BLOOD AND DILUTIONS OF CULTURE* | | | | | RESULTS WITH SERUM AND DILUTIONS OF CULTURE | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Undiluted | 1:5 | 1:25 | 1:125 | 1625 | Undiluted | 1:5 | 1:25 | 1:125 | 1:625 |
| Human 1 | +† | − | − | − | − | + | + | − | − | − |
| Human 3 | − | − | − | − | − | + | + | − | − | − |
| Human 6 | + | + | + | − | − | + | + | + | + | − |

* A twenty-four hour serum broth culture of normal meningococci (strain 124).

† + = growth; − = no growth (sterile).

blood was found to possess a similar or slightly higher bactericidal activity than guinea-pig blood.

Whole blood of persons was found somewhat more bactericidal than the corresponding fresh active sera, as shown in the protocols given in table 6.

Fresh active human and guinea-pig sera were more bactericidal than after heating at 56° C. for thirty to sixty minutes, although the latter are bactericidal to a degree, and showign thereby that the total bactericidal activity of normal serum is not dependent entirely upon complemental bacteriolysis.

TABLE 7

*The bactericidal activity of antimengitis sera alone and in combination with normal serum*

| COMPLEMENT FREE ANTIMENINGITIS SERA | DILUTIONS OF CULTURE* | | | | | |
|---|---|---|---|---|---|---|
| | Undiluted | 1:5 | 1:25 | 1:125 | 1:250 | 1:500 |
| Antimeningococcus serum (A) undiluted | + | − | − | − | − | − |
| Antimeningococcus serum (A) 1:10..... | + | ± | − | − | − | − |
| Antimeningococcus serum (A) 1:100.... | + | + | − | − | − | − |
| Antimeningococcus serum (A) 1:100 + human serum complement‡..... | − | − | − | − | − | − |
| Antimeningitis serum (B) undiluted..... | + | − | − | − | − | − |
| Antimeningitis serum (B) 1:10..... | + | + | − | − | − | − |
| Antimeningitis serum (B) 1:100..... | + | + | − | − | − | − |
| Antimeningitis serum (B) 1:100 + guinea-pig complement..... | + | + | + | + | + | + |
| Controls..... | + | + | + | + | + | + |
| Plates†..... | Unc. | 2700 | 1620 | 960 | 900 | 120 |

* Culture prepared by washing off twenty-four hour serum dextrose agar slant growths with 2 cc. of sheep serum broth, emulsifying and diluting with sheep serum water.

† Plates were prepared by drawing culture into the capillary tubes employed, expelling the culture, and then washing the cocci adhering to the inner wall into petri dishes with twelve changes of sheep serum broth; the number of colonies gives an idea of the number of cocci adhering to the inner wall of a pipet and subject to the bactericidal activity of the various sera.

‡ Four parts of antiserum 1:100 plus one part of undiluted normal serum.

With this technic the bactericidal activity of antimeningitis sera was more pronounced; in order to measure this activity, it was found necessary to use a larger number of meningococci than could be obtained in a twenty-four hour serum dextrose broth culture. The results of several experiments with antisera are shown in table 7; also an idea of the number of cocci exposed to the bactericidal substances of the sera.

As shown in table 7 dilutions of antimeningitis sera up to 1:100

were found bactericidal and this activity was sometimes, but not always, increased by the addition of fresh normal serum and particularly human serum.

## AGGLUTINATION OF MENINGOCOCCI IN RELATION TO THE BACTERICIDAL ACTIVITY OF NORMAL AND IMMUNE SERA

Inasmuch as agglutinins are regarded by many as aiding bacteriolysis and phagocytosis, we have studied the agglutinin content of horse antimeningitis and normal human and guinea-pig sera, for normal meningococci; furthermore laboratories engaged

TABLE 8

*Agglutination of meningococci* by normal human and guinea pig sera*

| | TEMPERATURE OF INCUBATOR† | FINAL DILUTIONS | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1:2 | 1:3 | 1:4 | 1:5 | 1:6 | 1:8 | 1:10 |
| | *deg. C.* | | | | | | | |
| Human serum | 55 | + | ± | − | − | − | − | − |
| Guinea-pig serum | 55 | + | + | ± | − | − | − | − |

* Normal meningococci strain 1.

† For sixteen to eighteen hours.

in the production of antimeningitis serum commonly employ the agglutination test as a measure of antibody response and we have sought to determine as a by study the relation between the agglutinin, opsonin and bactericidan content of the immune and normal sera.

The agglutinin content of all the various immune and many normal human and guinea-pig sera employed in this study, was determined with a macroscopic technic;[3] likewise after the addition of fresh normal human and guinea-pig sera to the immune sera to determine if these increased the agglutinating activity of the latter.

Normal human and guinea-pig sera were found to contain traces of agglutinin for normal meningococci as shown in table 8 with one serum of each.

[3] We are indebted to Dr. Shigeki Sekiguchi for assistance in conducting the agglutination tests.

The addition of fresh normal human serum to antimeningitis serum in amounts of 0.5 cc. of 1:5 dilution appeared, but not uniformly, slightly to influence agglutination as shown in table 9.

The addition of tricresol to fresh antimeningitis sera to the point of 0.25 per cent, followed by exposure of the sera to room temperature for a week, was usually followed by slight decrease in agglutinating activity, which may be ascribed to the loss of labile substances or to slight decrease in agglutinin or both; heating antimeningitis sera at 60°C. for thirty minutes was

TABLE 9

*The influence of normal serum upon the agglutination of meningococci by antimeningococcus serum**

| SERA | TEMPERATURE† | FINAL DILUTIONS 1: | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 60 | 80 | 100 | 120 | 160 | 200 | 240 | 320 | 400 | 480 | 640 |
| | *deg. C.* | | | | | | | | | | | |
| Antiserum alone | 37 | + | + | + | + | ± | ± | − | − | − | − | − |
| | 55 | + | + | + | + | + | + | + | ± | − | − | − |
| Antiserum+human serum 0.5 cc. (1:5) | 37 | + | + | + | + | + | + | + | − | − | − | − |
| | 55 | + | + | + | + | + | + | + | + | + | − | − |
| Autiserum + pig serum 0.5 cc. (1:5) | 37 | + | + | + | + | + | + | + | − | − | − | − |
| | 55 | + | + | + | + | + | + | + | ± | − | − | − |

* Strain of normal meningococci 124 employed.

† Incubated for sixteen to eighteen hours.

found to markedly decrease the agglutinating activity and this was not restored by the addition of normal serum. The results observed with a few of the sera tested are shown in table 10.

Incubation of the mixtures of serum and culture in the macroscopic tests at a temperature of 55°C. for at least sixteen hours was found to produce the most marked agglutination and usually higher than observed when the mixtures were incubated at 37°C. for the same period of time (the latter tests being conducted under sterile conditions to avoid the growth of contaminating bacteria which mask the reactions.

Despite a large amount of work with numerous repetitions of experiments, it is a difficult matter to draw deductions upon the

TABLE 10

*The influence of tricresol and heat upon the agglutinating activity of antimeningococcus sera**

| SERUM | FINAL DILUTIONS | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1280 |
| 2279; fresh and active | + | + | + | + | + | + | + | − |
| 2279; after tricresol | + | + | + | + | + | + | − | − |
| 2279; after heating† | + | + | ± | − | − | − | − | − |
| 2065; fresh and active | + | + | + | + | + | + | ± | − |
| 2065; after tricresol | + | + | + | + | + | + | − | − |
| 2065; after heating | + | + | ± | − | − | − | − | − |
| 2323; fresh and active | + | + | + | + | + | + | − | − |
| 2323; after heating | + | + | − | − | − | − | − | − |

* All tests were conducted with strain of normal meningococci 124 and at 55°C. for twenty hours.

† Heating at 60°C. for thirty minutes.

TABLE 11

*A comparison of the agglutinin, opsonin and bactericidan content of various antimeningitis and normal sera**

| SERA | AGGLUTINATION TITER | PHAGOCYTIC INDEX WITH DILUTED SERA | RESULTS OF BACTERICIDAL TESTS | | | | | | | CULTURE CONTROLS IN BACTERICIDAL TESTS |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1:4 | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | |
| Immune 2279, A... | 1:320 | (1:400)20 | 1200 | 240 | 140 | 180 | 180 | 240 | 120 | 600 |
| Immune 2065, B... | 1:320 | (1:400)15 | 8000 | 6000 | 4000 | 5400 | 5400 | 6000 | 6000 | 8000 |
| Immune Rl, C.... | 1:640 | (1:400)30 | 200 | 900 | 1080 | 1200 | 1300 | 1200 | 1500 | 7000 |
| Human, A..... | 1:3 | (1:4)20 | 900 | 1200 | 3700 | 3200 | 5400 | 8000 | 8000 | 7000 |
| Human, B..... | 1:4 | (1:4)227 | 1200 | 1400 | 1680 | 1800 | 3000 | 8000 | 8000 | 8000 |
| Guinea-pig, A..... | 1:3 | (1:3)14 | 7500 | 4300 | 3700 | 2600 | 6000 | 8000 | 8000 | 7000 |
| Guinea-pig, B..... | 1:3 | (1:3)16 | 9700 | 9000 | 8600 | 4800 | 7500 | 8500 | 8000 | 7000 |

* The antisera were tested about one week after bleeding and kept at a low temperature without the addition of tricresol. The normal sera were fresh and active.

relation between the agglutinins and opsonins and between the agglutinins and bactericidans of both immune and normal sera, owing largely to the unavoidable variation in the results of opsonic and more particularly, of bactericidal tests. A general review (see table 1) however, appears to warrant the following conclusions:

1. The opsonin content of normal serum bears no direct relation to the content of normal agglutinin; normal human and guinea-pig sera in a fresh active state possess well defined opsonic activity for meningococci and but very small amounts of agglutinin.

2. While normal human and pig sera are actively phagocytic, they are feebly bactericidal and, as stated, possess feeble agglutinating activities.

3. With the immune antibodies contained in various antimeningitis sera, we have found quite uniformly that those sera containing most agglutinin were also most likely to show the highest phagocytic and bactericidal activity. While agglutinins are not destructive it would appear that their production is a fair index of the amounts of the antimeningococcus opsonin and bactericidan present.

THE PROTECTIVE VALUE OF ACTIVE NORMAL HUMAN AND GUINEA-PIG SERA (COMPLEMENT) ALONE AND IN COMBINATION WITH ANTIMENINGITIS SERA AS STUDIED WITH THE PROTECTION TEST OF HITCHENS AND ROBINSON

*a. The protection test of Hitchens and Robinson.* On account of the irregular pathogenicity of the meningococcus, the animal protection test for measuring the curative value of antimeningitis serum has not been used extensively. Flexner (1) found young guinea-pigs of 125 grams to be the most susceptible animals and Amoss and Wollstein (8) consider a serum suitable for therapeutic use when it protects guinea-pigs of 90 to 100 grams weight against one fatal dose of living meningococci. Elser and Huntoon (9) and von Lingelsheim and Leuchs (10) found white mice most susceptible and the latter employed mice for protection tests.

More recently Hitchens and Robinson (5) have described a mouse test employing eight-hour meningococcus culture in sterile fresh guinea-pigs serum, diluted to four times its volume and injected intraperitoneally, which yielded them regular results in virulence tests. In their protection tests, the antiserum is in-

jected intraperitoneally in 0.5 cc. quantities two hours before the injection of varying amounts of culture. They have found this test specific and to parallel the extent of immunization more nearly than agglutination and complement fixation tests.

We have employed this technic with eight hour growth of meningococci on slants of serum-dextrose agar removed and diluted with fresh guinea-pig serum diluted to four times its volume with 0.85 per cent salt solution, in virulence and protection tests

TABLE 12

*Results of virulence tests at varying periods with meningococcus strain 124 suspended in 1:4 solution of active guinea-pig serum**

| DOSES | SEPTEMBER 20, 1917 | | OCTOBER 5, 1917 | | OCTOBER 9, 1917 | | OCTOBER 26, 1917 | | NOVEMBER 7, 1917 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Weight | Results | Weight | Results | Weight | Results | Weight | Results | Weight | Results |
| *cc.* | *gms.* | | *gms.* | | *gms.* | | *gms.* | | *gms.* | |
| 0.01 | 13 | D.* 3 days | 15 | D. 14 hrs. | 15 | S.† | 15 | D. 15 hrs. | 18 | D. 24 hrs. |
| 0.005 | 13 | D. 16 hrs. | 13 | D. 24 hrs. | 15 | S. | 14 | D. 14 hrs. | 16 | D. 14 hrs. |
| 0.0025 | 13 | D. 20 hrs. | 13 | S. | 13 | D. 13 hrs. | 12 | S. | 15 | S. 2 days |
| 0.00125 | 12 | D. 17 hrs. | 13 | D. 15 hrs. | 12 | S. | 12 | D. 15 hrs. | 20 | S. |
| 0.0006 | 13 | D. 24 hrs. | 12 | S. | 12 | D. 23 hrs. | 16 | S. | 24 | S. |
| 0.0002 | 12 | S.‡ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

* These tests were conducted with strain No. 124 transplanted every two days on serum dextrose agar with gradual loss of virulence.

† D. = died.

‡ S. = survived four days or longer.

with various antimeningitis sera, and our experiences may be summarized as follows:

1. White mice are quite susceptible to virulent meningococci and succumb with a meningococcus bacteriemia, the cocci being regularly found in the blood of the heart.

2. Virulence tests conducted with varying amounts of eight hour serum dextrose agar cultures suspended in and diluted 1:4 with active or heated guinea-pig serum or sheep serum broth and injected intraperitoneally into mice weighing from 10 to 25 grams without respect to the weight of the animals, frequently yielded irregular results; for this reason duplicate tests are ad-

visable for the determination of the minimal lethal dose and mice of about the same weight should be employed.

3. A particular source of error and variation in the results of virulence tests conducted on different days with the same strain is attributed to the variation in numbers of cocci injected, owing to variations in the amount of growth on different slants of serum dextrose agar. This error may be largely obviated by using twelve or more slants in preparing the suspension of cocci.

4. Our attempts to remove this source of error by securing more

TABLE 13

*Results of virulence tests in mice with emulsions of meningococcus 124 in 1:4 active and heated guinea-pig serum*

| EMULSION IN ACTIVE PIG SERUM | | | EMULSION IN HEATED PIG SERUM | | |
|---|---|---|---|---|---|
| Weight | Dose | Results | Weight | Dose | Results |
| *grams* | *cc.* | | *grams* | *cc.* | |
| 15 | 0.5 | Died in 17 hours | 15 | 0.5 | Died in 17 hours |
| 15 | 0.25 | Died in 17 hours | 14 | 0.25 | Died in 17 hours |
| 14 | 0.12 | Died in 17 hours | 14 | 0.12 | Died in 17 hours |
| 14 | 0.06 | Died in 17 hours | 14 | 0.06 | Died in 17 hours |
| 14 | 0.03 | Died in 17 hours | 14 | 0.03 | Died in 17 hours |
| 13 | 0.015 | Died in 17 hours | 14 | 0.015 | Died in 17 hours |
| 15 | 0.01 | Died in 17 hours | 13 | 0.01 | Died in 17 hours |
| 12 | 0.005 | Died in 17 hours | 12 | 0.005 | Died in 18 hours |
| 12 | 0.0025 | Died in 17 hours | 11 | 0.0025 | Died in 18 hours |
| 10 | 0.00125 | Survived 6 days | 11 | 0.00125 | Died in 4 days |
| 10 | 0.0006 | Survived 6 days | 11 | 0.0006 | Died in 5 days |

uniform growths in fluid culture media have so far proven unsuccessful.

5. Highly virulent strains of meningococci yielded more regular results than strains which had undergone some loss in virulence by reason of prolonged cultivation without animal passage (table 12); the protective tests were more regular in their results when highly virulent strains were employed.

6. For preparing the suspensions and dilutions of cocci it was not found necessary to use active guinea-pig serum; sheep serum broth was found satisfactory for this purpose. Comparative tests with heated and unheated (fresh) guinea-pig serum diluted

1:4 showed occasionally different results, the minimal lethal dose being less with the fresh active serum (table 13) and indicating the probable bactericidal effect of the active serum.

7. Our experiments have not been sufficiently numerous to warrant an opinion upon the relation between protection test in mice and such reactions in vitro as agglutination, bactericidal and opsonic tests for measuring the potency of antimeningitis serum, but we have tested different antimeningitis sera with the general result that those possessing the highest protective value also contained most agglutinin and opsonin. As previously stated the results of bactericidal tests in vitro were too irregular to permit comparison.

8. Even with a highly virulent strain or strains of cocci the results of protective tests on different days with the same antimeningitis serum varied to some extent. This was expected because animal tests cannot yield strictly regular and mathematically exact results, owing to variations in susceptibility in addition to other factors. On the basis of our experience with the protective test of Hitchens and Robinson we believe the following conditions important:

a. A highly virulent strain or strains of normal or para meningococci or both should be employed—the virulence being maintained by passage through mice.

b. A uniform suspension of cocci should be secured by washing off eight hour growths from twelve or more slants of serum dextrose agar with sheep serum broth and mixing thoroughly. For measuring the polyvalency of a serum twelve or more different cultures of meningococci and parameningococci may be mixed and employed.

c. Mice of approximately the same weight should be employed.

d. In conducting a test for the protective value of a serum, one or two sets of mice should be injected with the suspension of cocci alone, the doses being arranged according to the approximate virulence on the basis of former tests in order to determine the minimal lethal dose of the particular suspension being used in the protective tests. Owing to the likelihood of variation in results with different suspensions, it is not advisable to determine

the minimal lethal dose with one suspension and conduct the protective tests with a second. In other words the minimal lethal dose of each suspension used in the protective test should be determined at the same time and the results expressed according to the protection afforded by 1 cc. of serum over a period of 48 hours against multiples of the minimal lethal dose of culture.

TABLE 14

*The protection activity of antimeningitis serum alone and in conjunction with normal guinea-pig serum for virulent meningococci*

| WEIGHT | CULTURE* | ANTISERUM | GUINEA-PIG SERUM | RESULTS |
|---|---|---|---|---|
| *grams* | *cc.* | *cc.* | *cc* | |
| 25 | 0.5 | 0 | 0 | Died in 36 hours |
| 23 | 0.25 | 0 | 0 | Died in 2 hours |
| 2 | 0.125 | 0 | 0 | Died in 13 hours |
| 10 | 0.0625 | 0 | 0 | Died in 20 hours |
| 20 | 0.5 | 0.5 | 0 | Died in 24 hours |
| 10 | 0.25 | 0.5 | 0 | Died in 36 hours |
| 22 | 0.125 | 0.5 | 0 | Survived over 5 days |
| 25 | 0.0625 | 0.5 | 0 | Died in 15 hours |
| 24 | 0.5 | 0 | 0.5 | Died in 22 hours |
| 23 | 0.25 | 0 | 0.5 | Died in 20 hours |
| 23 | 0.125 | 0 | 0.5 | Survived over 5 days |
| 22 | 0 0325 | 0 | 0.5 | Died in 20 hours |
| 25 | 0.5 | 0.5 | 0.5 | Survived over 5 days |
| 20 | 0.25 | 0.5 | 0.5 | Died in 24 hours |
| 16 | 0..125 | .5 | 0.5 | Survived over 5 days |
| 16 | 0.0625 | 0.5 | 0.5 | Survived over 5 days |

* Strain 124 normal meningococci suspended in heated guinea-pig serum diluted 1:4.

9. While this protection test in its present state has not yielded us results as close and delicate as may be obtained with a satisfactory toxin in determining the antitoxic value of diphtheria antitoxin, we believe the test to be one of value as a means for determining and measuring the approximate protective and curative value of antimeningitis serum.

*b. The protective value of normal human and guinea-pig sera alone and in combination with antimeningitis serum.* The results of numerous experiments to determine the protective value of normal active human and guinea-pig sera alone and in combination

TABLE 15

*The protective activity of antimeningitis serum alone and in conjunction with normal guinea-pig serum for virulent meningococci*

| WEIGHT | CULTURE* | ANTISERUM | NORMAL GUINEA-PIG SERUM | RESULTS |
|---|---|---|---|---|
| *grams* | *cc.* | *cc.* | | |
| 15 | 0.01 | 0 | 0 | Died in 14 hours |
| 14 | 0.005 | 0 | 0 | Died in 24 hours |
| 12 | 0.0025 | 0 | 0 | Survived over 4 days |
| 12 | 0.00125 | 0 | 0 | Died in 15 hours |
| 16 | 0.0006 | 0 | 0 | Survived over 4 days |
| 16 | 0.01 | 0.5 | 0 | Survived over 4 days |
| 15 | 0.005 | 0.5 | 0 | Survived over 4 days |
| 13 | 0.0025 | 0.5 | 0 | Survived over 4 days |
| 12 | 0.00125 | 0.5 | 0 | Survived over 4 days |
| 12 | 0.0006 | 0.5 | 0 | Survived over 4 days |
| 16 | 0.01 | 0 | 0.5 | Died in 15 hours |
| 14 | 0.005 | 0 | 0.5 | Died in 20 hours |
| 14 | 0.0025 | 0 | 0.5 | Survived over 4 days |
| 13 | 0.00125 | 0 | 0.5 | Died in 21 hours. |
| 12 | 0.0006 | 0 | 0.5 | Died in 19 hours |
| 18 | 0.01 | 0.5 | 0.5 | Survived over 4 days |
| 14 | 0.005 | 0.5 | 0.5 | Survived over 4 days |
| 14 | 0.0025 | 0.5 | 0.5 | Survived over 4 days |
| 13 | 0.00125 | 0.5 | 0.5 | Survived over 4 days |
| 13 | 0.006 | 0.5 | 0.5 | Survived over 4 days |

* Strain 124 normal meningococci suspended in heated guinea-pig serum diluted 1:4.

with different antimeningitis sera may be summarized as follows; the protocols of several experiments are shown in tables 14, 15, 16, 17 and 18. In an effort to render the protection tests more delicate the technic of Hitchens and Robinson was modified in some experiments by using smaller doses of immune and normal serum.

1. Normal active human and guinea-pig sera were practically without demonstrable protective value in these tests employing white mice.

TABLE 16

*The protective activity of antimeningitis serum alone and in conjunction with normal human serum for virulent meningococci*

| WEIGHT | CULTURE* | ANTIMENINGITIS SERUM | HUMAN SERUM | RESULTS |
|---|---|---|---|---|
| *grams* | *cc.* | *cc.* | cc. | |
| 16 | 0.1 | 0 | 0 | Died in 13 hours |
| 17 | 0.1 | 0 | 0 | Died in 18 hours |
| 19 | 0.1 | 0 | 0 | Died in 19 hours |
| 14 | 0.1 | 0 | 0 | Died in 48 hours |
| 18 | 0.1 | 0 | 0 | Died in 13 hours |
| 18 | 0.1 | 0.2 | 0 | Died in 30 hours |
| 23 | 0.1 | 0.1 | 0 | Died in 24 hours |
| 20 | 0.1 | 0.05 | 0 | Died in 13 hours |
| 19 | 0.1 | 0.025 | 0 | Died in 24 hours |
| 16 | 0.1 | 0.0125 | 0 | Died in 3 days |
| 16 | 0.1 | 0 | 0.2 | Died in 13 hours |
| 18 | 0.1 | 0 | 0.2 | Died in 24 hours |
| 20 | 0.1 | 0 | 0.2 | Died in 24 hours |
| 23 | 0.1 | 0 | 0.2 | Died in 3 days |
| 20 | 0.1 | 0 | 0.2 | Died in 13 hours |
| 18 | 0.1 | 0.2 | 0.2 | Died in 24 hours |
| 16 | 0.1 | 0.1 | 0.2 | Died in 24 hours |
| 19 | 0.1 | 0.05 | 0.2 | Died in 3 days |
| 20 | 0.1 | 0.025 | 0.2 | Died in 13 hours |
| 20 | 0.1 | 0.0125 | 0.2 | Died in 24 hours |

* Strain 124 normal meningococci suspended in heated guinea-pig serum diluted 1: 4.

2. Occasionally the addition of normal human and guinea-pig serum to antimeningitis serum appeared to afford increased protection as shown in the protocol of one experiment with pig serum in table 14, but a general review of all experiments showed that no protective influence could be attributed to the addition of the normal to immune sera.

As previously stated it is scarcely to be expected that an animal test would bring out the increased protective value following the complementing of an immune serum if such actually does occur, because the body fluids of the test animal contains complement.

TABLE 17

*The protective value of active human serum (complement) alone and in combination with antimeningitis serum*

| WEIGHT | ANTIMENINGITIS SERUM | HUMAN SERUM | CULTURE* | RESULTS |
|---|---|---|---|---|
| *grams* | *cc.* | *cc.* | cc. | |
| 21 | 0.1 | 0 | 0.01 | Survived. |
| 24 | 0.1 | 0 | 0.05 | Survived. |
| 26 | 0.1 | 0 | 0.1 | Survived. |
| 16 | 0.1 | 0 | 0.2 | Survived. |
| 20 | 0.1 | 0 | 0.4 | Died in 24 hours |
| 24 | 0.1 | 0.1 | 0.01 | Survived |
| 24 | 0.1 | 0.1 | 0.05 | Survived |
| 24 | 0.1 | 0.1 | 0.1 | Survived |
| 19 | 0.1 | 0.1 | 0.2 | Died in 12 hours |
| 21 | 0.1 | 0.1 | 0.4 | Died in 12 hours |
| 19 | 0 | 0 | 0.01 | Died in 12 hours |
| 17 | 0 | 0 | 0.01 | Died in 12 hours |
| 18 | 0 | 0.1 | 0.01 | Died in 12 hours |
| 16 | 0 | 0.1 | 0.01 | Died in 12 hours |

* Strain 124 normal meningococci suspended in heated guinea-pig serum diluted 1:4.

We have also conducted combined in vitro-vivo tests by incubating in a water bath for one hour varying amounts of a suspension of virulent meningococci alone and in combination with antimeningitis serum and with antimeningitis and normal serum, followed by tests for virulence and bactericidal activity by intraperitoneal injection into white mice; the results of these experiments were quite irregular, but the addition of normal serum did not appear to influence the results.

TABLE 18

*The protective value of active guinea-pig serum (complement) alone and in combination with antimeningitis serum*

| WEIGHT | ANTIMENINGITIS SERUM | GUINEA-PIG SERUM | CULTURE* | RESULTS |
|---|---|---|---|---|
| *grams* | *cc.* | *cc.* | *cc.* | |
| 15 | 0 | 0 | 0.01 | Died* in 15 hours |
| 14 | 0 | 0 | 0.005 | Died in 15 hours |
| 12 | 0 | 0 | 0.0025 | Survived* |
| 12 | 0 | 0 | 0.00125 | Died in 15 hours |
| 16 | 0 | 0 | 0.0006 | Survived |
| 15 | 0.1 | 0 | 0.01 | Survived |
| 25 | 0.1 | 0 | 0.005 | Survived |
| 23 | 0.1 | 0 | 0.0025 | Survived |
| 23 | 0.1 | 0 | 0.00125 | Survived |
| 19 | 0.1 | 0 | 0.0006 | Survived |
| 20 | 0.1 | 0.1 | 0.01 | Survived |
| 23 | 0.1 | 0.1 | 0.005 | Survived |
| 26 | 0.1 | 0.1 | 0.0025 | Survived |
| 20 | 0.1 | 0.1 | 0.00125 | Survived |
| 20 | 0.1 | 0.1 | 0.0006 | Survived |
| 19 | 0.1 | 0.1 | 0.01 | Died in 12 hours |
| 17 | 0.1 | 0.1 | 0.01 | Died in 18 hours |

* Strain of normal meningococci 124 in heated pig serum 1: 4.

## SUMMARY

1. The bactericidal activity in vitro of different antimeningitis sera was found to be quite low. Fresh or active antimeningitis sera was somewhat more bactericidal than the same sera after inactivation by heating at 60°C. for thirty minutes.

2. Active normal human and guinea-pig sera are generally slightly bactericidal for meningococci.

3. The bactericidal activity of horse-antimeningitis and normal human and guinea-pig serum, is largely independent of complemental bacteriolysis.

4. The bactericidal activity in vitro of normal and immune sera was best shown by a pipet method employing small numbers of cocci and relatively large volumes of serum.

5. The addition of active normal human and guinea-pig serum to antimeningitis serum sometimes increased the bactericidal activity of the latter.

6. Whole human and guinea-pig blood was found slightly more bactericidal than the sera alone.

7. Normal human and guinea-pig sera frequently agglutinates meningococci in final dilutions up to 1:4, but not in higher dilutions.

8. Antimeningitis sera containing the largest amounts of agglutinin were found to possess most opsonin and apt to prove most bactericidal in vitro.

9. The mouse test of Hitchens and Robinson was found under certain technical conditions to be of value as a means for determining and measuring approximately the protective and curative value of antimeningitis serum.

10. Normal active human and guinea-pig sera were practically without demonstrable protective value in mice infected with virulent meningococci, although the addition of these normal sera to antimeningitis serum appeared in some experiments to slightly increase the protective power of the latter.

11. The addition of active normal human or guinea-pig serum (complement) to antimeningitis serum cannot be expected on the basis of our experiments, to greatly augment bactericidal activity because complemental bacteriolysis exerts but a minor rôle in the relatively feeble bactericidal activity of antimeningitis serum, but the addition of normal sera definitely increases opsonic activity (10) and since it would appear that a large part of the curative properties of antimeningitis serum is to be ascribed to the presence of opsonin, it is suggested as worthy of clinical trial to complement the antimeningitis serum by the addition of active human or guinea-pig serum prior to intraspinal injection and particularly in the treatment of severe and serum-resistant infections.

For this purpose human serum is superior to guinea-pig serum and may be obtained from the patient or a volunteer with the usual precautions for asepsis.

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# THE RELATION OF THE MENINGOCOCCIDAL ACTIVITY OF THE BLOOD TO RESISTANCE TO VIRULENT MENINGOCOCCI[1]

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In a previous communication (1) we have shown the greater bactericidal activity of whole blood for the meningococcus as compared with serum and briefly described a simple and convenient method for measuring the bactericidal activity of whole blood devised by Dr. Heist employing the many stemmed capillary pipet of Wright. Further experiences with this technic have demonstrated its simplicity; but a few drops of undefibrinated blood are required and the results are usually quite sharp and convincing. As is true with all bactericidal tests, however, the results observed with the blood of one person or lower animal tested on different days not infrequently yields slightly varying results, owing not only to probable fluctuations in the bactericidan content of the blood but more particularly to fluctuations in the numbers of viable meningococci in the culture employed; with careful attention to technic however, the latter error can be reduced to a minimum.

The object of the present investigation was to determine whether or not a relation exists between the meningococcidal activity of the blood and resistance to infection with virulent meningococci; furthermore whether or not the high natural immunity or resistance of certain of the lower animals to the meningococcus is to be ascribed in part to a higher meningococcidal activity of their blood.

Young and old guinea-pigs, rabbits and white mice were used in these experiments. Flexner (2) has found that guinea-pigs



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weighing from 175 to 200 grams are highly susceptible to virulent meningococci as compared with pigs weighing 350 to 400 grams or more and that young pigs are more susceptible than mice; Von Lingelsheim and Leuchs (3) and Kolle and Wassermann (4) have also found young guinea-pigs highly susceptible. Bettencourt and Franca (5) Elser and Huntoon (6) and Hitchens and Robinson (7) claim, however, that white mice are more highly susceptible and the latter employ these animals in a protection test with antimeningitis serum. All investigators who have worked with rabbits found them highly resistant; likewise all appear to agree that young guinea-pigs are more susceptible than older and heavier animals.

## EXPERIMENTAL

Our experiments were conducted with a single strain of meningococcus, the virulence and cultural characteristics of which was quite familiar to us; bactericidal tests were made with the whole blood of different series of young and old pigs, mice and rabbits followed by the intraperitoneal injection of the same culture in graded doses and according to the body weight of each animal into each series of animals; all animals were kept under observation for two to four days and after death the blood of the heart examined for meningococci. The strain of meningococcus employed was highly virulent and all animals succumbing within four days invariably showed the presence of meningococci in the blood of the heart. In order to render the virulence tests with different animals strictly comparable, all were injected on the same day with the same emulsion of meningococci and, as stated above, according to body weight. By experiments of this nature we have sought to determine whether the resistance or non-resistance of a certain species of animal bore a relation to the meningococcidal activity of the whole blood of this species and whether variation in resistance of different species of animals bore a direct relation to variation in bactericidal activity of the whole blood.

*Bactêricidal tests*

In the bactericidal blood tests the many-stemmed pipet devised by Wright for measuring the coagulation-time and anti-tryptic power of the blood (8) was employed after the method devised by Dr. Heist. Each pipet measures about 9 cm. in length and about 1 mm. or somewhat less in thickness and six are used at one time. In conducting the tests we have employed twenty-four hour serum broth cultures of a strain of meningococcus undiluted and in five dilutions prepared with sheep serum broth, namely, 1:5; 1:25; 1:250; 1:500 and 1:1000. These cultures are arranged in sterile tubules and allowed to run by capillary attraction into the six sterile and numbered pipets respectively; the pipets are now blown out and each loaded to the same level with blood secured by pricking the skin after cleansing with alcohol. The pipets are now sealed by dipping the top in paraffin and incubated for twenty-four hours when a smear is made of each and stained for meningococci. With care in the technic contaminations are rare and the results quite sharp and regular. The numbers of microörganisms exposed to the germicidal action of the whole blood are quite small being those which have adhered to the inner wall of each capillary tube. Controls were always included with each dilution of culture in which sheep serum broth was substituted for blood.

Not infrequently the blood of persons and lower animals was able to destroy all cocci in the undiluted culture and in order to measure the bactericidal activity, denser cultures were prepared by washing off twenty-four hour serum dextrose agar cultures with 2 cc. sheep serum broth and preparing further dilutions of this emulsion.

In order to estimate the number of viable meningococci in each dilution of culture adhering to the walls of the capillary tubes, plates were prepared by washing out the cocci with several changes of serum dextrose broth into sterile petri dishes followed by the addition of serum dextrose agar and counts at the end of twenty-four hours incubation. A series of experiments have shown that with the strain of meningococcus employed by us the

number of viable cocci of each dilution of culture adhering to the inner walls of the capillary pipets and subjected to the bactericidal activity of the blood, were about as follows:

| PARENT CULTURE | NUMBER OF COLONIES PER PLATE | | | | | |
|---|---|---|---|---|---|---|
| | Undiluted | 1:5 | 1:25 | 1:250 | 1:500 | 1:1000 |
| Twenty-four hour serum dextrose broth | 250 to 600 | 180 to 400 | 60 to 300 | 10 to 120 | Few | Few |
| Emulsion* | Unc. | Unc. | Unc. to 3200 | 200 to 800 | 100 | 60 |

* Prepared by washing off a series of twenty-four hour serum dextrose agar cultures with 2 cc. of sheep serum broth per tube and mixing.

1. As shown in table 1 the blood of white mice possesses but feeble bactericidal activity for meningococcci and these animals proved quite susceptible to infection with the particular strain of meningococcus employed in these experiments. With three exceptions all mice succumbed within twenty-four hours and meningococci were found in the blood of the heart. While the blood of a few mice proved slightly more bactericidal than the majority they were not found as a general rule to exhibit greater resistance to infection.

2. As shown in table 2 the blood of rabbits was found to possess high bactericidal activity for meningococci and these animals proved highly resistant to infection with the same strain injected intraperitoneally in doses of culture according to body weight comparable with those given the mice and other animals.

3. As shown in table 3 the blood of young guinea-pigs was found to possess slightly more bactericidal activity for meningococci than the blood of white mice although in virulence tests they were found generally equally susceptible to infection.

4. The blood of older guinea-pigs was slightly more bactericidal for meningococci than the blood of young pigs and distinctly more so than the blood of white mice. The older and heavier

TABLE 1

*Results of meningococcidal blood and virulence tests with white mice*

| WEIGHT | MENINGOCOCCIDAL TESTS* | | | | | | MENINGOCOCCIDAL TESTS† | | | | | | DOSE OF CULTURE PER 10 GRAMS | DOSE INJECTED | VIRULENCE TESTS | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Undiluted | 1:5 | 1:25 | 1:250 | 1:500 | 1:1000 | Undiluted | 1:5 | 1:25 | 1:250 | 1:500 | 1:1000 | | | Results | Heart |
| *grams* | | | | | | | | | | | | | *cc.* | *cc.* | | |
| 20.0 | ++‡ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | 0.05 | 0.1 | Died in 15 hours | + |
| 17.5 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | − | − | 0.05 | 0.088 | Died in 24 hours | + |
| 20.0 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | − | 0.025 | 0.05 | Lived 7 days | 0 |
| 17.0 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | − | + | − | 0.025 | 0.044 | Died in 15 hours | + |
| 20.0 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | − | + | 0.125 | 0.025 | Died in 72 hours | + |
| 17.0 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | − | − | 0.125 | 0.022 | Died in 15 hours | + |
| 16.0 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | − | − | − | − | 0.006 | 0.01 | Lived 7 days | 0 |
| 18.0 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | − | − | − | 0.006 | 0.011 | Died in 15 hours | + |

* Conducted with dense culture secured by washing off twenty-four hour serum dextrose agar cultures with 2 cc. sheep serum broth.

† Conducted with twenty-four hour serum broth culture.

‡ ++ = heavy growth; + = light growth; − = sterile.

TABLE 2

*Results of meningococcidal blood and virulence tests with rabbits*

| WEIGHT | MENINGOCOCCIDAL TESTS* | | | | | | MENINGOCOCCIDAL TESTS† | | | | | | DOSE OF CULTURE PER 10 GRAMS | DOSE INJECTED | VIRULENCE TESTS | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Undiluted | 1:5 | 1:25 | 1:250 | 1:590 | 1:1000 | Undiluted | 1:5 | 1:25 | 1:250 | 1:500 | 1:1000 | | | Results | Heart |
| *grams* | | | | | | | | | | | | | *cc.* | *cc.* | | |
| 1350 | ++‡ | ++ | ++ | + | + | + | − | − | − | − | − | − | 0.05 | 6.75 | Lived§ | 0 |
| 1650 | ++ | ++ | − | − | − | − | − | − | − | − | − | − | 0.025 | 4.125 | Lived | 0 |
| 1800 | ++ | ++ | − | − | − | − | − | − | − | − | − | − | 0.0125 | 2.25 | Lived | 0 |
| 2800 | ++ | ++ | − | − | − | − | − | − | − | − | − | − | 0.006 | 1.68 | Lived | 0 |
| 2100 | ++ | + | − | − | − | − | − | − | − | − | − | − | 0 | 0 | 0 | 0 |
| 1400 | ++ | + | − | − | − | − | − | − | − | − | − | − | 0 | 0 | 0 | 0 |

* Conduced with dense culture secured by washing off twenty-four hour serum dextrose agar cultures with 2 cc. sheep serum broth.

† Conducted with twenty-four hour serum broth culture.

‡ ++ = heavy growth; + = light growth; − = sterile.

§ Lived over seven days.

animals were likewise more resistant to infection with this strain of meningococcus when infected intraperitoneally with doses according to body weight.

*Meningococcidal activity of whole human blood*

Bactericidal tests were also made with the bloods of different persons of varying ages healthy and normal or suffering with various ailments; unfortunately we have not been able to study the bactericidal activity of the blood with this technic of persons ill or convalescent from meningococcus meningitis, owing to the

TABLE 3

*Results of meningococcidal blood and virulence tests with young guinea-pigs*

| WEIGHT | MENINGOCOCCIDAL TESTS* | | | | | | DOSE OF CULTURE PER 10 GRAMS WEIGHT | DOSE INJECTED | VIRULENCE TESTS | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Undiluted | 1:5 | 1:25 | 1:250 | 1:500 | 1:1000 | | | Result | Heart |
| *grams* | | | | | | | *cc.* | *cc.* | | |
| 125 | ++ | ++ | ++ | − | − | − | 0.05 | 0.62 | Died in 15 hours | + |
| 145 | ++† | ++ | ++ | − | − | − | 0.025 | 0.35 | Died in 15 hours | + |
| 140 | ++ | ++ | ++ | + | + | − | 0.0125 | 0.175 | Died in 15 hours | + |
| 90 | ++ | ++ | ++ | − | − | − | 0.006 | 0.06 | Died in 17 hours | + |
| 140 | ++ | ++ | ++ | + | + | − | 0 | 0 | 0 | 0 |
| 103 | + | − | − | − | − | − | 0 | 0 | 0 | 0 |

* Conducted with twenty-four hour serum dextrose broth culture.

† ++ = heavy growth of meningococci; + = light growth; − = sterile.

absence of cases in the hospitals to which we had access at the time this work was being conducted. It is highly probable however that the blood acquires increased bactericidal activity during and after the infection or active immunization with vaccine, which persists for a variable period of time.

The results of a number of these tests are shown in tables 5, 6 and 7 and may be summarized as follows:

1. The average meningococcidal activity of human blood has been found much less than that of rabbit blood and comparable to the bactericidal activity of the blood of young guinea-pigs and mice. If a relation exists between the bactericidal activity of the blood and resistance to meningococcus infection, persons are

TABLE 4

*Results of meningococcidal blood and virulence tests with older guinea-pigs*

| WEIGHT | MENINGOCOCCIDAL TESTS* | | | | | | DOSE OF CULTURE PER 10 GRAMS WEIGHT | DOSE INJECTED | VIRULENCE TESTS | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Undiluted | 1:5 | 1:25 | 1:250 | 1:590 | 1:1000 | | | Results | Heart |
| *grams* | | | | | | | *cc.* | *cc.* | | |
| 300 | ++† | ++ | + | + | − | − | 0.05 | 1.5 | Died in 48 hours | + |
| 395 | + | + | + | − | − | − | 0.025 | 1.0 | Died in 15 hours | + |
| 310 | + | + | − | − | − | − | 0.0125 | 0.4 | Died in 15 hours | + |
| 285 | + | + | − | − | − | − | 0.006 | 0.2 | Died in 24 hours | + |
| 390 | + | − | − | − | − | − | 0 | 0 | 0 | 0 |
| 362 | − | − | − | − | − | − | 0 | 0 | 0 | 0 |
| Over 300 | + | + | − | − | − | − | 0 | 0 | 0 | 0 |

* Conducted with twenty-four hour serum dextrose broth culture.

† ++ = heavy growth; + = light growth; − = sterile.

TABLE 5

*Meningococcidal activity of the whole blood of adults*

| NUMBER (SEX) | AGE | MENINGOCOCCIDAL TESTS* | | | | | | MENINGOCOCCIDAL TESTS† | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Undiluted | 1:5 | 1:25 | 1:250 | 1:500 | 1:1000 | Undiluted | 1:5 | 1:25 | 1:250 | 1:500 | 1:1000 |
| | *years* | | | | | | | | | | | | |
| 1 (M) | 38 | ++‡ | ++ | ++ | ++ | ++ | ++ | ++ | + | + | + | − | − |
| 2 (M) | 31 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | + | − | − | − |
| 3 (M) | 30 | + | + | + | − | − | − | − | − | − | − | − | − |
| 3 (M)§ | 30 | 0 | 0 | 0 | 0 | 0 | 0 | + | + | + | + | + | + |
| 4 (M) | 30 | ++ | ++ | ++ | + | + | + | + | − | − | − | − | − |
| 5 (M) | 17 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | − | − | − | − |
| 6 (M) | 22 | ++ | ++ | ++ | ++ | + | + | + | + | + | − | − | − |
| 7 (M) | 18 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | − | − | − | − | − |
| 8 (F) | 38 | ++ | ++ | + | − | − | − | − | − | − | − | − | − |
| 9 (F) | 31 | ++ | ++ | ++ | + | + | + | + | − | − | − | − | − |
| 10 (F) | 24 | ++ | ++ | ++ | ++ | + | + | + | + | − | − | − | − |
| 11 (F) | 24 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | − | − | − | − |
| 12 (M) | 28 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | − | − | − | − |
| 13 (F) | 31 | ++ | ++ | ++ | + | + | + | + | + | − | − | − | − |

* Conducted with dense culture secured by washing off twenty-four hour serum dextrose agar cultures with 2 cc. sheep serum broth.

† Conducted with twenty-four hour serum broth culture.

‡ ++ = heavy growth; + = light growth; − = sterile.

§ Bactericidal activity of the blood at the onset of a severe attack of nasopharyngitis.

about as susceptible as young guinea-pigs and mice and lack the high resistance of the adult rabbit.

TABLE 6

*Meningococcidal activity of the blood of normal children*

| NUMBER (SEX) | AGE | MENINGOCOCCIDAL TESTS* | | | | | | MENINGOCOCCIDAL TESTS† | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Undiluted | 1:5 | 1:25 | 1:250 | 1:500 | 1:1000 | Undiluted | 1:5 | 1:25 | 1:250 | 1:500 | 1:1900 |
| 1 (M) | 15 months | ++‡ | ++ | ++ | ++ | ++ | ++ | + | + | + | + | − | − |
| 2 (M) | 18 months | ++ | + | + | + | + | + | ++ | + | + | + | + | − |
| 3 (M) | 6 months | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | + |
| 4 (F) | 12 months | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | + | + | + |
| 5 (M) | 10 months | ++ | ++ | ++ | + | + | + | + | + | + | + | + | + |
| 6 (M) | 11 months | ++ | ++ | ++ | ++ | ++ | + | + | + | + | + | + | + |
| 7 (M) | 1–2 years | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | + | + | + |
| 8 (F) | 1–2 years | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | + | + | + | + |
| 9 (F) | 1–2 years | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | + | + |
| 10 (F) | 1–2 years | ++ | ++ | ++ | + | ++ | + | ++ | + | + | + | − | − |
| 11 (M) | 1–2 years | ++ | ++ | ++ | + | ++ | + | + | + | + | − | − | − |
| 12 (M) | 1–2 years | ++ | ++ | ++ | ++ | + | + | + | + | − | − | − | − |
| 13 (M) | 1–2 years | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | + |
| 14 (F) | 1–2 years | ++ | ++ | + | + | + | + | + | + | + | + | + | + |
| 15 (M) | 1–2 years | ++ | ++ | ++ | ++ | ++ | + | + | + | + | + | + | + |
| 16 (M) | 3 years | ++ | ++ | ++ | + | + | + | + | + | + | + | − | − |
| 17 (M) | 3½ years | ++ | ++ | + | + | + | + | + | + | − | − | − | − |
| 18 (F) | 3½ years | ++ | ++ | ++ | + | + | + | + | + | + | + | + | + |
| 19 (M) | 3 years | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | + | + | + | + |
| 20 (M) | 3 years | ++ | ++ | ++ | ++ | ++ | ++ | + | + | − | − | − | − |
| 21 (M) | 4 years | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | + | + | − | − |
| 22 (M) | 4 years | ++ | ++ | + | + | + | + | − | − | − | − | − | − |
| 23 (M) | 4½ years | ++ | ++ | ++ | ++ | + | + | + | + | + | + | − | − |
| 24 (M) | 5 years | ++ | ++ | ++ | ++ | + | + | + | − | − | − | − | − |
| 25 (M) | 5 years | ++ | ++ | ++ | ++ | + | + | ++ | + | + | + | − | − |
| 26 (M) | 10 years | ++ | + | + | + | − | − | − | − | − | − | − | − |

* Conducted with dense culture secured by washing off twenty-four hour serum dextrose agar cultures with 2 cc. sheep serum broth.

† Conducted with twenty-four hour serum dextrose broth culture.

‡ ++ = heavy growth; + = light growth; − = sterile.

2. A general review of the results of the bactericidal tests indicates that the meningococcidal activity of the blood of very young children is less than that of adults.

3. Considerable variation in bactericidal activity of the blood exists among different persons; the blood of the same person appears to vary from day to day under normal conditions although a definite statement on this point cannot be made because of the factor of variation and error in the technic and particularly in reference to the number of viable microörganisms present in the cultures employed. It would appear however, that the meningococcidal activity of the blood is decreased during acute infections, as shown in table 5 with the blood of no. 3 (M) before and after an acute infection of the upper air passages.

TABLE 7

*Meningococcidal activity of the blood of sick children*

| NUMBER (SEX) | AGE | DIAGNOSIS | MENINGOCOCCIDAL TESTS* | | | | | | MENINGOCOCCIDAL TESTS† | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Undiluted | 1:15 | 1:25 | 1:250 | 1:500 | 1:1000 | Undiluted | 1:5 | 1:25 | 1:250 | 1:500 | 1:1000 |
| 1 (M) | 11 months | Bronchitis | ++‡ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | + | + |
| 2 (M) | 6 years | Broncho-pneumonia | ++ | ++ | ++ | + | + | + | + | − | − | − | − | − |
| 3 (M) | 3 years | Bronchitis | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | + |
| 4 (M) | 3 years | Pertussis | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | − | − | − | − |
| 5 (M) | 3½ years | Pertussis | ++ | ++ | ++ | ++ | ++ | + | + | + | − | − | − | − |
| 6 (M) | 1 year | Pertussis | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | + | + | + |
| 7 (F) | 1 year | Malnutrition | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | + | + | + | + |

* Conducted with dense culture secured by washing off twenty-four hour serum dextrose agar cultures with 2 cc. sheep serum broth.

† Conducted with twenty-four hour serum dextrose broth culture.

‡ ++ = heavy growth; + = light growth; − = sterile.

## CONCLUSIONS

1. The bactericidal blood test described possesses the advantages of employing the whole blood and requiring but a few drops of blood easily obtained from the finger; the technic is comparatively simple and the results usually quite definite. A degree of error, however, is present as is true of all bactericidal tests because of variation in the numbers of viable microörganisms in the various dilutions of culture employed.

2. Bactericidal tests with a strain of virulent normal meningococci and the blood of adult white mice, adult rabbits and young and older guinea-pigs, have shown that the blood of rabbits possesses most bactericidal activity; the blood of old guinea-pigs was found somewhat more bactericidal than the blood of young guinea-pigs and white mice. The blood of young guinea-pigs was generally slightly more bactericidal than the blood of adult white mice.

3. In the virulence tests consisting in the intraperitoneal injection of the same culture in graded doses and according to body weight, rabbits were found most resistant; young guinea-pigs were about as susceptible as adult mice while older guinea-pigs were somewhat more resistant than either.

4. The results of these experiments indicate therefore, that there is a general parallelism between the meningococcidal activity of the blood and resistance to meningococci and that at least one factor in natural immunity to the meningococcus is the presence of bactericidal substances in the blood.

5. The results of our studies indicate that the meningococcidal activity of blood of children is somewhat less than that of adults; the bactericidal activity of the latter is comparable to the meningococcidal activity of the blood of mice and young guinea-pigs.

6. It is probable that certain persons possess sufficient natural immunity to the meningococcus to afford protection against meningitis and it is highly desirable to discover a simple and accurate clinical test or measure of this resistance comparable to the Schick test for antitoxic immunity in diphtheria, as a means for encouraging active immunization with meningococcus vaccines; the bactericidal test as described while being simple and fairly rapid does not meet with these requirements by reason of the possibilities of error and variation, although it possesses definite value as a strictly laboratory procedure in special investigations.

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# EXPERIMENTS ON THE PRODUCTION OF ANTI-POLIOMYELITIC SERUM IN RABBITS[1]

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One can find in the extensive literature on experimental poliomyelitis only a few scattered statements of experiments on the production of antipoliomyelitic serum in rabbits, and these statements, furthermore, are as contradictory as those regarding the question of the etiology of poliomyelitis and the susceptibility of rabbits to this disease. Flexner (1), in 1910, stated that rabbits which had been given injections of active poliomyelitis virus did not seem to yield immunity principles that had restraining or neutralizing effect on the virus either *in vitro* or within the body. Amoss (2), in 1917, reported that the serum of rabbits which he had injected intravenously with a heavy suspension of washed cultures of globoid bodies also failed to neutralize the filterable virus. On the other hand, Nuzum (3) claimed that he had succeeded in producing antipoliomyelitic serum in rabbits by immunizing them with strains of streptococci which he and several others believed to be the causative agents of poliomyelitis. He injected, besides other animals, four rabbits according to the rapid method described by Amoss and Wollstein, and tested the action of his polyvalent immune serum only on the streptococci which he used for immunization, and not on the filterable virus. But, like a number of other investigators, we have found that there is no etiological relationship between these streptococci and poliomyelitis. Moreover, Amoss and Eberson (4) have shown that the antipoliomyelitic serum prepared by Rosenow by the injection of similar organisms exhibited no neutralizing effect on the filterable virus. It

[1] Read before the American Association of Immunologists, March 29, 1918.

is this difference of opinion coupled with the scarcity of detailed reports which has led us to re-examine the problem.

## TECHNIQUE

The presence or absence of antipoliomyelitic substances in the serum of rabbits which had received repeated injections of the poliomyelitis virus was determined by attempting to neutralize the virus with the serum. Mixtures of such serum and poliomyelitis virus were prepared in sterile test tubes, kept at 37.5°C. for one hour and then in the ice box for fifteen to twenty minutes. The control mixtures were similarly prepared and treated at the same time, except that either normal rabbit serum or sterile physiological salt solution was mixed with the poliomyelitis virus in place of the serum of injected rabbits. The mixtures were given to monkeys by intracerebral injection of 0.5 or 0.8 cc.

The rabbits used in our first experiment were immunized by giving them eight subcutaneous injections of the poliomyelitis virus at weekly intervals, and those used in the second experiment were immunized with seven subcutaneous injections at weekly intervals followed by four at five day intervals. All of the animals were bled nine days after the last injection.

### *Experiment 1*

April 25, 1917. Two mixtures were prepared—mixture A contained 1 cc. of virus previously passed through a Berkefeld-filter and 2 cc. of serum of injected rabbits and mixture B contained 1 cc. of the same virus and 2 cc. of normal rabbit serum. Both mixtures were kept for one hour at 37.5°C. and for fifteen hours in the ice box.

April 26. The following two monkeys (Macacus rhesus) received intracerebral injections of 0.8 cc. of the above mixtures.

*a. Control monkey 16.* Received 0.8 cc. of mixture B.

April 27 to May 5. No symptoms.

May 6. Paralysis, which ended in death on the 11th.

Histopathological examination. Changes of poliomyelitis.

*b. Monkey 15.* Received 0.8 cc. of mixture A.

April 27 to July 17. No symptoms.

July 18. Paralysed, and was killed by ether.

Histopathological examination. Changes of poliomyelitis.

This experiment seems to indicate that the serum of injected rabbits, although it did not protect monkey 15 from the fatal effect of the poliomyelitis virus, had a least delayed the development of paralysis and death.

*Experiment 2*

In order to get more definite results, we have used in this experiment sterile physiological salt solution instead of normal rabbit serum in the mixtures which we gave to the control monkeys.

September 13, 1917. Two sets of injections were prepared—the first set consisted of mixtures C and D, each of which contained 0.5 cc. of a 5 per cent emulsion of poliomyelitis cord and 2 cc. of sterile physiological salt solution, and the second set consisted of mixtures E, F and G, each of which contained 0.5 cc. of the same cord emulsion and 2 cc. of serum of injected rabbits. The virus used in this experiment was a very virulent one; 0.1 cc. of a 5 per cent emulsion of the cord being a fatal dose. In this experiment all the mixtures were kept at 37.5°C. for one hour and then in the ice box for twenty hours.

September 14. The following five monkeys (Macaccus rhesus) received intracerebral injections of 0.5 cc of the above mixtures. Since 0.1 cc. of the cord emulsion which we used was a fatal dose—each of the five monkeys thus received one fatal dose of the virus plus 0.4 cc. of serum or sterile physiological salt solution. Immediately before the injection of the mixtures, monkeys 200, 197 and 199 each received 10 cc. of the serum of injected rabbits intravenously.

*a. Control monkey 581.* Received 0.5 cc. of mixture C (virus plus salt solution).

September 15 to 19. No symptoms.

September 20. Paralysis, which ended in death on the 21st.

Histopathological examination. Changes of poliomyelitis.

*b. Control monkey 179.* Received 0.5 cc. of mixture D (virus plus salt solution).

September 15 to 21. No symptoms.

September 22. Paralysis, which ended in death on the 24th.

Histopathological examination. Changes of poliomyelitis.

*c. Monkey 200.* Received 0.5 cc. of mixture E (virus plus serum).

September 15 to 21. No symptoms.

September 22. Paralysis, which ended in death on the 23d.

Histopathological examination. Changes of poliomyelitis.

*d. Monkey 197.* Received 0.5 cc. of mixture F (virus plus serum).
September 15 to 17. No symptoms.
September 18. Looked sick.
September 19. Paralysis, which ended in death on the 21st.
Histopathological examination. Changes of poliomyelitis.
*e. Monkey 199.* Received 0.5 cc. of mixture G (virus plus serum).
September 15 to 18. No symptoms.
September 19. Paralysis of left front leg. Weakness of right front leg.
September 20. Paralysis of all extremities. It was injected intravenously with 10 cc. of the serum of injected rabbits.
September 21. Found dead in the morning.
Histopathological examination. Changes of poliomyelitis.

This experiment shows clearly that the serum of rabbits immunized with eleven subcutaneous injections of the potent poliomyelitis virus had no more restraining or neutralizing effect on the virus than had ordinary physiological salt solution.

## DISCUSSION AND CONCLUSION

Our two experiments may be said to be somewhat contradictory in that the serum of the presumably immunized rabbits delayed the development of symptoms and death in one instance and showed no difference from sterile physiological salt solution in the other. This discrepancy is slight and, we think, may be explained by the difference in the individual resistance of the monkeys to poliomyelitis rather than by the difference in the action of the serum. It is well known that monkeys at times show very wide variations in the incubation periods, from as short as three and a half days to as long as forty-six days. The latter instance was recorded by Leiner and v. Wiesner in one case in which a virus that had been passed through a Riechel-filter was used. We, therefore, interpret the delayed development of symptoms and death of monkey 15 (see experiment 1) merely as a prolonged incubation period—fifty-three days—because this monkey, after death, showed typical changes of poliomyelitis—one of the criteria by which a sure diagnosis of poliomyelitis can at present be made.

In conclusion we may say that rabbits could not be immunized with poliomyelitis virus to produce serum which could restrain or neutralize the effect of the virus.

These experiments were done in collaboration with Dr. Hans Zinsser for whose advice, suggestions and aid the writer wishes to make grateful acknowledgment.

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# THE STUDY OF PROBLEMS OF IMMUNITY BY THE TISSUE CULTURE METHOD

## II. THE TISSUE CULTURE AS A MEANS FOR QUANTITATIVELY ESTIMATING TOXIN AND ANTITOXIN AND DETERMINING THE DISTRIBUTION OF ANTITOXIN IN PASSIVELY IMMUNIZED ANIMALS.

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In a previous paper Suzuki (1) has shown the value of the tissue culture as a means for a more detailed study of the nature of the conditions of natural immunity in animals. The present paper describes other experiments outlined for the purpose of studying more carefully the general distribution and action of antitoxic substances. It has been shown by Suzuki that chickens' tissues are sensitive in the tissue culture to the action of diphtheria toxin when this is added to the medium. Antitoxin when injected into chickens protects the chickens against lethal doses of toxin. The distribution of the antitoxin within these animals became of interest for analysis. Again, it seemed evident that the tissue culture method would be of use for the quantitative standardization of toxins. It thus became of interest to test the neutralizing power of antitoxin for toxin by this method.

In these experiments the tissue cells of young chickens and chick-embryos have been studied. The medium has consisted of plasma prepared from the blood of young or adult chickens, diluted with the substances to be tested. The cultures were prepared in the same way as those described in previous papers: Fragments of tissue or single cells are placed on the surface of a cover glass and covered with a layer of medium about 0.5 mm. in

thickness. A hollow slide rimmed with vaselin is inverted over the cover so that tissue and plasma is sealed in the hollow air chamber. After clotting had taken place the slides were inverted and the cover glasses sealed more completely by rimming them with paraffin. The fragments used in all these experiments were approximately 1 mm. in diameter unless otherwise indicated.

The toxin was obtained from the Board of Health of the city of New York. We are very much indebted to Dr. Anna Williams for her kindness in sending us toxin for this purpose. The antitoxin was purchased in the market.

## EXPERIMENTS

In the first series of experiments the sensitiveness of the cells of the heart muscle of eleven and twelve day chick-embryos to diphtheria toxin were tested. The results of these experiments are given in table 1. In the upper horizontal line the number of times the toxin is diluted in the medium is indicated. The primary dilutions were made in a 0.9 per cent NaCl solution and 1 part of this solution was then added to 1 part of plasma. The control medium contained 1 part of a 0.9 per cent solution of NaCl and 1 part of plasma. The sensitiveness of the cells of chick embryos to diphtheria toxin varies inversely with the age of the embryos.

The sign + indicates that a good growth of cells is observed. The sign ± indicates the growth of a few cells or the cells have grown and disintegrated. The sign − indicates that no growth is observed.

According to these experiments the tissue of eleven and twelve day chick embryos are sensitive to the particular toxin used. Active growth is noted only in those cultures where the toxin was diluted 2500 times.

In the second series of experiments the effect of horse's serum (diphtheria antitoxin) was tested. This serum as it is obtained in the market contains 0.4 per cent of tricresol. A small quantity of tricresol when added to the culture does not affect to any extent the growth of the cells. The antitoxin was diluted in the same way as that described for the toxin, table 1.

TABLE 1

*Tissue: Fragments of the heart muscle of chick-embryos eleven and twelve days old and a young chicken five weeks old*

*Medium: Plasma prepared from the blood of an adult chick, 1 part; various dilutions of diphtheria toxin in 0.9 per cent solution of* NaCl, *1 part*

*Control: The same plasma, 1 part; 0.9 per cent solution of* NaCl *1 part*

| TISSUE | TOXIN—NUMBER OF TIMES DILUTED[1] | | | | | | | | | | | | | | | | | CONTROLS | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 25 | 40 | 50 | 100 | 150 | 200 | 250 | 300 | 350 | 400 | 450 | 500 | 1000 | 1500 | 2000 | 2500 | 5000 | | |
| Twelve day chick embryos | − | − | − | | | | | | | | | | | | | | | + | + |
| | − | − | − | | | | | | | | | | | | | | | + | + |
| | − | − | − | − | − | − | | | | | | | | | | | | + | + |
| | − | − | − | − | − | − | | | | | | | | | | | | + | + |
| | | | | | − | − | − | − | − | | | | | | | | | + | + |
| | | | | | − | − | − | − | − | | | | | | | | | + | + |
| | | | | | | | | − | − | − | − | − | | | | | | + | + |
| | | | | | | | | − | − | − | − | − | | | | | | + | + |
| | | | | | | | | | | | − | − | − | + | + | + | + | + | + |
| | | | | | | | | | | | − | − | − | + | + | + | + | + | + |
| Eleven day chick-embryo | | | | | | | | | | | − | − | − | + | + | + | + | + | + |
| | | | | | | | | | | | − | − | − | + | + | + | + | + | + |
| Five weeks old chicken | | | | | | | | | | | − | − | − | − | − | − | + | + | + |
| | | | | | | | | | | | − | − | − | − | − | − | + | + | + |

TABLE 2

*Tissue: Fragments of the heart muscle of twelve day old chick-embryos*

*Medium: Plasma prepared from the blood of a normal chicken, 1 part; various dilutions of diphtheria antitoxin (horse's serum) in 0.9 per cent* NaCl *solution, 1 part*

*Control medium: Plasma, 1 part, 0.9 per cent* NaCl *solution, 1 part*

| | NUMBER OF TIMES ANTI-TOXIN IS DILUTED | | | | | | CONTROL |
|---|---|---|---|---|---|---|---|
| | 25 | 50 | 100 | 150 | 200 | 250 | |
| Series 1 growth | + | +++ | +++ | +++ | ++ | +++ | +++ |
| Series 2 growth | + | +++ | ++ | +++ | +++ | +++ | +++ |

[1] The toxin dilutions indicated in the tables are the dilutions of the toxin in the 0.9 per cent NaCl solution. The total dilution in the medium is two times these amounts.

The result of these experiments are given in the preceding table, table 2. The figures represent the number of times the antitoxin is diluted.

Having thus ascertained that a considerable concentration of horse's serum (diphtheria antitoxin containing 0.4 per cent tricresol) does not effect the growth of the cells it was possible to test as shown in the following table the effect of various dilutions of diphtheria toxin on the growth of cells in a medium containing a constant and known quantity of diphtheria antitoxin.

In table 3 the results of these experiments are given. After each culture, the amount of the dilution of toxin is indicated and the extent of the growth is indicated by the number of + marks.

TABLE 3

*Tissue: Fragments of the heart muscle of a twelve day chick embryo*
*Medium: Plasma prepared from the blood of a normal adult chicken; plasma, 1 part 1 per cent diphtheria antitoxin in 0.9 per cent* NaCl *solution, 1 part; various dilutions of diphtheria toxin in 0.9 per cent* NaCl *solution, 1 part*
*Control medium: Plasma, 1 part; 1 per cent diphtheria antitoxin and 0.9 per cent* NaCl *solutions, 1 part; 0.9 per cent* NaCl *solution, 1 part*

| | NUMBER OF TIMES TOXIN IS DILUTED | | | | | | | | CONTROL |
|---|---|---|---|---|---|---|---|---|---|
| | 20 | 50 | 100 | 300 | 500 | 800 | 1000 | 2000 | |
| Series 1 growth..... | + | ++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| Series 2 growth..... | + | ++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |

According to these experiments a 1 per cent solution of diphtheria antitoxin, the kind which we are using, neutralized an equal amount of a 1 per cent solution of the diphtheria toxin which we are testing.

In the fourth series of experiments an attempt was made to establish a passive immunity for the tissue in the culture (table 4).

The original control cultures contained medium consisting of 1 part of chicken plasma + 1 part isotonic 0.9 per cent NaCl solution. The original cultures of the experiment contained a medium consisting of the same chicken plasma 1 part + 1 part of a 1 per cent solution of diphtheria antitoxin in 0.9 per cent NaCl solution.

After forty-eight hours of active growth in the original cul-

tures the fragment of tissue from all the cultures including the controls were transplanted to a medium containing the same chicken plasma 1 part + 1 part of a 1 per cent solution diphtheria toxin in 0.9 per cent NaCl solution, subculture no. 1.

After twenty-four hours the fragments were again transplanted to the same medium subculture no. 2. A third transplant was made again after another twenty-four hours, subculture no. 3.

There was a slight growth of cells about the fragments transplanted from the original control cultures. These cells after a

TABLE 4

*Tissue: Fragments of the heart muscle of twelve day old chick embryo*
*Medium: Plasma obtained from the blood of an adult chicken—diluted one-half a indicated in text*

| EXPERIMENT | ORIGINAL CULTURES | SUB-CULTURES | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| 1 | +++ | +++ | +++ | − |
| 2 | +++ | ++ | +++ | − |
| 3 | +++ | +++ | +++ | − |
| 4 | +++ | +++ | +++ | − |
| 5 | +++ | +++ | ++ | − |
| 6 | +++ | +++ | ++ | − |
| 7 | +++ | +++ | ++ | − |
| 8 | +++ | +++ | + | − |
| 9 | +++ | +++ | − | − |
| 10 | +++ | +++ | ++ | − |
| Control 1 | +++ | + | − | − |
| Control 2 | +++ | + | − | − |

few hours rounded off. No activity was seen in the second transplant.

In the fifth series of experiments the growth of the cells was tested in a plasma prepared from the blood of a 3 pound chicken which had received forty-eight hours previously 2 cc. of diphtheria antitoxin into its wing vein. The results of the experiment are given in table 5.

These experiments show that the blood of the passively immunized chicken contains substance capable of neutralizing the diphtheria toxin.

In the sixth series of experiments (table 6) an attempt was made to transmit the passive immunity from the plasma of the adult chicken used in experiment 6, table 6 to fragments of heart muscle of a chick embryo.

Cultures 1 to 10 were prepared by placing fragments of heart muscle 1 mm. in diameter in a medium consisting of 1 part of the plasma of the passive immunized animal (obtained forty-eight hours after the injection of 2 cc. of antitoxin) and 1 part of 0.9 per cent NaCl solution.

TABLE 5

*Tissue: Fragments of the heart muscle of a twelve day old chick-embryo*
*Medium: Plasma prepared from the blood of a chicken forty-eight hours after it has received an injection into its wing vein of 2 cc. of diphtheria antitoxin. Plasma, 1 part; various dilutions of diphtheria toxin in 0.9 per cent* NaCl *solution, 1 part*
*Control A: Plasma; of the experiment, 1 part; 0.9 per cent* NaCl *solution, 1 part*
*Control B: Normal chicken plasma, 1 part; 1 per cent diphtheria toxin in 0.9 per cent* NaCl *solution, 1 part*

| | NUMBER OF TIMES TOXIN IS DILUTED | | | | | CONTROL A | CONTROL B |
|---|---|---|---|---|---|---|---|
| | 100 | 80 | 50 | 20 | 10 | | |
| Series 1 growth..... | +++ | +++ | +++ | +++ | + | +++ | − |
| Series 2 growth..... | +++ | +++ | +++ | +++ | + | +++ | − |

After forty-eight hours the fragments of tissue were transplanted to a medium containing 1 part of normal chicken plasma + 1 part of a 1 per cent solution of diphtheria toxin and 0.9 per cent NaCl, subcultures.

*Control A.* Cultures 11 and 12 were prepared in the same way. The medium in the subcultures contained, however, no diphtheria toxin.

*Control B.* Cultures 13 and 14 were prepared originally with a medium consisting of 1 part of normal plasma + 1 part of a 0.9 per cent NaCl solution. In the subcultures these fragments of tissue had been placed in a medium consisting of normal plasma 1 part + a solution containing 1 per cent diphtheria toxin and 0.9 per cent of NaCl. No growth was noted in these subcultures while the growth was active in all the other cultures of the series.

Having established this fact it became of interest to know through how many transplants the cells would continue to grow in the presence of diphtheria toxin after they had been thus passively immunized by the plasma obtained from the blood of a passively immunized chicken.

In cultures 1 to 12, table 7, fragments of the heart of a twelve day chick embryo were placed in a medium consisting of 1 part of plasma obtained from the passive immunized chicken (ex-

TABLE 6

*Tissue: Fragments of the heart muscle of a twelve day old chick-embryo*

| EXPERIMENT | ORIGINAL CULTURES | SUB-CULTURES |
|---|---|---|
| 1 | +++ | +++ |
| 2 | +++ | +++ |
| 3 | +++ | +++ |
| 4 | +++ | +++ |
| 5 | +++ | +++ |
| 6 | +++ | +++ |
| 7 | +++ | +++ |
| 8 | +++ | +++ |
| 9 | +++ | +++ |
| 10 | +++ | +++ |
| Control A 11 | +++ | +++ |
| Control A 12 | +++ | +++ |
| Control B 13 | +++ | – |
| Control B 14 | +++ | – |

periment 6) forty-eight hours after injection of the diphtheria antitoxin + 1 part of 0.9 per cent NaCl solution, original cultures.

After forty-eight hours the fragments were transplanted to a medium consisting of 1 part of normal plasma + 1 part of a 1 per cent diphtheria toxin in 0.9 per cent NaCl solution. After twenty-four hours the fragments were again transplanted into fresh drops of the same medium. This was continued until five subcultures had been made.

The original cultures of control A, cultures 13 to 19 were prepared the same as those in the experiment. The medium

used in the subcultures was different. It consisted of normal plasma 1 part + 0.9 per cent NaCl solution 1 part.

TABLE 7

*Tissue: Fragments of the heart muscle of a twelve day old chick-embryo*

| MEDIUM | ORIGINAL CULTURES | | SUB-CULTURES GROWTH | | | | |
|---|---|---|---|---|---|---|---|
| | Number | Growth | | | | | |
| Original cultures immune chicken plasma, 1 part plus 1 part of a 0.9 per cent solution of NaCl | 1 | + | + | + | + | + | − |
| | 2 | + | + | + | + | + | − |
| | 3 | + | + | + | + | − | − |
| | 4 | + | + | + | + | + | − |
| | 5 | + | + | −* | | | |
| | 6 | + | + | + | + | − | − |
| Subcultures, normal chicken plasma 1 part plus 1 part of a 1 per cent diphtheria toxin in 0.9 per cent NaCl solution | 7 | + | −* | | | | |
| | 8 | + | + | + | + | − | − |
| | 9 | + | + | + | + | + | − |
| | 10 | + | + | −* | | | |
| | 11 | + | + | + | + | + | − |
| | 12 | + | + | + | + | + | − |
| *Control culture A* | 13 | + | + | + | + | + | + |
| | 14 | + | + | + | −* | | |
| Original culture immune chicken plasma 1 part plus 1 part of 0.9 per cent solution of NaCl | 15 | + | + | + | + | + | + |
| | 16 | + | + | + | + | + | + |
| Subculture, normal chicken plasma, 1 part plus 1 part of a 0.9 per cent solution of NaCl | 17 | + | + | + | + | + | + |
| | 18 | + | + | + | −* | | |
| | 19 | + | + | + | + | + | + |
| *Control culture B* | 20 | + | − | − | − | | |
| Original culture normal, chicken plasma, 1 part plus 1 part of a 0.9 per cent solution of NaCl | 21 | + | − | − | − | | |
| | 22 | + | − | − | − | | |
| Subculture, normal chicken plasma 1 part plus 1 part of a 1 per cent diphtheria toxin in 0.9 per cent NaCl solution | 23 | + | − | − | − | | |
| | 24 | + | − | − | − | | |

* Culture is contaminated with bacteria.

In the original cultures of control B cultures 20 to 24 a medium of normal plasma 1 part + 0.9 per cent NaCl solution was used. The fragments from these cultures were transplanted in the same medium used in the subcultures of the experiment, cultures 1 to 12.

No growth was seen in any of the subcultures of control B. No growth was seen in any of the fifth series of subcultures of the experiment. The cells on the other hand were growing actively in the fifth subculture of control A, cultures 13 to 19. Burrows and Neymann (2) find that cells grow actively from fragments of heart muscle of twelve-day chick embryos through from 6 to 8 transplants in a medium of normal plasma 1 part + 0.9 per cent NaCl solution when the transplants are made seventy-two hours apart. Growth ceases after this time.

Table 8 gives the results of a series of experiments very similar to those given in table 7. The same normal and immune plasmata were used. The experiment differs in that different quantities of diphtheria toxin were added to the different cultures. The growth of the subcultures is alone indicated. Controls A and B were prepared in exactly the same manner as those in experiment 7, table 7.

By these experiments it is possible to show that the tissue cells of chick-embryos are able to resist otherwise lethal doses of diphtheria toxin after they have remained a short time in the plasma of a passively immunized chicken. It was interesting that the cells ceased to grow in the cultures as quickly when small amounts of toxin are added to the medium as when larger amounts are added.

In the next series of experiments it became of interest to ascertain whether it is possible to study by this method the distribution of the antitoxin substances in the animal.

Two young chickens five weeks old were selected. They were pullets of one hatching. They were selected from twenty of the hatching and were as nearly alike as one could obtain chickens. Into the wing vein of one of them 2 cc. of diphtheria antitoxin was injected. The other was used as control. The injected chicken is designated A. The control B. After forty-eight hours blood was taken from each and plasmata A and B respectively were prepared. A piece of artery was also taken from each of the chickens, tissue A and B respectively.

About fragments of the arteries of this age embryo the cells commence to grow only after a considerable latent period. In

TABLE 8

*Tissue: Fragments of the heart muscle of an eleven day chick-embryo*

*Medium: Original culture not indicated in the table. Plasma obtained from an adult chicken which has received forty-eight hours previously 2 cc. of diphtheria antitoxin into its wing vein. Plasma, 1 part; 0.9 per cent NaCl solution, 1 part. In subcultures indicated in the table: normal chicken plasma, 1 part; various dilutions of diphtheria toxin in 0.9 per cent; NaCl solution, 1 part*

*Medium: Control A. Original culture not indicated in the table. Plasma prepared from the blood of the passively immunized chicken, 1 part; 0.9 NaCl solution, 1 part; subcultures: Normal chicken plasma, 1 part; 0.9 per cent NaCl solution, 1 part*

*Medium: Control B. Original culture not indicated in the table, normal plasma, 1 part; 0.9 per cent NaCl solution, 1 part. Subcultures: Normal plasma, 1 part; 1 per cent diphtheria toxin in 0.9 per cent NaCl solution, 1 part*

| EXPERIMENT | GROWTH OF SUB-CULTURES | | | | |
|---|---|---|---|---|---|
| | Number of times toxin is diluted | | | | |
| | 1 | 2 | 3 | 4 | 5 |
| 100 | + | + | + | − | − |
| | + | + | + | + | − |
| 200 | + | + | + | + | − |
| | + | + | + | + | − |
| 400 | + | + | + | −* | |
| | + | + | + | + | − |
| 600 | + | + | + | + | − |
| | + | + | + | + | − |
| 1000 | + | + | + | + | − |
| | + | + | + | + | − |
| Control A. No toxin | + | + | + | + | + |
| | + | + | + | + | + |
| Control B. 100 | + | − | − | | |
| | + | − | − | | |

the table (table 9) the growth was recorded each day for a period of three days.

In the first series of cultures, no. 1, table 9, fragments of tissue A were planted in a medium consisting of 1 part of plasma A +

1 part of 0.9 per cent NaCl solution control, or 1 part of 0.9 per cent NaCl solution containing varying amounts of diphtheria toxin. On the third day the cells were growing actively in all these cultures; 2 cultures were made in each of the media tested.

In the second series no. 2 fragments of tissue A were tested in a medium containing 1 part of plasma B + 1 part of 0.9 per cent NaCl solution or 1 part of a 0.9 per cent NaCl solution contain-

TABLE 9

| MEDIUM | GROWTH AFTER | NUMBER OF TIMES DILUTED | | | | | CONTROL |
|---|---|---|---|---|---|---|---|
| | | 100 | 500 | 1000 | 3000 | 5000 | |
| | *days* | | | | | | |
| (1) Toxin is added to plasma A and used in culture containing tissue A | 1 | − −* | − − | − − | − − | − − | − − |
| | 2 | ± + | ++ + | + ± | ± − | + ± | + + |
| | 3 | + ++ | ++ + | +++ + | + ± | + + | ++ ++ |
| (2) Toxin is added to plasma B and used in culture containing tissue A | 1 | − − | − − | − − | − − | − − | − − |
| | 2 | − − | − ± | + ++ | − + | − + | + ± |
| | 3 | − − | − + | + ++ | ± + | − + | ++ + |
| (3) Toxin is added to plasma B and used in culture containing tissue B | 1 | − − | − − | − − | − − | − − | − − |
| | 2 | − − | − − | − − | − − | − − | + + |
| | 3 | − − | − − | − − | − − | − − | ++ ++ |

* The cells from the fragments of the arteries of this aged chicken grow slowly.

ing varying amounts of diphtheria toxin. The growth of the cells of tissue A was not active in the presence of plasma B and toxin. No growth is seen where as much as 1 per cent of toxin is contained in the medium.

In the third series of experiments no. 3 tissue B was tested in the same manner in plasma B. No growth was noted except in the control cultures.

A second series of experiments of this kind was also performed, table 10. In this series two young chickens similar to those used in the other were obtained. Into the wing vein of one of them

0.5 cc. of diphtheria antitoxin was injected. This chicken was designated as C. The other chicken D was kept for control. Blood together with a piece of artery was taken from each after forty-eight hours and 4 series of cultures were prepared. In the first series no. 1 the tissues C were tested in plasma C, 1 part + 0.9 per cent NaCl solution 1 part or 0.9 per cent NaCl solution which contain varying amounts of diphtheria toxin as indicated in the table. In the second series no. 2 fragments of tissue D were tested in a medium consisting of 1 part of plasma C 1 part of

TABLE 10

| | GROWTH AFTER | NUMBER OF TIMES DILUTED | | | | | CONTROL |
|---|---|---|---|---|---|---|---|
| | | 100 | 500 | 1000 | 3000 | 5000 | |
| | *days* | | | | | | |
| (1) Toxin is added to plasma "C" and used in cultures containing tissue C | 1 | − − | − − | − − | − − | − − | − − |
| | 2 | ⊥ − | ⊥ − | + ⊥ | ⊥ + | + + | + + |
| | 3 | + ⊥ | + + | + + | + + | + + | + + |
| (2) Toxin is added to plasma "C" and used in cultures containing tissue D | 1 | − − | − − | − − | − − | − − | − − |
| | 2 | ⊥ ⊥ | − ⊥ | − ⊥ | − ⊥ | ⊥ + | ⊥ + |
| | 3 | + + | − + | − + | − + | + + | + + |
| (3) Toxin is added to plasma "D" and used in cultures containing tissue C | 1 | − − | − − | − − | − − | − − | − − |
| | 2 | − − | − ⊥ | − ⊥ | − − | − + | + ⊥ |
| | 3 | − − | − + | − + | − − | + + | + + |
| (4) Toxin is added to plasma "D" and used in cultures containing tissue D | 1 | − − | − − | − − | − − | − − | − − |
| | 2 | − − | − − | − − | − − | − − | ⊥ + |
| | 3 | − − | − − | − − | − − | − − | + + |

⊥, Only a few cells are seen to grow.

0.9 per cent NaCl solution or 1 part of 0.9 per cent NaCl solution which contained varying amounts of diphtheria toxin. In the third series of experiments no. 3 tissue C was tested in a medium similar to the others but which contained plasma D. In the fourth series no. 4, tissue D was tested in a media containing plasma D.

Active growth is seen in no. 1 and good growth is seen in no. 2. In no. 3 active growth is seen only when very small amounts of toxin are added to the medium. No growth is seen in no. 4. The growth is active in the controls of all 4 series.

After the results of these experiments had been obtained the question arose, "Do the cells of passively immunized animals have any increase resistances to the toxin." Is not the slight increased resistance which they show due to the plasma which is

TABLE 11

*Tissue: Fragments of the artery of chickens A and B cut into very small pieces and teased apart with needles until single cells are liberated. These are washed three times in 0.9 per cent NaCl solution*

*Medium: Plasmata prepared from the blood of chickens A and B. One of the plasmata, 1 part; various dilutions of toxin in 0.9 per cent NaCl solution and cell emulsion, 1 part*

*Control medium: The corresponding plasma, 1 part; 0.9 per cent NaCl solution and cell emulsion, 1 part*

| TISSUE | MEDIUM | TOXIN NUMBER OF TIMES DILUTED | | | | | | | CONTROL | REMARKS |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 20 | 100 | 500 | 1000 | 2000 | 6000 | 10000 | | |
| A | A | − | ± | ++ | ++ | − | +++ | | ++ | Fragments |
| | | − | ± | − | − | − | − | | − | |
| | | − | − | − | + | − | + | | + | Single cells |
| | | − | + | − | + | + | + | | + | |
| A | B | − | − | ± | ++ | + | − | | − | Fragments |
| | | − | − | − | ± | ± | +±± | | ++ | |
| | | − | − | − | + | − | + | | + | Single cells |
| | | − | − | − | + | + | − | | + | |
| B | A | ++ | − | ++ | + | + | − | | + | Fragments |
| | | − | + | ++ | ± | + | + | | − | |
| | | + | − | + | + | + | − | | ± | Single cells |
| | | − | + | ± | − | + | + | | + | |
| B | B | − | − | − | − | − | ± | + | − | Fragments |
| | | − | − | − | − | − | + | − | +++ | |
| | | − | − | − | − | − | − | + | + | Single cells |
| | | − | − | − | − | − | + | + | + | |

possibly retained in the fragments. To prove this it was necessary to study the reaction of carefully washed single cells and very small fragments.

In experiment 11 two chickens similar to those used in 9 and which had been treated in similar manner were selected. The

experiment was performed in the same manner except that single cells or very small fragments which had been washed once or as many as three times in 0.9 per cent NaCl solution were tested. The method of washing described in the preceding paper (Suzuki) (1).

The chickens are designated as A and B respectively. They were one and one-half months old. A weighed 340 grams, B weighed 345 grams. A received forty-eight hours previous to the taking of plasma and tissue 1 cc. of diphtheria antitoxin into a wing vein.

The results of this experiment is given in table 11. As indicated in the table it has ben demonstrated that both the plasma and the cells of animals injected previously with diphtheria antitoxin have a greater resistance to diphtheria toxin than normal chicken tissue and plasma.

## CONCLUSIONS

Thus it has been possible to show that the tissue culture has a very definite value for the study of toxic and antitoxic substances. It broadens the possibility for the investigation of these substances and their properties. One toxin has alone been investigated. A study of this one has indicated, however, the value of the method for determining the neutralizing power of a plasma for any given toxin. Chicken tissues have been used in these experiments. The tissues of other animals may be similarly investigated.

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# THE STUDY OF PROBLEMS OF IMMUNITY BY THE TISSUE CULTURE METHOD

## I. A STUDY OF THE CELLS AND BLOOD PLASMA OF ANIMALS WHICH ARE NATURALLY RESISTANT AND OTHERS WHICH ARE SUSCEPTIBLE TO DIPHTHERIA AND TETANUS TOXINS.

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Although it has been appreciated for a long time that the tissue culture method may be used for solving many problems in immunity and infection only a few of those problems for which it is particularly suited have so far been studied. A few years ago Drs. Coca and Burrows undertook to ascertain by this method whether the natural immunity of the rat for diphtheria toxin is due to peculiarities of the cells of these animals or whether it is due to the existence of substances present in the serum, blood plasma or generally distributed throughout the body of the animal. Owing to circumstances which arose this work was not completed. Dr. Burrows asked me to undertake this problem and I am indebted to him for the general method of experimentation used and the preparation of the manuscript.

If we are to come to a definite and comprehensive understanding of the true nature of toxins and antitoxic substances we must accumulate more careful quantitative data concerning the physical-chemical properties of these substances. Consequently, a study of the distribution of protective substances in the organism will aid in this direction. The tissue culture method allows such studies. In the present paper the author wishes to describe a method for determining the presence and studying the properties

[1] This work was commenced in the Pathological Laboratory, Johns Hopkins University and completed in this laboratory.

of the protective substance in animals that are naturally immune and in those that are susceptible to bacterial and other toxins.

Rats are resistant even to large doses of diphtheria toxin while chickens and guinea-pigs are susceptible. The connective tissue cells of rats grow readily in their own plasma and in the plasma of guinea-pigs and chickens. In turn the connective tissue cells of chickens and guinea-pigs grow readily in their own plasma and in the plasma of either of the other two animals respectively.

It was thus possible, by the addition of varying quantities of diphtheria toxin to the various plasmata, to test the relative resistance of the tissue cells of each of these three animals in each of the three plasmata.

It has been shown that the growth of cells in the tissue culture is proportional to the size of fragments provided the fragments are not greater than 1 mm. in diameter. The growth is also proportional to the cellular content of the fragments. This proportion to the cellular content means not only the number of cells per unit area in the fragment but also the kinds of cells present. Again the cells of embryos, foetuses and young animals grow more readily than those of adults. The growth not varies only with these factors but also with the amount of fibrinogen present in the plamatic medium. Diluting the plasma with a liquid substance causes variations in the growth (Burrows (1) ).

For this reason the control cultures in these experiments were plasma diluted one-half with isotonic sodium chloride solution. The toxin was diluted in isotonic NaCl solution and one part of diluted toxin added to one part of plasma was the medium in the experiments. In each case a tissue has been selected from which a large number of fragments of similar cellular content can be obtained.

The toxin used in these experiments was obtained from the Board of Health of New York City. A few of the samples contained preservative and others did not. All toxins were tested for the presence of bacteria. Fresh toxin was used and it was filtered when it arrived in the laboratory and it was handled at all times most carefully so that it did not become contaminated

with bacteria. A large number of the various bacteria that might easily gain entrance, grow readily in the cultures and prevent the growth of the cells. These when present can, however, be detected and need not lead to error. The cultures were made by placing small fragments of tissue 1 mm. in diameter in a drop of liquid medium which was spread over a small part of

TABLE 1

*Tissue: Fragments of the heart muscle of rats, one to five days old*
*Medium: Plasmata prepared from the blood of adult rats, guinea-pigs, and chickens. One of the plasmata, 1 part; diphtheria toxin pure or diluted in 0.9 per cent NaCl solution, 1 part*
*Control medium: The plasma corresponding to the experiment, 1 part; 0.9 per cent NaCl solution, 1 part*

| MEDIUM | TOXIN—NUMBER OF TIMES DILUTED* | | | | | | | | | | | | | | | | | | | | CONTROL | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | 5 | | 10 | | 20 | | 50 | | 80 | | 100 | | 150 | | 200 | | 250 | | 300 | | | |
| | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B |
| Rat plasma..... | − | − | − | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | − | − | − | − | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | − | − | + | − | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | − | − | − | − | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Guinea-pig plasma | − | − | − | − | − | − | − | − | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | − | − | − | − | − | − | − | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | − | − | − | − | − | − | − | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Chicken plasma | − | − | − | − | − | − | − | − | − | − | − | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | − | − | − | − | − | − | − | − | − | − | − | ± | + | + | + | + | + | + | + | + | + | + | + | + |
| | − | − | − | − | − | − | − | − | − | − | ± | − | + | + | + | + | + | + | + | + | + | + | + | + |

*The toxin dilution, as indicated in the tables, is the dilution of the toxin in the salt solution. The absolute dilution in the medium is two times this amount.

the surface of a cover glass. The drop of medium was spread so that it formed a layer 0.5 mm. in thickness. A hollow ground slide previously rimmed with vaseline was immediately inverted over the cover glass so that the fragment and medium lay in the closed hollow air chamber. After the medium had clotted the

slide was inverted and the cover was rimmed with paraffin to hold it firmly to the slide and to make the sealing more complete. The slides were incubated at the temperature most suitable for the tissue which is being investigated. The cultures containing chicken tissue were incubated at 39.4°C. while those containing the tissues of rats and guinea-pigs were placed in an incubator carefully regulated at 37°C.

TABLE 2

*Tissue: Fragments of the heart muscle of young guinea-pigs, varying in age from one to five days, and fragments of the ovaries of adult guinea-pigs*

*Medium: Plasmata prepared from the blood of adult guinea-pigs and rats. One of the plasmata, 1 part; diphtheria toxin pure or diluted in 0.9 per cent NaCl solution, 1 part*

*Control medium: 0.9 per cent NaCl solution, 1 part; the plasma corresponding to the experiment, 1 part*

| MEDIUM | TISSUES | TOXIN—NUMBER OF TIMES DILUTED | | | | | | | | | | | | | | | | | | | | | | CONTROL | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | | 5 | | 10 | | 20 | | 50 | | 80 | | 100 | | 150 | | 200 | | 250 | | 300 | | | |
| | | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B |
| Guinea-pig plasma | Ovary | − | − | − | − | − | − | − | − | − | − | − | − | + | + | + | + | + | + | + | + | + | + | + | + |
| | Heart | − | − | − | − | − | − | − | − | − | ± | − | ± | + | + | + | + | + | + | + | + | + | + | + | + |
| | Ovary | − | − | − | − | − | − | − | − | − | − | − | − | + | + | + | + | + | + | + | + | + | + | + | + |
| | Heart | − | − | − | − | − | − | − | − | − | − | − | ± | + | + | + | + | + | + | + | + | + | + | + | + |
| Rat plasma | Ovary | − | − | − | − | − | − | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | Heart | − | − | − | − | ± | ± | ± | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | Heart | − | − | − | − | ± | − | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | Ovary | − | − | − | − | − | − | ± | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |

In all these experiments the tissues of young animals or foetuses have been used. The particular tissues tested have been heart muscle and ovaries. The plasma was prepared from the blood of adult animals by the method described by Burrows (2).

In the first series of experiments the growth of cells from fragments of heart muscle of young rats were tested in the plasma of rats, guinea-pigs and chickens to which isotonic NaCl solution had been added in the proportion of one part of NaCl solution

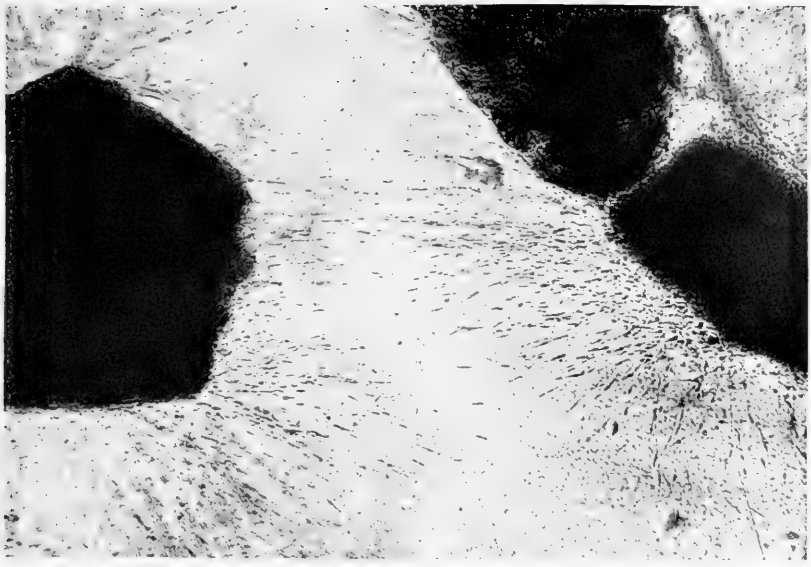

Fig. 1. Cells Growing from Fragments of the Heart Muscle of a One Day Old Rat in Rat's Plasma Containing 50 Times Diluted Diphtheria Toxin

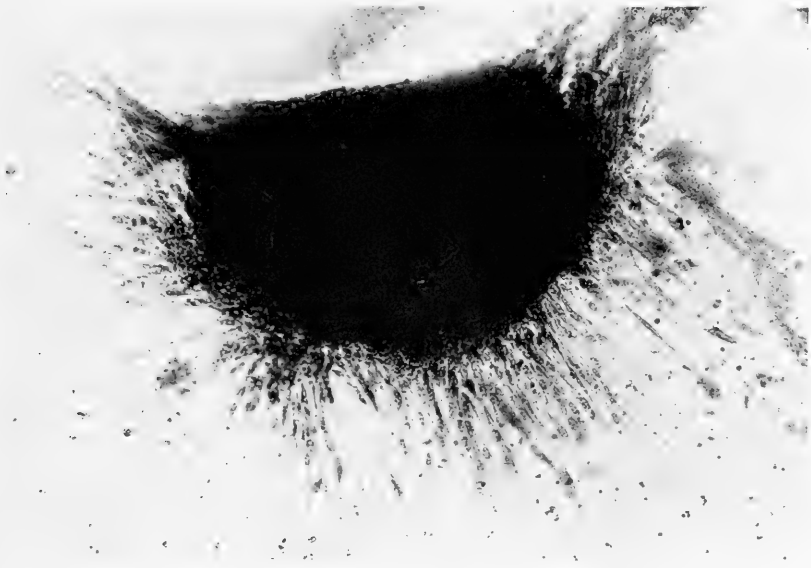

Fig. 2. Cells Growing from a Fragment of the Ovary of a Half Grown Guinea-Pig in the Plasma of a Guinea-Pig Containing 200 Times Diluted Diphtheria Toxin

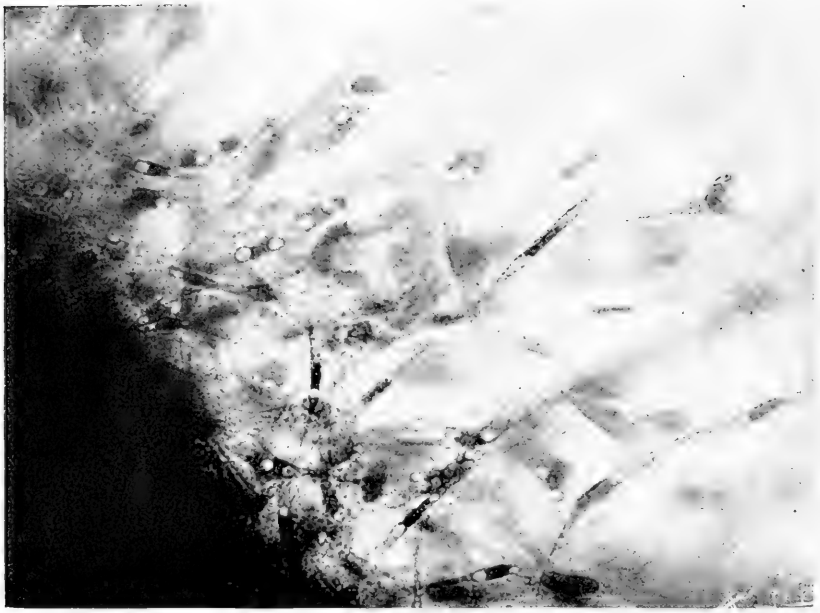

FIG. 3. CELLS GROWING FROM A FRAGMENT OF THE HEART MUSCLE OF A TWELVE DAY OLD CHICK-EMBRYO IN THE PLASMA OF A CHICKEN CONTAINING 100 TIMES DILUTED TETANUS TOXIN

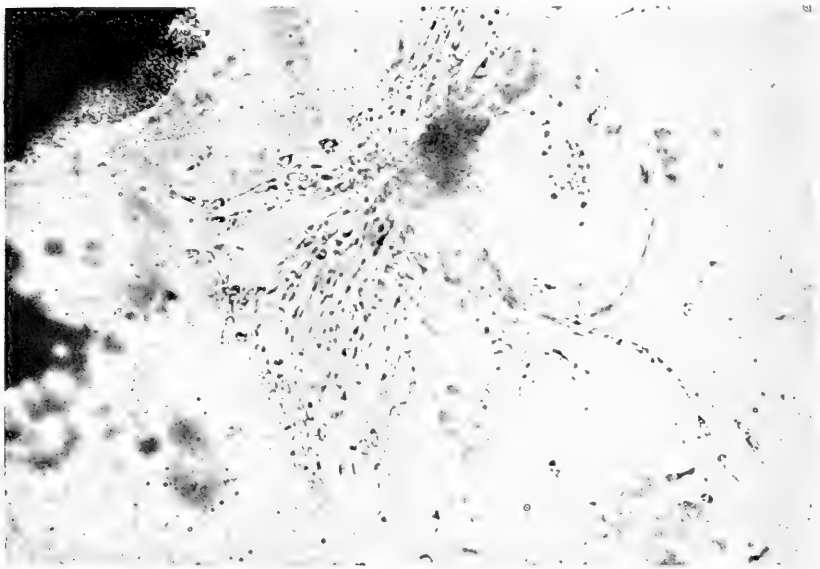

FIG. 4. CELLS GROWING FROM A VERY SMALL FRAGMENT OF THE OVARY OF A GUINEA-PIG IN THE PLASMA OF A GUINEA-PIG ONE PART PLUS ONE PART OF A 0.9 PER CENT NaCl SOLUTION

to one part of plasma and in the plasma of these same animals diluted with equal parts of the various dilutions of diphtheria toxin, table 1. The sign − indicates that no growth of cells is noted in the cultures. The cells of the fragment are dead. The sign ∸ indicates that only a few cells are noted to grow. The sign + indicates a good growth of cells (fig. 1). In many of

TABLE 3

*Tissue: Fragments of the heart muscle of fifteen and sixteen day old chick embryos*
*Medium: Plasmata prepared from the blood of adult chickens and rats. One of the plasmata, 1 part; diphtheria toxin pure or diluted in 0.9 per cent NaCl solution, 1 part*
*Control medium: 0.9 per cent NaCl solution, 1 part; the plasma corresponding to the experiment, 1 part*

| MEDIUM | TOXIN—NUMBER OF TIMES DILUTED | | | | | | | | | | | | | | | | | | | | | | CONTROL | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | 5 | | 10 | | 20 | | 50 | | 80 | | 100 | | 150 | | 200 | | 250 | | 300 | | | |
| | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B |
| Chicken plasma. | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | + | + |
| | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | + | + |
| | TOXIN—NUMBER OF TIMES DILUTED | | | | | | | | | | | | | | | | | | | | | | | |
| | 1000 | | 2000 | | 3000 | | 4000 | | 5000 | | | | | | | | | | | | | | | |
| | − | − | − | − | ∸ | + | ∸ | + | + | + | | | | | | | | | | | | | + | + |
| | − | − | − | − | − | ∸ | + | ∸ | + | + | | | | | | | | | | | | | + | + |
| Rat plasma..... | − | − | − | − | − | − | − | − | − | − | − | − | + | + | + | + | + | + | + | + | + | + | + | + |
| | − | − | − | − | − | − | − | − | ∸ | + | + | + | + | + | + | + | + | + | + | ∸ | + | + | + | + |
| | − | − | − | − | − | − | − | − | ∸ | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | − | − | − | − | − | − | − | − | ∸ | ∸ | + | + | + | + | + | + | + | + | + | + | + | + | + | + |

the cultures where larger amounts of toxin were used the cells disintegrated after a short period of active growth. This did not take place in the control cultures (fig. 4) and in the cultures marked +.

In table 2 guinea-pig tissue, heart muscle and ovaries (fig. 2), were tested. The plasmata used in these experiments were prepared from the blood of adult guinea-pigs and rats. The dilu-

tions of toxin and plasma and the method of notation are the same as those used in the table indicating the results of experiments with rat tissue, table 1.

In the third series of experiments, recorded in table 3, the growth of cells from fragments of heart muscle of fifteen and sixteen day chick embryos was studied in the plasma of chickens and rats.[3]

TABLE 4

*Tissue: Fragments of the heart muscle of chick embryos fourteen to sixteen days old*
*Medium: Plasmata prepared from the blood of adult rats, guinea-pigs and chickens. One of the plasmata, 1 part; tetanus toxin pure or diluted in 0.9 per cent NaCl solution, 1 part*
*Control medium: Corresponding plasma, 1 part; 0.9 per cent NaCl solution, 1 part*

| MEDIUM | TOXIN—NUMBER OF TIMES DILUTED | | | | | | | | | | | | | | | | | | | | | | CONTROL | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | 5 | | 10 | | 20 | | 50 | | 80 | | 100 | | 150 | | 200 | | 250 | | 300 | | | |
| | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B |
| Chicken plasma. | − | − | + | + | + | + | + | + | + | + | + | + | + | + | | | | | | | | | + | + |
| | − | − | + | + | + | + | + | + | + | + | + | + | + | + | | | | | | | | | + | + |
| | − | − | + | + | + | + | + | + | + | + | + | + | + | + | | | | | | | | | + | + |
| | − | − | + | + | + | + | + | + | + | + | + | + | + | + | | | | | | | | | + | + |
| Guinea-pig plasma..... | − | − | − | − | ± | + | + | + | + | + | + | + | + | + | | | | | | | | | + | + |
| | − | − | − | − | − | + | + | + | + | + | + | + | + | + | | | | | | | | | + | + |
| | − | − | − | − | ± | + | + | + | + | + | + | + | + | + | | | | | | | | | + | + |
| Rat plasma..... | − | − | − | − | − | ± | + | + | + | + | + | + | + | + | | | | | | | | | + | + |
| | − | − | − | − | − | − | + | + | + | + | + | + | + | + | | | | | | | | | + | + |
| | − | − | − | − | − | ± | + | + | + | + | + | + | + | + | | | | | | | | | + | + |
| | − | − | − | − | − | + | + | + | + | + | + | + | + | + | | | | | | | | | + | + |

[3] The amount of diphtheria toxin necesary to kill the cells of chickens when added in NaCl solution and chicken plasma varies with the age of the embryos used. Heart muscle was used in all these experiments. In the particular experiment table 1 fifteen and sixteen day chick embryos were used. In other experiment where fragments of the heart muscle of eleven and fourteen day chick embryos have been used the cells resisted as much as $\frac{1}{3000}$ part of toxine. The younger enbryonic tissue resists a greater dose of toxine than do the older ones.

Burrows and Neymann in their experiments with amino acids used heart muscle from the young embryo. They found it killed readily. Recently they have made many experiments with these tissues and the larger number have shown that the tissues of young embryos are more resistant. (Unpublished notes, Burrows and Neymann.)

In each experiment and its control, fragments of the same organ and the same plasma were used. Fragments of the same heart were used in corresponding experiments with rat and chicken plasma.

The first table shows definitely that rat tissue resists, in its own plasma, a greater amount of diphtheria toxin than it does in guinea-pigs plasma and a still greater amount than it does in

TABLE 5

*Tissue: Fragments of the heart muscle of rats one to five days old*
*Medium: Plasmata prepared from the blood of adult rats, guinea-pigs and chickens. One of the various plasmata, 1 part; tetanus toxin pure or diluted in 0.9 per cent NaCl solution, 1 part*
*Control medium: The plasma corresponding to the experiment, 1 part; 0.9 per cent NaCl solution, 1 part*

| MEDIUM | TOXIN—NUMBER OF TIMES DILUTED | | | | | | | | | | | | | | | | | | | | | | CONTROL | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | 5 | | 10 | | 20 | | 50 | | 80 | | 100 | | 150 | | 200 | | 250 | | 300 | | | |
| | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide A |
| Rat plasma..... | − | − | − | − | − | + | + | + | + | + | + | + | + | + | + | + | | | | | | | + | + |
| | − | − | − | − | − | − | + | + | + | + | + | + | + | + | + | + | | | | | | | + | + |
| | − | − | − | − | − | − | + | − | + | + | + | + | + | + | + | + | | | | | | | + | + |
| Guinea-pig plasma..... | − | − | − | − | − | − | − | − | − | − | − | − | + | + | + | + | + | + | + | + | + | + | + | + |
| | − | − | − | − | − | − | − | − | − | − | − | − | + | + | + | + | + | + | + | + | + | + | + | + |
| | − | − | − | − | − | − | − | − | − | − | − | − | + | + | + | + | + | + | + | + | + | + | + | + |
| Chicken plasma. | − | − | + | + | + | + | + | + | + | + | + | + | + | + | + | + | | | | | | | + | + |
| | − | − | − | − | + | + | + | + | + | + | + | + | + | + | + | + | | | | | | | + | + |
| | − | − | − | + | + | + | + | + | + | + | + | + | + | + | + | + | | | | | | | + | + |

chicken's plasma. Rat plasma, tables 2 and 3, not only protects the cell of rats but likewise it protects the cells of guinea-pigs and chickens. Guinea-pig cells grow in a medium containing one part of rat plasma and one part of NaCl solution with 40 times diluted diphtheria toxin. In their own plasma they can resist no more than 200 times diluted toxin. Chicken cells show active growth in plasma of rats one part plus NaCl solution containing toxin 200 times diluted. In its own plasma the chicken's

tissue used in one of the experiments showed growth only when the toxin was diluted 5000 times.

In the same way the rat tissue survived in its own plasma a large dose of toxin; it was least resistant in the plasma of the chickens, the animal most susceptible to this poison.

Tables 4 and 5 indicate the results of a study of effect of tetanus toxin on the growing cells, Fig. 3. The experiments were performed in exactly the same manner as those recorded in tables 1, 2, 3 except for the substitution of the tetanus toxin.

Chickens are resistant to tetanus toxin. In a like manner as the rat plasma protected the susceptible chicken and guinea-pigs cells against large doses of diphtheria toxin so chicken plasma protects the cells of the rat and guinea-pig against a considerable dosage of tetanus toxin. Again, chicken tissue is less resistant to large doses of tetanus toxin when cultivated in a plasma of a less resistant animal than when it is cultivated in its own plasma.

## EXPERIMENTS TO DETERMINE CELLULAR RESISTANCE

It is true that the tissues of none of these animals grow quite so actively in the foreign plasma as in their own plasma. These variations are not great. The arrangement of the experiments controls for variations of this kind.

Although this method is suitable for demonstrating the presence of protective substances in the plasma of an animal it is not an accurate method for determining the relative resistance of the cells. Tissue fragment such as the ones used in the experiments must also contain plasma which in the case of the resistant animal must protect the cells of that animal. Before one may determine the actual resistance of the individual cells these cells must be studied after they have been washed free from all external fluids. Burrows has noted that individual cells do not grow in a tissue culture. In the tissue culture one cell grows at the expense of another one disintegrating. On the other hand isolated connective tissue cells may show movement and thus comparable metabolic activities. These cells live and move through the utilization of substances stored within them (3).

TABLE 6

*Tissue:* *Fragments of the heart muscle and subcutaneous tissue of fifteen and sixteen day old chick-embryos*

*Medium:* *Plasma prepared from the blood of an adult chicken, 1 part; various dilutions of diphtheria toxin in 0.9 per cent NaCl solution, 1 part*

*Control medium:* *The plasma corresponding to the experiment, 1 part; 0.9 per cent NaCl solution, 1 part*

| MEDIUM | TISSUE | | TOXIN—NUMBER OF TIMES DILUTED | | | | | | | | | | | | | | | | | | | | | | | | CONTROL | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 600 | | 1000 | | 2000 | | 4000 | | 6000 | | 8000 | | 10000 | | 12000 | | 14000 | | 16000 | | 18000 | | 20001 | | | |
| | | | A | B | A | B | A | B | A | B | A | B | A | B | A | B | A | B | A | B | A | B | A | B | A | B | A | B |
| Fifteen days chick embryo. Heart muscle | Washed 3 times in salt solution | F | − | − | − | − | − | − | − | − | + | + | | | | | | | | | | | | | | | + | + |
| | | S | − | − | − | − | − | − | − | − | + | − | | | | | | | | | | | | | | | + | + |
| | Washed once | F | − | − | − | − | − | − | − | − | + | + | | | | | | | | | | | | | | | + | + |
| | | S | − | − | − | − | − | − | − | − | + | + | | | | | | | | | | | | | | | + | + |
| Sixteen days chick embryo. Heart muscle | Washed 3 times | F | | | − | − | − | − | − | − | − | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | | S | | | − | − | − | − | − | − | − | − | − | − | − | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | Washed once | F | | | − | − | − | − | − | − | − | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | | S | | | − | − | − | − | − | − | − | − | − | − | + | + | + | + | + | + | + | + | + | + | + | + | + | + |

F, Fragment; S, single cells.

Another series of experiments has been performed to determine the resistance of the cells. Fragments of tissue were carefully cut up into very small pieces with sharp scissors and further teased apart with needles. This was done in salt solution. The salt solution cell mixture was centrifuged. The supernatant fluid was poured off and more salt solution added. Cells and small fragments were washed in this way one or more times.

TABLE 7

*Tissue: Fragments of the heart of a seven day old rat*

*Medium: Plasma prepared from the blood of an adult chicken, 1 part; diphtheria toxin diluted in 0.9 per cent NaCl solution, 1 part*

*Control medium: Plasma corresponding to the experiment, 1 part; 0.9 per cent NaCl solution, 1 part*

| MEDIUM | | TOXIN—NUMBER OF TIMES DILUTED | | | | | | | | | | | | CONTROL | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 200 | | 400 | | 800 | | 2000 | | 3060 | | 4000 | | | |
| | | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B |
| Tissue washed once in salt solution | Fragment | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | | + | + | + | + | + | + | + | + | + | − | + | + | + | + |
| | | − | − | − | − | − | − | + | − | + | − | + | + | + | + |
| | Single cells | + | + | + | − | + | + | + | + | + | + | + | + | + | + |
| Tissue washed 3 times in salt solution | Fragment | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | | − | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | | − | − | − | + | + | + | + | + | − | + | + | + | + | + |
| | | − | − | − | − | − | + | + | + | − | + | − | − | + | + |
| | Single cells | − | − | + | + | − | + | + | + | − | + | + | + | + | + |

The individual cells and small fragments were then added to plasma and toxin. The cellular emulsion was added to the plasma and toxin in such a way that relative proportions of plasma and toxin cell solutions remained the same as in the other experiments.

In the experiments shown in table 6 heart muscle cells and connective tissue cells of chick embryos fifteen and sixteen days of age were tested in a medium containing chicken plasma. The

experiments are recorded in the horizontal columns. Small fragments and cells washed once or as many as three times with NaCl solution were tested. The number of times the toxin was diluted in the medium used in each particular culture is indicated in the top horizontal column. The cultures were made in duplicate, A and B, respectively, and the + mark indicates

TABLE 8

*Tissue: Fragments of the heart muscle of a nineteen day chick embryo and a rat six days old*

*Medium: Plasma prepared from the blood of an adult chicken, 1 part; tetanus toxin diluted with 0.9 per cent NaCl solution, 1 part*

*Control medium: Plasma corresponding to that in the experiment, 1 part; 0.9 per cent NaCl solution, 1 part*

| MEDIUM | | | TOXIN—NUMBER OF TIMES DILUTED | | | | | | | | | | | | CONTROL | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 200 | | 400 | | 800 | | 2000 | | 3060 | | 4000 | | | |
| | | | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B | Slide A | Slide B |
| Tissue washed 3 times in salt solution | Chicken tissue 19 day chick embryo | F | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | | | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | | | − | − | + | + | + | + | + | + | + | + | + | + | + | + |
| | | S | + | + | + | + | − | + | + | + | + | + | + | + | + | + |
| | Rat tissue 6 days suckling | F | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | | | − | − | + | + | + | + | + | + | + | + | + | + | + | + |
| | | | − | − | − | − | + | + | + | + | + | + | + | + | + | + |
| | | S | − | − | + | + | + | + | + | + | + | + | + | + | + | + |

F, Fragments; S, Single cells.

where growth or movement is observed in a culture. The sign − indicates that no activity was observed.

In the experiments shown in table 7 the tissue of a young rat, heart muscle is tested, in the plasma of chickens. The experiments were performed in the same manner as those recorded in table 6.

In the experiments shown in table 8 the growth of washed chicken cells and rat cells (heart muscle) were tested in a medium containing chicken plasma and various dilutions of tetanus toxin.

## SUMMARY AND CONCLUSIONS

By these experiments it has been possible to show definitely that the natural immunity of rats for diphtheria toxin and chickens for tetanus is due to two factors—a special resistance of at least certain of the cells of these animals and further to the existence of neutralizing substances in their plasma. The plasma of these animals protects not only the cells of these animals against lethal doses of toxin but it also protects the cells of susceptible animals.

So far heart muscle and ovary or in other words connective tissue cells have alone been studied. The resistance of many other tissue may be similarly investigated; such as, nervous tissue, other epithelial tissues and lymphoid tissue.

A sufficient number of experiments have not been made to give quantitative results. The method allows this study and a further investigation into the nature of the cellular resistance and the mechanism of neutralization as indicated in the plasma tests.

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# A STUDY OF THE IMMUNIZING PROPERTIES OF BACTERIAL VACCINES PREPARED AFTER VARIOUS METHODS[1]

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It is now generally recognized and conceded that bacteria, vaccines may exert both specific and non-specific effects when administered to persons and lower animals by parenteral injection; by specific effects we refer to the production of specific antibodies as agglutinins and lysins for the bacterial protein while non-specific effects refer to the temperature and leucocytic response, the mobilization of ferments and other reactions studied and described by Jobling and Petersen (1), and others.

For purposes of therapeutic immunization bacterial vaccines are generally prepared by suspending the microörganisms in isotonic sodium chloride solution followed by a destruction of their vegetative power by heating at 56 to 60°C. for one hour. It has been claimed, however, that heating even to 53°C. tends to alter or destroy in a measure the essential antigenic immunizing or specific properties of a vaccine and various other means for their preparation has been proposed. The ideal vaccine is probably the living microörganism as little changed by artificial means as possible; Sanborn (2) in a summary of the literature on the use of killed and living vaccines, concludes that unheated vaccines have proven superior. In order to avoid the destructive influence of heat Casselman (3) and more recently Kisskalt (4) have advocated the use of phenol killed vaccines, which had previously been employed for dysentery vaccine by Gay; others have em-

[1] Aided by a grant from the Pediatric Society.

ployed ether, galactose, sodium fluoride, iodine and other chemical substances. In the preparation of antibacterial sera as antimeningococcus and antipneumococcus serum, killed cultures produce an antibody response, but more recently it has been shown that smaller doses of living microörganisms produce a quicker and more pronounced reaction and if this experience can be borne out in active immunization in man, it will appear as support for the contention that the superior vaccines are those produced with the least possible change in the bacterial protoplasm. It cannot be questioned, however, that when vaccines are prepared in such manner that the bacterial protein undoubtedly undergoes some more or less profound chemical change, as in the method of preparation advised by Loeffler, which consists in heating the cultures to 120° to 150°C. followed by drying or pulverizing, such vaccines possess some degree of antigenic activity capable of eliciting both specific and non-specific responses. Nevertheless, an important question at issue would appear to be the determination of which of the differently prepared vaccines is capable of eliciting the best specific response.

Active immunization by means of sensitized vaccines, that is to say, by cultures that have been first treated with an immune serum and then killed, was introduced by Besredka in 1902. Gay and Claypool (5), in an extended series of experiments, have studied the comparative immunizing value of a number of different vaccines of the typhoid bacillus in rabbits that were subsequently infected in such manner as to induce a carrier state among the controls, and they have found that untreated bacteria killed and precipitated by alcohol, dried, ground, and employed in weighed amounts, do not protect as well as typhoid bacilli that have been sensitized with a strong immune serum, washed and subsequently treated in the same manner; furthermore, alcohol-killed, sensitized cultures were found to protect almost as well as the living sensitized cultures of Metchnikoff and Besredka, and the sediment of alcoholic killed, sensitized cultures protected better than the living cultures. On the basis of these results Gay and Claypool have advocated the latter vaccine in the prevention and treatment of typhoid fever.

We have prepared vaccines of *B. typhosus* after various methods including living, heat-killed and chemically killed preparations and have sought to make a comparative study of their immunizing properties in rabbits by means of subcutaneous injections in doses similar to those employed among persons and according to body weight; the antibody response was studied by means of the agglutination and complement-fixation reactions and the non-specific reaction was followed by means of total leucocyte counts and temperature observations. The results of our limited studies are summarized in this communication.

## TECHNIC

All vaccines but one (alcohol-killed sensitized sediment) were prepared with the same strain of *B. typhosus*, immediately counted and diluted to provide two parts, namely, one portion containing 20,000,000 bacilli per cubic centimeter for purposes of injection and the second portion, containing 2,000,000,000 bacilli per cubic centimeter, for agglutination and complement fixation tests. The following vaccines were prepared:[2]

1. Autolysate vaccine consisting in suspending washed bacilli in sterile distilled water. After three days at room and one day at refrigerator temperature the vaccines were found to have become sterile.

2. Heat killed vaccine secured by two exposures of one hour each at 56°C.

3. Tricresol killed vaccine secured by the addition of tricresol to 0.25 per cent and incubating at 37°C. Both vaccines were found sterile at the end of twenty-four hours.

4. Mercurophen[3] killed vaccines secured by the addition of mercurophen 1:5000 and incubating at 37°C. Both vaccines were found sterile at the end of twenty-four hours.

5. Living vaccines freshly prepared as needed and in the same strengths.

[2] Attempts were also made to prepare a vaccine killed with X-rays, but complete sterilization could not be secured.

[3] Mercurophen is one of the new mercurial germicides of superior value prepared in the Dermatological Research Laboratories of the Philadelphia Polyclinic.

6. Alcohol killed sensitized sediment vaccine kindly furnished by Dr. Gay.

All rabbits were carefully weighed before each injection and given 1 cc. of the first five mentioned vaccines or 20,000,000 bacilli per kilogram of body weight, by subcutaneous injection every five days. These doses were equivalent to 1,200,000,000 bacilli per 60 kgm. or the weight of a young adult. The vaccine of alcohol-killed sensitized sediment was furnished by the California State Board of Health already suspended in such proportions that 1 cc. was the dose for an adult; the rabbits received 0.016 cc. per kilogram of body weight or the equivalent of 1 cc. per 60 kgm. of weight.

Leucocyte counts and temperature observations were generally made just before and three and twenty-four hours after the administration of the vaccines.

Agglutination tests were made at intervals during the course of immunization, the macroscopic technic being employed with incubation at 55°C. for eighteen to twenty-four hours.

In the complement fixation tests[4] each antigen was titrated just before the tests were conducted with each serum and used in amounts corresponding to one-third the anticomplementary unit. An antisheep hemolytic system was employed and the results recorded after the following scheme:

++++ = complete inhibition of hemolysis
+++ = 75 per cent inhibition of hemolysis
++ = 50 per cent inhibition of hemolysis
+ = 25 per cent inhibition of hemolysis
+ = less than 25 per cent inhibition of hemolysis
− = complete hemolysis

In conducting these tests all sera were heated at 62°C. for thirty minutes in order to prevent the tendency for non-specific fixation of complement on the part of rabbit serum as studied and emphasized by Kolmer and his associates (6) and used in increasing doses with a constant dose of antigen; after heating at this temperature all sera yielded negative Wassermann reactions.

[4] We are indebted to Miss Sara M. Levy for aid in conducting these tests.

## RESULTS

The results of a set of experiments are expressed in tables 1 to 9 and the study may be briefly summarized as follows:

*Influence upon leucocytes.* Not infrequently the various animals showed different results with the same vaccine but in general the subcutaneous injection of all of the vaccines was usually followed within three hours by a slight increase in the total number of leucocytes with a return to usual or normal numbers within twenty-four hours. As shown in table 1 the vaccine of

TABLE 1

*The influence of various vaccines upon total leucocyte counts (subcutaneous injection)*

| VACCINES | FOURTH INJECTION | | | FIFTH INJECTION | | | SIXTH INJECTION | | |
|---|---|---|---|---|---|---|---|---|---|
| | Before | Three hours after | Twenty-four hours after | Before | Three hours after | Twenty-four hours after | Before | Three hours after | Twenty-four hours after |
| Autolysate | 11400 | 11000 | 11800 | 13200 | 13600 | 13000 | 12400 | 14800 | 16000 |
| Heat killed | 12800 | 12200 | 10000 | 11800 | 16000 | 12200 | 19000 | 19000 | 19800 |
| Heat killed | 13600 | 14400 | 15000 | 14800 | 20800 | 14600 | 17600 | 13000 | 15000 |
| Tricresol-killed | 12800 | 17400 | 14000 | 14800 | 13000 | 13200 | 12200 | 14400 | 13600 |
| Tricresol-killed | 10000 | 10000 | 10600 | 8000 | 9000 | 7800 | 8800 | 6600 | 16500 |
| Mercurophen-killed | 10600 | 10000 | 13000 | 7800 | 8000 | 8400 | 9200 | 12000 | 7600 |
| Mercurophen-killed | 8200 | 20600 | 12000 | 17000 | 15000 | 12600 | 13000 | 10800 | 19600 |
| Living | 5000 | 11200 | 8600 | 10600 | 8600 | 8300 | 11600 | 9400 | 11600 |
| Living | 11600 | 18800 | 17400 | 12400 | 15200 | 14200 | 13800 | 16600 | 0 |
| Alcohol-killed; sensitized | 18600 | 26600 | 18800 | 14000 | 20400 | 14400 | 9600 | 8700 | 12200 |
| Alcohol-killed; sensitized | 11800 | 26000 | 12800 | 15000 | 19000 | 15000 | 10000 | 20000 | 20000 |

alcohol-killed sensitized sediment produced the most constant and highest alterations in the total number of leucocytes. The production of leucocytosis is a desirable reaction generally considered at the present time as a non-specific result and not characteristic of typhoid vaccine alone.

*Influence upon temperature* The temperature reactions were similar to the leucocytic disturbances in so far that the results were not regular or uniform; practically all of the vaccines produced, at some time within three hours after injection, a slight increase of temperature which had largely subsided within twenty-four

hours. As a general rule an increase of temperature occurred with an increase of leucocytes and the greatest temperature reaction was observed among the rabbits treated with the alcohol killed sensitized vaccine (table 2).

*Agglutinin production.* While the production of agglutinin and lysin is not acceptable at the present time as a strict index of actual protection, nevertheless such production may be accepted as an index of the antigenic properties of the various vaccines and the degree of specific response elicited by them. Our interest in

TABLE 2

*The influence of various vaccines upon temperature.* (Subcutaneous injection)*

| VACCINES | FOURTH INJECTION | | | FIFTH INJECTION | | | SIXTH INJECTION | | |
|---|---|---|---|---|---|---|---|---|---|
| | Before | Three hours after | Twenty-four hours after | Before | Three hours after | Twenty-four hours after | Before | Three hours after | Twenty-four hours after |
| Autolysate | 104.0 | 102.2 | 98.3 | 100.0 | 103.0 | 101.0 | 102.2 | 102.0 | 102.2 |
| Heat killed | 100.3 | 101.1 | 102.0 | 101.3 | 101.2 | 100.0 | 101.1 | 102.3 | 101.0 |
| Heat killed | 101.0 | 101.1 | 100.2 | 101.1 | 102.0 | 101.3 | 102.2 | 100.0 | 100.0 |
| Tricresol-killed | 100.3 | 100.3 | 100.0 | 102.3 | 103.3 | 102.1 | 102.4 | 103.1 | 102.2 |
| Tricresol-killed | 103.1 | 102.1 | 100.3 | 101.3 | 102.2 | 100.4 | 102.4 | 103.1 | 102.2 |
| Mercurophen-killed | 99.1 | 103.3 | 99.4 | 100.0 | 103.3 | 101.1 | 101.3 | 101.4 | 101.3 |
| Mercurophen-killed | 101.3 | 103.1 | 100.0 | 103.0 | 103.1 | 102.4 | 103.4 | 102.0 | 102.2 |
| Living | 100.0 | 100.3 | 101.1 | 100.1 | 102.0 | 100.2 | 102.3 | 101.2 | 101.4 |
| Living | 100.0 | 101.0 | 99.2 | 102.0 | 102.3 | 102.0 | 100.0 | 102.4 | 101.1 |
| Alcohol-killed; sensitized | 99.3 | 101.3 | 101.1 | 100.1 | 102.3 | 101.0 | 102.3 | 104.0 | 101.2 |
| Alcohol-killed; sensitized | 102.0 | 103.4 | 99.3 | 103.0 | 104.1 | 103.1 | 102.3 | 103.1 | 102.0 |

* Rectal.

this phase of the study was centered mainly upon the following questions: (1) which vaccine produced most agglutinin and complement fixing antibody and (2) do the antibodies stimulated by the respective vaccines possess any degree of specificity for the particular antigen (vaccine) employed, as indicating the degree of chemical alteration in the protein of the typhoid bacillus during the various processes of manufacture of the different vaccines.

As was expected, individual rabbits were found to show well marked variations in antibody response to the same vaccine administered in the same manner and in dose corresponding to the body weight of each animal. After an analysis of our results we have tentatively formulated the following answers to the above mentioned questions, the results of one series of agglutination tests being shown in table 3, as an example of the high and low values observed. For measuring the agglutinin production

TABLE 3

*Results of cross agglutination tests with immune sera after six subcutaneous injections of the various vaccines*

| IMMUNE SERA | TITER WITH VARIOUS ANTIGENS | | | | | |
|---|---|---|---|---|---|---|
| | Autolysate antigen | Heat-killed antigen | Tricresol-killed antigen | Mercurophen killed antigen | Living antigen | Formalized antigen |
| Autolysate V.* antiserum | 1:4096 | 1:2048 | 1:1280 | 1:2560(?) | 1:2560 | 1:4096 |
| Heat-killed V. antiserum | 1:256 | 1:384 | 1:192 | 1:192(?) | 1:192 | 1:512 |
| Heat-killed V. antiserum | 1:1456 | 1:2912 | 1:2912 | 1:1456 | 1:2560 | 1:2560 |
| Tricresol-killed V. antiserum | 1:1024 | 1:1024 | 1:728 | 1:728 | 1:640 | 1:1456 |
| Tricesol-killed V. antiserum | 1:1456 | 1:1280 | 1:1024 | 1:1024 | 1:1024 | 1:1456 |
| Mercurophen-killed V. antiserum | 1:2912 | 1:2560 | 1:2560 | 1:5824 | 1:2560 | 1:4096 |
| Mercurophen-killed V. antiserum | 1:512 | 1:160 | 1:192 | 1:256 | 1:384 | 1:728 |
| Living V. antiserum | 1:128 | 1:192 | 1:96 | 1:256 | 1:320 | 1:320 |
| Living V. antiserum | 1:640 | 1:192 | 1:192 | 1:256 | 1:1024 | 1:1456 |
| Alcohol-killed sensitized V. antiserum | 1:192 | 1:160 | 1:128 | 1:160 | 1:192 | 1:384 |
| Alcohol-killed sensitized V. antiserum | 1:160 | 1:96 | 1:80 | 1:96 | 1:96 | 1:192 |

* V = vaccine.

we used a formalinized culture prepared according to the Oxford University method. This method consists in cultivating the strain of *B. typhosus* employed in our study, in flasks of broth for forty-eight hours and sterilizing the culture with 0.1 per cent of commercial (40 per cent) formalin in a cold dark chamber over a period of four to five days. This antigen was found of particular value in the macroscopic agglutination test and superior to any of the other preparations, including fresh living antigen, for routine work.

An analysis of the agglutinin production in our experiments as measured with this formalinized antigen and based not upon individual animals but upon averages, shows the following:

1. The vaccines of living and autolysed typhoid bacilli produced in general the greatest amount of agglutinin; the mercurophen and tricresol killed vaccines ranked second; the heat killed vaccines were third and the alcohol-killed sensitized sediment was fourth. Numerous researches such as those of Gay and Claypool (5), Garbat (7), Liebermann and Acel (8) and others indicate that sensitized vaccines in general produce less agglutinin than the untreated vaccines, and our results indicate the same; bacteriolytic tests were not made.

2. Reference to table 3 shows that with several of the vaccines there was apparently a slight degree of specificity of the immune agglutinin for the particular vaccine antigen used in immunization, as shown by the higher agglutinin titer with the homologous antigen. These results were, however, not constant and we have ascribed them largely to individual reactions rather than to inherent alterations of the chemical nature of the antigens during the process of manufacture. The question is one of considerable importance not only theoretically but also from a practical standpoint.

*Production of complement fixing antibody.* In conducting these tests we have had the following three questions in view: (1) which form of vaccine produces most complement fixing antibody; (2) does the antibody show any tendency to specificity for the particular antigen (vaccine) employed in the immunization and (3) which vaccine yields best results as antigen in the complement fixation tests? In tables 4 to 9 are shown the results of one set of tests with sera taken after six injections of each vaccine and examples of the data upon which we have drawn tentatively the following answers to the second and third questions.

1. In a general way the amount of complement fixing antibody in each serum bore a more or less direct relation to the agglutinin content. As based upon the results of complement fixation tests employing graded doses of each serum the order of antibody response to the various vaccines was as follows (named in order

of highest to lowest): living and autolysed; mercurophen-killed; tricresol-killed; heat-killed and alcohol-killed sensitized sediment.

TABLE 4

*Results of complement fixation tests with autolysate antigen**

| IMMUNE SERA | INCREASING DOSES SERUM† | | | | |
|---|---|---|---|---|---|
| | 0.005 cc. | 0.01 cc. | 0.05 cc. | 0.1 cc. | 0.8 cc. |
| Autolysate V. antiserum | − | − | ++ | ++++ | ++++ |
| Heat-killed V. antiserum | − | − | + | ++++ | ++++ |
| Tricresol-killed V. antiserum | − | − | − | ± | ++ |
| Tricresol-killed V. antiserum | − | − | − | ± | +++ |
| Mercurophen-killed V. antiserum | − | − | ++ | +++ | ++++ |
| Mercurophen-killed V. antiserum | − | − | − | − | + |
| Living V. antiserum | − | − | − | + | ++ |
| Living V. antiserum | − | − | − | + | + |
| Alcohol-killed sensitized V. antiserum | − | − | − | − | ++ |
| Alcohol-killed sensitized V. antiserum | − | − | − | − | +++ |

* Anticomplementary unit 0.4 cc.; used in dose of 0.15 cc.
† Serum controls completely hemolysed.

TABLE 5

*Results of complement fixation tests with heat-killed antigen**

| IMMUNE SERA | INCREASING DOSES SERUM† | | | | |
|---|---|---|---|---|---|
| | 0.005 cc. | 0.01 cc. | 0.05 cc. | 0.1 cc. | 0.2 cc. |
| Autolysate V. antiserum | − | − | − | − | + |
| Heat-killed V. antiserum | − | − | − | + | ++ |
| Tricresol-killed V. antiserum | − | − | − | − | − |
| Tricresol-killed V. antiserum | − | − | − | − | − |
| Mercurophen-killed V. antiserum | − | − | − | − | − |
| Mercurophen-killed V. antiserum | − | − | − | − | + |
| Living V. antiserum | − | − | − | − | − |
| Living V. antiserum | − | − | − | − | + |
| Alcohol-killed sensitized V. antiserum | − | − | − | − | + |
| Alcohol-killed sensitized V. antiserum | − | − | − | − | + |

* Anticomplementary dose 1 cc. (1:10); dose employed 0.3 (1:10).
† Serum controls completely hemolyzed.

2. As was found with the agglutinins the complement fixing antibodies occasionally showed a slight tendency toward specificity for homologous antigen as indicated by the fixation of com-

plement with the smallest amounts of serum; these results may have been individual reactions and proved so irregular that we

TABLE 6

*Results of complement fixation tests with tricresol-killed antigen**

| IMMUNE SERA | INCREASING DOSES OF SERUM† | | | | |
|---|---|---|---|---|---|
| | 0.005 cc. | 0.01 cc. | 0.05 cc. | 0.1 cc. | 0.2 cc. |
| Autolysate V. antiserum | − | − | − | + | ++ |
| Heat-killed V. antiserum | − | − | + | + | + |
| Tricresol-killed V. antiserum | − | − | + | + | + |
| Tricresol-killed V. antiserum | − | − | − | ± | + |
| Mercurophen-killed V. antiserum | − | − | − | − | − |
| Mercurophen-killed V. antiserum | − | − | − | − | − |
| Living V. antiserum | − | − | − | − | − |
| Living V. antiserum | − | − | − | + | + |
| Alcohol-killed sensitized V. antiserum | − | − | − | ± | ± |
| Alcohol-killed sensitized V. antiserum | − | − | − | − | ± |

* Anticomplementary unit 2 cc. of 1:10; used in dose of 0.7 cc. of 1:10.
† Serum controls completely hemolysed.

TABLE 7

*Results of complement fixation tests with mercurophen-killed antigen**

| IMMUNE SERA | INCREASING DOSES OF SERUM† | | | | |
|---|---|---|---|---|---|
| | 0.005 cc. | 0.01 cc. | 0.05 cc. | 0.1 cc. | 0.2 cc. |
| Autolysate V. antiserum | − | − | − | + | +++ |
| Heat-killed V. antiserum | − | − | + | ++ | ++ |
| Tricresol-killed V. antiserum | − | − | − | + | ++ |
| Tricresol-killed V. antiserum | − | − | − | + | ++ |
| Mercurophen-killed V. antiserum | − | − | + | ++ | +++ |
| Mercurophen-killed V. antiserum | − | − | − | + | ++ |
| Living V. antiserum | − | − | − | + | ++ |
| Living V. antiserum | − | − | − | − | + |
| Alcohol-killed sensitized V. antiserum | − | − | − | + | ++ |
| Alcohol-killed sensitized V. antiserum | − | − | − | − | + |

* Anticomplementary unit 1 cc. (1:10); used in dose of 0.3 cc. (1:10).
† Serum controls completely hemolysed.

are inclined to the belief that specific antibody response to the various antigens of typhoid bacilli was not elicited.

3. In reference to the antigenic sensitiveness of the various antigens employed in the complement fixation tests the results of

our experiments show that they may be arranged as follows (named in order of highest to lowest): living; autolysed; mercu-

TABLE 8

*Results of complement fixation tests with living antigen.**

| IMMUNE SERA | INCREASING DOSES SERUM† | | | | |
|---|---|---|---|---|---|
| | 0.005 cc. | 0.01 cc. | 0.05 cc. | 0.1 cc. | 0.2 cc. |
| Autolysate V. antiserum | ± | +++ | ++++ | ++++ | ++++ |
| Heat-killed V. antiserum | ± | ++++ | ++++ | ++++ | ++++ |
| Tricresol-killed V. antiserum | − | − | +++ | ++++ | ++++ |
| Tricresol-killed V. antiserum | ± | ± | ++ | ++++ | ++++ |
| Mercurophen-killed V. antiserum | − | ++ | ++++ | ++++ | ++++ |
| Mercurophen-killed V. antiserum | − | − | + | ++++ | ++++ |
| Living V. antiserum | − | + | ++ | ++++ | ++++ |
| Living V. antiserum | − | ± | + | ++++ | ++++ |
| Alcohol-killed sensitized V. antiserum | − | + | +++ | ++++ | ++++ |
| Alcohol-killed sensitized V. antiserum | − | ± | ++ | ++++ | ++++ |

* Anticomplementary unit 2 cc.; used in dose of 0.7 cc.
† Serum controls completely hemolysed.

TABLE 9

*Results of complement fixation tests with alcohol-killed sensitized sediment antigen**

| IMMUNE SERA | INCREASING DOSES SERUM† | | | | |
|---|---|---|---|---|---|
| | 0.005 cc. | 0.01 cc. | 0.05 cc. | 0.1 cc. | 0.2 cc. |
| Autolysate V. antiserum | − | − | − | + | ++ |
| Heat-killed V. antiserum | − | − | + | +++ | ++++ |
| Tricresol-killed V. antiserum | − | − | − | − | ± |
| Tricresol-killed V. antiserum | − | − | − | ± | ± |
| Mercurophen-killed V. antiserum | − | − | ± | ± | + |
| Mercurophen-killed V. antiserum | − | − | − | − | ± |
| Living V. antiserum | − | − | ± | ± | + |
| Living V. antiserum | − | − | − | − | ± |
| Alcohol-killed sensitized V. antiserum | − | − | − | ± | + |
| Alcohol-killed sensitized V. antiserum | − | − | ± | ± | ++ |

* Anticomplementary unit 2 cc.; used in dose of 0.7 cc.
† Serum controls completely hemolyzed.

rophen-killed; tricresol-killed; alcohol-killed sensitized sediment and heat-killed. Inasmuch as bacterial antigens are quite commonly heated during the course of their preparation, the results

indicating that heat apparently destroyed in large measure the complement fixing sensitiveness of bacterial antigen are of special importance.

## CONCLUSIONS

1. All vaccines of *B. typhosus* prepared in various ways and including living, autolysed, chemical and heat-killed and alcohol-killed sensitized sediment usually produced slight leucocytosis and slight increase of temperature when administered to rabbits by subcutaneous injection in doses according to body weight and comparable to those given to persons; the alcohol-killed sensitized sediment produced these non-specific reactions in highest degree.

2. Agglutinin and complement fixing antibodies were produced in highest degree by the administration of living and autolysed vaccines followed in order by the mercurophen-killed, tricresol-killed, heat-killed and alcohol-killed sensitized sediment vaccines.

3. While the agglutination and complement fixation reactions showed in some instances a tendency toward specificity on the part of these antibodies for the particular antigen (vaccine) of *B. typhosus* responsible for their production, the results generally do not indicate definitely the development of specificity to this degree.

4. An antigen of *B. typhosus*, prepared by cultivating the microörganism in plain neutral broth for forty-eight hours and sterilizing the culture with 0.1 per cent of commercial (40 per cent) in a cold dark place over a period of four to five days, proved of particular value in macroscopic agglutination tests.

5. The various vaccines of *B. typhosus* used as antigens in the complement fixation tests may be arranged as follows in order of antigenic sensitiveness: living and autolysed; mercurophen-killed; tricresol-killed; alcohol-killed sensitized sediment and heat-killed, the latter proving least antigenic.

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# THE BACTERICIDAL ACTION OF WHOLE BLOOD, WITH A NEW TECHNIQUE FOR ITS DETERMINATION[1]

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The various efforts that have been made to produce an immunity to lobar pneumonia due to the pneumococcus have failed to present sufficient proof of the value of prophylactic inoculation in man. Artificially produced immunity to this organism is not an impossibility, we know, because rabbits, horses and other susceptible animals may be rendered highly immune if the inoculations are given properly. This fact encourages us to hope that sufficient immunity can be conferred upon man. Our problem is to prepare and administer a vaccine in the proper manner.

The statistical method of determining the results of series of experimental vaccinations with pneumococci presents disadvantages in that it involves the inoculation of large numbers of individuals and observations extending over a long period of time.

A simple test that would show us promptly to what extent the tissues of the inoculated individual are responding to the vaccine would be of great assistance. With its aid we could tell what preparation of vaccine is most active, what dose is the optimum, and how the doses should be spaced.

It may be that certain human beings are not susceptible to pneumococcus infection. These it would be unnecessary to inoculate. A simple test that would show which individuals are immune and which are susceptible would be of great value.

[1] The pneumonia research was made possible through the kindness of Mr. Samuel S. Fels, the poliomyelitis research through the kindness of Mr. Jules E. Mastbaum.

The familiar serum tests which are employed with success in the case of many microörganisms have not been entirely satis factory when applied to the pneumococcus. Agglutination is the most promising. Lister (1), in South Africa, made use of agglutinins and opsonins to estimate the response of rabbits and men to inoculations of pneumococci. He placed most reliance upon agglutinins. When pneumococci are mixed in test tubes with the serum of a patient convalescing from pneumonia or the serum of an animal that has received injections of killed pneumococci, agglutination is slight. In order to demonstrate agglutinins and opsonins Lister mixed one volume of a suspension of pneumococci and one volume of washed human leukocytes with one, two or three volumes of the serum to be examined. These were mixed and incubated in capillary glass tubes. The mixtures were then blown out upon slides and stained. Lister found that agglutination of the stained bacteria could be easily seen. By counting the pneumococci ingested by the leukocytes he arrived at the opsonic index from the same slide. Even by the use of this delicate method Lister found that certain rabbits inoculated were actually immune, as shown by their surviving lethal doses of living pneumococci, before any trace of agglutinins appeared in their serum. In his own words,

> Rabbit No. 22, which had received 3 doses of only 50 million cocci, failed both to agglutinate and to opsonise strain 737 and gave but a trace of agglutination and a relatively small degree of phagocytosis with strain 3472; despite these facts the animal survived its test dose.

In another place when speaking of prophylactic inoculations in man, referring to a dose of 2,000,000,000 pneumococci, comprising five groups he says,

> Such a dose does not give rise to either agglutinins or opsonins demonstrable by the technique employed by me; the degree of protection conferred by this procedure appears nevertheless to have been statistically quite appreciable.

Agglutinins, even when searched for by Lister were tardy in making their appearance, and did not keep pace with the real immunity of the individual inoculated.

Complement fixation and tests for the presence of bactericidins in serum have proven of little value in connection with the pneumococcus.

In this paper we describe a test which gives some promise of helping us toward immunization against pneumonia, as well as against other diseases. Before describing such a test it would be better to work out its applications more fully than we have done, but the need for something which will help even a little toward the prevention of pneumonia is so acute at this time that the technique is set forth with the hope that others will help in proving or disproving its value. In addition to the technique of the test the experiments which led up to its adoption are briefly touched upon.

## A. UPON PNEUMOCOCCI IN RELATION TO THE NATURAL IMMUNITY OF THE PIGEON TO PNEUMOCOCCIC SEPTICAEMIA

At the extremes of the scale of susceptibility to pneumococcus septicaemia stand the white mouse and the pigeon. A 0.00001 cc. broth culture contains sufficient pneumococci to kill a mouse. The pneumococci centrifuged from 150 cc. of broth culture of the same organism cause no ill effect whatever when injected into a pigeon. Between the protoplasm of these two animals great differences must exist with respect to the pneumococcus. When we apply our living reagent, pneumococci, to the protoplasm of the mouse the mouse is destroyed: when we apply it to the protoplasm of the pigeon the pneumococci are destroyed.

Strouse (2) and Kyes (3) have shown by many careful experiments that there is no difference to be found between the action of pigeon serum and mouse serum upon pneumococci. In our experiments also the biological action in vitro of pigeon serum upon pneumococci did not differ from that of the mouse.

Our next step was to inject living pneumococci into mice and pigeons and observe carefully what took place. This was done by giving the injections intraperitoneally and withdrawing portions of the peritoneal exudate at frequent intervals for examination.

In the mouse within two hours after the injection the pneumococci had become quite numerous and for the first four hours they increased rapidly, more rapidly than in artificial culture medium, enumerations of the cocci withdrawn with the peritoneal exudate being made by Wright's blood corpuscle method. Then for a few hours the increase was checked. Often the number of cocci diminished slightly. This slowing coincided with the appearance in the exudate of the leukocytes. After the eighth hour multiplication began again and continued steadily until the mouse died. Eight hours after the injection large numbers of leukocytes were present. Many of these had ingested one or two pneumococci, seldom more than two. Phagocytosis was very limited. The leukocytes were not dead nor paralyzed because if dead streptococci were injected into the peritoneal cavity, even late in the pneumococcic infection, the leukocytes promptly seized them and practically every leukocyte would become filled with streptococci.

In the pigeon the injected pneumococci disappeared rapidly from the peritoneal exudate. The number injected was sufficiently large that for the first two hours after the injection many pneumococci were found in every portion of exudate withdrawn. After that time they began to disappear and before any considerable number of leukocytes had migrated into the peritoneal cavity not a single pneumococcus could be found. Some of the leukocytes contained a few pneumococci. However, the number within the cells bore no comparison to the number injected. If, when the pneumococci had disappeared from the exudate the pigeon was killed, the peritoneal cavity opened and smears made by pressing a glass slide against the surfaces of the organs very few cocci could be found either within or without the leukocytes.

Kyes (3) has shown what is the ultimate fate of the pneumococci which disappear from the peritoneal cavity of the pigeon. They are carried to the liver and spleen and ingested by certain fixed phagocytes, "hemophages," where they undergo intracellular digestion and disappear.

We thus have pictures of what follows the injection of pneumococci in the two species—mouse and pigeon. But the picture

is not complete because it furnishes no explanation why such opposite phenomena occur in the two animals. There must be some rapidly acting and powerful force or forces (some push or some pull, or a combination of the two) at work in the pigeon which determines the digestion of pnemococci in cells fixed far from the site of inoculation. This force is absent from the protoplasm of the mouse. Histological differences in the tissues of the two animals is not an adequate explanation of the immunity of one and the susceptibility of the other. If the immunity of an animal were dependent upon its cellular architecture, the virulence of organisms would be proportional to their size and shape, which is by no means true. It is difficult to evaluate the part played by the high body temperature of the pigeon. When we attempt to find what part the temperature plays in immunity by first artificially lowering the temperature and then injecting the bacteria, we must remember that procedures which interfere with the mechanism that regulates temperature may interfere likewise with other protoplasmic mechanisms, such as those which are concerned in immunity. When we lower the pigeon's temperature with pyramidon we cannot be sure we are lowering the temperature and doing nothing else. In a classical experiment of similar nature it has been shown, indeed, that we are doing other things which have a direct bearing upon the immunity of the animal. The fowl, naturally immune to anthrax, becomes susceptible when its temperature is lowered by cold or drugs. But the measures which lower the temperature diminish the activity of the leukocytes, phagocytosis, normally very active, becoming slight or absent (4).

That something is missing from our knowledge of immunity to the pneumococcus is suggested by Lister (1) who says: "Whatever importance is to be placed upon the presence of agglutinins and opsonins in the sera of inoculated animals (including man) I strongly suspect that there are other factors concerned in immunity against the Pneumococcus" (p. 12b). Kyes (3) says, when speaking of the importance of the phagocytosis of pneumococci which takes place in the liver and spleen of the pigeon: "at best the degree of this importance can be determined only

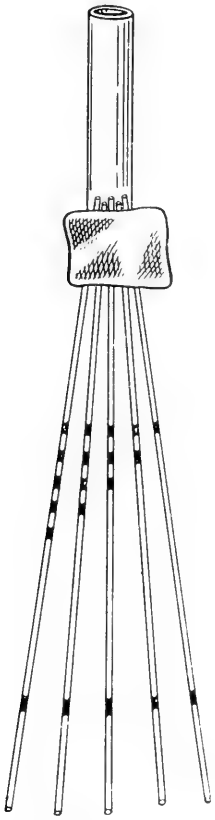
approximately, in view of the incompleteness of our present knowledge as to the many additional factors which may contribute to resistance." These missing factors do not seem to reside in the serum.

We therefore turned our attention to whole blood as it flows from the capillaries. At the moment of coagulation profound changes take place in the blood. Could there be a difference with respect to its action on the pneumococcus between the blood circulating in the capillaries and the serum which separates after coagulation? Wright (5) states that there is. Toward the end of his work on pneumonia in South Africa he observed that blood as it issues from the capillaries has a bactericidal action upon pneumococci, which is not found in defibrinated or citrated blood, nor in serum, but he did not have the opportunity of working further upon this line.

It was necessary to evolve a simple method of bringing blood as it flows fom the capillaries in contact with living pneumococci. To be of value such a test must be easy to carry out under adverse circumstances and have a sharp end point. Prof. B. F. Lacy suggested the utilization of surface tension, which gave us the clue. Before giving the results of our work with uncoagulated blood we will describe our method.

Heavy-walled glass tubing is drawn out into capillary tubing having an inside diameter of 0.5 to 1 mm. The capillary tubing is cut in 15 cm. lengths and the ends trimmed square by nicking with a fine file or with the edge of a carborundum pocket hone and breaking across. Five of these tubes are held side by side, palisade fashion, and a pellet of plasticine moulded closely about them near one end. A short piece of glass tubing stuck into the plasticine so that it encloses the five protruding ends makes a convenient handle. Pressure on the plasticine spreads out the capillary tubes into a fan shape. With a wax pencil a fiduciary mark is made upon each capillary tube about 5 cm. from its free end, and also each one is given an identification mark. Tube 1 receives one dot with the wax pencil, tube 2 two dots and so on. This many-stemmed pipette is a modification of that devised by Wright (6) for estimating the coagulation time of blood.

Five small, sterile test tubes are numbered from 1 to 5 and arranged in a row. They may be very conveniently held in a sloping position by thrusting their ends in a long strip of plasticine. In tube 1 is placed a quantity of a twenty-four hour cul-



Wright's Many-Stemmed Pipette Used in Estimating the Bactericidal Power of Whole Blood

ture of pneumococci in blood broth. In tube 2 one volume of the broth culture is diluted with 4 volumes of NaCl, making a 1:5 dilution. In a similar manner a 1:25 dilution is made in tube 3, and a 1:125 and a 1:625 in tubes 4 and 5. In other words dilutions of culture are made in multiples of five. Daily

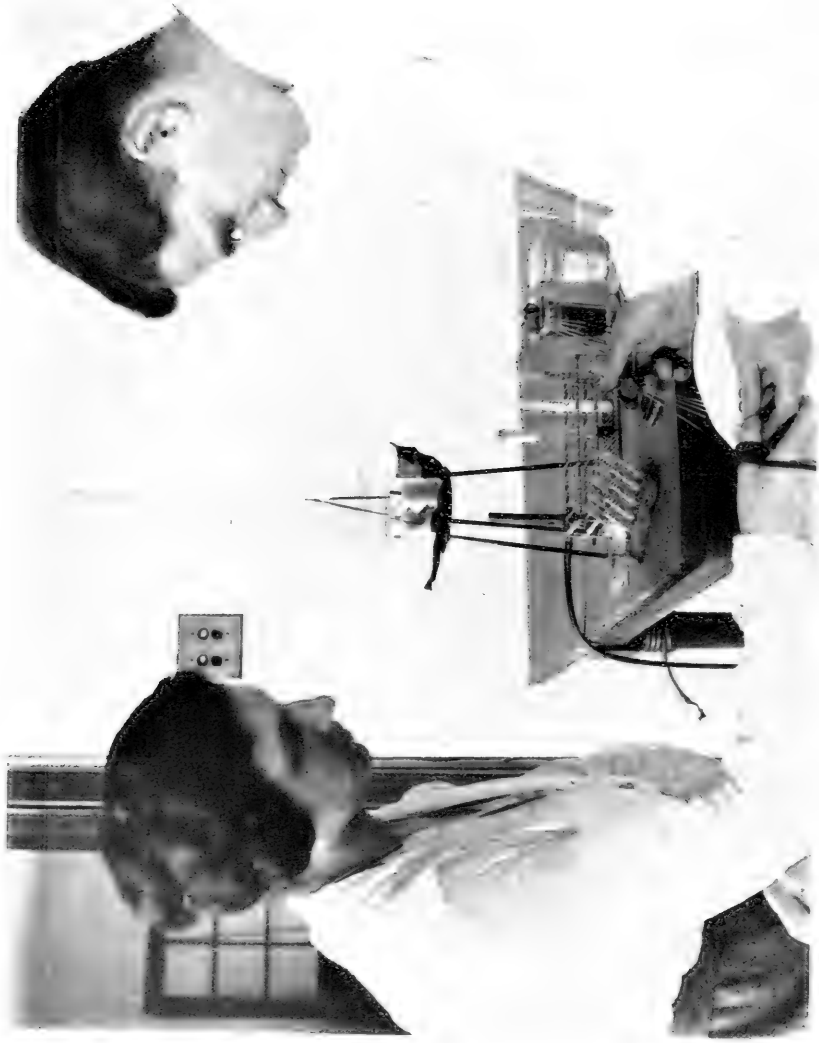


FIG. 1. METHOD OF FILLING MANY-STEMMED PIPET WITH HUMAN BLOOD FOR THE ESTIMATION OF ITS BACTERICIDAL POWER AND THE APPARATUS REQUIRED FOR THE TEST

variation in the number of pneumococci found in broth cultures is considerable. Some days the growth will be heavy and some days it will be light. It is in order to overcome this source of inaccuracy that we make such large steps in preparing our series of dilutions. A 1:5 dilution made from a heavy growth will contain fewer pneumococci than any undiluted light growth, providing the medium and incubation period is not varied. But if we used a 1:2 dilution in our second tube it might be that on some days, when the growth was good, the 1:2 dilution would contain more bacteria than the undiluted culture of a day when growth was light. This would cause very serious error when we came to compare one day's work with another. By using dilutions prepared in multiples of at least five, we may miss finer shades of differences, but our final results are more reliable.

The many stemmed pipette is taken up and the tip of tube 1 touched to the surface of the undiluted culture in test tube 1. The fluid runs up the slender tube by capillary attraction. When the upper level of the ascending fluid reaches the fiduciary mark, the tip of the tube is withdrawn from the test tube, the fluid which has run up the tube remaining in situ. The other capillary tubes are filled in the same manner from the test tubes bearing corresponding numbers. If now the tip of one of the capillary tubes filled with diluted culture is touched to a piece of moist cheese-cloth the column of fluid will run out, the adhesion between the cloth and the liquid used being greater than that between the same liquid and glass. If the tube should not completely empty itself gentle blowing into the other end of the pipette will start the downward flow again. In this manner each capillary tube is thoroughly emptied in turn.

It is evident that when the suspension of pneumococci flows out, a certain number of organisms will remain sticking to the glass. That suspension which contained the greatest number of organisms, to wit, no. 1, will leave the greatest number sticking to the wall of the glass tube. We have now a series of five tubes containing diminishing numbers of living pneumococci. The pneumococci are practically dry because what little diluent remains when we empty the tubes evaporates quickly. We are

ready to bring fresh, uncoagulated blood in contact with these pneumococci. The ear of a rabbit is rubbed briskly for a moment to increase the circulation and a vein is pricked. As the blood wells up the tip of capillary tube 1 is introduced into the drop and the blood flows up by capillary attraction. When the ascending column of blood has reached the fiduciary mark the tube is moved aside and tube 2 takes its place in the flowing blood. In less than a minute all five tubes are filed. It will be observed that we have introduced into each tube a volume of blood equal to the volume of culture fluid which we ran in at first. We are therefore able to express our results in terms of the action of a volume of blood upon the cocci deposited from an equal volume of culture fluid. This allows us to compare one series of tests with another series.

When all five tubes have been filled we seal the distal ends by dipping them in paraffine which has been melted and allowed to cool until it is on the point of solidifying. This method of closing the ends avoids bringing any appreciable amount of heat in contact with the freshly coagulated blood. The capillary tubes are then gently pulled out of the pellet of plasticine, dropped into a test tube and incubated for twenty-four hours, at the end of which time they are taken out, the tips broken off and a drop from each blown out upon a glass slide. All five drops can be placed in a row on the same slide. The dots which we placed upon the capillary tubes before beginning operations enables us to identify them now. The slide is fixed in formalin vapor, stained and examined under the microscope.

Under the microscope we see that some tubes contain no pneumococci. Numerous red and white blood corpuscles are present and well stained, and strands of fibrin are interlaced among the cells. Other tubes contain great numbers of pneumococci mixed with debris but very few cells; apparently the cells and fibrin have been digested by the pneumococci. These two pictures are practically the only ones encountered. Either the pneumococci have grown vigorously or they have not grown at all.

By using this method we can for the first time detect a difference with respect to the pneumococcus between the protoplasm

of the rabbit or mouse and that of the pigeon. When the five tubes are filled with mouse or rabbit blood the pneumococci grow vigorously in all five tubes. Even the very few cocci which clung to the wall of the tube filled with the 625–fold dilution multiply without hindrance. But when we use pigeons' blood we get a very different result. Either there is no growth in any tube, or only tube 1 shows a growth. The pigeons' blood contains substances or conditions which either killed the pneumococci or inhibited their growth in all tubes except those that contained the heaviest sowing of organism.

The chicken, like the pigeon, is practically immune to pneumococcic septicaemia. When we use chicken's blood, growth takes place in the first two or three tubes but not further. Chicken's blood is apparently less bactericidal than pigeons.

What is the nature of this substance or condition in the blood of the pigeon and chicken which is inimical to pneumococci? We can define a few of its properties. We have already seen that pigeons' serum exerts no bactericidal action upon pneumococci. Pigeons' blood clots so quickly that it is difficult to handle, but blood can be drawn from the heart of a chicken and defibrinated by shaking with glass beads. When we test this defibrinated blood upon the pneumococci in our many stemmed pipette, although the blood has been drawn less than ten minutes and is still warm it has lost its bactericidal activity. Defibrinated chicken-blood is just as good a culture medium for pneumococci as defibrinated rabbits' blood.

Our tests upon human blood have not been extensive as yet but they have brought out another property of this bactericidal activity. The bactericidal activity of normal human blood is usually less than that of the pigeon and chicken. Recovery from an attack of lobar pneumonia is paralleled by an increase of the bactericidal power of the blood of the patient, and this activity is specific to type. The blood of a patient who has recovered from a type II infection kills type II organisms but not type I organisms. Similarly the intravenous injection of dead pneumococci into rabbits is followed by the appearance of more or less bactericidal activity in the rabbits' blood which is specific

to type. In the case of man ample blood for the test may be obtained by applying a tourniquet to a finger and pricking the skin lightly.

### B. UPON THE GLOBOID BODIES OF POLIOMYELITIS IN RELATION TO THE NATURAL IMMUNITY OF THE RABBIT TO POLIOMYELITIC INFECTION

Let us turn to another disease, acute anterior poliomyelitis. For the sake of discussion let us admit that the globoid bodies of Flexner and Noguchi are the cause of poliomyelitis. Pneumonia and poliomyelitis have certain points in common when they are examined from the view-point of the immunologist. The serum of a man who has recovered from pneumonia will protect a mouse against a lethal dose of pneumococci. The serum of a man who has recovered from an attack of poliomyelitis will protect a monkey against a lethal dose of virus. Pneumococcic immune serum has but little, if any, more agglutinative, bactericidal, or opsonic action upon pneumococci than normal serum. Poliomyelitic immune serum has but little, if any, more agglutinative, bactericidal or opsonic action upon globoid bodies than normal serum. In poliomyelitis as in pnemococcic infections we have animals which are susceptible and those which are immune. Human beings and monkeys are susceptible, rabbits and other animals are, at least to a certain extent, immune.

We have tested human and rabbit's blood upon globoid bodies in the same way we tested pigeon and mouse blood upon pneumococci. This test has been made possible by the use of Smillies (7) hydrogen-nitrogen anaerobic jar. The tubes of the many stemmed pipette are filled with a suspension of globoid bodies. The suspension is drained out and blood from man run into one set of tubes and from a rabbit into another set. The tubes are placed in the jar, from which the oxygen is removed, and incubated for ten or twelve days. At the end of the incubation time a smear is made from each capillary tube and stained with dilute Giemsa. The results are no less striking than when the blood of pigeon and mouse are tested upon pneumococci. In human blood the globoid bodies grow vigorously—much more vigorously

than in any artificial culture medium we have used. In the rabbit's blood on the contrary no growth whatever takes place. We would expect, by analogy, that did we use a concentration of globoid bodies heavy enough they would grow in rabbit's blood as the pneumococcus grows in pigeon's blood when sufficient cocci are seeded.

Matsunami and Kolmer (8) employing our method, which we described to them in a personal communication, have reported finding a similar parallel between the varying susceptibility of laboratory animals to meningococcic infection and the bactericidal action of their blood in vitro.

Incidental to our work with uncoagulated blood we observed that contaminations seldom occurred. We have made little mention of precautions to insure the sterility of our tests. Only moderate precautions are necessary. In spite of our having, at times, used animal's blood as it dripped from the fur, it was seldom that we found a contaminated tube. This is in marked contrast with the difficulty of avoiding chance contaminations when doing bactericidal tests with serum. A few experiments were performed to see if this freedom from contaminations was due to chance or to the uncoagulated-blood medium. We found that *B. subtilis* refused to grow in the uncoagulated blood in our capillary tubes even when heavy suspensions were used for seeding. Other nonpathogenic organisms were tested. They came from sputum, from otitis media and from venereal lesions. They quite uniformly failed to grow in uncoagulated blood.

We may formulate a tentative hypothesis to cover the facts which we have observed: When small numbers of bacteria are planted in fresh, uncoagulated blood only those bacteria grow and multiply which are pathogenic for the species from which the blood is drawn. Further, the number of given organisms destroyed by blood from different species is, to a certain extent, proportional to the natural immunity of those species to the organism. This likewise appears to be true of the immunity induced by inoculations.

Any mechanism of immunity existing in the blood may be brought to bear upon the bacteria when our method is used.

Agglutinins, opsonins and other anti-bacterial factors, natural or artificially induced, have equal chance to come into play. Our test does not tell us whether it is the phagocytes which destroy the pneumococci, whether it is some substance in the blood having a chemical action, or whether it is something entirely different, or whether it is a combination of mechanisms. We have already seen that in ridding the body of pneumococci the leukocytes do not take the conspicuous part they take in ridding it of streptococci for instance. Still, in certain tubes where the blood of patients with pneumonia was brought in contact with pneumococci, many leukocytes were observed to be well filled with bacteria, although the pneumococci had eventually gained the mastery. Therefore we cannot be sure that, under certain conditions, the leukocytes do not play a very considerable part in pneumococcic immunity. Phagocytosis as the chief factor in immunity stands upon a very firm basis. Before we give an important place to another factor, in any particular instance, it is our duty to examine the facts carefully and see if they may not be fitted into the phagocytosis theory. When we are seeking for an explanation why uncoagulated blood is more active than defibrinated blood or serum, we must remember, as Wright (9) points out, that when we allow blood to clot in our glass tubes a semi-solid is formed. In the fibrin meshes of this clot the phagocytes are able to move in any direction and of course they may move toward the pneumococci on the walls of the tube, and devour them. In the case of fresh defibrinated blood the phagocytes quickly settle to the bottom and the bacteria floating above them may multiply unrestrictedly. But if we accept this explanation in the case of pneumococci it would be equally true of mouse and rabbit blood and the blood of the birds. Mouse and rabbit phagocytes would have the same opportunity to migrate upon the fibrin support toward the pneumococci as do the phagocytes of the pigeon, yet the mouse and rabbit phagocytes fail to prevent the multiplication of the pneumococci. Therefore it remains that there must be some essential difference between the blood of the mouse and the pigeon with respect to the pneumococci. It is with this difference that we are concerned, be it

chemical or physical in nature, or of such a nature that it cannot be classed as either chemical or physical.

We have spoken of the action of pigeon blood upon pneumococci as bactericidal, using that term in its broadest meaning to denote the killing of bacteria. We do not mean to suggest that it is or is not identical with that property possessed by certain serums of killing certain bacteria, for instance the ability of immune typhoid serum to kill typhoid bacilli.

## SUMMARY

That the serum of a pigeon does not differ in its action upon pneumococci from the serum of a mouse or rabbit would appear from our experiments as well as from those of previous investigators.

We have found, however, that if small numbers of living pneumococci are seeded, by a suitable method, in pigeon's blood before it coagulates the pneumococci fail to multiply. On the contrary if pneumococci are seeded in mouse's or rabbit's blood before it coagulates the pneumococci grow with great vigor. The evidence points to the presence in the uncoagulated blood of the pigeon of a bactericidal factor which is absent from the blood of the mouse or rabbit.

Fresh defibrinated blood of both immune and susceptible species is an excellent culture medium for the pneumococcus, which shows that the bactericidal factor disappears during the process of coagulation.

Pneumococci fail to multiply in the blood of a rabbit which has been suitably inoculated with killed pneumococci, the reaction being specific to type.

The blood of a man that has recovered from lobar pneumonia prevents the growth of pnemococci belonging to the type which caused his disease.

The globoid bodies of poliomyelitis grow readily in uncoagulated human blood. They fail to grow when seeded in uncoagulated rabbit blood.

Many of the non-pathogenic bacteria usually met with as contaminations in bacteriological work, as for instance the *B. subtilis*, fail to grow when seeded in uncoagulated blood.

The tentative hypothesis advanced to cover the facts observed is that when small numbers of bacteria are seeded in blood before it has had time to coagulate only those bacteria grow and multiply which are pathogenic for the animal from which the blood is taken.

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# COMPLEMENT FIXATION WITH PROTEIN SUBSTANCES

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The classical studies of Wells and Osborne on the biological reaction of vegetable proteins (1) have demonstrated that the specificity of the anaphylactic reaction depends upon the chemical structure of the protein molecule, and if the molecule be split—whether by acid hydrolysis or peptic digestion—its anaphylactic specificity is lost.

Whether or not these findings are applicable also to the complement fixation reaction is the aim of the introductory studies recorded below. The substances employed consisted of proteins, split proteins, racemized proteins, and animal tissues. These substances were introduced parenterally into rabbits and specific complement fixing antibodies sought for in the blood of these animals at definite intervals. Knowing the chemical nature of the immunizing substance, we were thus able to judge whether proteins of various origin and chemical constitution are able to call forth in the animal organism, specific complement fixing antibodies, and if so, to what degree a protein molecule may be modified or split and still retain this property.

## INTRODUCTION

Complement fixation with purified proteins have been reported by several investigators. Dunbar (2) obtained specific complement fixing sera on injection of pollen from various plants. White and Avery (3) demonstrated the presence of complement-fixing antibodies in the serum of rabbits immunized with edestin. More

recently, Lake, Osborne and Wells (4) have further established the fact that purified vegetable proteins call forth the production of specific complement-fixing antibodies.

In our studies, two vegetable proteins of high purity—edestin from hempseed and phaseolin from kidney bean—and three animal proteins of relative purity—casein, lactalbumin and gelatin—were employed. Casein and lactalbumin are found side by side in milk, and it seemed worth while to see whether these substances could be interchanged as antigens. Gelatin was used because of its peculiar chemical construction. This protein, as is well known, lacks cystine, tyrosin and tryptophane and from the standpoint of nutrition it behaves somewhat like a carbohydrate. Furthermore Vaughan (5) has been unable to obtain a soluble toxin from it and according to Wells (6) it does not produce anaphylactic antibodies. Whether or not, therefore, it will call forth complement-fixing antibodies is of considerable interest.

When turning to the question of the production of antibodies on injections with split proteins, one encounters a field relatively unexplored. According to Krause (7), split protein products have not the power to produce specific precipitin antibodies. Wells (8) investigated the different fractions obtained from tryptic and peptic digestion of egg white with a view of calling forth anaphylactic antibodies in guinea-pigs. His findings indicate that the proteoses and peptones as well as the lower products of hydrolysis obtained from this protein, could not sensitize guinea-pigs, either with egg white or the digestive products themselves. This investigator concludes that as far as anaphylaxis is concerned, nothing less than the entire protein molecule can act as an antigen.

The same conclusion is reached in the later studies of Wells and Osborne (9). On the other hand, proteoses from various seeds, according to these investigators, produce strong anaphylactic reactions in animals. The explanation being that these so-called proteoses of vegetable origin are not in any way similar to products of peptic digestion or acid hydrolysis of proteins. In fact these vegetable proteoses are chemically similar to soluble

animal proteins, which explains their high anaphylactic specificity.

We were unable to find a record of complement fixation studies with split protein products. It seemed therefore worth while to investigate the question whether the immunological behavior of such substances in so far as the complement fixation reaction is concerned, would be similar to that of the precipitin and anaphylactic reactions.

The following split proteins were employed in this series: (1) Proteose from acid digestion of racemized casein; (2) Proteose from gliadin by acid digestion; (3) Proteose formed during the preparation of racemized zein; (4) Proteose formed during the racemization of edestin; (5) Vaughan's crude soluble poison from casein, and (6) Insoluble residue remaining from preparation of Vaughan's crude soluble poison from casein.

Racemized proteins also furnish good material for immunological study. These modified proteins, it will be recalled, are prepared by digesting protein in large quantities of normal sodium hydroxide and permitting the mixture to stand for a long interval, during which the plane of polarization of the protein is changed. Ten Broeck (10) has investigated the immunological properties of racemized egg albumin in rabbits and guinea-pigs and obtained negative results. Underhill and Hendrix (11), in their physiological studies of these proteins, found that purified racemized proteins show no poisonous action. It seemed worth while to add these modified proteins to our series with the view of determining whether they have the power to call forth complement-fixing antibodies when injected in rabbits. The following racemized proteins were employed; racemized zein, edestin casein, and egg albumin.

Finally, it was desired to obtain some data with regard to the specificity of animal tissues; the question being, will liver tissue or muscle tissue injected into rabbits call forth the production of specific complement-fixing antibodies? In other words, will the immunological behavior of animal tissues be similar to that of blood, the specificity of which is well established? Guinea-pig's muscle and heart tissue were employed in one case with a

view of determining whether rabbits will produce specific antibodies against these and whether heart and skeletal muscle may be interchanged as antigens. In another series, rabbit muscle and liver were employed; this with a view of determining whether an animal's own tissue may act as a foreign antigen. This problem was investigated by Bradley and Sansum (12) by means of the anaphylactic reaction. These investigators found a low order of sensitization on injecting extracts of guinea-pig tissues in guinea-pigs. But, as is well known, weak and "border-line"—anaphylactic reactions present many difficulties in the interpretation of results. It was hoped therefore that the complement fixation reaction might furnish a better medium for the study of the specificity of tissues.

## EXPERIMENTAL

Varying modes of immunization for the study of antibody production are reported by different investigators. White and Avery (3) in their immunity studies with edestin injected in a rabbit, 50, 100, 50 and 150 mgm. of edestin at intervals of five, six, and one days respectively, bleeding the animal 19 days later. Lake, Wells and Osborne (4) it appears, observed no hard and fast rule with regards to the method of immunization. In most cases they employed about 3 grams of protein; fractions of which they injected at varying intervals and extended their entire period of immunization from about five to six weeks.

It seemed important, in attempting immunity studies with a variety of substances of different chemical nature, that some standard method be adhered to. It was soon found that 5 injections at 48-hour intervals and final bleeding six days after last injection, was ample time for the production of complement-fixing antibodies. The quantities of protein employed were 50, 75, 100, 125 and 150 mgm., respectively. Later, a still simpler method was employed in a number of cases, giving three injections at twenty-four hour intervals and bleeding eight days after the last injection. The quantity of protein employed in this case being 50, 75, and 100 mgm., respectively.

Table 1 gives an outline of the two methods of immunization employed. Both of these methods produced complement-fixing antibodies in rabbits with purified proteins, and they will be referred to in the text as method 1 and method 2.

Of the proteins employed in immunization, edestin and phaseolin were kindly furnished to us by Dr. Thomas B. Osborne; the casein was a Kahlbaum product; the lactalbumin, an "Arlco" preparation, and the gelatin was of the type generally employed in bacteriological work. The racemized proteins and proteoses, as well as Vaughan's crude soluble poison from casein, and the insoluble residue remaining from its preparation, were kindly furnished to us by Prof. Frank P. Underhill. The preparation of these protein derivatives is given by Underhill and Hendrix

TABLE 1

| IMMUNIZATION METHOD NO. 1 INJECTIONS GIVEN AT 48-HOUR INTERVALS | | | IMMUNIZATION METHOD NO. 2 INJECTIONS GIVEN AT 24 HOUR INTERVALS | | |
|---|---|---|---|---|---|
| Number of injections | Quantity of protein injected | Time of bleeding | Number of injections | Quantity of protein injected | Time of bleeding |
| | *mgm.* | | | *mgm.* | |
| First..... | 50 | Six days after | First..... | 50 | Eight days after |
| Second..... | 75 | last injection | Second..... | 75 | last injection |
| Third..... | 100 | | Third..... | 100 | |
| Fourth..... | 125 | | | | |
| Fifth..... | 150 | | | | |

(13) in their recent studies on the physiological action of these products.

The guinea-pig's muscle and heart and the rabbit's liver and muscle were prepared in the following manner: After bleeding the animals very nearly to completion the tissues were removed, freed from connective tissue, and washed in distilled water. They were then placed in about ten times their quantity in water, slightly acidified with acetic acid. This was done in order to coagulate the proteins and remove the extractives. After ten minutes boiling, the tissues were placed in fresh water and again boiled for ten minutes. This process was repeated three times. After the last boiling, the tissues were dried at 40°C. and ground into a powder form, when they were ready for use.

The injections were made intravenously with the exception of a few instances where intraperitoneal injections were resorted to. Those of the proteins or protein derivatives which are insoluble in water, were rendered soluble just before the injection by the addition of a few drops of normal sodium hydroxide.

Before beginning the injections the sera of the experimental animals were, in a number of cases, tested for complement-fixing antibodies, the proteins intended for injections being used as antigens. Kolmer and Trist (14) recently reported a large number of cases where normal rabbits gave positive complement-fixation reactions with lipoidal and bacterial antigens, without previous immunization with these substances. In no case, however, did we find non-specific binding with the proteins and protein derivatives employed in these studies.

The bleeding was performed under anesthesia and the sera were obtained—after the blood had been permitted to remain over night in the ice-box—practically free from corpuscles.

In table 2 is recorded the kind and quantity of protein injected, also the dates of the injections and bleedings.

The complement-fixation test as it is employed in the Laboratories of the Health Department of New York City was adopted in this work. The following is a description of the method in detail:

*Complement* The complement employed was fresh guinea-pigs serum obtained in the following manner: The pig was rendered unconscious by a moderate blow at the junction of the brain and cord. The blood was then obtained by incising with pointed scissors the anterior chest wall directly into the heart. After permitting the blood to clot at room temperature, it was placed in the ice box over night; the serum collected and mixed with sterile saline in the proportion of 1–10. This 10 per cent solution in saline served as complement, and it was freshly prepared for each series of experiments.

*Sheep's cells.* The blood employed was obtained from the jugular vein of sheep, collected in sterile bottles containing glass beads. After defibrination by shaking, it was freed from serum in the following manner: In a 15-cc. graduated cent ifuge tube, the blood was poured to the 8 cc. mark, and made up to about 15 cc. with saline and mixed.

TABLE 2

*Protocol of immunization*

| NUMBER OF EXPERIMENT | DATE OF INJECTIONS | TYPE OF PROTEIN AND QUANTITY INJECTED | NUMBER OF EXPERIMENT | TYPE OF PROTEIN AND QUANTITY INJECTED | DATE OF BLEEDING |
|---|---|---|---|---|---|
| | | Phaseolin (rabbit A) | | Edestin (rabbit B) | |
| | *1915* | *mgm.* | | *mgm.* | *1915* |
| 1 | October 26 | 50 | 2 | 50 | |
| | October 28 | 75 | | 75 | |
| | October 30 | 100 | | 100 | November 9 |
| | November 1 | 125 | | 125 | |
| | November 3 | 150 | | 150 | |
| | | Casein (rabbit C) | | Lactalbumin (rabbit D) | |
| 3 | December 9 | 5 | 4 | 5 | |
| | December 11 | 5 | | 5 | |
| | December 13 | 5 | | 5 | December 23 |
| | December 15 | 5 | | 5 | |
| | December 17 | 5 | | 5 | |
| | | Lactalbumin (rabbit E) | | Casein (rabbit F) | |
| | *1916* | | | | *1916* |
| 5 | January 4 | 50 | 6 | 50 | |
| | January 6 | 75 | | 75 | |
| | January 8 | 100 | | 100 | January 18 |
| | January 10 | 125 | | 125 | |
| | January 12 | 150 | | 150 | |
| | | Guinea-pig heart (rabbit G) | | Guinea-pig muscle (rabbit H) | |
| 7 | January 4 | 50 | 8 | 50 | |
| | January 6 | 75 | | 75 | |
| | January 8 | 100 | | 100 | January 19 |
| | January 10 | 125 | | 125 | |
| | January 12 | 150 | | 150 | |
| | | Vaughan's soluble poison (rabbit I) | | Insoluble residue from Vaughan's poison (rabbit J) | |
| 9 | January 25 | 50 | 10 | 50 | |
| | January 27 | 75 | | 75 | |
| | January 29 | 100 | | 100 | February 8 |
| | January 31 | 125 | | 125 | |
| | February 2 | 150 | | 150 | |

TABLE 2—*Concluded*

| NUMBER OF EXPERIMENT | DATE OF INJECTIONS | TYPE OF PROTEIN AND QUANTITY INJECTED | NUMBER OF EXPERIMENT | TYPE OF PROTEIN AND QUANTITY INJECTED | DATE OF BLEEDING |
|---|---|---|---|---|---|
| | | Proteose from digestion of racemized casein (rabbit K) | | Proteose from gliadin by acid digestion (rabbit L) | |
| | *1916* | *mgm.* | | *mgm.* | *1916* |
| 11 | February 29 | 50 | 12 | 50 | |
| | March 1 | 75 | | 75 | March 11 |
| | March 2 | 100 | | 100 | |
| | | Proteose from preparation of racemized zein (rabbit M) | | Proteose from preparation of racemized edestin (rabbit N) | |
| 13 | February 29 | 50 | 14 | 50 | |
| | March 1 | 75 | | 75 | March 11 |
| | March 2 | 100 | | 100 | |
| | | Racemized zein (rabbit O) | | Racemized edestin (rabbit P) | |
| 15 | February 29 | 50 | 16 | 50 | |
| | March 2 | 75 | | 75 | |
| | March 4 | 100 | | 100 | March 14 |
| | March 6 | 125 | | 125 | |
| | March 8 | 150 | | 150 | |
| | | Rabbit liver (rabbit Q) | | Rabbit muscle (rabbit R) | |
| 17 | March 4 | 50 | 18 | 50 | |
| | March 6 | 75 | | 75 | |
| | March 8 | 100 | | 100 | March 18 |
| | March 10 | 125 | | 125 | |
| | March 12 | 150 | | 150 | |
| | | Racemized casein (rabbit S) | | Racemized egg albumin (rabbit T) | |
| 19 | March 11 | 50 | 20 | 50 | |
| | March 12 | 75 | | 75 | March 21 |
| | March 13 | 100 | | 100 | |
| | | Phaseolin (rabbit U) | | Edestin (rabbit V) | |
| 21 | March 30 | 50 | 22 | 50 | |
| | March 31 | 75 | | 75 | April 9 |
| | April 1 | 100 | | 100 | |
| | | Gelatin (rabbit W) | | | |
| 23 | March 30 | 50 | | | |
| | March 31 | 75 | | | April 9 |
| | April 1 | 100 | | | |

This was centrifuged for about five minutes at 2500 revolutions. Clear fluid was now pipetted off, fresh saline added, and again centrifuged as above This washing was repeated five times (fresh saline being added each time). The sixth or final washing was carried out by centrifuging for ten minutes at 2500 revolutions. The amount of packed cells in the graduated centrifuge tube was now read, and the sediment was made up into a 5 per cent suspension in saline. This 5 per cent suspension, also, was freshly prepared for each series of determinations.

*Amboceptor.* Antisheep's cells amboceptor was employed throughout the work and was obtained by immunizing rabbits with sheep cells. After thoroughly washing the cells in the manner stated above, 50 per cent suspensions in saline were made up and injected intravenously in rabbits at 48 hour intervals. The following quantities were used in the immunization:

First injection 0.5 cc. of 50 per cent suspension of washed sheep cells.
Second injection 1.0 cc. of 50 per cent suspension of washed sheep cells.
Third injection 1.5 cc. of 50 per cent suspension of washed sheep cells.
Fourth injection 2.0 cc. of 50 per cent suspension of washed sheep cells.
Fifth injection 3.0 cc. of 50 per cent suspension of washed sheep cells.

Rabbits were bled six days after the last injection to test the strength of the amboceptor. If a 1–1000 dilution in saline brought about complete hemolysis of the red cells in the presence of complement during one hour at 37°C. it was considered sufficiently strong to be used in our hemolytic system. Rabbits were then completely bled under ether anasthesia. Blood was obtained under sterile precautions by inserting pointed scissors directly into the heart. Serum was removed after complete clotting and, inactivated at 56°C. for thirty minutes for three successive days. Stock solutions of 1–100 in saline were then prepared, and 1–1000 dilutions were freshly prepared from the stock solutions for each series of tests.

*Antigen.* One hundred milligrams of the protein material in 100 cc. of saline served as the antigen. This was either a solution or a suspension depending on whether the protein substance employed was soluble or insoluble.

*Standardization of the hemolytic system.* Freshly prepared 5 per cent suspension of sheep's cells, 10 per cent guinea-pigs complement, and 1–1000 amboceptor were placed in a series of 11 Wassermann tubes in the following amounts:

Tube 1 contained 0.1 cc. amboceptor, 0.1 cc. complement, 0.1 cc. sheep's cells.

Tube 2 contained 0.09 cc. amboceptor, 0.1 cc. complement, 0.1 cc. sheep's cells.

Each succeeding tube contained the same amount of complement and sheep's cells, but the quantity of amboceptor decreased by 0.01 cc. in each case. Thus, the tenth tube contained 0.1 cc. complement, 0.1 cc. red cells, and only 0.01 cc. amboceptor.

The eleventh control tube contained complement and cells only, as a test for natural amboceptor in the guinea-pig's serum.

The total volume of each tube was made up to 0.3 cc. with saline and the rack of tubes was placed in the water bath at 37°C. for one hour. The smallest amount of amboceptor that caused complete hemolysis at the end of one hour was considered the unit for that system. If, for example, the tube containing 0.05 cc. of amboceptor was completely hemolyzed at the end of that time, 0.05 cc. was the unit and three times this quantity; i.e., 0.15 cc. of amboceptor was used to sensitize each 0.1 cc. of sheep's cells. Experience has shown that optimum results are obtained by using three units of amboceptor.

*Antigen titration.* These titrations were carried out in a series of 12 Wassermann tubes. The first six of these tubes contained 0.01 cc. of immune rabbit serum, 0.1 cc. of complement, and antigen in the following proportions: First tube contained 0.25 cc.; second tube 0.20 cc.; third tube, 0.15 cc.; fourth tube 0.10 cc.; fifth tube 0.05 cc.; sixth tube 0.025 cc. The five following tubes contained the same quantities of antigen and complement as the first five tubes, but they contained no immune serum. These served as the antigen controls. The 12th or last tube contained 0.02 cc. of immune serum and 0.1 cc. of complement but no antigen; this served as the immune serum control.

The volume in each of these twelve tubes was now made up to about 0.3 cc. with normal salt solution, and the rack placed in water bath for half hour at 37°C. After this time sensitized sheep's cells, previously prepared, were added and again placed in the water bath for about ten minutes. The absence of hemolysis in any of the tubes containing immune serum, antigen, complement and sensitized cells proved the presence of complement fixing antibodies in the rabbit's sera tried, providing no hemolysis took place after remaining in the ice-box over night.

Antigens that are somewhat related to one another were interchanged in practically all cases, the same immune serum being employed. Thus, with phaseolin immune serum, both phaseolin

and edestin were employed as antigens. This was done in order to find some possible cases of non-specific binding.

The following is the record of a specific complement-fixation reaction, indicating also the results obtained:

(1) Phaseolin antigen with phaseolin immune serum, prevented hemolysis of sensitized cells, indicating the presence of complement binding antibodies. (2) Phasolin antigen with edestin immune serum did not prevent hemolysis of sensitized cells; i.e., hemolysis was complete. (3) All control tubes wherein, either antigen or immune serum was left out, were also hemolyzed.

In all cases where negative results were obtained double quantities of immune serum were employed and wherever the antigen was not lytic or anticomplementary, double quantities of the antigen also were used, without, however, any indications for complement binding being found. On the other hand, those proteins which gave positive results, produced fixation of complement in all cases in the Wassermann tubes containing 0.10 cc. of antigen (0.10 mgm. of protein), and in some cases, also, in those tubes which contained no more than 0.05 cc. and 0.025 cc. of antigen (0.05 mgm. and 0.025 mgm. of protein).

Immunization of rabbits with egg albumen with a view of calling forth the production of complement-fixing antibodies was also attempted. Two rabbits were employed, one receiving the injections according to method 1, the other according to method 2. But strange as it may seem, particularly in view of the fact that egg albumen is so extensively used in immunization studies, both animals were found dead in their cages. One animal died about twelve hours after the second injection, and the other about one hour after the third injection. It is likely that the quantities employed for injections, although apparently without poisonous effects, in the case of other proteins, were too large in the case of egg albumen.

TABLE 3

*Record of results of complement fixation tests employing specific and non-specific immune serum*

| NUMBER OF EXPERIMENT | DATE | PROTEIN EMPLOYED FOR INJECTION | METHOD OF IMMUNIZATION (TABLE 1) | ANTIGEN EMPLOYED IN COMPLEMENT FIXATION TEST | IMMUNE SERUM EMPLOYED IN COMPLEMENT FIXATION TEST | COMPLEMENT BINDING |
|---|---|---|---|---|---|---|
| | *1915* | | | | | |
| 1 | November 10 | Phaseolin | No. 1 | Phaseolin | Phaseolin | Positive |
| | | Phaseolin | No. 1 | Phasolin | Edestin | Negative |
| 2 | November 10 | Edestin | No. 1 | Edestin | Edestin | Positive |
| | | Edestin | No. 1 | Edestin | Phaseolin | Negative |
| 3 | December 24 | Casein | * | Casein | Casein | Negative |
| | | Casein | * | Casein | Lactalbumin | Negative |
| 4 | December 24 | Lactalbumin | * | Lactalbumin | Lactalbumin | Negative |
| | | Lactalbumin | * | Lactalbumin | Casein | Negative |
| | *1916* | | | | | |
| 5 | January 19 | Lactalbumin | No. 1 | Lactalbumin | Lactalbumin | Positive |
| | | Lactalbumin | No. 1 | Lactalbumin | Casein | Negative |
| 6 | January 19 | Casein | No. 1 | Casein | Casein | Positive |
| | | Casein | No. 1 | Casein | Lactalbumin | Negative |
| 7 | January 20 | Guinea-pig heart | No. 1 | Guinea-pig heart | Guinea-pig heart | Negative |
| | | Guinea-pig heart | No. 1 | Guinea-pig heart | Guinea-pig muscle | Negative |
| 8 | January 20 | Guinea-pig muscle | No. 1 | Guinea-pig muscle | Guinea-pig muscle | Negative |
| | | Guinea-pig muscle | No. 1 | Guinea-pig muscle | Guinea-pig heart | Negative |

| | 1916 | | | | | | |
|---|---|---|---|---|---|---|---|
| 9 | February | 9 | Vaughan's poison from casein | No. 1 | Vaughan's poison from casein | Vaughan's poison from casein | Negative |
| | | | Vaughan's poison from casein | No. 1 | Vaughan's poison from casein | Insoluble residue from Vaughan's poison | Negative |
| 10 | February | 9 | Insoluble residue from Vaughan's poison | No. 1 | Insoluble residue from Vaughan's poison | Insoluble residue from Vaughan's poison | Negative |
| | | | Insoluble residue from Vaughan's poison | No. 1 | Insoluble residue from Vaughan's poison | Vaughan's poison from casein | Negative |
| 11 | March | 12 | Proteose from digestion of racemized casein | No. 2 | Proteose from digestion of racemized casein | Proteose from digestion of racemized casein | Negative |
| | | | Proteose from digestion of racemized casein | No. 2 | Proteose from digestion of racemized casein | Proteose from digestion of gliadin | Negative |
| 12 | March | 12 | Proteose from digestion of gliadin | No. 2 | Proteose from digestion of gliadin | Proteose from digestion of gliadin | Negative |
| | | | Proteose from digestion of gliadin | No. 2 | Proteose from digestion of gliadin | Proteose from digestion of racemized casein | Negative |
| 13 | March | 12 | Proteose from digestion of racemized zein | No. 2 | Proteose from digestion of racemized zein | Proteose from digestion of racemized zein | Negative |
| | | | Proteose from digestion of racemized zein | No. 2 | Proteose from digestion of racemized zein | Proteose from digestion of racemized edestin | Negative |
| 14 | March | 12 | Proteose from digestion of racemized edestin | No. 2 | Proteose from digestion of racemized edestin | Proteose from digestion of racemized edestin | Negative |
| | | | Proteose from digestion of racemized edestin | No. 2 | Proteose from digestion of racemized edestin | Proteose from digestion of racemized zein | Negative |

TABLE 3—*Continued*

| NUMBER OF EXPERIMENT | DATE | | PROTEIN EMPLOYED FOR INJECTION | METHOD OF IMMUNIZATION (TABLE 1) | ANTIGEN EMPLOYED IN COMPLEMENT FIXATION TEST | IMMUNE SERUM EMPLOYED IN COMPLEMENT FIXATION TEST | COMPLEMENT BINDING |
|---|---|---|---|---|---|---|---|
| | *1916* | | | | | | |
| 15 | March | 15 | Racemized zein | No. 1 | Racemized zein | Racemized zein | Negative |
| | | | Racemized zein | No. 1 | Racemized zein | Racemized edestin | Negative |
| 16 | March | 15 | Racemized edestin | No. 1 | Racemized edestin | Racemized edestin | Negative |
| | | | Racemized edestin | No. 1 | Racemized edestin | Racemized zein | Negative |
| 17 | March | 19 | Rabbit liver | No. 1 | Rabbit liver | Rabbit liver | Negative |
| | | | Rabbit liver | No. 1 | Rabbit liver | Rabbit muscle | Negative |
| 18 | March | 19 | Rabbit muscle | No. 1 | Rabbit muscle | Rabbit muscle | Negative |
| | | | Rabbit muscle | No. 1 | Rabbit muscle | Rabbit liver | Negative |
| 19 | March | 22 | Racemized casein | No. 2 | Racemized casein | Racemized casein | Negative |
| | | | Racemized casein | No. 2 | Racemized casein | Racemized egg albumin | Negative |
| 20 | March | 22 | Racemized egg albumin | No. 2 | Racemized egg albumin | Racemized egg albumin | Negative |
| | | | Racemized egg albumin | No. 2 | Racemized egg albumin | Racemized casein | Negative |
| 21 | April | 10 | Phascolin | No. 2 | Phascolin | Phascolin | Positive |
| | | | Phascolin | No. 2 | Phascolin | Edestin | Negative |
| 22 | April | 10 | Edestin | No. 2 | Edestin | Edestin | Positive |
| | | | Edestin | No. 2 | Edestin | Phascolin | Negative |
| 23 | April | 10 | Gelatin | No. 2 | Gelatin | Gelatin | Negative |

* Methods of immunization not adhered to. Only 5 mgm. of protein injected during each of five intervals (see table 2—experiments 3 and 4).

## CONCLUSIONS

A series of protein substances of various origin and chemical constitution were tested with regard to their ability to call forth complement-fixing antibodies in rabbits. Two simple methods of immunization which extended eleven and sixteen days respectively, and which gave positive results with vegetable proteins, were employed throughout the series. The complement fixation test used was the one standardized and employed in the Health Department of New York City.

Two vegetable proteins of high purity—edestin and phaseolin—gave positive complement-fixation reactions. Two animal proteins of relative purity, one of which was a compound protein (casein), and the other a simple protein (lactalbumin), also gave positive complement-fixation reactions. The latter two proteins however, gave negative results at first, as is indicated in table 3 (experiments 3 and 4). The reason for this, was because the quantitative relations of the injections as tabulated in methods 1 and 2, were not abided by. Instead of beginning the injections with solutions containing 50 mgm. of protein and increasing each injection by 25 mgm., solutions were injected which contained not more than 5 mgm. each.

In this regard, therefore, there appears to be an important quantitative difference between the production of anaphylactic antibodies, where a fraction of a milligram often suffices (0.0000001 gram in the case of edestin (3)), and the production of complement-fixing antibodies where much larger quantities of protein are required for immunization. Positive results were obtained with casein and lactalbumin when the experiments were carried out in accordance with method 1.

Gelatin, which lacks cystine, tyrosine, and tryptophane, and has been shown to lack anaphylactogenic properties, gave negative results also in our hands. It is likely that the deficiency of this protein in aromatic radicals, is responsible for its lack of antigenic properties. These radicals, as has been shown by a number of workers (15), appear to be particularly important in immunity reactions. For, as is well known, the mere lack of

amino acids does not prevent a protein from being an antigen. The studies of Wells and Osborne have long demonstrated that such proteins as zein, hordein, and gliadin although lacking one or more amino acid radicals (glycocoll, tryptophane, and lysine), are nevertheless strong antigens.

Four proteoses, as well as Vaughan's soluble poison from casein and the insoluble residue remaining from its preparation, gave negative results. This number is evidently too small to enable one to say that the same would be true in the case of other split proteins. However, in view of the consensus of opinion that split proteins are unable to call forth precipitin and anaphylactic antibodies, it appears likely, by virtue of our negative finding, that split proteins also lack the power of producing complement-fixing antibodies.

Four racemized proteins also gave negative results in our hands. These modified proteins appear to undergo a profound molecular rearrangement during the prolonged alkali digestion. It will be recalled also that they are not split by digestive ferments *in vitro*. Their inability, therefore, to call forth antibody production is, perhaps, to be expected.

Finally, negative results were also obtained with various animal tissues employed in this series. When attempting to immunize animals with animal tissue, several difficulties are encountered. It is virtually impossible to wash them entirely free from extractives, red and white cells, and blood serum. Furthermore we have more than one type of cells to contend with in the tissue itself (parenchymatous, endothelial, connective tissue cells, etc.). These factors would tend to make tissues non-specific antigens. We thus find that the so called antisera produced on injection of various tissues, reported in the literature, contained not only antibodies against the tissues injected, but against leucocytes, red cells, and endothelium as well.

Why the findings in this laboratory were totally negative, is perhaps explained by the fact that the tissues were subjected to boiling temperature during their process of preparation for the injections. It is likely that had they not been boiled, they would have produced in rabbits non-specific immune sera.

SUMMARY

1. Vegetable and animal proteins, injected into rabbits, called forth the production of complement-fixing antibodies in these animals.

2. (a) Gelatin; (b) racemized proteins; (c) proteoses; (d) Vaughan's soluble poison from casein; (e) insoluble residue remaining from the preparation of Vaughan's poison from casein; and (f) animal tissues, did not produce complement fixing antibodies when injected into rabbits.

3. What has been found to be true in the case of other immunity reactions, appears to be true, also, in the case of the complement fixation reaction, namely that *the specificity of the complement fixation reaction depends upon the chemical structure of the protein molecule and if the molecule be split or modified by racemization, its specific complement binding power is lost.*

The writers wish to express their indebtedness to Prof. William H. Park for his helpful criticism throughout this work.

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# A NOTE ON THE RELATION BETWEEN PROTEOLYSINS AND HAEMOLYSINS

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This note is a report of studies undertaken with a view of determining whether specific proteolysins are produced in animals on protein injections, if a procedure simulating the production of specific haemolysins be adhered to.

The question of the presence of specific proteolytic substances in the blood of animals injected with proteins, is an old one and no attempt will be made here to go over the range of literature. Suffice it to recall that the so-called Abderhalden reaction for cancer and pregnancy, which was based on the assumption of the presence of such proteolysins in the blood (2), is now discarded. More recently, Taylor and Hulton (1) also, studied this problem from a biochemical viewpoint. They injected various proteins into rabbits with a view of detecting the presence of proteolytic ferments in the blood of these animals, obtaining negative results.

Although various attempts have been made from time to time to attack this problem from an immunological viewpoint (2), there is, to our knowledge, no work on record where a procedure was observed similar to that recorded in this paper. Generally speaking the procedure adapted for calling forth proteolysins in the animal organism was similar to that observed in the laboratories of the Department of Health of New York City, when attempting to produce haemolytic antibodies, except that some purified protein was substituted for red cells. After proper immunization with a given protein, the serum was tested for specific proteolysins by digesting it with its protein antigen and guinea-pig complement for a definite interval at 37°C. and subsequently determining the increase in amino acid nitrogen.

Two purified proteins, edestin from hempseed and phaseolin from kidney bean (kindly furnished by Dr. Thomas B. Osborne) were used in these experiments. Two rabbits weighing approximately 5 pounds each, served as the experimental animals. The injections were made intravenously.

The following is a table of the quantity of protein injected and time of injections.

| DATE, 1915 | RABBIT A | RABBIT B |
|---|---|---|
| | Quantity of phaseolin injected | Quantity of edestin injected |
| | *mgm.* | *mgm.* |
| October 26 | 50 | 50 |
| October 28 | 75 | 75 |
| October 30 | 100 | 100 |
| November 1 | 125 | 125 |
| November 3 | 150 | 150 |

Both animals were bled under anesthesia, on November 9, six days after last injection. The sera were separated from the clots with much care to prevent hemolysis, and were divided into two portions, one-half being inactivated at 56°C. for a half hour, the remaining half being used in an unheated form.

Our measure of ferment action consisted in the amino acid increase after incubation for six hours at 37°C. of mixtures of the rabbits' serum with guinea-pig's complement and the specific proteins.

The amino acids were determined by means of the Van Slyke micro amino apparatus. The advantage of this procedure over other measurements of proteolytic action are well pointed out by Van Slyke and his co-workers in their recent paper on the Abderhalden reaction (3). "First, it is quantitative, and permits accurate results with the small amounts of material available. Secondly it is specific for proteolysis; it permits one to follow the chemical change which is characteristic of protein hydrolysis."

The principle of this gasometric method (4) for the determination of amino-nitrogen, is based on the fact that aliphatic amino groups react with nitrous acid with the liberation of nitrogen gas.

$$\text{Thus } R.NH_2 + HNO_2 = ROH + H_2O + N_2.$$

The quantity of nitrogen gas liberated serves as the measure of the amount of amino nitrogen present in the unknown solution.

Another factor was essential for the accurate determinations of free amino nitrogen, namely, the removal of the proteins from the serum. The method adapted for this purpose was first suggested by Rona and Michaelis (5) and it has since been successfully employed by other investigators (6) (7).

Briefly stated the procedure was as follows: 2 cc. of serum were diluted to 20 cc. in a beaker and heated to boiling. 1.5 cc. of colloidal ferric hydrate were added drop by drop the mixture being shaken with each addition. Precipitation of proteins was then complete. 1.0 cc. of a 20 per cent $MgSO_4$ solution was added to coagulate the excess of iron.

The solution was then filtered through a hardened paper into a 100 cc. evaporating dish, the filtrate being water clear. After the filtration was completed the precipitate was washed by means of a hot water wash bottle into the original beaker, about 20 cc. of water being used in the process.

The mixture was again heated to boiling and the contents of the beaker were filtered into the first filtrate the same filter paper being used. Finally, the filtrate was evaporated nearly to dryness and it was redissolved in 0.5 cc. of water just previous to the amino nitrogen determination.

The digestive mixture consisted uniformly of 2 cc. each 1 cc. of rabbit's serum, 0.5 cc. of complement and 0.5 cc. of protein suspension containing 0.005 gram of protein. This quantity of protein was found to be sufficient in view of the fact that by means of the micro-amino apparatus one can measure accurately small fractions of a milligram of amino nitrogen.

The following tabulation gives the procedure in detail with the results obtained.

*Tube A* contained 1 cc. serum rabbit A (phaseolin), 0.5 cc. guinea-pig's complement, 0.5 cc. phaseolin suspension in saline. Placed in the incubator for six hours, after which the protein was precipitated

and the amino nitrogen determined. *Quantity of amino nitrogen gas found = 0.310* cc.

*Tube B* (control) contained same constituents of tube A, except that the amino nitrogen was determined immediately, without incubation. *Quantity of amino nitrogen gas found = 0.310 cc.*

*Tube C* contained same constituents as tube A except that the serum was first inactivated for one-half hour at 56°C. The complement and phaseolin suspension were then added and placed in the incubator for six hours. *Quantity of amino nitrogen gas found = 0.320 cc.*

*Tube A′* contained 1.0 cc. serum rabbit B, 0.5 cc. complement, 0.5 cc. edestin suspension in saline. Placed in incubator for six hours, after which the protein was precipitated and amino nitrogen determined. *Quantity of amino nitrogen gas found = 0.360 cc.*

*Tube B′* (control) contained the same constituents as tube A′ except that the amino nitrogen was determined immediately, without incubation. *Quantity of amino nitrogen gas found = 0.345 cc.*

*Tube C′* contained the same constituents as tube A′ except that the serum was first inactivated for one-half hour at 56°C. The complement and phaseolin suspension were then added and placed in the incubator for six hours. *Quantity of amino nitrogen gas found = 0.360 cc.*

These findings indicate that the serum of rabbits immunized against protein, possesses no greater proteolytic activity than normal serum.

## CONCLUSIONS

An attempt was made to find whether proteolytic substances are produced in rabbits on protein injections, if a procedure simulating the production of haemolytic substances in these animals, be resorted to. Proteolysis was determined by observing the increase in amino acid nitrogen after digesting mixtures of the immune serum, the specific protein and complement for a given period. The results gave no evidence of any increase in amino acids under these conditions, which would indicate that haemolysis and proteolysis are probably two distinct phenomena.

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# THE INFLUENCE OF ARSPHENAMINE AND MERCURIC CHLORID UPON COMPLEMENT AND ANTIBODY PRODUCTION

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Numerous investigations within recent years have indicated that certain drugs may induce a state of temporary immunity to trypanosome infections by stimulating the antibody producing tissues, the leucocytic mechanism or both, or, combine with antibodies and render the latter more active.

Ehrlich and Shiga (1) have shown that mice infected with caderas and treated with one or more injections of trypan-red, developed a temporary immunity which could not be ascribed to an antibody response following infections with the parasites alone or to the presence of unexcreted dye, but rather to the presence of antibodies in response to the stimulating influence of the drug; later Ehrlich (2) demonstrated the same phenomenon with *T. brucei* and Halberstaedter (3) in similar studies found the immunity highly specific, that is, mice infected with dourine and treated with trypan-red developed an immunity to dourine alone and not to other trypanosomes as *T. brucei* or vice versa. Corroborative evidence of the apparent effect of this and other drugs upon antibody production was given later by the extensive work of Terry (4) who found that a strong immunity against surra of India was obtained by injecting mice with dyes either alone or in combination with acetylatoxyl. That the action of the drugs is indirect rather than wholly trypanocidal, was seemingly shown by the fact that large intraperitoneal injections of surra and caderas were capable of infecting mice when introduced as early as twenty-four hours after the drug and before the latter had been wholly excreted.

Further indications of the possible important relation of drugs to immunity is shown in the reports of several homeopathic physicians as in that Watters (5), who claimed that the administration of calcium

sulphide increased the opsonic index to staphylococci; of Mellon (6) who found that the administration of baptisia influences favorably the production of group agglutinins for typhoid and other closely related bacteria and that veratrum viride increased the opsonic index to pneumococci; of Wheeler (7) who claims that phosphorus increases the opsonic index of human serum to the tubercle bacillus; of Wesselhoeft (8) whose experiments were interpreted as indicating the curative effects of quinine in malaria, could not be ascribed entirely to its parasiticidal activity but probably in part to a favorable influence upon the production of anti-plasmodial antibodies; and of Hooker (9) who showed that the administration of phosphoric acid, arsenious anhydrid and mercuric chlorid homeopathically to normal persons, resulted in the elaboration of agglutinins and complement fixing antibodies for *B. typhosus, B. paratyphosus A and B* and *B. dysenteriae.* In several of these investigations the drugs alone were administered to healthy persons and the appearance of an increase of certain group antibodies in the blood serum was interpreted as an increase of normal or natural antibody and an indication of the possible stimulating influence of these drugs upon antibody producing tissues and a means of their curative value in certain diseases. Likewise the investigations of Arkin (10) concerning the influence of drugs upon phagocytosis may be mentioned in this connection; medicaments which have an inhibitory action upon oxidative processes as chloral, morphine and ether were found to depress phagocytosis while mercuric and other chlorids, colloidal metals, strychnine, arsenic and others, were found to stimulate phagocytosis *in vitro* and *in vivo.*

Following the introduction and encouraging results of arsenical compounds in the experimental chemotherapeusis of protozoan infections, several investigators have a studied their possible influence upon antibody production and particularly the influence of dioxydiamidoarsenobenzol (salvarsan), with the result that a general impression exists that part of the curative influence of dioxydiamidoarsenobenzol in spirochaetic and trypanosome infections, is to be ascribed to the influence of the drug in stimulating the production of protective and curative antibodies in addition to its powerful parasiticidal activity. Aggazzi (11) found that arsenious acid, atoxyl and arsenophenylglycin increased the output of typhoid agglutinin; Friedberger and Masuda (12) claim that salvarsan increases the content of normal agglutinins and hemolysins in the serum; Boehncke (13) found that the administration of salvarsan may be followed by an increase of diphtheria anti-

toxin and of various bacteriolysins, opsonins and precipitins, but not of complement binding substances; Weisbach (14) also claims that the administration of salvarsan results in an increase of agglutinin and hemolysin, while Reiter (15) was unable to note any such influence, his experiments indicating that large doses of the drug lowers resistance to various bacteria.

As further indications of the probable important relation of certain drugs to immunity, are several reports indicating that their administration may be followed by an increase of complement in the serum. Weil and Duport (16) have reported that the intravenous administration of sodium bicarbonate to rabbits resulted in an increase of serum complement; Fenyvessy and Freund (17) claim similar results with the intravenous administration of calcium chlorid and Ciuca (18) found that the injection of tartar emetic and salvarsan was followed by an increase of serum complement in normal and trypanosome-infected animals, while the administration of atoxyl caused a decrease of complement in the serum of normal animals and in a proportion of trypanosome-infected animals.

## EXPERIMENTAL

Since the results of these investigations have indicated that salvarsan may exert an important effect upon complement and spirocheticidal antibodies, we have conducted a series of experiments for the purpose of a further study of the probable influence of arsphenamine[1] (arsenobenzol) and mercury upon antibody and complement production, as follows:

1. A study of their probable influence upon the production of immune antisheep and antihuman hemolysins and agglutinins for sheep and human erythrocytes in rabbits.

2. Upon the production of immune typhoid agglutinin in rabbits.

3. Upon hemolytic complement and normal antisheep hemolysin in rabbit serum.

[1] Arsphenamine is the trade name proposed by the Federal Trade Commission for salvarsan and its substitutes. Throughout this study, the arsphenamine prepared by Dr. J. F. Schamberg, Dr. Geo. W. Raiziss, and Dr. John A. Kolmer in the Dermatological Research Laboratories of the Polyclinic and known as arsenobenzol, was employed in alkaline solution.

4. Upon normal typhoid agglutinin and hemolytic complement in human serum.

We have included a study with mercury bichlorid, because salts of mercury do not appear to have been previously employed in experiments of this nature, while their curative influence in syphilis is well known.

Additional experiments similar to those recorded in this paper but with the employment of animals infected with trypanosomes are being conducted, inasmuch as the results may vary according to the nature of the stimulant (erythrocytes, typhoid bacilli or trypanosomes) used, but we have considered it advisable to record the work finished under the above plan.

*I. The influence of arsphenamine and mercury upon the production of immune hemolysins and hemagglutinins*

*Experiment 1.* In this experiment the sera of six large healthy rabbits were given preliminary tests for the presence of antisheep hemolysin and sheep agglutinin and 1 cc. of a 5 per cent suspension of washed sheep cells per kilogram of body weight injected intravenously every three days followed two hours later by the intravenous administration to rabbits 1 and 2 of arsphenamine in dose of 0.01 gram per kilo (equivalent to 0.6 per 60 kilos) and to rabbits 3 and 4, of bichlorid of mercury in dose of 0.0001 gram per kilo (equivalent to 0.006 gram or about $\frac{1}{10}$ grain per 60 kilos); rabbits 5 and 6 were controls and received injections of cells only. All animals were bled from an ear three days after each injection of cells and drugs, the sera separated and inactivated and the titer of hemolysin determined in the presence of 1 cc. of 1:20 dilution of guinea-pig's complement and 1 cc. of 2.5 per cent suspension of washed sheep cells; the agglutinin titer was determined with 1 cc. of 2.5 per cent suspension of sheep cells alone; both readings were made after incubation of 38°C. for two hours.

The results of this experiment are shown in tables 1 and 2 and may be summarized as follows:

TABLE 1

*Influence of arsphenanine and mercury upon the production of immune antisheep hemolysin*

| ANIMAL NO. | DRUG | DOSE PER KILO | HEMOLTYIC TITER AFTER INJECTION OF CELLS AND DRUGS | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Preliminary injection | First injection | Second injection | Third injection | Fourth injection | Fifth injection | Sixth injection | Seventh injection |
| 1 | Arsenobenzol..... | 0.01 | Less 1: 10 | 1: 25 | 1: 10 | 1: 1000 | 1: 5000 | 1: 10000 | 1: 5000 | 1: 5000 |
| 2 | Arsenobenzol..... | 0.01 | Less 1: 10 | Less 1: 10 | Less 1: 10 | 1: 1000 | 1: 2500 | 1: 5000 | 1: 5000 | 1: 5000 |
| 3 | Bichlor. mercury..... | 0.0001 | Less 1: 10 | Less 1: 10 | 1: 25 | 1: 1000 | 1: 2500 | 1: 10000 | 1: 5000 | 1: 2500 |
| 4 | Bichlor. mercury..... | 0.0001 | Less 1: 10 | 1: 50 | 1: 25 | 1: 1000 | 1: 5000 | 1: 5000 | 1: 2500 | 1: 5000 |
| 5 | Control..... | 0 | Less 1: 10 | 0 | 1: 50 | 1: 1000 | 1: 2500 | 1: 10000 | 1: 12500 | 1: 25000 |
| 6 | Control..... | 0 | Less 1: 10 | 0 | 1: 100 | 1: 166 | 1: 400 | 1: 10000 | 1: 16000 | Died |

TABLE 2

*Influence of arsphenanine and mercury upon the production of immune antisheep hemagglutinin*

| ANIMAL NO. | DRUG | DOSE PER KILO | HEMAGGLUTININ TITER AFTER INJECTION OF CELLS AND DRUGS | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Preliminary injection | First injection | Second injection | Third injection | Fourth injection | Fifth injection | Sixth injection | Seventh injection |
| 1 | Arsenobenzol..... | 0.01 | Less 1: 10 | Less 1: 10 | Less 1: 10 | 1: 50 | 1: 50 | 1: 125 | 1: 166 | 1: 166 |
| 2 | Arsenobenzol..... | 0.01 | Less 1: 10 | Less 1: 10 | Less 1: 10 | 1: 100 | 1: 166 | 1: 125 | 1: 125 | 1: 125 |
| 3 | Bichlor. mercury..... | 0.0001 | Less 1: 10 | Less 1: 10 | Less 1: 10 | 1: 100 | 1: 100 | 1: 125 | 1: 50 | 1: 50 |
| 4 | Bichlor. mercury..... | 0.0001 | Less 1: 10 | Less 1: 10 | Less 1: 10 | 1: 50 | 1: 100 | 1: 50 | 1: 50 | 1: 166 |
| 5 | Control..... | 0 | Less 1: 10 | Less 1: 10 | Less 1: 10 | Less 1: 10 | 1: 10 | 1: 50 | 1: 25 | 1: 166 |
| 6 | Control..... | 0 | Less 1: 10 | Less 1: 10 | Less 1: 10 | Less 1: 10 | Less 1: 10 | 1: 16 | 1: 16 | Died |

1. In the production of hemolysin neither arsphenamine nor bichlorid of mercury appeared to exert a stimulating influence, inasmuch as the amount of hemolysin produced in the drug treated animals was not higher than that in the controls; on the contrary they appeared to produce less hemolysin. It is possible that the doses of arsphenamine and mercury were too large for a three day interval of administration, but all animals appeared to stand the injections well and the majority gained slightly in weight during the course of the experiment.

2. Both drugs however, appeared to increase the output of hemagglutinin to a slight extent, as shown in table 2 by the more rapid production of this antibody in the drug treated animals.

*Experiment 2.* In this experiment a series of six rabbits were bled and the inactivated sera were tested for normal antisheep hemolysin and hemagglutinin. Each animal was then given a daily intravenous injection of 1 cc. of a 1 per cent suspension of washed sheep cells per kilogram of body weight followed immediately with an intravenous injection of the following to the first four animals:

Rabbit 27. 0.004 gram arsphenamine per kilo (0.24 gram per 60 kilos).

Rabbit 28. 0.001 gram arsphenamine per kilo (0.06 gram per 60 kilos).

Rabbit 29. 0.00004 gram bichlorid mercury per kilo (0.0024 gram or about $\frac{1}{30}$ grain per 60 kilos).

Rabbit 30. 0.00002 gram bichlorid mercury per kilo (0.0012 gram or about $\frac{1}{60}$ grain per 60 kilos).

All animals were bled from the ear twenty-four hours after the injection of cells and drugs and the hemolysin content of the inactivated sera was determined by titration in the presence of 1 cc. of 1:20 guinea-pig's complement and 1 cc. of 2.5 per cent suspension of sheep cells; the results of hemagglutination tests were read after incubation at 55°C. for twelve to sixteen hours.

The results of this experiment are shown in the tables 3 and 4 and may be summarized as follows:

1. Generally considered the daily administration of cells and drugs did not appear to result in a greater production of hemolysin

TABLE 3

*Influence of arsphenanine and mercury upon the production of immune antisheep hemolysin*

| ANIMAL NO. | DRUG | DOSE PER KILO | HEMOLYTIC TITER AFTER INJECTION OF CELLS AND DRUGS | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Preliminary injection | First injection | Second injection | Third injection | Fourth injection | Fifth injection | Sixth injection | Seventh injection |
| 27 | Arseno-benzol.. | 0.004 | 1: 10 | 1: 20 | 1: 40 | 1: 40 | 1: 40 | 1: 40 | 1: 500 | 1: 1000 |
| 28 | Arseno-benzol.. | 0.001 | 1: 20 | 1: 40 | 1: 40 | 1: 100 | 1: 125 | 1: 250 | 1: 10000 | 1: 25000 |
| 29 | Bichlor. mercury | 0.00004 | Less 1: 10 | 1: 20 | 1: 20 | 1: 100 | 1: 125 | 1: 500 | 1: 5000 | 1: 16000 |
| 30 | Bichlor. mercury | 0.00002 | 1: 20 | 1: 40 | 1: 40 | 1: 166 | 1: 250 | 1: 2500 | 1: 50000 | 1: 50000 |
| 31 | Control... | 0 | Less 1: 10 | 1: 10 | 1: 20 | 1: 40 | 1: 40 | 1: 500 | 1: 16000 | Died |
| 32 | Control... | 0 | 1: 10 | 1: 20 | 1: 20 | 1: 100 | 1: 40 | 1: 500 | 1: 16000 | 1: 50000 |

TABLE 4

*Influence of arsphenanine and mercury upon the production of immune antisheep agglutinin*

| ANIMAL NO. | DRUG | DOSE PER KILO | AGGLUTININ TITER AFTER INJECTION OF CELLS AND DRUGS | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Preliminary injection | First injection | Second injection | Third injection | Fourth injection | Fifth injection | Sixth injection | Seventh injection |
| 27 | Arseno-benzol | 0.004 | 1: 3 | 1: 3 | 1: 3 | 1: 3 | 1: 10 | Less 1: 3 | 1: 20 | 1: 20 |
| 28 | Arseno-benzol | 0.001 | 1: 3 | Less 1: 3 | Less 1: 3 | 1: 3 | 1: 3 | Less 1: 3 | 1: 40 | 1: 40 |
| 29 | Bichlor. mercury.... | 0.00004 | 1: 3 | Less 1: 3 | Less 1: 3 | 1: 10 | 1: 10 | 1: 40 | 1: 125 | 1: 125 |
| 30 | Bichlor. mercury.... | 0.00002 | Less 1: 3 | Less 1: 3 | Less 1: 3 | Less 1: 3 | 1: 20 | 1: 40 | 1: 166 | 1: 250 |
| 31 | Control..... | 0 | 1: 3 | Less 1: 3 | Less 1: 3 | 1: 3 | 1: 10 | 1: 20 | 1: 40 | Died |
| 32 | Control..... | 0 | Less 1: 3 | 1: 3 | 1: 3 | 1: 3 | 1: 10 | 1: 10 | 1: 40 | 1: 125 |

than was observed in the controls receiving cells alone; rabbits 27 and 29 receiving the larger doses of arsphenamine and mercury produced less hemolysin than the animals receiving the smaller doses, indicating that the larger doses tended to depress antibody production even though the general health and body weight of all animals was maintained at a normal level.

2. Likewise both drugs did not appear appreciably to increase agglutinin production; on the other hand the larger doses of both arsphenamine and mercuric chlorid appeared to limit agglutinin production, as was true of hemolysin production just described.

*Experiment 3.* In this experiment a series of six large healthy rabbits were bled and the inactivated sera tested for normal antihuman hemolysin and hemagglutinin. Each animal was then given daily intravenous injections of washed human cells in dose of 1 cc. of a 5 per cent suspension per kilogram of body weight; immediately after these injections the animals were injected intravenously with the following:

Rabbits 13 and 14. 0.001 gram arsphenamine (0.06 gram per 60 kilos).

Rabbits 15 and 16. 0.00001 gram bichlorid mercury (0.0006 gram or about $\frac{1}{100}$ grain per 60 kilos).

Each animal was bled every three days and the inactivated sera titrated for hemolysin with 1 cc. of 1: 20 guinea-pig's complement and 1 cc. of a 2.5 per cent suspension of human cells; also for hemagglutinin employing a 1 per cent suspension of cells and reading the results after incubation at 55°C. for twelve to sixteen hours.

The results are shown in tables 5 and 6 and may be summarized as follows:

1. The production of antihuman hemolysin was slight with all animals including the controls; neither drug appeared to influence hemolysin production.

2. Both drugs appeared slightly to increase the output of hemagglutinin, as shown by a somewhat quicker response on the part of the drug treated animals and the final higher titer of the serum of two (nos. 14 and 15).

TABLE 5

*Influence of daily injection of arsphenanine and mercury upon the production of immune antihuman hemolysin*

| ANIMAL NO. | DRUG | DOSE PER KILO | HEMOLYTIC TITER AFTER INJECTION OF CELLS AND DRUGS | | | |
|---|---|---|---|---|---|---|
| | | | Preliminary injection | After two injections | After five injections | After nine injections |
| 13 | Arsenobenzol... | 0.001 | Less 1: 10 | Less 1: 10 | Less 1: 10 | Died |
| 14 | Arsenobenzol.... | 0.001 | Less 1: 10 | Less 1: 10 | Less 1: 10 | Less 1: 10 |
| 15 | Bichlor. mercury..... | 0.00001 | Less 1: 10 | Less 1: 10 | Less 1: 10 | Less 1: 10 |
| 16 | Bichlor. mercury..... | 0.00001 | Less 1: 10 | Less 1: 10 | Less 1: 10 | Less 1: 10 |
| 17 | Control..... | 0 | Less 1: 10 | Less 1: 10 | Less 1: 10 | Less 1: 10 |
| 18 | Control..... | 0 | Less 1: 10 | Less 1: 10 | Less 1: 10 | Died |

TABLE 6

*Influence of daily injections of arsphenanine and mercury upon the production of immune antihuman agglutinin*

| ANIMAL NO. | DRUG | DOSE PER KILO | AGGLUTININ TITER AFTER INJECTION OF CELLS AND DRUGS | | | |
|---|---|---|---|---|---|---|
| | | | Preliminary injection | After two injections | After five injections | After nine injections |
| 13 | Arsenobenzol..... | 0.001 | Less 1: 10 | Less 1: 10 | 1: 50 | Died |
| 14 | Arsenobenzol..... | 0.001 | Less 1: 10 | Less 1: 10 | 1: 15 | 1: 250 |
| 15 | Bichlor. mercury.... | 0.00001 | Less 1: 10 | Less 1: 10 | 1: 10 | 1: 500 |
| 16 | Bichlor. mercury.... | 0.00001 | Less 1: 10 | Less 1: 10 | 1: 16 | 1: 166 |
| 17 | Control..... | 0 | Less 1: 10 | Less 1: 10 | Less 1: 10 | 1: 166 |
| 18 | Control..... | 0 | Less 1: 10 | Less 1: 10 | Less 1: 10 | Died |

## *II. The influence of arsphenamine upon normal antisheep hemolysin*

*Experiment 4.* In this experiment we have studied the influence of a single large dose of arsphenamine administered intravenously to rabbits, upon the total hemolytic activity (normal hemolysin and complement) of their sera for sheep cells and upon the normal hemolysin alone; as shown by Kolmer and Williams (19) the sera of about 70 per cent of normal rabbits contain antisheep hemolysin and about the same percentage (63 per cent) was found in the present series.

Each rabbit was bled immediately before, three hours and again eighteen hours after the intravenous injection of arsphenamine in dose of 0.06 gram per kilo (equivalent to 3.6 grams per 60 kilos of body weight). The serum was separated and its hemolytic activity determined by titrating varying amounts of active serum with a constant dose of 1 cc. of 2.5 per cent suspension of washed sheep cells. Each serum was then heated at 55°C. for thirty minutes and its content of normal antisheep hemolysin determined in a titration employing 1 cc. of 1:20 dilution of guinea-pig's complement and 1 cc. of 2.5 per cent sheep

TABLE 7

*The influence of arsphenanine upon the total hemolytic activity of the sera of normal rabbits*

| NO. | DOSE PER KILO | RESULTS WITH SERA BEFORE INJECTION | | | | | | RESULTS WITH SERA THREE HOURS AFTER INJECTION | | | | | | RESULTS WITH SERA EIGHTEEN HOURS AFTER INJECTION | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.3 cc. | 0.2 cc. | 0.15 cc. | 0.1 cc. | 0.08 cc. | 0.05 cc. | 0.3 cc. | 0.2 cc. | 0.15 cc. | 0.1 cc. | 0.08 cc. | 0.05 cc. | 0.3 cc. | 0.2 cc. | 0.15 cc. | 0.1 cc. | 0.08 cc. | 0.05 cc. |
| | *gram* | | | | | | | | | | | | | | | | | | |
| 606 | 0.06 | C | C | C | M | S | S | C | M | M | S | S | N | C | M | M | M | S | N |
| 607 | 0.06 | C | M | M | M | M | S | C | M | M | M | M | S | C | M | M | M | S | S |
| 608 | 0.06 | C | M | M | S | N | N | S | S | N | N | N | N | M | M | S | N | N | N |
| 611 | 0.06 | C | M | M | S | S | S | M | M | S | S | S | S | C | M | S | S | S | S |
| 618 | 0.06 | C | M | M | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| 622 | 0.06 | M | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |

C = complete hemolysis; M = marked hemolysis; S = slight hemolysis; N = no hemolysis.

cells. The results in both tests were read after incubation in a water bath at 38°C. for an hour. The results of a few of these tests are shown in tables 7 and 8 and the whole has been summarized as follows:

*a*. The total hemolytic activity of the sera of 25 rabbits tested three hours after the preliminary tests and injection of arsphenamine showed: No change with the sera of 10; a decrease with the sera of 15.

*b*. Tested eighteen hours after the injection of arsphenamine, the sera of 22 of these rabbits surviving showed: No change with

the sera of 9; an increase of hemolytic activity with the sera of 2; a decrease of hemolytic activity with the sera of 11.

*c.* The content of normal antisheep hemolysin in the sera of 30 rabbits tested three hours after the preliminary tests and injection of arsphenamine showed: No change with the sera of 27; a slight decrease with the sera of 3.

*d.* Tested eighteen hours after the injection of arsphenamine, the sera of 25 of these rabbits surviving showed: No change with the sera of 22; a slight decrease with the sera of 3.

From these experiments it would appear that a single large dose of arsphenamine tends to decrease the content of hemolytic

TABLE 8

*The influence of arsphenamine upon the antisheep hemolysin in the sera of normal rabbits*

| NO. | DOSE PER KILO | RESULTS BEFORE INJECTION | | | | | RESULTS THREE HOURS AFTER INJECTION | | | | | RESULTS EIGHTEEN HOURS AFTER INJECTION | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.2 cc. | 0.1 cc. | 0.08 cc. | 0.05 cc. | 0.03 cc. | 0.2 cc. | 0.1 cc. | 0.08 cc. | 0.05 cc. | 0.03 cc. | 0.2 cc. | 0.1 cc. | 0.08 cc. | 0.05 cc. | 0.03 cc. |
| | *gram* | | | | | | | | | | | | | | | |
| 598 | 0.06 | S | S | S | N | N | S | S | S | N | N | S | S | S | N | N |
| 604 | 0.06 | S | N | N | N | N | S | N | N | N | N | S | S | S | N | N |
| 610 | 0.06 | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| 611 | 0.06 | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N |
| 612 | 0.06 | S | N | N | N | N | N | N | N | N | N | N | N | N | N | N |
| 615 | 0.06 | S | N | N | N | N | N | N | N | N | N | N | N | N | N | N |

S = slight hemolysis; N = no hemolysis.

complement, normal hemolysin or both and particularly the complement, for a period of at least eighteen hours following the administration of drug. Smaller doses of arsphenamine however, administered to persons, may produce an increase of hemolytic complement following a primary depression, as shown in the following experiment.

### *II. The influence of arsphenamine upon hemolytic complement of persons*

*Experiment 5.* The fresh active sera of 20 persons suffering with syphilis were titrated in varying doses to determine their

content in hemolytic complement[2] with a constant unit of antisheep hemolysin and 1 cc. of 2.5 per cent suspension of washed sheep cells; each person was then given an intravenous injection of 0.4 to 0.6 gram arsphenamine in the clinic of Dr. Jay F. Schamberg in the course of their regular treatment. Blood was drawn one and eighteen hours later and the complement content was determined with each fresh serum and the same unit of hemolysin and dose of cells. The results observed with the sera of 19 persons secured one hour after the administration of arsphenamine were as follows (a few being given in table 9 as examples):

TABLE 9

*The influence of arsphenanine upon the hemolytic complement of adult syphilitic persons*

| NO. | DOSE GIVEN | RESULTS WITH SERA BEFORE INJECTION | | | | | | RESULTS WITH SERA ONE HOUR AFTER INJECTION | | | | | | RESULTS WITH SERA EIGHTEEN HOURS AFTER INJECTION | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.3 cc. | 0.2 cc. | 0.15 cc. | 0.1 cc. | 0.08 cc. | 0.05 cc. | 0.3 cc. | 0.2 cc. | 0.15 cc. | 0.1 cc. | 0.08 cc. | 0.05 cc. | 0.3 cc. | 0.2 cc. | 0.15 cc. | 0.1 cc. | 0.08 cc. | 0.05 cc. |
| | *gram* | | | | | | | | | | | | | | | | | | |
| B. B. | 0.6 | C | M | M | S | S | N | C | M | M | S | S | N | C | C | M | S | S | N |
| S. S. | 0.6 | C | M | S | S | N | N | C | M | S | N | N | N | C | C | C | C | M | S |
| G. G. | 0.6 | C | C | M | S | N | N | C | C | M | S | S | N | C | C | C | C | C | M |
| H. S. | 0.6 | C | C | C | S | S | S | C | C | M | S | S | S | C | C | C | M | S | S |
| P. C. | 0.6 | C | C | C | C | M | S | C | C | C | C | M | S | C | C | C | M | M | S |
| S. H. | 0.6 | C | C | C | M | S | S | C | C | C | M | S | N | C | C | C | C | C | M |

C = complete hemolysis; M = marked hemolysis; S = slight hemolysis; N = no hemolysis.

No change with the sera of 15; a decrease of hemolytic activity (complement) with the sera of 4.

Seventeen of these persons returned to the clinic on the following day, and their sera secured about eighteen hours after the administration of arsphenamine showed: No change with the sera of 5; an increase of hemolytic activity (complement) with the sera of 9; a decrease of hemolytic activity (complement) with the sera of 3.

[2] As the majority of these sera also contained normal antisheep hemolysin and as this was not removed, the results indicate the influence of the drug upon the total hemolytic activity of the sera rather than upon complement alone.

## *IV. The influence of arsphenamine and mercury upon immune and normal typhoid agglutinin*

*Experiment 6.* In this experiment the sera of a series of six rabbits were titrated for typhoid agglutinin and then each animal was given intravenously 1 cc. of a heat-killed monovalent typhoid vaccine per kilogram of body weight; each cubic centimeter of vaccine contained two billion bacilli. The vaccine was administered every three days and it was followed two hours

TABLE 10

*Influence of arsphenanine and mercury upon the production of immune typhoid agglutinin*

| ANIMAL NO. | DRUG | DOSE PER KILO | AGGLUTININ TITER AFTER INJECTIONS OF VACCINE AND DRUGS | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Preliminary injection | First injection | Second injection | Third injection | Fourth injection | Fifth injection | Sixth injection |
| | | *gram* | | | | | | | |
| 7 | Arsenobenzol.... | 0.01 | Less 1:4 | Less 1:4 | Died | 0 | 0 | 0 | 0 |
| 8 | Arsenobenzol.... | 0.01 | Less 1:4 | Less 1:4 | Less 1:4 | Less 1:4 | 1:64 | 1:512 | 1:2048 |
| 9 | Bichlor. mercury..... | 0.0001 | Less 1:4 | Less 1:4 | Died | 0 | 0 | 0 | 0 |
| 10 | Bichlor. mercury..... | 0.0001 | Less 1:4 | Less 1:4 | Less 1:4 | 1:768 | 1:1024 | 1:6144 | 1:8192 |
| 11 | Control ..... | 0 | Less 1:4 | Less 1:4 | 1:24 | 1:1024 | 1:1024 | 1:6144 | 1:12000 |
| 12 | Control..... | 0 | Less 1:4 | Less 1:4 | 1:24 | 1:768 | 1:768 | 1:2048 | 1:3072 |

later by the intravenous administration to the first two rabbits of arsphenamine in dose of 0.01 gram per kilo (equivalent to 0.6 per 60 kilos) and to the third and fourth rabbits, of bichlorid of mercury in dose of 0.0001 gram per kilo (equivalent to 0.006 gram or about $\frac{1}{10}$ grain per 60 kilos). Each animal was bled three days after the injections and the inactivated serum was titrated for typhoid agglutinin in a macroscopic test, the results being read after incubation at 55°C. for twelve to sixteen hours.

The results of this experiment are shown in table 10 and indi-

cate that the influence of both drugs was an inhibition on agglutinin production; possibly this effect was due to the use of two large doses of both arsphenamine and bichlorid.

*Experiment 7.* The sera of 24 persons suffering with syphilis were titrated for their content in normal typhoid agglutinin and the tests repeated one and again eighteen hours after the first dose of 0.6 gram of arsphenamine received in the treatment of their infection. The results of these tests showed no increase of normal agglutinin after the one injection of arsphenamine.

## SUMMARY

1. Numerous studies indicate that several drugs and particularly arsenic compounds, may stimulate antibody production and that their curative effect in the treatment of disease and particularly syphilis and experimental trypanosomiasis, may be ascribed to this influence upon antibody production in addition to their direct parasiticidal influence.

2. In our experiments, the intravenous administration of arsphenamine to normal rabbits in doses varying from 0.01 gram to 0.004 gram per kilogram of body weight (equivalent to 0.6 to 0.24 gram per 60 kilos), did not result in an increased output of immune hemolysin to sheep cells, but rather suppressed hemolysin production; smaller doses did not appear to retard hemolysin production to sheep's and human cells but likewise they did not result in an increase. The smaller doses, however, generally produced a slight increase of agglutinin for sheep's and human cells.

3. Similar results were observed with mercuric chloride; large doses appeared to suppress hemolysin and agglutinin production while smaller doses tended to increase the production of hemagglutinins but not that of the hemolysins.

4. A single large dose of arsphenamine (0.06 gram per kilo) administered intravenously to rabbits reduced the hemolytic activity of their sera within a period of twenty-four hours after injection, probably by an influence upon the hemolytic complement; the administration of a smaller dose (0.6 gram) to syphilitic

persons as part of the treatment of their infection, was found generally to produce a depression in the hemolytic activity of the serum as tested one hour after injection followed by a general increase within eighteen hours.

5. Large doses of arsphenamine and mercuric chlorid to rabbits tended to limit agglutinin production for typhoid bacilli; single doses of arsphenamine (0.6 gram) in adults did not influence the amount of normal typhoid agglutinin in their sera.

6. The general result of these experiments indicates that while massive doses of arsphenamine and mercuric chlorid tend to suppress antibody production and cause a decrease in hemolytic complement, smaller doses tend to increase the production of antibody (agglutinins) and augment the complement content after a primary decrease. It is probable that both drugs administered in the treatment of syphilis, owe part of their therapeutic efficacy to their influence upon increasing antibody production and complement.[3]

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# PROCEEDINGS OF THE AMERICAN ASSOCIATION OF IMMUNOLOGISTS

## Fifth Annual Meeting, Held at the New Medical Laboratories and in the Hygiene Laboratory of the University of Pennsylvania, Philadelphia

*March 29–30, 1918*

The President, Dr. John A. Kolmer, in the Chair.

1. Report of the Council. Executive Session.

2. The Rôle of Immunity in the Conduct of the Present War

*John A. Kolmer* (President's address, see this volume, page 371).

3. Experiments on the Production of Antipoliomyelitic Serum in Rabbits

*Edgar H. Tsen* (see this volume, page 213).

4. Active Immunity in Experimental Poliomyelitis

*Harry L. Abramson* (see this volume, page 437).

DISCUSSION

*Arthur F. Coca:* It is important to emphasize the concluding remarks of Dr. Abramson. The conditions under which Dr. Abramson was working to demonstrate immunity were highly artificial and the immunity attained was possibly more than was necessary to constitute an absolute resistance to the natural infection as occurring in human beings. In the artificial experiments the mechanism of resistance at the natural atria of infection was circumvented. It would have been hard to foresee, from the guinea-pig experiments, how little antitoxin is necessary in order effectively to prevent the natural infection with diphtheria in human beings.

*William H. Park:* I wish to say a word in line with Dr. Coca's remarks. In test animals, one species might react, and one might not react. In regard to protective sera: The rabbit gives very feeble protective serum; the monkey gives better serum, but the horse gives as good, or even a better serum than the monkey. Perhaps Dr. Abramson will say a word on that question. In my own experiments

I first used virus from the brain, digested by trypsin, as suggested by Dr. Neustadter and then later untreated virus. Different species of animals show different periods of development of antibodies and also a different degree.

*John A. Kolmer:* Has the serum been used in the treatment of human infections?

*Harry L. Abramson:* I wish to add a word about rabbit poliomyelitis. I spent about six months inoculating rabbits with material from human cases and experimental poliomyelitis with rather discouraging results. Only a few of a large number of young animals inoculated intracerebrally, perineurally and intraperitoneally exhibited flaccid paralyses. The presence of paralysis was considered the only reliable evidence of a possible poliomyelitic infection. The pathologic findings in the cords of these animals were not like that of human poliomyelitis.

The sera of these animals were not tested for the presence of immune substances. However, an attempt was made to determine whether injections into monkeys of emulsions of cords from rabbits that exhibited flaccid paralyses would prevent the experimental disease. In one instance an animal so treated was paralyzed after an incubation of fourteen days whereas the control animal was paralyzed in six days. It is problematical whether this prolongation of the incubation period in the treated animal was due to any degree of immunity conferred by the injections of rabbit's cord or whether it was simply an instance of variability in incubation period. The test virus used was that of the 1916 epidemic, which is quite variable.

As regards an anti-poliomyelitic horse serum, two animals have been subjected to injections of polio-virus emulsions over a period of three to six months. Both have neutralized *in vitro* a 5 per cent emulsion of the highly virulent Rockefeller strain in the proportion of one part of serum to one part of virus.

I have had no personal experience in the use of this anti-poliomyelitic horse serum in the treatment of the human disease. Dr. Neustadter of New York treated three cases, in all of which there were rather severe reactions. It is very difficult to draw conclusions as to the value of serum therapy in poliomyelitis because of the great difficulty in prognosis in this disease. Large numbers of unselected cases must be treated and the mortality figures compared with that of an equal number of unselected cases in the same outbreak before one can make any positive statements on this matter.

Rosenow's anti-poliomyelitic horse serum was subjected to the *in vitro* neutralization test and was found to neutralize the Rockefeller virus in proportion of one part of serum to one part of virus.

### 5. Types of Meningococci Concerned in Epidemic Infections

*A Parker Hitchens and C. H. Robinson:* The troops in this country are now experiencing the same conditions that the British troops experienced at the beginning of the war. The work of English investi-

gators is here of value, especially that of Gordon on the meningococci. Gordon typed the different strains of these organisms as has been done with the pneumococci, into four different groups. This work has been followed in the present experiments, and 100 cultures, received from the first of the year, have been typed according to the Gordon method. The strains have been received from several concentration camps in this country. The cultures, from spinal fluids or from naso-pharyngeal swabs—were Gram negative cocci. They corresponded to a greater or less degree to Gordon's types; namely, I, parameningococcus; II, normal meningococcus: III and IV, Comparatively rarely found in this country. The grouping of the strains varied with different camps. Camp Jackson had 1 strain of type I; 9 strains of type II; 1 strain of type III. Great Lakes camp showed: type I, 1 strain; type II, 28 strains; 2 Flavus; 2 undermined. It was shown that each camp had a predominating type, but whether this was a true epidemic was not determined. The work was undertaken with the hope that specific sera might be evolved to counteract the types. Gordon has had excellent results with his specific strains. He found that if specific serum were given before the third day of the disease the mortality was only 9 per cent: if on the seventh day the mortality was 50 per cent. In testing the types the precipitin test has given valuable results, which were confirmed by cultural methods. Agglutination of strains seems to give distinct differences which follow the types as laid down by specific sera, but what the relationship is, is not yet conclusive. A rapid method of typing the meningococci was sought, so as to expedite the application of specific therapy.

6. The Influence of Normal Human and Guinea-Pig's Serum (Complement) Alone and in Combination with Antimeningitis Sera upon Virulent Meningococci.

*John A. Kolmer, Toitsu Matsunami and Ikuzo Toyama* (see this volume, page 157).

7. The Relation of the Meningococcidal Activity of the Blood to Resistance to Virulent Meningococci

*Toitsu Matsunami and John A. Kolmer* (see this volume, page 201).

DISCUSSION

*William H. Park:* I would like to ask a question as to the use of "Specific therapy" in the treatment of cerebro-spinal meningitis Could one get a monovalent serum for one type which would be appreciably stronger than the polyvalent serum for the same type? If one or two types could be injected into the horse at once and a nearly equally good antiserum produced for each, the advantage would be much greater than if one used a different horse for each type. Is there evidence that

a polyvalent serum had been produced? If not, it would be advantageous to produce a serum for one or two types. In the pneumococcus serum it is advisable to make antisera against distinct types, because the microörganisms are not closely related; but in this case a polyvalent serum against two or more types would seem to be equally potent.

*Dr. Hitchens:* Dr. Park's point is very well taken and I agree up to a certain point. There might, however, be a greater possibility of reducing the death rate through the use of specific sera. It is possible that there may be immunological variations of meningococci within the serological types. It is well known that with the best balanced sera obtainable one finds cases of meningitis to resist their action. The meningococci isolated from spinal fluids in these cases are resistant in culture to the action of the polyvalent serum, although belonging to one of the well known fixed types. These may be serum-fast strains or not agglutinable strains. The Rockefeller Institute agglutination work would show that meningococci can not be accurately and sharply grouped within certain types, but follow the order of a chromatic scale. There are an almost innnumerable number of variations, although the variations are slight. The problem of getting a polyvalent serum is therefore difficult. It is felt, however, that work should follow the lines of making a polyvalent type serum and find out whether this would not confer more resistance in the refractory cases. It will be found necessary for this purpose to use many strains of meningococci. One can not select single strains of the four types and immunize a horse to these four strains because there are an infinite number of variants within the types. In one case where the spinal fluid gave a specific reaction with the precipitin test, the patient did not do well with the polyvalent serum. With a specific serum the case recovered.

In regard to Dr. Matsunami's paper, those who are interested in active immunization against cerebro-spinal fever will welcome the technic of Dr. Heist. If even a low grade of immunity can be demonstrated, this technic will be of considerable assistance.

*William H. Park:* Dr. Hitchens has not exactly answered my question. Can not one horse be injected with two or three types, as well as with one type alone? In New York we are using the combined serum, and we have obtained a reduction of mortality to 22 per cent. It will not do to take the time to type individual strains as in pneumonia. Has it been proved that one can not produce a serum from a single horse that would answer to several strains? If not, there must be a horse for each strain. This would be very confusing from the laboratory standpoint.

*H. Parker Hitchens:* I thought I had replied to Dr. Park. If the number of antigens could be limited to four or five there would be no reason to think of specific sera. It is not possible to produce sera equivalent to each four strains, as high as for a single strain. In an antiserum for all strains it may be as high as against a single strain. If there were only five variants it would not be necessary to think of specific sera; but, if, as has been suggested, there is a chromatic scale of

variation—forty to sixty different varieties, one can not obtain sera as high for individual variants, as if they were split into groups of four or five. There is, however, as yet, no positive evidence in support of this theory.

*Charles Krumwiede:* I have been working on the precipitin test for the diagnosis of type. From my experience I anticipated considerable cross reaction between groups one and three and between groups two and four. Before progress can be made, an easier way of differentiation eliminating the necessity of absorption to obtain an accurate determination of type will have to be found. However, the test might differentiate, with some degree of accuracy, groups one and three from groups two and four. Nevertheless, there is danger of a misleading reaction due to group antibodies even though the strain does not belong to any of the four groups. I have found, as has Dr. Kolmer, that horse serum is rich in normal opsonins and it is difficult for this reason to standadize therapeutic sera by determining the opsonic content. Attempts to differentiate between "normal" and "immune" opsonins by heating and reactivation have failed.

## 8. The Nature of Antianaphylaxis

*J. Bronfenbrenner and M. J. Schlesinger:* The convulsion in eclampsia and epilepsy as well as the characteristic symptoms of asthma, hay fever and certain dermatoses have been interpreted by a number of investigators as anaphylactic phenomena. Our earlier experimental studies of anaphylaxis (1) led us to the conclusion that the latter are due to the liberation of proteolytic enzymes in the blood of the individuals and experimental animals affected. We found that the activation of these proteolytic enzymes is controlled by the mechanism based upon the balance of the proteolytic ferments and antiferments in the blood (2). The actual measurements of antitryptic index in the individuals during eclamptic and epileptic seizures, confirmed our view that a decrease of antitryptic powers of the blood leads to proteolysis *in vivo*, and vice versa, that recovery from anaphylaxis is accompanied by a marked rise of antitryptic power of the blood (3). Since it was shown by several investigations that sensitized animals can be protected against anaphylaxis by special treatment, we started out to study the mechanism of this protection with the view of finding, if possible, a theoretical basis for the methods of prevention of clinical anaphylaxis in man. It is an established fact that the injection of a sublethal dose of antigen preliminary to the test injection prevents shock in sensitized animals. The explanation of this phenomenon offered by Friedberger was that the sublethal dose of antigen exhausts the serum of its specific antibody by combining with it, and thus prevents the formation of anaphylatoxin when a subsequent test injection is made. There are, however, some phenomena in antianaphylaxis which cannot be adequately explained on the basis of exhaustion of antibody. Thus, for instance, it was noticed that already a few days after the injection

of a sublethal dose of antigen into hypersensitive animal, its hypersensitiveness partly returns and that, in general, the length of the antianaphylactic state depends upon the size of the vaccinating injection. Moreover, the experiments of Anderson and Frost suggested that antibody is present in the blood of antianaphylactic animals long before they return to the state of hypersensitiveness. Our own experiments conducted with the view of determining whether the exhaustion of antibody was the underlying mechanism in the experiments of Friedberger led us to the conclusion that such was not the case. We found that animals sensitized simultaneously against two proteins and receiving a vaccinating injection of one of them become resistant to the test injection of the second protein, though there could be no question of exhaustion of the second antibody. We found further that guinea-pigs receiving a large sublethal dose of any anaphylatoxin prepared *in vitro* become resistant to a subsequent test injection of several lethal doses of the same or any other anaphylatoxin. Moreover, direct experiments *in vitro* by means of the Abderhalden reaction show definitely that the antibody is not exhausted from the blood of sensitized animals receiving a sublethal dose of antigen. That the state of antianaphylaxis in experimental animals is not due to the changes in antibody concentration in their blood is also suggested by the fact that animals can be rendered resistant to anaphylactic shock by a number of nonspecific methods. Our analysis of the mechanism of the antianaphylaxis produced in experimental animals by a number of such nonspecific methods brought out the following conclusions. Introduction of certain poisonous substances, which may cause destructive changes in the tissues, in quantities not sufficiently large to kill the animal outright, is followed by the death of tissues immediately affected by poison. In this process the intracellular ferments are set free, and together with the ferments thrown out from the surrounding fixed cells as well as from the blood serum and leucocytes digest the dead material. The split products of such digestion exert antagonistic action, and retard or stop further activity of the proteolytic ferments. The time of appearance, the rate of their increase and the length of time during which these split products remain in the blood, determine the antitryptic titer of such blood. We have tested with this point in view the effects of ether, chloroform, alcohol, choral, morphine and scopolamine, and we found that the power of these substances to protect the animals against anaphylaxis is strikingly parallel with their power to increase the antitryptic titer in the blood of these animals. We found that the animals were protected against anaphylaxis only so long as the antitryptic titer resulting from the treatment remained above the normal. The same relation between the power of substances administered to protect against anaphylaxis and to increase the antitryptic titer of the blood of treated animals was found also in the cases of treatment with $BaCl_2$, $CaCl_2$ as well as with lecithin and cholesterin.

It was suggested by several investigators that starvation or exposure to low temperature may also protect the sensitized animals against

anaphylaxis. Chemical studies by various investigators suggest very strongly that there is a certain amount of analogy between the changes in metabolism of animals during starvation and anaphylaxis. We found accordingly that the antitryptic index of the blood or starving guinea-pigs is above normal. As for the protective influence of low temperature, our measurements have shown that it is not connected with the antitryptic balance, but is merely due to the fact that the whole process of anaphylaxis becomes slow under the influence of cold and the symptoms become, accordingly, less acute.

### 9. Studies on So-Called Cellular Anaphylaxis

*W. P. Larson and E. T. Bell.*

#### DISCUSSION

*John F. Anderson:* I wish to ask Dr. Bronfenbrenner whether my impression is correct that if the animal were sensitized to more than one protein there would be an inhibitory effect from the injection of a sublethal dose.

*J. Bronfenbrenner:* In the experiments with animals sensitized to two antigens in which one of the antigens is injected in order to vaccinate against the effect of subsequent injection of the second antigen, one has to be very careful properly to select the vaccinating dose. If the amount of antigen injected is but a small fraction of the lethal dose, it may not protect sufficiently, but if the amount inoculated is sufficiently close to the minimum lethal dose, so that it may even cause slight anaphylaxis, the animal is temporarily protected against at least one and a half or two lethal doses of the second antigen.

*Arthur F. Coca:* Dr. Larson's results contradict those of some previous work of my own; that is, they seem to show that my conclusions, as far as they pertain to the percentage of residual blood after perfusion, were wrong. This, however, does not invalidate my chief conclusion, which was that the site of the anaphylactic reaction is in the tissue cells, because in the same paper evidence is presented proving that if as little as 50 per cent of the actively sensitized guinea-pig's blood is removed by perfusion the residual 50 per cent will not, in half of the animals, contain an amount of the sensitizing antibodies sufficient to sensitize a single guinea-pig. The experiments in perfusion of passively sensitized guinea-pigs were still more conclusive inasmuch as Weil had shown that the blood of such animals contains no demonstrable antibodies forty-eight hours after the sensitizing injection.

Dr. Bronfenbrenner's experiments illustrate what has been called nonspecific antianaphylaxis. For the purpose of Dr. Bronfenbrenner's argument it is necessary to show that the desensitization observed is specific; otherwise the experiments can throw no light on the nature of specific "antianaphylaxis." In general it should be borne in mind that any humoral theory of anaphylaxis is at a disadvantage in not being able to explain the latent period in passive sensitization.

*G. H. A. Clowes:* I can substantiate Dr. Anderson's position. Six years ago he had tried desensitization against hay fever in patients sensitive to spring and autumn fever. Timothy and ragweed were used and it was found that the skin test was strongly specific. Patients desensitized to timothy were not altered in their reaction to ragweed. Immunity developed only against the agent employed. The matter of antitryptic reaction is related to the matter of surface tension. The soaps, lecithins, calcium chloride and barium chloride play a part in this question. The nonspecific interference is a matter of surface tension I myself have obtained a marked desensitization to hay fever with a marked antitryptic index. This was followed up with care on account of its relation to the cancer question. The high antitryptic index was coincident with a maximum desenaitisation following a slight anaphylactic shock which fell appreciably a few months after the hay fever period. Anaphylactic effects appear to be due to increased permeability of the protoplasmic film.

*J. Bronfenbrenner:* Of course the the time which is allowed to elapse between the vaccinating and the test injections in my experiments is a very essential element. The test injection must follow closely enough, so that the antitryptic index of the vaccinated animal is still above the normal at the time of the test injection. Another equally important factor is the method of injection. If the vaccinating injection is given intraperitoneally or subcutaneously—the results can never be as sharp as in the case of intravenous inoculations.

Dr. Coca is quite right in making a distinction between specific and nonspecific phenomena in antianaphylaxis, but in my experiments I did not intend to study this question. My problem was to see whether the mechanism of antianaphylaxis in the experiments of Friedeberger was that of exhaustion of antibody, and if not whether the mechanism in this case as well as in other instances of antianaphylaxis is the same. I suspected that there must be only one essential process (or set of processes) underlying all the phenomena of antianaphylaxis, because it was found empirically that the specific anaphylaxis can be regularly checked by such nonspecific treatment as the administration of ether or the injection of $BaCl_2$. While the experiments of Bronfenbrenner do not prove that the entire mechanism of the phenomena can be reduced to the question of the control of ferment action in the blood, they show, nevertheless, conclusively that in all the cases of antianaphylaxis studied by him, the high antitryptic index of the blood was a part of the symptom-complex of antianaphylaxis in guinea-pigs, and vice versa; whenever the antitryptic index of the blood in the guinea-pigs was raised (by whatever method) the animals were refractory to shock.

Dr. Larson's experiments seem to me exceedingly valuable. It was very interesting to see so clearly demonstrated that the perfusion of organs is very irregular. Dr. Larson himself has drawn conclusions as to the importance of his observation in relation to the question of cellular anaphylaxis as contrasted with humoral anaphylaxis. There is

very little doubt but that the cells of the body take part in what we observe as final symptoms of anaphylactic reaction. What is the exact part played by the cells is not yet well understood, but it seems that in all of the experiments in which the behavior of the tissue was studied during the anaphylactic shock the presence of the serum in such tissues was not definitely excluded. While such was the feeling of several investigators all along, Dr. Larson's experiments for the first time demonstrated that such was the case. I would like to ask Dr. Larson whether he can offer any suggestions as to the reason why the fluid with which the organ is perfused does not reach certain parts of the tissues.

### 10. Experimental Pollinosis in Guinea-Pigs

*Henry L. Ulrich.*

#### DISCUSSION

*G. H. A. Clowes:* I do not feel competent to discuss Dr. Ulrich's paper as a whole, but I am interested in the results. In experiments made with human material, numerous attempts have been made with a variety of means to induce sensitization in individuals that were not previously sensitive, but without result. This might give a clue to the work upon this mysterious condition.

*John F. Anderson:* A good many years ago it was demonstrated that animals could be sensitized by instillations in the nose so as to respond with symptoms of anaphylaxis. Has Dr. Ulrich confused the sensitization to protein with the sensitization to pollen. These are two entirely different propositions and opened a field for large experimentation.

*Arthur F. Coca:* No doubt we all recognize this work of Dr. Ulrich as revolutionary. Despite the innumerable injection into human beings of various kinds of foreign protein no instance of the experimental production of a condition corresponding to hay fever has yet been reported. In a series of experiments with Dr. Cooke and Dr. Flood I was unable to produce the usual condition of experimental anaphylaxis in guinea-pigs with strong extracts of pollen.

*A. Parker Hitchens:* I would like to ask Dr. Ulrich what pollen was used and what method of extraction? All the pollen I have used was very badly contaminated with bacteria of different kinds. The bacterial contamination might play a large part in the phenomena which resulted.

*G. H. A. Clowes:* I found contamination by bacteria and minute insects. Reactions to these might be introduced. I never succeeded in getting reactions in normal individuals.

*Geo. H. Smith:* We instilled pollen into the nose and mucous membrane of normal animals and also we tried to injure the mucous membranes to see whether they would respond more after injury. The animals were shut up in glass cages in which pollen was kept circulating by means of the electric fan. No sensitization was obtained.

*Henry L. Ulrich:* The pollen was washed in acetone and the sterile pollen was injected into the peritoneal cavity. It never showed any growth. It was used dry in powder form.

### 11. A Skin Reaction to Pneumotoxin

*Charles Weiss:* This study has been instituted with the idea of using the endocellular toxin of the pneumococcus (hemotoxin) rather than the bacterial emulsion. The protein free pneumococci were dissolved in sodium cholate. A specific reaction was obtainable in guinea-pigs and also in persons suffering from lobar pneumonia. The guinea-pigs were sensitized with a sublethal dose of pneumotoxin. The animals survived the injection and there was a true pneumotoxin reaction, which was different from the reaction to pneumococcus protein or autolysates of it. A vaccine of dead pneumococci was tried and three animals reacted to it. It was found that heating pneumococci at 56°C. for one-half hour was not sufficient to destroy the endocellular pneumotoxin. The skin test would appear to be a true test for the presence of pneumotoxin in the body of the animal. By various chemical tests it is found that the pneumotoxin is a true protein, and in human cases with lobar pneumonia there was a positive reaction demonstrable before the crisis and a negative reaction after the crisis. In children the reaction was most characteristic. Other persons tested, suffering with tuberculosis, appendicitis, skin broncho-pneumonia or with acute or chronic infections not of pneumococcic origin, as well as healthy adults and children did not react.

The reaction is regarded as similar to the tuberculin reaction and is indicative of a state of allergy to pneumotoxin. Sensitization to the toxin presumably takes place with its liberation (by the action of normal body enzymes upon pneumococci normally localized in the lung alveoli) at the time of the prolonged chilling due to exposure. Failure to elicit the reaction during convalescence indicates the establishment of a temporary immunity or the disappearance of excess of toxin. This skin test does not seem to be of value as a method of serological type diagnosis but may aid in differential diagnosis between appendicitis or tuberculosis and pneumonia (especially in children). It is also of interest because of its bearing on the mechanism of the crisis.

### 12. The Influence of Arsenobenzol and Mercury upon Antibody Production

*Ikuzo Toyama and John A. Kolmer:* The possibility of certain drugs acting as antigens have been the theme of several studies by different workers. The antigenic action of drugs may account for acquired drug tolerance and also aid in resistance to infection by stimulating antibody production against microparasite apart from direct action of the drug on the parasite. The latter phase of the subject is the one particularly dealt with by the authors. Considerable evidence would point to the

conclusion that many drugs exert a stimulating action on antibody production by the tissues. Such drugs as arsenous anhydrid, phosphoric acid and mercuric chlorid, administered by mouth, are found to act in this manner. Salvarsan appears to stimulate agglutinin production, according to some workers. The present experiments were conducted on rabbits, to determine whether small daily doses of arsenobenzol and mercuric chlorid tend to increase antibody response to alien erythrocytes or to typhoid bacilli. After a series of experiments, five in all, on a large number of rabbits, and control animals, it was found that no increase of antibody production was shown, after injections of arsenobenzol or mercuric chlorid. On the contrary, it would seem that there is a lowering of antibody production, probably due to lessening of resistance by toxic effects. Further experiments, however, are in progress, upon the action of these drugs in experimental trypanosome infections. It is felt that such work should be done upon human sera, as work upon the sera of lower animals may not be a true index in human cases. The importance of the subject demands careful experimental work in this direction.

## JOINT SESSION WITH THE AMERICAN ASSOCIATION OF PATHOLOGISTS AND BACTERIOLOGISTS

### 1. A Contribution to the Bacteriology of B. fusiformis; Its Morphologic Phases and Their Significance

*Ralph R. Mellon.*

### 2. The Various Immunological Reactions in Glanders

*G. Benjamin White:* During an epidemic of glanders in a herd of ninety-five horses an excellent opportunity was afforded for observations on the results of various immunological tests. The following observations may be reported.

In all cases where glanders lesions were found at autopsies the horses had given positive subcutaneous mallein tests whereas the eye test and the complement fixation and agglutination tests were in some cases positive and in some cases negative. Horses giving positive eye tests always gave a positive subcutaneous test but were either positive or negative by complement fixation or agglutination. Complement fixation and agglutination tests were sometimes positive with a negative ophthalmic, negative subcutaneous and no lesions at autopsy.

It was found that the subcutaneous injection of mallein never sensitizes a horse to the extent that it will react positively to a second subcutaneous test nor does such an injection produce sensitization of the mucous membranes. The eye test, therefore, in such cases cannot be changed from negative to positive with the subcutaneous injection of mallein even when the injection is repeated several times. Such injections, however, do cause complement fixation and agglutination to

change from negative to positive. The injection of dead mallei bacilli produces no general or eye sensitiveness but does change a negative complement fixation and agglutination to positive. It is felt, therefore, that a positive subcutaneous mallein test shows the presence of a glanders lesion. The lesion, however, may be a healed, or inactive, one.

The subcutaneous injection of mallein renders glandered animals insensitive to subsequent injections for a period of about four weeks. No horses were found refractory when tested five weeks after the first injection.

The similarity between immunity reactions in glanders and those in tuberculosis is pointed out.

DISCUSSION

*William H. Park:* There is no absolute relation between the complement fixation, agglutination and mallein tests in horses. Very few New York horses would pass all three tests. This has been a serious difficulty in determining what horses should be used for food. It has been found necessary to require both serum reactions to be positive or a definite mallein reaction, before reporting the horse as infected. This has been done and it has been found to admit sufficient horse meat to alleviate the food shortage.

*A. Parker Hitchens:* It is my feeling that many more horses react to the subcutaneous test than those having glanders. I apply the subcutaneous test, as well as eye test and complement fixation, and I depend upon complement fixation. I have found that we must accept some horses that give positive subcutaneous tests. We have had no trouble with those animals. Reading of the subcutaneous test requires considerable skill; the symptoms vary. Just what is a positive subcutaneous reaction and what is not, is open to question and depends largely upon the individual making the examination.

*G. Benjamin White:* I quite agree with Dr. Hitchens that a horse may give a positive subcutaneous test and yet have no *active* glanders. A healed lesion may be present and it has been my experience that in such a case the horse develops no active glanders unless subjected to some particularly severe strain.

### 3. Persistence of Active Immunity in Those Immunized Against Diphtheria

*William H. Park:* In testing different species of animals, some have been found to have no immunity; some are entirely immune and again there is a group in which some are immune while others are not. Guinea-pigs have no natural immunity whereas horses almost always possess antitoxic immunity. The guinea-pig requires from four to eight weeks to develop immunity after toxin-antitoxin injections while the horse can be immunized very rapidly and nearly always produced considerable antitoxin. The guinea-pig loses its immunity in about nine months;

in the horse at the end of twelve months the developed immunity drops to the original level, i.e., $\frac{1}{50}$ to $\frac{1}{4}$ unit. Human beings differ from both horses and guinea-pigs in that some are immune while others are not. Dr. Zingher has made interesting studies in immunizing groups of babies. One group was of immune babies with immune mothers; these remained immune after the period of passive immunity from the mother had passed. Another group was from mothers that were not immune; these became immune after the injections.

Dr. Zingher tested children in institutions and found that fourteen months after immunization, those that had received but one injection of antitoxin were as immune as those that had had received two or three injections. The development of the immunity, however, was slower. Results observed by Dr. Rosenberg are very encouraging; the very great majority of children remained immune. It seems as though the acquired immunity to diphtheria will prove to be as permanent as natural immunity. Of 404 immunized children tested, 396 remained immune. Two of these had never become immune.

From these results it seems practical to immunize infants; this will give protection at the period of life when there is the greatest danger.

DISCUSSION

*Alfred F. Hess:* The use of toxin-antitoxin mixtures has made a great difference in the Hebrew infant asylum in New York City. Formerly there were about 15 cases of diphtheria a year in the institution with three or four deaths, but since the use of toxin-antitoxin was introduced there has been no diphtheria. Of 150 cases injected and followed for from one to two years, 98.75 per cent have remained immune. As regards the best time for immunization, early injection should be made because, while 80 per cent of infants have derived a passive immunity from the mother and are thus immune during the first few months of life, yet this immunity is for the most part lost in the later months of the first year. Immunization with toxin-antitoxin injections will, I believe, entirely do away with diphtheria in institutions.

*John A. Kolmer:* What is the nature of the reaction following the injection of toxin-antitoxin? Is a single dose advocated or is it wise to give a second one?

*William H. Park:* Children receiving only one injection develop immunity more slowly than those receiving two or three injections; 90 per cent of the former become immune by the end of six months whereas an equal percentage of the latter are immune by the end of the third month. For immediate protection antitoxin alone must be given. The toxin-antitoxin method is being used in the Navy as diphtheria is not always recognized at once and furthermore if the seamen are immunized one does not need to worry about carriers. In a few cases the injections cause a slight toxic effect. If the inert protein in the mixture can be controlled the procedure will become entirely harmless.

*John F. Anderson:* Is there a quick deterioration in the mixture?

*William H. Park:* So-called "standard" toxin that has become relatively stable through long standing, is used for the toxin-antitoxin mixtures; the mixtures can, therefore, be kept for months without appreciable change.

### 4. A Simple Method for Blood Cultures

*John G. Wurts and S. W. Sappington.*

### 5. A Bacteriological Study of Post-Operative Pneumonia

*Miriam P. Olmstead:* One hundred and thirty cases of post-operative pneumonia have been studied and a pathogenic organism has been recovered from at least thirty-one, a percentage of 23.8. Two cases had a Pneumococcus I in the blood stream, one of these Pneumococcus I in pre- and post-operative sputum. One had a Pneumococcus II in the sputum, pre- and post-operative, and the urine was precipitated by Pneumococcus II serum. In two cases with an atypical II in pre- and post-operative sputum, the etiology was established, in one by a positive agglutination test of the patient's serum, and in the other by a urine precipitin reaction with Type II serum. Pneumococcus III was established as the inciting factor in five cases; one had a positive blood culture, from one the organism was obtained by lung cultures at autopsy, the blood of two agglutinated the Pneumococcus III recovered from the post-operative sputum, the urine of these two and one other, who had Pneumococcus III in the pre-operative sputum specimen, gave a precipitin reaction with Pneumococcus III serum In eighteen cases a Pneumococcus IV was found to be the inciting factor. It was recovered from the blood once and seventeen cases gave a positive agglutination reaction with strains from the sputum. In one of these cases the urine precipitated with Pneumococcus IV serum. A hemolytic streptococcus was found in the blood stream of one case, and a hemolytic streptococcus in the sputum of another gave a positive thread reaction with the patient's serum. A mucoid streptococcus was found in sputum and chest fluid of one case. Some of these organisms were undoubtedly the inciting factors of other post-operative pneumonia cases, but in the absence of a positive blood culture or an immunological reaction, the significance of their presence in the sputum is uncertain. The most common inciting factor, as Whipple (Surgery, Gynecology and Obstetrics, 1918, xxvi, 29) has stated, is a Pneumococcus IV. It is at least suggestive that a pneumococcus, usually a IV, was recovered from either a pre-operative or a post-operative sputum specimen in ninety-seven of the one hundred and thirty cases studied, i.e., 74.6 per cent, while a pneumococcus was recovered from the sputum of only 32.2 per cent of all the surgical cases examined before operation, and of 50 per cent of the cases that subsequently developed post-operative pneumonia.

DISCUSSION

*Alfred F. Hess:* Did pneumonia develop among cases where the pneumococcus was absent from the pre-operative sputum; if so, how many were there? In what percentage of cases was there a discrepancy in the type of pneumococcus where pneumonia developed?

*John A. Kolmer:* How many patients, with pneumococci in the blood stream, died? How many showing a precipitin reaction died? I am under the impression that persons showing the precipitin reaction have rather a bad prognosis.

*Miriam P. Olmstead:* In reply to Dr. Hess I would say that pneumonia developed in 44 cases from whose pre-operative sputum a pneumococcus was not recovered. In 14.9 per cent of the cases studied, there was discrepancy in the pre- and post-operative sputum findings. Type IV was present in 8 of these but the strains recovered were found to belong to different groups. It is probable that in at least some cases both strains were present in the sputum at the same time, but that, owing to the similarity of the colonies, only one strain was isolated. In reply to Dr. Kolmer, I would say that 2 patients with pneumococci in the blood stream died; one had a type I, the other type III organism. There were no fatal cases among those whose urine gave a precipitin reaction.

*Augustus B. Wadsworth:* I am greatly interested in this work. The fact that pneumonia and not a general pneumococcemia without local lesions developed in man is an indication of some degree of insusceptibility or immunity against pneumococcus infection. In my experiments on rabbits pneumonic lesions developed only when the animal was partially immunized. Forthermore, the prevalence of pneumococcus infection of the upper respiratory tract suggests that different individuals acquire varying degrees of immunity from time to time. It is thus generally assumed that man possesses varying degrees of pneumococcus immunity, and the demonstration of the specific reactions of pneumococcus immunity in the serum of the normal healthy human subject is of interest.

### 6. The Active Immunization Against Pneumonia

*R. Kohn.*

### 7. Production of Pneumococcus Antiserum and the Corresponding Curves Obtained by Protection and Agglutination Tests

*G. Benjamin White:* On account of the unusual demand for anti-pneumococcus serum every effort had been put forth to speed up and increase the production of this serum. The problem had three phases; first, to produce a potent serum; second, to bring the horses to production in the shortest possible time; and, third, to carry out the immunizations with the least detriment to the horses. The basic plan of immuni-

zation originated by Cole and his co-workers was followed. The first two phases have been studied with success and experiments on the third phase are now under way.

The best type of horse is the medium heavy animal of the draft type with considerable spirit.

A single strain of the type I organism appears to be sufficient. It is doubtful whether it is necessary to have a strain of high virulence. The stock cultures are carried on blood agar while the cultures used for injections are grown for sixteen to eighteen hours in meat infusion broth with a reaction of about plus 0.3. The cultures are centrifuged, the sediments are not washed but are emulsified in physiological salt solution and injected into the horses intravenously immediately after preparation. Where killed cultures are to be given, the emulsions are devitalized by heating for three-quarters of an hour at 56°C.

Various plans of immunization were tried and that suggested by Cole was found to give excellent results. A slight modification of this brought a horse to production in the record time of twenty-eight days.

The dosage is regulated by the temperature reaction following the injection of the day before. When the horses have reached the stage of production, injections of live pneumococci are given once each week and the horses are bled six days after every second injection, 7500 cc. being taken at a time.

No parallelism was found between the agglutinative and the protective titers and the former is found to be less stable than the latter. All serum produced is released for distribution on its protective titer regardless of its agglutinative power.

DISCUSSION

*A. Parker Hitchens:* I congratulate Dr. White on the results obtained in the rapid immunization of the horses. This is an especially important point at this time. In regard to bleeding, what was the plan of bleeding as regards the time of injections? What was the interval between the injections?

*John F. Anderson:* We have been using a method of six daily injections, in three courses, with seven-day intervals. The horse can be bled in thirty-two days. A standard has been made to protect against 0.1 cc., 0.2 cc. and 0.3 cc. of culture.

*George W. McCoy:* I am glad to hear Dr. White bring out the point of the unreliability of the agglutinating power as compared with the protective value. We have had sera sent back marked N.G. because they did not agglutinate type I pneumococci although they possessed a high protective power. All commercial sera, before being sent out are tested by the hygienic laboratory.

*Augustus B. Wadsworth:* Had the high protective serum any agglutinating power at all?

*George W. McCoy:* It had no agglutinating power at all.

*Augustus B. Wadsworth,* Was it controlled?

*George W. McCoy:* Yes.

*William H. Park:* In the South of France there was a great deal of pneumonia among the African troops. At one camp the men were vaccinated by Borrel with cultures from cases of pneumonia in the camp. In 18 days the pneumonia cases ceased to develop. The men in a distant camp were given the same vaccine, but the results were negative. The supposition was that the pneumonias in the second camp was due to a different type. The cessation of cases may have been a coincidence, but it is striking that the cessation of the epidemic in the camp followed the use of the vaccine.

*G. Benjamin White:* The horses are bled six days after the second injection.

We have had four interesting instances of horses dying from pneumococcus infection during immunization. Three horses died from lobar pneumonia due to type I and at the time of death their serum showed both good protective and agglutinative power. Another horse died of a pneumococcus (type I) endocarditis with pneumococcus bacteremia during the course of immunization. The organism isolated from the blood was found to be serum-fast.

A similar paradox was observed in the case of a horse immunized against paratyphoid B. Three weeks after the end of the course of immunization the animal was found to have a bacteremia and from the blood paratyphoid bacilli, the B type, were isolated. The organism was found to have acquired no serum-fastness. The serum of this horse at the time of death agglutinated both the hemotogenous strain and the stock strain in a dilution of 1: 5,000.

## 8. A Rapid Simple Method for the Determination of Type of Pneumococcus in Sputum of Lobar Pneumonia

*Charles Krumwiede, Jr.*

### DISCUSSION

*O. W. H. Mitchell:* I have gotten the same results by the method of extracting with sand. I am convinced as to the specificity of the reaction. The rapidity and simplicity of the method makes it an excellent one. Our sera are furnished by the N. Y. State laboratory. The tests have been checked by Dr. Wadsworth and invariably when the sputum has reacted definitely my report has been corroborated.

Young rats have been found easier to procure than mice for the pneumonia work. Children keep them as pets and a few inquiries generally result in getting as many as are necessary. Half grown rats are preferable to mice. The peritoneal cavity is larger.

## 9. On the Influence Exerted by Salts on the Electrical Resistance and Permeability of Tissues

*B. H. A. Clowes:* I have recently demonstrated by electrical conductivity experiments that tumor tissues are more permeable than normal

tissues in both plants and animals. I have also produced artificial membranes made by saturating filter paper with emulsions of oil in soap which exhibited variations in electrical conductivity when exposed to various antagonistic salts similar to those exhibited by laminaria and other marine organisms experimented with by Osterhout.

Both laminaria tissue and emulsion membranes when exposed to the influence of NaCl exhibit a rise in permeability. If subsequently transferred to $CaCl_2$ for a short period they exhibit a fall in permeability, and alternating variations in permeability within certain well defined limits can be effected in both cases by alternating treatments with NaCl and $CaCl_2$. These experiments are paralleled by experiments on the surface tension of soap films previously reported. If an aqueous soap or NaOH solution is allowed to flow from a capillary pipette through olive oil a given number of drops is obtained. If NaCl is added to the solution the number of drops is greatly increased. If $CaCl_2$ is added to the solution the number of drops is diminished but if NaCl is mixed with $CaCl_2$ in certain balanced ratios in which they occur in the blood, sea-water, etc. the number of drops approximates that given by the original soap solution. These effects are all attributable to the influence of electrolytes on the state of dispersion and consequently the permeability of interfacial soap films. A practical demonstration as to how the electrolytes in question may control the permeability of emulsions is obtained by shaking a suitable emulsion of oil dispersed in water by means of soap with increasing proportions of $CaCl_2$. The conductivity which serves as an index of permeability remains approximately constant up to a critical point at which the emulsion of oil in water is converted into one of water in oil. At this point the resistance rises to an enormous extent owing to the transformation from an emulsion which is permeable to water to one which is impermeable. NaCl, alkalis, etc. exert an effect the reverse of that of $CaCl_2$ promoting the permeability of emulsions and also of tissues.

A further proof that these experiments on conductivity soap films and emulsions actually afford an index of the permeability to water and water-borne substances is afforded by introducing layers of a suitably constituted emulsion into long glass tubes, supported by filter paper and tightly fitting rubber tubes and passing various solutions through this emulsion diaphragm and determining the rate of flow. Distilled water, sea-water and properly balanced mixtures of NaCl and $CaCl_2$ flow through at nearly the same rate of speed. NaCl flows a great deal faster than the balanced solution and $CaCl_2$ considerably slower, the relative rate of flow corresponding remarkably with the number of drops obtained in the surface tension experiments.

From the above experiments it appears probable that the mechanism controlling the permeability of the protoplasm is dependent upon an extremely delicately balanced emulsion of soaps, lipoids and fats and that proteins simply afford the mesh or net-work in the capillary spaces of which the influence exerted by electrolytes on the permeability of emulsions would be accentuated. Pathological changes are frequently

attributable to disturbances in the balance of soap and fats in emulsions. For example, fatty degeneration appears to be simply the aggregation of fatty globules under the influence of surface tension changes to a point at which they become readily visible.

Anaphylaxis and sensitization similarly appear to depend upon changes in permeability. A point particularly to emphasize is that the permeability of any given structure is not dependent simply upon the size of the pores of the filter but upon substances like soaps which lower the surface tension in the capillary spaces exerting an effect analogous to that of a lubricant.

DISCUSSION

*James Ewing.* Yesterday after listening to the philosophical presentation of Dr. Clowes I suggested that it would be well for the speaker to attempt an interpretation of his observations in terms intelligible to the pathologist and the immunologist. It has seemed to me that we were on the verge of seeing a great light, which never dawned. One wishes to know how physical systems have a direct bearing on pathological conditions such as fatty degeneration. I am not prepared to admit that the fatty changes are as simple as the interactions of these systems of emulsions. This line of work corresponds to that done by Novy. The phenomena of anaphylaxis will probably be found to fall in line with all of these observations. I feel that I can congratulate Dr. Clowes on having made his experiments intelligible—to the pathologist.

*Henry Ulrich:* I feel that surface tension is a vital factor in the formation of spores. *B. subtilis* will not grow on media with lowered surface tension. *B. anthracis* entirely loses its pathogenicity when grown on media with lowered surface tension. It will then produce no symptoms whatever. When blood serum is added to culture media, the tension is lowered. The tension has to be suited to the growth of the organism. This work of Dr. Clowes is very suggestive.

*J. Bronfenbrenner:* In my opinion one can not emphasize too much the usefulness of physical methods in the study of biologic phenomena. Already several years ago Ascoli noticed the changes of surface tension in the mixtures of antibody-containing sera with suitable antigens, and he proposed to use the measurements of surface tension of such mixtures for diagnostic purposes. Though his results were not very sharp, there is no doubt that the application of more recent methods of measurement of surface tension will corroborate his conclusions. In my own experience in the study of various questions in immunity, I was amply convinced of the necessity of applying physico-chemical methods to the study of these problems. In my study of the Abderhalden reaction, I noticed that the surface tension of the serum undergoing autodigestion always decreased, and vice versa, whenever the surface tension of serum was diminished, it underwent autodigestion. In collaboration with Dr. Fleisher I also noticed that the refractive index of serum increased during the process of autodigestion, thus indicating that the dispersion of the colloidal particles of the serum increases during this process. It

is more than likely that the mechanism of the anaphylactic shock, or rather the nature of the cellular response to the humoral reaction in anaphylaxis, may be largely a surface reaction, affecting the permeability of vital cells, or of their electro-conductivity. There is also a great deal of evidence that the whole question of ferment action may be bound up with the question of colloidal dispersion. In general, the phenomena to which Dr. Clowes has called our attention are of fundamental importance in the study of various biological problems.

*G. H. A. Clowes:* I feel immensely interested in the observation about anthrax spores. That corresponds with my own findings. Anyone who has studied amoeboid movement, spore formation karyokinesis, budding, etc., side by side with emulsions will be convinced as to the importance of surface tension. I have produced from emulsions objects that looked exactly like leucocytes and I have shown them to pathologists who thought they were leucocytes. Dr. Bronfenbrenner has mentioned the myostagmin reaction, which was Ascoli's work. He let drops fall into the air instead of into oil. Ferment action appears to be definitely due to surface tension changes. Perfect contact must first be effected, then dispersion or aggregation may occur.

10. Experiments Upon the Chemotherapy and Chemoserotherapy of Pneumococcus Infection

*John A. Kolmer, Edward Steinfield and Charles Weiss.*

11. Studies on the Toxicity of Pneumonic Lungs

*John A. Kolmer, Charles Weiss and Edward Steinfield.*

DISCUSSION

*A. Parker Hitchens:* Has Dr. Kolmer taken into consideration the great increase of toxicity in tissues that undergo autolysis? A piece of liver digested in sterile salt solution for twenty-four hours becomes extremely toxic.

*Ralph R. Mellon:* Do these extracts of empyema fluid bear any relation to the aggressins of the disease?

*Charles Weiss:* It is very difficult to decide whether or not the toxicity of the pneumonic exudate is due to the fact that the tissue was undergoing autolysis. The hemolytic properties of the pneumonic exudate may not have been specific for pneumonia. They have been attributed to various fatty acids. Specific anaphylactic results were obtained by sensitizing guinea-pigs to normal and pneumonic exudates. The latter are rich in fibrin and the reaction may have been specific to the fibrin. But the experiments indicate that at least part of the toxicity and hemolytic activity of the exudate was due to the presence of toxins liberated from the pneumococci.

*James Ewing:* This work is an important step in the right direction. I do not feel competent to offer any criticism, but I wonder why the work of immunologists turn always to specific toxins and away from products of tissue changes which the pathologist is interested in. I believe that immunologists have been held back by exclusive attention to Ehrlich's theories.

*G. H. A. Clowes:* Were these substances soluble in fats and lipoids or in water? This is of vital importance in determining their characteristics.

*John A. Kolmer:* We have no means of absolutely controlling this work in relation to aggressins. We tried to ascertain whether this toxic substance would retard phagocytosis of pneumococci *in vitro* and we found that it does so to a slight extent.

*Charles Weiss:* I have isolated albumin, globulin, uric acid and lipoid substances from the extracts, but this work is still under way.

*H. G. Wells:* There is an error in the last remark. Uric acid is formed only in the liver. Xanthin is probably what the speaker meant.

*John A. Kolmer:* Mr. Weiss referred to the literature on the subject.

12. The Properties of Pneumotoxin and its Probable Rôle in the Pathology of Lobar Pneumonia

*Charles Weiss and John A. Kolmer.*

## Final Session

1. The Examination of the Blood Preliminary to the Operation of Blood Transfusion

*Arthur F. Coca* (see this volume, page 93).

### discussion

*John A. Kolmer:* I desire to describe briefly a microscopic method which I have employed during the past year with very satisfactory results and which is fashioned after a microscopic technic described by Lee.

A small amount of blood is obtained from the finger of the patient and each of the donors in small and separate test tubes to supply a few drop of serum; also a few drops from each in small test tubes containing 1 cc. of a 1 per cent sodium citrate salt solution to supply a suspension of cells. The sera are separated and the cells washed once with the centrifuge, although the latter is not absolutely necessary. In setting up the tests, hanging drop slides are employed as in the Widal reaction. On a series or cover glasses, two loopsful of the recipient's serum is mixed with one loopful of corpuscle suspension from each of the donors; in a second series, one loopful of the recipient's suspension of cells is mixed with two loopsful of serum from each donor. The preparations are examined microscopically with the low power objective, fifteen minutes later.

Controls are included with the cell suspension of the recipient and each donor. Agglutination is well marked when it occurs and easily read. With this method no attempt has been made to group the bloods but it is extremely simple and it has been found very practical. I wish to ask Dr. Coca's opinion of the practical value of these tests inasmuch as surgeons occasionally express themselves as believing that the tests are not essential to successful transfusion, although he personally does not share this view according to experience.

*Arthur F. Coca:* The choice of methods may depend upon what apparatus is at hand. The procedure that I have described is quite as easy as that of blood counting and all of the apparatus required for it is available in any laboratory in which blood counting is done. Dr. Kolmer's method requires much more time, much more blood and more apparatus than the one that I have described. His second series of mixtures is unnecessary to the purpose in view.

So far as I am aware the deaths that have occurred as a result of transfusion have happened when the compatibility test had not been made.

## 2. The Isolation, Purification and Concentration of Immune Hemolysin

*M. Kosakai* (see this volume, page 109).

### DISCUSSION

*William H. Park:* Has Dr. Kosakai been able to separate antibodies from the bacteria?

*John A. Kolmer:* I should like to ask Dr. Kosakai whether it is possible, with his method, to separate the hemagglutinins from the hemolysins. It is desirable, particularly, where the anti-human hemolytic system is to be used, to produce a serum preparation that is free from agglutinins. Was Dr. Kosakai's final solution free from agglutinin?

*M. Kosakai:* I have not attempted to separate anti-bacterial antibodies from the bacteria with my method. I have not been able to separate the hemagglutinins from the hemolysins.

## 3. A Rapid Simple Method for the Extraction of Precipitin Antigen from Bacteria

*Charles Krumwiede, Jr.* (see this volume, page 1).

### DISCUSSION

*George H. Smith:* Can this "precipitin antigen" be employed also for immunization?

*Charles Krumwiede:* I have not tried to produce an immune serum with an antigen prepared in this way from bacteria. An extract made in this manner from meat did not stimulate the production of antibodies.

### 4. A Method of Preparing Bacterial Antigens

*J. C. Small* (see this volume, page 413).

#### DISCUSSION

*John A. Kolmer:* Several years ago I experimented with bacterial antigen prepared by different methods, working with the typhoid colon group. The results of the complement-fixation tests were similar to those of Dr. Small but with that method I was not able to differentiate between paratyphoid A and paratyphoid B. Were rabbits used for the production of the immune sera and if so was the serum used active or was it heated? In previous work I found that the serum of some rabbits when heated at 56°C. for one-half hour develops the property of fixing complement in a nonspecific manner; for this reason animals should be tested in a preliminary way before immunization is begun and those that do not show this phenomenon may be selected. Otherwise the nonspecific fixation may be avoided by heating the serum at 62°C. instead of 56°C. for thirty minutes as was done by Meyer and Boermer with the serum of mules.

*Charles Weiss:* Has the speaker found that the results are different after the removal of the lipoids from what they were without this procedure? In regard to the heating of antigen it has been found in working with streptococcus antigen that heating destroys the anticomplementary qualities but at the same time it weakens the antigenic properties of the preparation.

*G. H. A. Clowes:* In reference to the question of heating is it the experience of the members present that the quantitative activity of the serum is diminished by heating at 62°C.?

*John A. Kolmer:* The heating of immune sera at 62°C. may cause slight deterioration of the specific antibodies present. The deterioration is, however, difficult to measure. Investigations have shown that nonspecific inhibition of complement is especially likely with bacterial antigens.

*M. A. Wilson:* Our experience has been that there is no deterioration of the antigenic preparation if it is heated.

*Hassow von Wedel:* I have used tubercle bacilli antigen that has been heated at intervals five or six times without noting any difference in its antigenic value.

### 5. A Contribution to the Study of Complement Fixation in Tuberculosis

*M. A. Wilson* (see this volume, page 345).

### 6. A Contribution to the Study of Complement Fixation in Tuberculosis

*Hassow von Wedel* (see this volume, page 35).

DISCUSSION

*Paul Lewis:* I have been much interested and instructed by these papers. The results may be valuable from a diagnostic point of view. I can add some data from my own experience from work done within the last year. At first I used bacterial suspensions or the autolysate which latter I found, lost rapidly in anticomplementary effect. Testing with the same serum day after day, I found that one frequently does not get the same result on two days running. I think this may have been because the reaction between antiserum and antigen in those cases was a weak one and that the readings were only permanently positive with the strongest sera. Experiment showed that by prolonging the fixation period more durable reactions could be obtained. Four hours proved to be the maximum period which it was practicable to use owing to irregular deterioration of the complement in longer exposures. Using a four-hour incubation period I have titrated numbers of sera to determine the amount of complement fixed. In certain instances this may reach 2 cc. of the usual 1 + 9 dilution of guinea-pig's complement. It has been found that the tuberculous sera can be well preserved by mixing with an equal quantity of neutral glycerine. Pooled serum from a number of cases giving a strong deviation, thus preserved has been used over a period of six months to study the properties of various antigens.

*G. H. A. Clowes:* In working on cancer cases and also on syphiltic cases eight years ago I attributed the development of an increase in complement fixing power in serum to changes in the colloidal particles, due to their being held in the ice-box for some time. I wish to ask what was the temperature of the ice-box used. Was the serum frozen?

*Hassow von Wedel:* I am not able to answer Dr. Clowes's question. The ice-box probably had a fluctuating temperature.

*G. H. A. Clowes:* I have seen complement deviation variations as high as 10 to 1 It is easy to vary the surface of a particle by changes in physical conditions or by variations of the hydrogen ion content. This can frequently be determined by the ultramicroscope which shows variations in the size of the particles.

*William H. Park:* I feel that, at present, this test should be used for primary cases; later we may be in position to use it on a diagnostic basis.

*John A. Kolmer:* I wish to ask whether the peculiar property of human serum of developing fixation powers after standing was found also for the Wassermann and gonococcus-fixation tests.

*Miriam Olmstead:* Did the guinea-pigs that gave a fixable complement for the tuberculosis antigen also give a fixable complement for the gonococcus antigen? How many specimens giving positive gonococcus fixation were used for the tuberculosis tests?

*M. A. Wilson:* We killed, at one time, 10 guinea-pigs and we found that of the sera all were fixable in the meningococcus fixation; four were fixable in the gonococcus fixation and only one was fixable in the tuberculosis fixation.

## 7. A Study of Controlled Postmortem Wassermann Reactions: a Supplementary Report on 400 Cases

*Stuart Graves: 1.* Post mortem Wassermann tests confirmed ante-mortem Wassermann tests in 97 per cent of 68 controlled cases. A four plus positive reaction in a specimen obtained sixty hours post mortem confirmed a four plus reaction obtained ante mortem in the same case—one with anatomic and clinical evidence of syphilis. A negative reaction in a specimen taken twenty-two hours post mortem confirmed a negative ante mortem reaction.

2. In 91.2 per cent of the cases showing anatomic lesions of syphilis and presenting evidence of syphilis in their histories the sera post mortem gave positive Wassermann reactions.

3. The fact that only 2.5 per cent of the sera were anticomplementary or otherwise unfit for use compares favorably with the fact that 1.14 per cent of 6000 ante mortem specimens were similarly unfit.

4. Only 2.6 per cent of 378 cases showing anatomic evidence of syphilis gave negative reactions.

5. The reactions conformed to the anatomic and historic evidence in 304 of 378 cases or 80.4 per cent, which is considerably lower than it would have been if satisfactory histories and physical examinations had been recorded in Class V.

6. There is no logical reason for supposing that acute infections or malignant tumors cause positive Wassermann reactions.

7. The positive reaction appeared in 2.7 times as many negroes as whites, in 1.7 times as many males as females and in only 11 white females or 6.5 per cent.

8. The Wassermann reaction, carried out on post-mortem blood according to the methods followed in this investigation, is practically as reliable a test for syphilis as when performed with ante-mortem specimens and is of great value in pathological anatomy and in medico-legal cases.

### Discussion

*John A. Kolmer:* I wish to endorse all that Dr. Graves has said inasmuch as my own results coincide entirely with his experience. I am familiar with the published reports just described tending to discredit the practical value of the Wassermann reaction and I think it is particularly unfortunate that reports published with this object in view should reach the practitioner of medicine. No one that has dealt with pathological findings would deny that a pathologist cannot exclude syphilis on the basis of autopsy findings alone. Spirochaetes might not produce much tissue reaction and yet suffice to produce antibodies, which would be indicated by the Wassermann reaction. In the last four or five years much has been learned of the technic of the Wassermann reaction rendering it a test of great diagnostic value. While we must welcome attempts to point out sources of error, criticisms should be carefully controlled.

8. Observations on the Intraspinous Auto-Salvarsanized Serum Therapy of Cerebrospinal Syphilis

*Benjamin A. Thomas.*

9. Experiments upon the Passive Transfer of Antibodies to the Cerebrospinal Fluid

*John A. Kolmer and Shigeki Sekiguch* (see this volume, page *101*.)

DISCUSSION

*A. Parker Hitchens:* The question whether the antibodies find their way from the blood to the spinal fluid is important, especially in cerebrospinal fever. In severe cases of epidemic meningitis in the army camps the antimeningitis serum has been given by intravenous as well as by intraspinous injection. The results of this treatment are encouraging. Dr. Kolmer's work would show that specific treatment by the intravenous route should be of value in meningeal infection.

*J. Bronfenbrenner:* It is still not settled definitely whether the antibodies normally pass directly from the blood into the spinal fluid. This passage is even more questionable in such pathological conditions in which the pressure in the spinal canal is much greater than in the blood vessels. The fact that the introduction of antimeningitis serum into the circulation seems to produce clinical improvement could possibly be due rather to another mechanism than the direct passage of antibodies from the blood into the spinal canal. Recent investigations of English workers lead them to the conclusion that the meningococcus produces a soluble toxin. Such a toxin may be forced out of the spinal canal into the circulation on account of the pressure in the spinal canal, and so cause a certain amount of general toxemia aggravating the specific symptoms of meningitis. The therapeutic serum may contain a certain amount of antitoxin and to that extent may improve the clinical condition of the patient when introduced intravenously.

*A. Parker Hitchens:* About every three hours during the severe stage of the disease the spinal fluid is removed, and with the rapid filling up of the canal again there may be some effect.

*John A. Kolmer:* Our paper simply deals with the passage of antibody from the blood to the spinal fluid under normal conditions. In the experimental animals it was found necessary to have a large amount of antibody in the circulation.

10. Vaccine Dosage

*Joseph Head.*

11. The Vaccine Treatment of Acne, with Special Reference to the Rôle of Bacillus coli

*Albert Strickler and Jay F. Schamberg:* Thousands of cases of this disease never come to the physician at all; it is only when suffering from

severe forms with great disfigurement, that the patients seek advice. The lesions are chiefly in the sebaceous glands, which are very active at puberty. Puberty is, in fact, the primary predisposing cause. Anemia and constipation are found to be pretty constant accompaniments of acne. The condition often develops after typhoid fever, and intestinal intoxication evidently plays a rôle in the etiology. In the treatment of acne by vaccines, it was found that there is a complement fixation in 63 per cent of the cases with an antigen prepared from a colon bacillus isolated from the intestinal tract of the patient. The fixation is higher when the antigen is from the patient. It was resolved to treat 50 cases solely with vaccines prepared from an autogenous colon bacillus. These cases were controlled by cases treated with other methods such as vaccines from other germs, therapeutic and hygienic measures. The *B. coli* vaccines were found to possess better curative effects than any other mode of treatment.

DISCUSSION

*Jay F. Schamberg:* I would like to emphasize one or two points. Vaccines prepared from *B. acne* and from staphylococci have been in use for many years, and in some cases they have given brilliant results; in others they have failed to respond. The present experiments were carried out in a hospital clinic upon a large number of patients. 63 per cent gave positive complement fixation with the strains of *B. coli* used for the vaccines. A large percentage of patients responded to a remarkable degree. It is likely that at puberty, when there is great developmental activity, there is liability to infection from the intestinal tract. The activity of the intestinal organisms may then produce noxious effects. The complement-fixation tests would seem to incriminate especially the colon bacillus.

*A. Parker Hitchins:* It is an important point to find out which strain of *B. coli* is the chief factor in producing the disease. Has any work been done on the sera in this respect?

*Albert Strickler:* In regard to the present work, I personally welcome an active case as I feel that a good deal could be done for the patient. Formerly I rather dreaded to see such patient come, as the results of treatment were so often disappointing.

*Jay F. Schamberg:* No attempt has been made as yet to differentiate strains. The complement-fixation test merely showed that there is greater specificity to the *B. coli* from active cases.

## 12. Lipo-Vaccines

*Eugene R. Whitmore and E. Fennel*

DISCUSSION

*A. Parker Hitchens:* This plan for the preparation of bacterial vaccines is likely to be of immense value. If one can give the entire treat-

ment in one day, the economic value of the procedure is obvious. At Camp Beauregard I saw a detachment injected with meningitis lipovaccines; the reactions were very severe, as were also those from triple typhoid vaccine. The meningitis vaccine caused severe headache and insomnia. All the symptoms disappeared the next day. I feel great confidence in the outcome of the work of Colonel Whitmore and Lieutenant Fennel.

*Lieutenant Fennel:* There was a very severe reaction to the first vaccine we used. Since the improvement in technic the severity of the reaction was much lessened.

## 13. A Study of the Immunizing Properties of Bacterial Vaccines Prepared After Various Methods

*M. W. Perry and Sara Levy.*

### DISCUSSION

*Lieutenant Fennel:* I wish to ask Mr. Perry whether he titrated the agglutinable culture by the standard set by Oxford. If so, the results will be of considerable value for the army work.

*John A. Kolmer:* The culture was secured from a German university sixteen years ago and was standardized for agglutination work. It is highly susceptible to agglutination. It is not standardized by the Oxford method.

# A CONTRIBUTION TO THE STUDY OF THE COMPLEMENT FIXATION REACTION IN TUBERCULOSIS.[1]

M. A. WILSON

*From the Research Laboratory of the Department of Health, New York City*

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## I. ON THE STANDARDIZATION OF COMPLEMENT

In our study of complement fixation in tuberculosis we have found a point of technique that has increased the efficiency of the test. It has to do with the standardization of the guinea-pig's serum to determine the value of the complement. In this preliminary report, we shall describe the method or standardizing the complement, the preparation of our tuberculosis antigen, and the diagnostic test, with such results as we have obtained thus far in our study.

### *Technique*

All reagents are used in one tenth the classic Wassermann volumes. Fixation period, one hour, 37°C.

*Serum.* The patient's serum is inactivated for thirty minutes at 56°C. 0.02 cc. and 0.01 cc. of the undiluted serum are used in the test, and 0.04 cc. for control of anticomplementary action.

*Antigen.* Two antigens have been used. One was made from 12 stock cultures of human tubercle bacilli, the other from strain 305 (used for tuberculin production.) Those antigens are suspensions in 0.9 per cent saline of dried bacilli, from which all constituents soluble in alcohol and ether have been removed. The bacilli were grown in glycerin-broth. The polyvalent antigen cultures were grown for three weeks, and the monovalent ones for three months. The broth cultures were killed by heating them in the Arnold sterilizer for one hour. The cultures were

[1] Preliminary report.

then filtered through filter paper. The filtrate was discarded, and the residue was placed in absolute alcohol, in the proportion of one volume of residue to 10 volumes of alcohol. This mixture was shaken thoroughly by hand, and was placed in the ice-box for two weeks. It was then filtered through paper and the filtrate was discarded: The residue was washed in absolute alcohol and the sediment obtained by centrifugalization was washed in ether. After a further centrifugation the ether was discarded and the centrifuge tube containing the residue was plugged and placed in the dark at room temperature over night. By this simple procedure the residue was dried within twenty-four hours. The dried powder was emulsified in a large mortar with 0.9 per cent saline in the proportion of 1 gram of powder in 200 cc. of saline. This gave a concentrated emulsion convenient for storing as a stock antigen. The emulsion was heated for one hour at 80°C. The antigen was now ready for use, and it was standardized to be used in such a dilution that 0.1 cc. contained two standard fixation units and one fourth, or less, of the anti-complementary dose. The unit was determined by titrating varying amounts of the antigen with 0.01 cc. of a known positive tuberculosis serum, and two hemolytic units[2] of a complement known to be potent for tuberculosis fixation. The standard dilution of the two antigens employed is 1: 50. This makes a final dilution of dried bacilli 1: 10,000. These antigens are not anticomplementary in the amount used in the test.[3] They have given uniform and constant fixation reactions. The tests reported will show that they are specific and stable. They were made ten months ago and are perfectly efficient today.

[2] As determined with the use of one-tenth of the standard amount (i.e., 0.1 cc. instead of 1 cc.) of 5 per cent sensitized sheep's corpuscles.

[3] In some instances a sample of antigen that has been standing in the ice-box for some time has been found to have increased in its anticomplementary action. This change is not accompanied by any deterioration of the "antigenic" property of the preparation and, as we have found, it can be removed by heating the diluted preparation for one-half hour at 56°C. The change is not of frequent occurrence; however, as a routine precaution, we heat all of our diluted antigen before using it in the tests.

*Complement.* Guinea-pig's serum, twenty-four or forty-eight hours old; pooled from six to ten pigs. Before the pooling, the serum from each pig is tested for its hemolytic strength, for antisheep amboceptor, for anticomplementary reaction with a heterologous serum and for fixability with the combination of tuberculosis antigen and tuberculosis serum. This last test we emphasize as an essential, if uniform results are to be obtained with different lots of complement; it has proven beyond a doubt that although a guinea-pig's serum may react perfectly in all other respects it may fail to be fixed by tuberculosis antigen and serum. Of the pigs we have tested 64 per cent failed in fixability, while they were perfectly good in other respects. If in a pool of six or ten complements, there are several strongly fixable, the presence of the negative complements in the pool may not appreciably affect the test; on the other hand, if most of the complements are negative the fixability of the pool will not serve to give a true reaction with the patient's serum. In such a case a four plus reaction may drop to a two or three plus reaction and a two or one plus reaction may become negative.

Tables 1 and 2 show the variation in fixability of the guinea-pig's serum.

*Indicator for the fixation reaction.* 0.1 cc. of a 5 per cent suspension of sheep cells sensitized with three standard units of antisheep amboceptor.

*Controls for diagnostic fixation reaction:*

Antigen—for anticomplementary reaction.
for specificity.
for potency.

Serum—for anticomplementary reaction.
for specificity.
for natural antisheep amboceptor.

Complement—for stability (system control).
Sensitized cells—for stability (reading control).

TABLE 1

| PIG NO. | DATE BLED | COMPLEMENT FIXATION REACTION | | |
|---|---|---|---|---|
| | | Tuberculosis | Meningococcus | Gonococcus |
| 1 | November 13 | No fixation | Complete fixation | Complete fixation |
| 2 | November 13 | No fixation | Complete fixation | Complete fixation |
| 3 | November 13 | Complete fixation | Complete fixation | Complete fixation |
| 4 | November 13 | No fixation | Complete fixation | Complete fixation |
| 5 | November 13 | Weak fixation | Complete fixation | Complete fixation |
| 6 | November 13 | No fixation | Complete fixation | No fixation |
| 7 | November 13 | No fixation | Complete fixation | No fixation |
| 8 | November 13 | Weak fixation | Complete fixation | Complete fixation |
| 9 | November 13 | No fixation | Complete fixation | Complete fixation |
| 10 | November 13 | Weak fixation | Complete fixation | No fixation |
| 11 | November 13 | Complete fixation | Complete fixation | Complete fixation |
| 12 | November 13 | Complete fixation | Complete fixation | Weak fixation |

TABLE 2

*Showing the number of guinea-pigs serums efficient for tuberculosis complement fixation*

| NUMBER OF PIGS BLED | NUMBER EFFICIENT FOR COMPLEMENT FIXATION | | |
|---|---|---|---|
| | Tuberculosis | Meningococcus | Gonococcus |
| 10 | 1 | 10 | 4 |
| 3 | 3 | 3 | 3 |
| 11 | 1 | 11 | 6 |
| 8 | 8 | 8 | 8 |
| 11 | 3 | 10 | 8 |
| 6 | 1 | no test | 1 |
| 6 | 3 | 6 | 1 |
| 10 | 3 | 10 | 8 |
| 5 | 0 | 4 | 4 |
| 11 | 4 | 9 | 6 |
| 4 | 1 | 4 | 4 |
| 10 | 5 | 10 | 8 |
| 8 | 3 | 8 | 6 |
| 10 | 5 | 9 | 8 |
| 6 | 1 | 5 | 6 |
| 10 | 4 | 10 | 7 |
| 129 | 46 | 117 | 88 |

The pooled serum is titrated with cells sensitized with 3 standard units of amboceptor. The reaction is read at the end of fifteen minutes. Two hemolytic units of the pooled complement are used.

### *Results*

The results of tests of serums from 344 cases are given in table 3.

TABLE 3

| | PULMONARY ACTIVE | BONE AND JOINT DISEASES | TUBERCULAR GLANDS | POLIOMYELITIS SPINAL FLUIDS | CASES HAVING NO SYMPTOMS OF TUBERCULOSIS |
|---|---|---|---|---|---|
| | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| Strongly positive | 67 | 12 | 35 | 0 | 0 |
| Weakly positive | 25 | 10 | 23 | 0 | 0 |
| Negative | 8 | 78 | 42 | 100 | 100 |

### *Conclusions*

1. Not all guinea-pigs' serums are efficient for tuberculosis complement fixation.

2. The serum from each guinea-pig should be tested for fixability with tuberculosis antigen plus tuberculosis serum before pooling the complement for diagnostic tests.

## II. OBSERVATION OF THE VON WEDEL REACTION

Dr. von Wedel, working independently in our laboratory, found that some serums from active tuberculosis cases gave a negative complement fixation reaction when the test was made on the first day after bleeding, and when the same specimens were tested a week later, having stood in the ice-box during the interval, they gave a positive reaction. This occurred repeatedly on later bleedings from the same patient. The complement used for all tests had been previously tested for fixability with tuberculosis antigen plus tuberculosis serum: therefore, the negative reaction in the first test was not due to an inefficient complement. Serums from non-tubercular cases gave no fixation at any period after bleeding, and this fact rules out the question of non-specificity of the later positive reaction following the early negative phase.

The controls for anticomplementary reaction in the patient's serum and in the antigen were all completely hemolyzed.

This early negative phase was not demonstrated in the serums from all tubercular cases; but the percentage was so large as to be significant.

Having personally observed the accuracy of Dr. von Wedel's technique, and the many repeated tests he made to discover the possibility of an error, I was convinced of the verity of the reaction and of the necessity for making the later test before the tuberculosis antibody content of all serums can be determined.

All tubercular serums tested, throughout the remainder of my study, will be given the early and later tests.

# A CONTRIBUTION TO THE STUDY OF THE COMPLEMENT FIXATION REACTION FOR TUBERCULOSIS

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The complement fixation reaction for tuberculosis has occupied the attention of many investigators for the past seventeen years, and it has been studied mainly from the standpoint of its possible value to clinical medicine in the diagnosis and prognosis of this disease. The methods commonly employed for diagnosing tuberculosis leave much to be desired and there is little doubt that many cases escape detection until marked damage has been done. A test, therefore, that will give a sure and early diagnosis, is of the utmost value both to the patient and to the general public.

The results of the early work with this complement fixation test were of such a contradictory nature that they were of little practical value. However, the reports of recent investigators seem to promise that this test will be of marked value to the clinician.

The following is a brief review of some of the more important investigations made during the past few years.

Widal and LeSourd (1) appear to be the first who used the complement fixation reaction in attempting to arrive at a more certain method of diagnosing tuberculosis. Bordet and Gengou (2) in 1903 demonstrated the presence of antibody capable of uniting with tubercle bacilli and fixing complement in the sera of tuberculous animals. Wassermann and Bruck (3) in 1906 also demonstrated the presence of an antibody to tuberculin in patients treated with tuberculin. Caulifield (4), Laird (5), Hammer (6), Calmette and Massol (7) using various forms of bacillary emulsions as antigens, obtained results which were

very inconclusive, ranging from 33 per cent to about 97 per cent of positive results in cases of tuberculosis. Much (8), using various acid-fast bacteria as antigens, with sera from tuberculous and healthy persons, obtained fixation in 77 per cent of the healthy cases, in other words, a large number of non-specific fixations. Frazer (9), using various antigens found that 96.6 per cent of sera from normal individuals gave no fixation with antigens made from living bacilli, and that with this antigen 42.3 per cent of sera from tuberculous individuals gave positive results. She states that the most reliable antigen is prepared from living human bacilli, and thinks that the complement fixation test made with living bacillary antigen is of more value in the diagnosis of tuberculosis than any other reaction thus far discovered. Dudgeon, Meek and Weir (10) state that all of their cases that had been treated with tuberculin gave positive results. Products of the bacilli were found to be very satisfactory as antigens. With an alcoholic antigen, (11) prepared from tubercle bacilli they obtained 89.3 per cent of positive results. Bronfenbrenner (12) using an antigen of tubercle bacilli grown by Besredka on egg broth, obtained a very high percentage of positive results, but also obtained quite a large percentage of non-specific positive results and 24 positive reactions with syphilitic sera. McIntosh, Fildes and Radcliffe (13) criticized Besredka's (14) antigen and concluded after testing many antigens, that the living bacillary emulsion was best. Inman (15) and Kuss, Lercdde, and Rubenstein (16) found the antigen non-specific. Stimson (17), using a variety of antigens, reported a small number of cases with but fair results. Corper (18), in 1916 using an autolysate as antigen and also a bacillary emulsion antigen, concludes that the complement fixation test for tuberculosis is not absolute, being positive in only about 30 per cent of all clinically definite cases of tuberculosis, both active and inactive. In 1917, Corper and Sweany (19) comparing their autolysate antigen with the bacillary antigen of Miller, (20) concluded that there is not a great deal of difference between the results obtained with these antigens and that it is impossible by means of this test absolutely to differentiate active from inactive tuberculosis. They prefer the autolysate to the bacillary antigen. They obtained 65 non-specific cross fixations out of 92 specimens of sera that gave positive Wassermann reactions. Slack, Burns, Castleman and Bailey (21) state that it appears from their observations that the complement fixation reactions are specific for tuberculosis, and that they obtain no cross fixation with positive Wassermann sera.

Of recent investigators, Craig (22) and Miller (23) obtained the best results. Craig reports the results of 166 examinations on cases of pul-

monary tuberculosis, in which he used an alcoholic extract antigen of human tubercle bacilli. He obtained 92.6 per cent of positive results in active cases, and 66.1 per cent in inactive cases. Out of 150 cases of syphilis, which were free from lesions in the lungs, none gave positive reactions. All of 100 other examinations (various diseases) gave negative results. However, Lucke (24) and other investigators report that alcoholic extract antigens, as used in Craig's work, have many times proven inpracticable and worthless, and as his description of the preparation of his antigen was wanting in detail, the described preparation was frequently found impossible. Miller reports 100 per cent of positive results in active cases and 100 per cent of negative of results in non-tubercular cases.

A great number of various antigens have been used and a great diversity of results has been obtained, reaching from 30 per cent efficiency up to Miller's claim of 100 per cent. There seems to be no uniformity in the findings and conclusions of any two observers; even the most promising of all these investigations, that is, Craig's and Miller's, are of limited practical value, as serologists have had great difficulty in using their antigens, and therefore have been unable to reproduce their results.

The main object of this preliminary study of the complement fixation reaction for tuberculosis was, therefore, with the use of the perfected Wilson antigen, which is easy to prepare and which can be kept for a long period of time without becoming anticomplementary, to find a method that would give specific positive results in active tubercular cases and which would not give non-specific results in negative cases.

I have made 1078 complement fixation tests on 200 specimens of blood serum taken from cases with no clinical history of tuberculosis and from patients with active, inactive and primary pulmonary tuberculosis. This study was made in conjunction with Miss Wilson in the New York City Board of Health Research Serological Laboratory. The cases were practically all from the Westchester County Hospital and I have the complete clinical data on all the cases. The clinical data consist of age; past and present temperature, pulse and respiration records; sputum reports and physical symptoms with clinical diagnoses.

## TECHNIQUE

The technique employed was similar to that originally used by Wassermann with the following modifications.

At first the tests were carried out in both one-quarter and one-tenth the original Wassermann volume, but as I found no difference in the results, I have since continued to use the one-tenth Wassermann volume only.

*Complement.* The pooled blood serum from six to ten healthy guinea-pigs was used as complement; in addition, we used serum from separate guinea-pigs untested for its complement fixation value; serum from separate guinea-pigs after having been tested for complement fixation value and serum from six to eight guinea-pigs, all of which had been specially tested for complement fixation value. All these complements were titrated with 2.5 per cent sheep cells, sensitized with three units of anti-sheep amboceptor; the unit was recorded at the end of fifteen minutes. Exactly 2 units were used in the regular test.

*Antigen.* The Wilson antigen, which was used throughout this study, is simply a suspension of tubercle bacilli killed with heat, extracted with alcohol and ether, and dried. The complete technique of preparing this antigen is given by Miss Wilson in her paper on the study of the complement fixation reaction for tuberculosis in the current issue of this journal.

*Sheep's cells.* A 5 per cent suspension of sheep cells, which had been washed five times in sterile saline was used, after having been sensitized with an equal volume of amboceptor in the water bath for half an hour.

*Amboceptor.* Three units were used in the tests.

*Fixation period.* After the patients' serum, complement, antigen and saline were mixed, the mixtures were incubated in the water bath at 37.5°C. for one hour. The sensitized cells were then added and the reading was made in exactly fifteen minutes. Six series of tests have been made for the purpose of comparing the results after the following different methods of incubation: one hour in the water bath; two hours in the water bath; two hours in the water bath followed by two hours in the ice-box;

and four hours in the ice-box. Apparently the one hour water bath incubation gives the most uniform results. The ice-box incubation gives by far the weakest fixation. The two-hours in the water bath followed by two hours in the ice-box gives almost the same results as the two hour water bath incubation alone which gave quite a number of anti-complementary reactions in the tests where we doubled the regular Wassermann amount of patients' sera. These comparisons, of course, are too few to allow a positive statement as to which is the best method of incubation. A large number of comparisons will be made in the near future to determine this question.

Results were reported as plus minus if any degree of fixation was observed; 1 plus if marked fixation in the first antigen tube; 2 plus if complete fixation in first tube; 3 plus if complete fixation in first tube and marked fixation in second tube, 4 plus if complete fixation in both tubes.

During 1913, Dr. Cyrus W. Field and the writer carried out a series of 730 Wassermann reactions in the Bellevue Hospital Laboratories, using the regular amount of serum prescribed by Wassermann and also twice, three times, four times and five times that amount. These amounts of patient's serum were tested in all of the 730 cases, our controls being carried out with double the amounts of serum used with the antigen. Discarding all those cases that were anticomplementary in the regular Wassermann amounts, and considering only those which ordinarily would be considered as not anticomplementary, we found that we had no anticomplementary and no non-specific reactions with double the usual amount of serum. Three times the usual amount of serum gave about 1 per cent of anticomplementary reactions, four times the usual amount of serum gave about 5 per cent of anticomplementary reactions and five times the usual amount of serum gave about 25 per cent anticomplementary reactions.

As these results were so favorable, and as several other investigators have made favorable reports on the use of larger quantities of patients' sera, I have made all my tests since January 1 with the regular Wassermann amount and with double that

amount of patient's serum; that is, 0.04 cc. of serum in the first antigen tube with 0.08 cc. of serum in its control tube; 0.02 cc. of serum in the second antigen tube with 0.04 cc. of serum in its control tube, and 0.01 cc. of serum in the third antigen tube.

All the specimens of sterile sera from 95 known non-tubercular cases, gave negative results with 0.04 cc. of patient's serum in the antigen tubes. Four contaminated specimens of serum from known non-tubercular cases, gave non-specific weakly positive reactions. Forty-nine sera from cases with active tuberculosis gave strong positive reactions with double the amount of serum and only 43 gave strong positive reactions with the regular amount of serum. Ten sera gave positive reactions of 2 plus to 4 plus when double the amount of serum was used, and only doubtful or negative results with the single amount. Of the type II cases, 46 gave positive results with double the amount and only 41 with the single amount. Of the type IV cases, 6 gave positive results with double the amount and only 2 with the regular amount. We, therefore, had an appreciable number of cases which gave definite positive results with the double amount and negative or doubtful results with the single amount of patient's serum.

The patients were bled Thursday afternoons and the sera were separated from the clots Thursday evenings or Friday mornings and inactivated Friday mornings. Each specimen of serum was tested the morning after it was removed from the patient and this serum was retested week after week for from four to five weeks with an interval of seven days between each test, together with specimens from new patients each week. We, therefore, have a record in many instances of eight weekly complement fixation tests on the same specimens of sera kept under as nearly sterile precautions as was possible in the ice chest at about 8°C.

By this proceedure, I made a very interesting observation which may possibly account for some of the wide discrepancies in the various complement fixation results reported by the different workers. The complement fixation results on sera from positive cases made the first day after taking the specimens from the patients were in a very large percentage of cases negative or

weakly positive; while in most instances, seven days later these same sera gave a strongly positive reaction and continued to give this strongly positive reaction week after week with unvarying regularity. None of the non-tubercular sera gave a positive reaction the second, third, or fourth week after the specimen had been obtained from the patient.

Of all positive sera in our series, only 12 gave a 3 or a 4 plus reaction the first day tested. After being preserved in the ice-box under sterile precautions for seven days, 49 sera gave 3 or 4 plus reactions. Of 82 sera from tubercular cases of all types that gave negative or doubtful reactions the first day, the results on the seventh day were positive in 37. The reactions apparently did not change after the sixth day.

TABLE 1

*Results obtained with the same specimens of serum one day, seven days, fourteen days, and twenty-one days after the blood sera were removed from the patients*

| SERUM NO. | FIRST DAY* | SEVENTH DAY | FOURTEENTH DAY | TWENTY-FIRST DAY |
|---|---|---|---|---|
| 13 | − | 4+ | 4+ | 4+ |
| 25 | − | 4+ | 2+ | 3+ |
| 14 | − | 4+ | 2+ | + |
| 61 | + | 4+ | 4+ | 4+ |
| 67† | − | − | − | − |

* This refers to the day after the blood was removed from the patient.

† The serum was obtained from a normal non-tubercular individual.

I have attempted to find out on just what day the positive result first appears in most of these delayed reactions, but heretofore I have been able to carry out only two series of daily titrations and from these I could not draw any positive conclusions. This work, however, will be continued and when our final results are published, I hope that they will include information upon this important question. The phenomenon just described apparently bears no relationship to the type of case, as I find it occurring in the old active cases, in the primary cases and in the inactive cases. In order to see whether the phenomenon was possibly due to anticomplementary reaction, I have used four times the usual amount of patient's serum in the serum controls without, however,

obtaining any more anticomplementary results with this large amount of serum at the end of the first week than on the first day. This phenomenon may be due to the presence of some inhibitory substance which disappears from the serum upon standing for several days.

Stimson mentions the rôle possibly played by the inhibiting substances in the patient's serum, which are stated by Caulfield, Calmette and his co-workers to occur in certain tubercle sera of tubercular individuals and which have the effect of producing negative reactions in sera that contain anti-bodies. They, however, did not state whether these inhibitory substances disappeared from the sera upon standing. This is a point that will require rigid investigation.

Various specimens of serum taken from the same patient at different times gave complement fixation results which were comparable. I took duplicate specimens from one to six weeks apart on 31 cases. The results on 24 were identical. The remaining 7 gave results that were closely alike; the slight differences being, perhaps, explained by the variations in the physical condition of the patients.

## CLASSIFICATION OF THE CASES

The types of tuberculosis cases have been variously classified by many investigators. Noted classifications were made by Williams, of Brompton Hospital, Cornet, L. Bard, Koeniger, Turban, (25-26), Meissen (27), and Walther L. Rathbun (28). Rathbun classifies tuberculosis as incipient, moderately advanced and far advanced. The following broad classification seems to be necessary both from the clinical and from the laboratory standpoint. Types II, III and IV must be separated, for they give entirely different clinical pictures and also entirely different complement fixation results.

*Type I.* Primary cases; very few physical symptoms present; no tubercle bacilli found in the sputum or found only after the examination of many specimens by the antiformin method.

*Type II.* Active cases; patient expectorating tubercle bacilli, the diseased area being walled off incompletely or not at all.

*Type III.* Active cases in the last stage; patient in a dying condition.

*Type IV.* Partially inactive cases; that is, cases expectorating tubercle bacilli but having very marked fibrous formation with the consequent complete walling off of the diseased area from the body proper.

*Type V.* Inactive cases.

*Type VI.* Cases reported as suspicious but expectorating no tubercle bacilli and having no symptoms of tuberculosis.

*Type VII.* Non-tubercular cases.

TABLE 2

*Comparison of results in the various types of cases*

| TYPE OF CASE | NUMBER IN EACH TYPE | NUMBER OF POSITIVE RESULTS | NUMBER OF DOUBTFUL RESULTS | PERCENTAGE OF POSITIVE RESULTS | PERCENTAGE OF DOUBTFUL RESULTS | PERCENTAGE OF POSITIVE RESULTS WHICH WERE 3+ OR 4+ | NUMBER OF POSITIVE RESULTS ON THE FIRST DAY | NUMBER OF POSITIVE RESULTS ON THE SEVENTH DAY | NUMBER OF 3+ RESULTS ON THE FIRST DAY | NUMBER OF 3+ RESULTS ON THE SEVENTH DAY |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 6 | 6 | 0 | 100 | 0 | 83 | 1 | 6 | 1 | 5 |
| 2 | 47* | 46 | 1 | 98 | 2 | 90 | 15 | 46 | 10 | 41 |
| 3 | 11 | 3 | 3 | 27 | 27 | 100 | 1 | 3 | 1 | 3 |
| 4 | 19 | 6 | 5 | 31 | 26 | 0 | 1 | 6 | 0 | 0 |
| 5 | 12 | 3 | 0 | 25 | 0 | 0 | 0 | 3 | 0 | 0 |
| 6 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | 99 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

* Two sera anticomplementary.

In table 2 I have grouped the results of the complement-fixation tests according to the clinical type of the cases examined.

Of 6 type I cases, all gave positive complement fixation reactions, or 100 per cent of positive results. Of 49 type II cases, 46 were positive, 1 was doubtful and 2 were anticomplementary, or omitting the 2 anticomplementary sera 98 per cent were positive and 2 per cent were doubtful.

Of 11 type III cases 3 were positive, 3 were doubtful and 5 were negative; or 27 per cent were positive and 27 per cent were doubtful. Of 19 type IV cases, 6 were positive, 5 were doubtful and 8 were negative; or 31 per cent were positive and 26 per cent were doubtful.

Of 12 type V cases, 3 were positive, 9 were negative; or 25 per cent were positive. Of 4 type VI cases, all were negative. Of 92 sterile type VII sera, all were negative. The contaminated type VII cases gave weak non-specific reactions.

The clinical diagnoses were all made either by Dr. Rosenberg, who was formerly diagnostician for tuberculosis at the Westchester County Hospital, or by Dr. Slade, diagnostician for tuberculosis for the New York City Board of Health.

As Dr. Rosenberg left the Westchester County Hospital before my work was completed, some of the latter cases were diagnosed and classified by the internes in the hospital. All of these latter cases were examined and reclassified by Dr. Slade thus the above diagnoses were all made by expert diagnosticians. Dr. Slade's diagnoses were made after my results had already been recorded.

In attempting to determine whether the temperature, pulse and respiration records of the patients bore any direct relationship to the complement fixation reactions, I have compared my laboratory findings with the record charts, and I find that the cases giving a 4 plus reaction had an average temperature of 99.4; pulse, 94; and respiration, 27. The patients giving a 2 plus reaction had an average temperature of 99; pulse, 85; respiration, 24. Those giving a doubtful or negative reaction had an average temperature of 99; pulse, 95; and respiration, 25. 40 per cent of the 4 plus cases and only 10 per cent of the doubtful and negative cases had a temperature of over a hundred. In other words a large percentage of cases having a high temperature and respiration records gave strongly positive reactions; vice-versa, a large percentage of cases having low temperature and respiration records gave negative reactions. Forty per cent of the 4 plus, 10 per cent of the 2 plus and only 4 per cent of the doubtful and negative cases had a respiration record of 30 or over. I also attempted to see whether, possibly, the age of the patient had any effect on our reactions and found that the average age of the patients giving a 4 plus reaction was 36; the average age of those giving a 2 plus reaction was 43; the average age of those giving a 1 plus reaction was 35; the average age of those giving a plus-minus reaction was 40. As all of the groups contained patients both young and old, no conclusions could be made.

Miss M. A. Wilson has observed that the serum of a large percentage of guinea-pigs is unsuitable for use in the complement fixation test for tuberculosis because the complement of these sera is not fixed, under the usual conditions of the test, with the sera of tuberculous individuals.

A similar irregularity in the guinea-pigs' sera, when employed in the Wassermann test, was reported by Noguchi and Bronfenbrenner (29) in their article on the "Variation of the complement activity and fixability of guinea-pig's sera in Wassermann work." They state that positive patient's sera will often fix complement from some guinea-pigs and will not fix complement from other guinea-pigs, but that there is no definite relationship between the complementary activity and the fixability of a given specimen of guinea-pig's serum. However, the irregularity observed by the latter authors was relatively infrequent as compared with that reported by Miss Wilson, who found that for the complement-fixation test in meningococcus and gonococcus infection and in tuberculosis, respectively about one-tenth, one-third and two-thirds of all guinea-pigs supply inefficient complement.

With the purpose of further studying this phenomenon I carried out a number of tests with sera from actively tubercular patients, at first, in two series; one with pooled guinea-pigs' sera that had not been tested as to fixability, the other with pooled guinea-pigs' sera, each of which had been separately so tested and found satisfactory. The pooled serum was derived, in each case, from six or eight guinea-pigs. These parallel tests gave practically the same results. In a few instances I obtained a 2 plus instead of a 1 plus reaction, or a 3 plus reaction with the tested complement and a 2 plus reaction with the untested complement; but this difference was not regularly encountered and in a few instances, in fact, better results were obtained with the pooled untested complement than with the specially tested complement.

This experiment being inconclusive I then carried out a series of tests with sera from seven frank tubercular cases (six of which I had already examined) testing each guinea-pig's complement

separately against each positive serum. The results of these tests are shown in table 3.

Serum 1, tested with complement from guinea-pig 1, gave negative results in the antigen tubes containing 0.02 cc. and 0.04 cc. of patient's serum on the first and fourth days after taking the specimen from the patient. On the sixth day and again on the seventh day complement from guinea-pig 2 gave a 3 plus reaction with 0.02 cc. of patient's serum and a 4 plus reaction with double that amount. Tests made on the ninth and eleventh days with complement from guinea-pig 3, gave negative results

TABLE 3

*Showing variations in flexibility of the complement of different guinea-pigs' sera in the complement-fixation test in tuberculosis*

| PATIENT'S SERUM NO. | COMPLEMENT NO. 1, FIRST TEST | | COMPLEMENT NO. 1, LATER TEST ‡ | | COMPLEMENT NO. 2, FIRST TEST | | COMPLEMENT NO. 2, LATER TEST ‡ | | COMPLEMENT NO. 3, FIRST TEST | | COMPLEMENT NO. 3, LATER TEST ‡ | | POOLED TESTED COMPLEMENT FIRST TEST | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | −* | −† | ± | − | 4+ | 2+ | 4+ | 3+ | − | − | − | − | 4+ | 2+ |
| 2 | − | − | − | − | 4+ | 3+ | 4+ | 3+ | 1+ | − | − | − | 4+ | 2+ |
| 3 | − | − | − | − | 4+ | 2+ | 3+ | 2+ | − | − | − | − | 1+ | ± |
| 4 | − | − | − | − | 4+ | 4+ | 4+ | 4+ | 1+ | − | | | 4+ | 3+ |
| 5 | 2+ | − | 3+ | 1+ | 4+ | 4+ | 4+ | 4+ | 4+ | − | 4+ | − | 4+ | 4+ |
| 6 | − | − | − | − | 4+ | 1+ | 3+ | 1+ | − | − | − | − | ± | − |
| 7 | − | − | 2+ | − | 4+ | 4+ | 4+ | 4+ | − | − | − | − | 3+ | 1+ |

* The results in the first column were obtained with 0.04 cc. of patient's serum.

† The results in the second column were obtained with 0.02 cc. of patients' serum.

‡ Complement was preserved in the interim with an equal amount of 18 per cent salt solution and kept in contact with ice.

with all amounts of patient's serum. On the fourteenth day, the pooled complement, made from sera of six tested guinea-pigs, gave results which were practically the same as those obtained when complement from guinea-pig 2 was used, that is, a 2 plus reaction with 0.01 cc. and 0.02 cc. of patient's serum and a 4 plus reaction with 0.02 cc. and 0.04 cc. of patient's serum. On the sixteenth day, complement from an additional guinea-pig which may be designated 4 gave a plus-minus and a 1 plus reaction.

Patients' sera 2, 3, 4, 6 and 7 gave almost identical results.

Serum 5 gave a 1 plus and a negative result with complement

1, a 4 plus reaction with complement 2, negative results with complement 3, a 4 plus reaction with the pooled complement and a 1 plus reaction with complement 4, when the regular amount of patient's serum was used.

The results of this limited experiment confirm those reported by Miss Wilson, inasmuch as only one of the three individual guinea-pigs' sera was found to be suitable for use in the test. In view of the fact that with this one serum (guinea-pig 2) the different patients' sera reacted differently and since the otherwise unsatisfactory guinea-pig's serum 3 was fixed with the double amount of one of the patients' sera (serum 5) it would seem advisable in testing guinea-pigs' sera as to fixability, to test them with a strongly reacting patient's serum, or, better, with two such sera and to use the latter in the usual quantity, not with the double quantity.

H. J. Corper (19) states that in his series of cases, he used 92 sera with positive Wassermann reactions and of these, 65 gave cross fixation with all tubercle bacterial antigens. He, therefore, concludes that in the presence of a positive Wassermann reaction, the presence of a positive complement fixation test for tuberculosis is of no practical value. He also states (18) that, while the most reliable investigators concede that a suspension of living tubercle bacilli is the only one of the many antigens used, that is of specific value, the objections to the bacillary emulsion are the small leeway between the antigenic and the anticomplementary dose, the turbidity produced in the tubes and the fairly high percentage of non-specific reactions.

My own experiences are in disagreement with all of Corper's conclusions. First, because in my series of cases there were 26 specimens from patients that gave positive Wassermann reactions but offered no physical symptoms of tuberculosis. None of these gave any cross fixation with the Wilson tuberculosis antigen. Secondly, in no instance did I obtain a positive reaction using double the regular amount of sterile patient's serum in my entire series of known non-tubercular cases. Thirdly I have never found the Wilson antigen to be anticomplementary in four times the dose used in the test, if the antigen is heated at

55°C. for one half hour just before being used. Fourthly, my tests in the active tubercular cases gave 98 per cent of positive reactions. Fifthly, the very slight turbidity produced by the antigen in no way interferes with the reading of the results.

## CONCLUSIONS

1. The tubercle bacillus antigen of Miss Wilson is not anti-complementary in four times the amount capable of producing positive complement fixation with sera from the great majority of cases of active tuberculosis.

2. Pooled complement from at least six guinea-pigs should be used in making the tests, or the complement from single pigs should be tested for its complement fixation value with known positive sera.

3. Double the original Wassermann amount of patients' serum should be used.

4. No report should be made until the sera has been tested, after having been kept under sterile conditions in the ice chest for from four to six days, preferably six days.

5. My results seem to indicate that if the afore-mentioned modifications of the original complement fixation tests are used, 100 per cent of non-tubercular cases will give absolutely negative results; nearly 100 per cent of the primary and active cases will give positive results with the exception of the dying cases; and about 25 per cent of the partially inactive and inactive cases will give only weak positive results.

Before final percentage results can be arrived at, it will be necessary to make many more tests on a large number of sera from active, inactive and incipient pulmonary tubercular cases and a large number of control sera from non-tubercular cases.

I hope to report on the results of about three thousand tests in the fall.

I wish to take this opportunity to thank Dr. Wm. H. Park and Dr. Russell, chief of the staff of the Westchester County Hospital for their invaluable assistance and advice, without which this study could not have been made.

CHART 4

*Clinical and laboratory records of all our tuberculosis patients*

| NAME | SPUTUM REPORT* | TYPE OF DIAGNOSIS | AGE | TEMPERATURE | PULSE | RESPIRATION† | COMPLEMENT-FIXATION RESULTS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | ONE DAY△ | | One week△ | | LATER□ | |
| | | | | | | | 2X Ser.‡ | Reg. S.§ | 2XS. | Reg. S. | 2XS. | Reg. S. |
| Mrs. Duffy | ± | 1 | 18 | 99 | 84 | 30 | | 4+ | | 0 | | 0 |
| I. Anderson | ± | 1 | 36 | 98 | 86 | 24 | | − | | 4+ | | 3+ |
| Fillapo | − | 1 | 24 | 97 | 104 | 20 | + | − | | 3+ | 4+ | 2+ |
| | − | 1 | 24 | 98 | 104 | 20 | + | ± | 4+ | 2+ | 3+ | + |
| | − | 1 | | | | | + | − | 4+ | 2+ | | |
| May Cooper | ± | 1 | 40 | 100 | 102 | 28 | | − | | + | 2+ | ± |
| Mrs. Baer | + | 2 | 33 | 101 | 100 | 26 | 2+ | + | 4+ | 4+ | | |
| Conklin | + | 2 | 45 | 97 | 88 | 28 | 2+ | ± | 4+ | 2+ | | |
| | + | 2 | | 98 | 80 | 24 | 2+ | − | 4+ | 4+ | 3+ | + |
| | + | 2 | | | | | 2+ | − | 4+ | 4+ | | |
| Dolan | + | 2 | 32 | 100 | 120 | 28 | 4+ | 4+ | 4+ | 4+ | 4+ | 2+ |
| | + | 2 | | | | | 4+ | 2+ | 4+ | 3+ | | |
| Barton | + | 2 | 30 | 99.8 | 115 | 29 | 4+ | 2+ | 4+ | 3+ | | |
| | + | 2 | | | | | 4+ | 2+ | 4+ | 3+ | | |
| Blauth | + | 2 | 53 | 101 | 104 | 32 | | − | | 4+ | | |
| | + | 2 | | 101 | 112 | 30 | | 3+ | | 4+ | | 4+ |
| | + | 2 | | | | | 4+ | 2+ | 4+ | 4+ | 4+ | 4+ |
| | + | 2 | | | | | 2+ | − | 4+ | 4+ | | |
| Scheehan | + | 2 | 26 | 97 | | | | − | | 4+ | | 2+ |
| | + | 2 | − | − | − | − | | − | | 3+ | | + |
| Loffran | + | 2 | 50 | 99 | 116 | 26 | | + | | 4+ | | 4+ |
| | + | 2 | | 96 | 68 | 20 | + | ± | | 3+ | 4+ | 2+ |
| Commisky | + | 2 | 25 | 98 | 84 | 20 | | 4+ | | 4+ | 4+ | 4+ |
| | + | 2 | -- | − | − | − | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ |
| Eckman | + | 2 | 45 | 102 | 98 | 32 | 4+ | + | 2+ | + | 4+ | 4+ |
| | + | 2 | | 98 | 80 | 20 | − | − | 4+ | 4+ | 4+ | 3+ |
| J. Cerpus | + | 2 | 35 | 99 | 78 | 20 | 4+ | 2+ | 4+ | 4+ | | |
| Murphy | + | 2 | 38 | 97 | 90 | 28 | 4+ | 3+ | 0 | 0 | 4+ | 4+ |
| Jos. Thompson | + | 2 | 42 | 100 | 130 | 36 | | − | | 4+ | | 4+ |
| R. Douglas | + | 2 | 46 | 101 | 135 | 30 | | − | | 2+ | | 4+ |
| J. Ackerman | + | 2 | 35 | 98 | 100 | 30 | | − | | 4+ | | 4+ |
| | + | 2 | | 98 | 80 | 22 | + | − | 4+ | 4+ | | |
| Gournack | + | 2 | 40 | 101 | 136 | 30 | | − | | 4+ | | |
| Parez | + | 2 | 23 | 99 | 92 | 24 | | − | | 4+ | | 4+ |
| John Hurst | + | 2 | 43 | 100 | 84 | 24 | | − | | 4+ | | 4+ |
| Rivella | + | 2 | 47 | 98 | 80 | 30 | | − | | 4+ | 4+ | 2+ |
| D. Bonis | + | 2 | 23 | 98 | 84 | 20 | | 4+ | | 4+ | | + |

CHART 4—*Continued*

| NAME | SPUTUM REPORT* | TYPE OF DIAGNOSIS | AGE | TEMPERATURE | PULSE | RESPIRATION† | COMPLEMENT-FIXATION RESULTS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | One day△ | | One week△ | | Later□ | |
| | | | | | | | 2X Ser.‡ | Reg. S.§ | 2X S. | Reg. S. | 2X S. | Reg. S. |
| Shields | + | 2 | 25 | 99 | 76 | 24 | — | — | 2+ | ± | | |
| | + | 2 | — | — | — | — | — | — | 4+ | 4+ | 4+ | 2+ |
| Mannix | + | 2 | 39 | 99 | 108 | 24 | | 4+ | | 4+ | | |
| Monroe | + | 2 | 50 | 98 | 84 | 26 | — | — | 3+ | + | 2+ | — |
| | + | 2 | | 97 | 72 | 22 | — | — | 4+ | + | ± | — |
| Smith | + | 2 | 35 | 98 | 84 | 20 | — | — | 3+ | + | | |
| May Hide | + | 2 | 23 | 102 | 116 | 28 | | — | | 4+ | | ac |
| | + | 2 | — | — | — | — | | — | | ac | | ac |
| F. Ross | + | 2 | 27 | 102 | 120 | 36 | | — | | 4+ | | 2+ |
| | + | 2 | — | — | — | — | | — | | | | ac |
| McEwen | + | 2 | 34 | 101 | 122 | 28 | | ± | | 2+ | | 4+ |
| | + | 2 | | 102 | 104 | 29 | — | — | 4+ | 2+ | + | ± |
| Lustigo | + | 2 | 53 | 98 | 86 | 32 | | 2+ | | 3+ | | 4+ |
| Kiernan | + | 2 | 52 | 97 | 76 | 20 | | — | | — | 4+ | 2+ |
| Taylor | — | 2 | 39 | 98 | 88 | 24 | | — | | ± | 2+ | — |
| | + | 2 | | 97 | 76 | 22 | — | — | ± | — | | |
| Mocia | + | 2 | 40 | 98 | 76 | 22 | — | — | — | — | + | — |
| | + | 2 | | 100 | 80 | 20 | — | — | 2+ | + | | |
| Mrs. Rich | + | 3 | 32 | 99 | 86 | 29 | + | ± | 4+ | 3+ | | |
| | + | 3 | — | — | — | — | — | — | 4+ | 4+ | 4+ | 2+ |
| Aug. Perilla | + | 3 | 19 | 102 | 132 | 30 | | 4+ | | 4+ | | 4+ |
| Wood | + | 3 | 44 | 100 | 100 | 28 | ± | — | — | — | — | — |
| Chas. Doyle | + | 3 | 30 | 98 | 84 | 20 | — | — | ± | — | | |
| | + | 3 | | | | | — | — | ± | — | | |
| | — | 3 | | | | | — | — | — | — | | |
| Kouwart | — | 3 | 34 | 99 | 102 | 30 | | — | | — | | — |
| | ± | 3 | | 97 | 76 | 22 | — | — | — | — | | |
| Rogner | + | 3 | 20 | 104 | 142 | 28 | | — | | — | | |
| | + | 3 | — | — | — | — | — | — | — | — | — | — |
| Monahan | + | 4 | 55 | 98 | 72 | 22 | — | — | + | — | | |
| | + | 4 | | 97 | 72 | 20 | — | — | — | — | | |
| | + | 4 | | | | | — | — | — | — | | |
| W. Ahren | + | 4 | 53 | 98 | 112 | 26 | | — | | — | | — |
| C. Bennett | + | 4 | 43 | 98 | 90 | 28 | | — | | — | ± | — |
| | + | 4 | | 98 | 84 | 24 | — | — | ± | — | | — |
| Fr. Rousso | + | 4 | 31 | 101 | 110 | 24 | | — | | — | | — |
| | + | 4 | | — | — | — | | — | | — | — | — |
| J. Burns | + | 4 | 44 | 98 | 80 | 22 | | ± | | | 2+ | + |
| Pohamis | + | 4 | 26 | 99 | 104 | 28 | | + | | 0 | ac | ac |

CHART 4—*Concluded*

| NAME | SPUTUM* | TYPE OF DIAGNOSIS | AGE | TEMPERATURE | PULSE | RESPIRATION† | COMPLEMENT-FIXATION RESULTS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | One day△ | | One week△ | | Later□ | |
| | | | | | | | 2X Ser.‡ | Reg. S.§ | 2X S. | Reg. S. | 2X S. | Reg. S. |
| Tody | + | 4 | 30 | 100 | 116 | 29 | − | − | − | − | | |
| | + | 4 | | | | | − | − | ± | − | | |
| Ida Ford | − | 4 | 30 | 100 | 88 | 28 | ac | ac | − | − | | |
| | − | 4 | | | | | − | − | + | − | − | − |
| Lena Stokes | + | 4 | 35 | 99 | 86 | 22 | ± | − | − | − | | |
| J. Burfurnack | + | 4 | 35 | 99 | 86 | 22 | − | − | − | − | | |
| | + | 4 | | | | | − | − | ± | − | − | − |
| Neary | − | 4 | 41 | 98 | 92 | 24 | | − | 2+ | ± | ± | − |
| | − | 4 | − | | | | − | − | − | − | + | ± |
| Bradshall | − | 5 | 32 | 99 | 120 | 26 | | − | | − | − | − |
| | − | 5 | 32 | 97 | 76 | 28 | − | − | + | + | ± | − |
| W. Bennett | − | 5 | 54 | 98 | 96 | 24 | | − | | − | − | − |
| Blohm | − | 5 | 44 | 97 | 104 | 26 | | − | − | − | − | − |
| Brundage | ± | 5 | 43 | 98 | 68 | 22 | − | − | − | − | | |
| McKillegat | − | 5 | 65 | 98 | 92 | 28 | | − | | + | | ± |
| Rigunioa | − | 5 | 47 | 97 | 76 | 22 | | − | | − | − | − |
| Tripoda | − | 5 | 20 | 99 | 72 | 20 | | − | | − | − | − |
| Neil | − | 5 | 49 | 98 | 100 | 26 | | − | | − | − | − |
| Leno Fiorella | − | 5 | 15 | 99 | 116 | 24 | − | − | − | − | | |
| | − | 5 | | | | | − | − | − | − | − | − |
| May Sedden | − | 5 | 50 | 98 | 88 | 22 | 2+ | ± | | | | |
| Wm. Webb | − | 6 | 34 | 97 | 80 | 22 | | − | | − | | − |
| Odell | − | 6 | 35 | 98 | 90 | 24 | | − | | − | − | − |
| Mrs. Hegil | − | 6 | 40 | 97 | 80 | 22 | | − | − | − | − | − |
| Spudds | − | 6 | 22 | 99 | 70 | 24 | − | − | − | − | | |

* Sputum report obtained within two weeks of the time the blood specimen was removed.

† Temperature, pulse and respiration were taken the same afternoon the blood was removed.

‡ Refers to the use of double the regular Wassermann amount of patient's sera.

§ Refers to the use of the regular Wassermann amount of patient's sera.

△ Refers to the time the reaction was made after the specimen was removed from the patient.

□ These results were obtained from two to six weeks after the specimens were removed from the patients.

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# THE RÔLE OF IMMUNITY IN THE CONDUCT OF THE PRESENT WAR[1]

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When the history of the present great war is written a notable victory over the common enemy, disease, will be recorded as one of the greatest triumphs in this greatest of all conflicts. In all probability this triumphs over disease will also be recorded as the most important single factor in explanation of the stamina and long-sustained man-power of the involved nations; never before in the history of the world have so many men been engaged in combat with such freedom from internal deterioration due to disease not only among the warriors in preparation and at the line of battle, but also among the supporting civilian population; history records many instances of cessation of wars and sieges due to disease among offenders or defenders or both and a remarkable freedom from pestilence in the present conflict has undoubtedly played a prominent rôle in permitting it to reach the dimensions of the greatest of all wars.

This triumph over disease is due in most part to prevention by sanitary measures, specific immunization and improved methods of treatment of the inevitable and unavoidable sick and injured. With the exception of small-pox, in which disease the science of immunity long ago contributed the most important and one essential means of prevention in the form of cow-pox vaccination, sanitary measures embracing the proper disposal of infectious material and the prevention of the spread of infectious diseases by the processes of isolation and quarantine and including the maintenance of individual resistance by proper

[1] Presidential address at the Fifth Annual Meeting of the American Association of Immunologists held at the University of Pennsylvania on March 29 and 30, 1918.

food, work, rest and play, has played the most important rôle, with the science of micro-parasitology and immunity embracing a knowledge of the parasitic causes of so many of the acute infectious diseases and specific immunization of several by means of vaccines and sera, a close second worthy of the division of honor and credit.

Mention has just been made of cow-pox vaccination in the prevention of small-pox; history shows that without this immunological discovery and process great wars would be impossible and particularly one of the present dimensions involving so many countries and millions of men and offering splendid facilities for the rapid dissemination of the virus; the prevention of typhoid and paratyphoid fever by means of active immunization with vaccines while not as successful as cow-pox vaccination, must be credited with a great measure of success in the prevention of these diseases formerly so widely prevalent among armies; certain measures of success which in some instances are quite marked, have also attended the prevention of bubonic plague, bacillary dysentery, cholera and rabies by means of active immunization.

The prevention and treatment of tetanus and diphtheria with their respective antitoxic sera have proven most valuable immunological procedures and particularly so in the prevention of tetanus at a time when the modern earth digging methods of war have widely distributed the bacillus and rendered practically every wound regardless of severity and location a real danger and menace to life; likewise in the treatment of epidemic cerebrospinal meningitis a potent antiserum has proven conclusively that it is the best means of treatment, its free and intelligent use resulting in a considerable reduction in the percentages of death and the disabling sequelae. In the treatment of that dreaded disease, pneumonia, "The Captain of the Men of Death," the science of immunity has contributed a means for the serologic diagnosis of the type of pneumococcus present and prepared a serum for the treatment of type 1 infections which has proven its worth and right to a prominent place in the modern treatment of this disease. Still more recently the science of immunity has pro-

duced for the toxins of the gas-producing bacillus which has played havoc among so many of our wounded heroes in the past and present, a serum that bids fair to prove of value in the prevention and treatment of this dangerous infection.

Immunological reactions are also proving of practical value at the present time in the diagnosis of several diseases and particularly the serological reaction in the diagnosis of syphilis, which disease menaces all peoples at present and particularly in the future, by reason of its wide dissemination and insidious nature rendering all persons regardless of age and sex vulnerable and liable to its attack. Furthermore in the treatment of this "Third Great Plague," the newly developed branch of chemotherapy in the field of immunity, has contributed a most remarkable remedy in the form of dioxydiaminoarsenobenzol or the popularly known "606," and our hopes for the present prevention of syphilis and protection of the future and unborn peoples, resides in large part in the treatment of the infected until they are rendered less infectious even if not completely cured, by the widespread and more free employment of this and other anti-syphilitic remedies. To this end all efforts made to lower its cost and thereby facilitate its use in the treatment of the poor and of large numbers of persons, are to be welcomed as commendable and a work of first rank importance.

Therefore, while the science of immunity has contributed considerable that is of practical value in the diagnosis and treatment of various diseases of particular importance in relation to the present war, much and indeed more, remains to be accomplished of which mention may be made of but a few of the more pressing problems as follows: The discovery of a test of effective natural immunity to pneumonia and meningococcus meningitis, if such immunity exists, comparable to the Schick test for immunity to diphtheria, as a means of encouraging and facilitating active immunization with vaccines in the prevention of these diseases; a test for natural immunity to tetanus, which may be developed along the lines of the Schick test if some means can be devised for removing the danger of the spore; a means of specific immunization against measles, acute anterior polio-

myelitis, syphilis and gonorrhea and an improvement of our means for active immunization against cholera, plague, dysentery and typhoid fever, not to forget that problem of problems, namely, the discovery of a means of specific immunization and treatment for tuberculosis.

At out meeting last year the Association officially passed resolutions offering to our federal government the services of our members and laboratories in the conduct of our great war; before and since then not a few of our members have enlisted for active duty in the federal service and at least one has given up his life as a sacrifice to duty; many and probably all members of our Association are more or less intimately associated in some work having a direct bearing upon the problems of health and disease and particularly those menacing or likely to menace the health of our armies abroad and at home; to all the Association would hold up in pardonable pride the accomplishments of the science of immunity in the past and wish all God-speed in their work for the present and future for the health and happiness of mankind for all time and everywhere.

# ON THE MODE OF ACTION IN VITRO AND THE PREPARATION OF HEMOLYTIC ANTIBODIES

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This work was undertaken with the idea, first, of studying the mechanism of amboceptor action *in vitro*, and secondly, of ascertaining, if possible, what part of the red blood cells is responsible for their antigenic property. Inasmuch as our work has now been brought to a necessary close, we are bringing together in this article our findings even though our conclusions at this stage cannot be far-reaching.

Many of the general principles applying to the mode of action of hemolytic antibodies have been worked out by Muir (1) who showed that the combination of amboceptor and cells is a weak one, easily dissociable, and occurs according to a law apparently resembling the law of mass action. He showed, further, that amboceptor is not destroyed by hemolysis but remains bound to the receptors of the hemolyzed cells from which it can dissociate in the same manner as from the whole cells, such dissociation being more marked at incubator temperature.

By analogy with the law of mass action, if the amount of unhemolyzed cells is very large in proportion to the amount of hemolyzed receptors the dissociation from the hemolyzed portion will be practically complete, and almost the entire amount of amboceptor at any one time will be resident on the unhemolyzed cells. This was found to be so in experiments of which the following is an example.

A relatively large quantity (1 cc.) of washed sheep cells, concentrated by rapid centrifugation for ten minutes, was diluted

to 40 cc., and 80 units[1] of amboceptor added, followed by three units of complement. After incubating for thirty minutes a hemolysis of about 10 per cent of the cells had occurred. The liquid was centrifuged again at the same speed and for the same period as before, the volume of concentrated cells was measured, and the sediment was then made up to a 5 per cent suspension. A quantity of untreated cells was then taken, equal to the amount of cells remaining after the partial hemolysis just described, and to this 80 units of amboceptor and enough salt solution to secure a 5 per cent suspension were added. The amount of complement necessary completely to hemolyze 0.5 cc. of each of these two suspensions was determined and was found to be the same. We thus concluded that both samples of cells contained the same amount of amboceptor, showing first, that under these conditions, dissociation, as might be expected from chemical reasoning, is negligible from the unhemolyzed cells, and secondly, that amboceptor is not only set free during hemolysis, but is quantitatively unaltered. The results are noted in table 1.

TABLE 1

*Showing the action of amboceptor to be "progressive"*

| | COMPLEMENT 1:10 | | | | | |
|---|---|---|---|---|---|---|
| | 0.10 cc. | 0 11 cc. | 0.12 cc. | 0.13 cc. | 0.14 cc. | 0 |
| Treated cells..... | +++ | +++ | +++ | ++++ | ++++ | – |
| Untreated cells ..... | +++ | +++ | +++ | ++++ | ++++ | – |

++++ indicates complete hemolysis.
– Indicates no hemolysis.

[1] Throughout this paper the "unit" of amboceptor is taken as the smallest amount of inactivated immune rabbit serum which hemolyzed completely 0.5 cc. of 5 per cent red cell suspension, with the addition of 0.05 cc. of complement, after one hour's incubation at 37°C. and in a total volume of 2.5 cc. The "unit" of complement is the smallest amount of guinea pig serum which completely hemolyzes in one hour at 37°C. 0.5 cc. of 5 per cent red cell suspension in the presence of two (2) units of amboceptor, in a total volume of 2.5 cc. The 5 per cent red cell suspension is prepared by centrifuging washed sheep cells rapidly at a definite speed for ten minutes and then bringing up the measured sediment to the proper dilution with 0.85 per cent NaCl solution.

Should the amount of hemolyzed cells be large or should several washings with salt solution be made, the amboceptor on the cells so treated will be smaller in amount than on the control series, showing a considerable loss by dissociation.

If the hemolyzed receptors are heavily loaded with amboceptor, it is easy to conceive that this should in part dissociate, and undissolved red cells being still present, that the dissociated amboceptor should attach itself to these, causing their hemolysis. The action of amboceptor would therefore not cease with the hemolyzing of one cell, but would be continuous, and as the amount of hemolyzed receptors increased and the amount of unhemolyzed receptors decreased, the hemolyzed receptors would become less saturated with immune body, and consequently would split off less, thus causing the velocity of the reaction to decrease, a phenomenon well recognized in hemolytic work, for the last traces of unhemolyzed cells disappear very slowly. That this is not chiefly due to the deterioration of complement at incubation temperature can be shown by the fact that the system still contains that component, usually in considerable amounts. Furthermore, the velocity of deterioration of complement in the presence of amboceptor is practically the same as in pure salt solution (as far as our rough immunological methods will permit us to measure). The products of complement deterioration, so called "complementoid," likewise do not inhibit hemolysis when used in quantities comparable to the amounts of complement ordinarily used in hemolytic work. On the other hand, laked cells, whether dissolved by amboceptor and complement, or by distiled water and subsequently made isotonic are capable of greatly inhibiting the reaction.

In table 2, A is guinea-pig's serum heated to 56°C. for thirty minutes and represents "complementoid." B represents 100 per cent cells laked with distilled water, made isotonic, and diluted with salt solution to ten times the original volume. Each tube contains 0.5 cc. of 5 per cent cells, two units of amboceptor and one unit of complement, the total volume being 2.5 cc. An additional control for A not tabulated and containing 1 cc. of heated serum but no complement showed no hemolysis.

This observation under B of table 2 coincides with the work of Muir (2), who showed that the receptors of the red cells are not destroyed by hypotonicity. They are, therefore, capable of binding amboceptor just as the whole cells do, and their addition to the system will cause a diminution in the amount of amboceptor available for hemolytic purposes.

Bordet (3) found that the stroma of hemolyzed red cells is capable of fixing antibody, and he was able, by injecting them into an animal, to produce antibodies hemolytic for the whole cells. Stewart (4) confirmed this but found the hemolytic prop-

TABLE 2

*Showing the absence of inhibiting effect on hemolysis by heated guinea-pig serum, and the presence of such effect by isotonic laked cell products*

| | AMOUNTS OF A OR A ADDED TO HEMOLYTIC SYSTEM (CC.) | | | | | |
|---|---|---|---|---|---|---|
| | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 | 0.6 |
| A. Heated serum 1–10..... | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| B. Laked cell products 10 per cent..... | + | + | + | + | + | + |
| | AMOUNTS OF B OR B ADDED TO HEMOLYTIC SYSTEM (CC.) | | | | |
| | 0.7 | 0.8 | 0.9 | 1.0 | 0 |
| A. Heated serum 1–10..... | ++++ | ++++ | ++++ | ++++ | ++++ |
| B. Laked cell products 10 per cent.. | Tr. | Tr. | Tr. | Tr. | ++++ |

\+ Signifies perceptible hemolysis.
Tr. a trace of hemolysis.
++++ signifies complete hemolysis.

erty of the antiserum less marked than the agglutinative property. By filtration of the laked cells through a Berkefeld filter, Muir (5) was able to retain all of the stroma and with it practically all of the receptors. A repetition of this process confirmed the result, the filtrate in our work showing absolutely no amboceptor-binding power. If, however, the stroma is prepared[2] and sus-

[2] For the preparation of stroma the washed cells were laked in distilled water, centrifuged at low speed to remove leucocytes, etc., and the solution then made slightly hypertonic with NaCl. Vigorous centrifugation then brings down the greater part of the stroma, which can be washed and repeatedly centrifuged until fairly free from hemoglobin. They form a reddish sticky mass.

pended in salt solution, it possesses remarkably active amboceptor-binding properties. As proof of this latter statement we briefly review one experiment. To 1 cc. of diluted stroma, representing not more than the equivalent of 4 cc. of 5 per cent cells,[3] 8 units of amboceptor were added and salt solution up to 8 cc. This mixture was kept at room temperature for thirty minutes and it was then centrifuged at high speed. To 2 cc. of the supernatant fluid, 0.5 cc. of 5 per cent cells and 2 units of complement were added. This mixture was incubated at 37°C. for thirty minutes. There was no hemolysis. The mixture was again centrifuged at high speed and to the supernatant fluid 0.5 cc. of sensitized cells were added. On incubating at 37°C. complete hemolysis promptly occurred.

The amboceptor-binding properties of the stroma were found by us to be partially destroyed by heating to 65°C. for thirty minutes, markedly diminished at 70° and completely destroyed at 80°. These results differ from Muir's (6) in that he found evidence of amboceptor-binding even after heating to 100° for one hour. Our results point to the protein nature of the antigenic substance.

Dried stroma on being resuspended in salt solution act in the same manner as the moist freshly-prepared material, while ox cell stroma is without effect, showing the species specificity of the reaction.

Rabbits were then injected intravenously with the suspensions of stroma from sheep cells and it was observed that the production of both hemolysins and agglutinins was marked. Since the animals so treated receive very little protein in comparison with those injected with whole cells, it was thought possible to increase the quantity of injected material. Accordingly the stroma of approximately 25 cc. of concentrated sheep cells, suspended in about 10 cc. of salt solution, was injected each time, and apparently with little if any ill effect. Table 3 shows the details in the case of two of the rabbits.

[3] The calculation was made on the assumption that all the stroma had been secured by our process. We intentionally overestimate the amount.

TABLE 3

*Showing the production of hemolysins and agglutinins by the use of stroma as antigen*

| | DATE | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | April 16 | April 19 | April 22 | April 25 | April 29 | May 2 | May 6 | May 9 | May 13 | May 18 | May 22 | May 25 |
| **Rabbit 1** | | | | | | | | | | | | |
| Weight in grams | 1420 | 1340 | 1375 | 1350 | 1430 | 1525 | 1450 | 1540 | | | | |
| Number of injection | 1 | 2 | 3 | 4 | 5 | | | | | | | |
| Hemolytic titer | | 1–100* | 1–1000 | 1–2500 | 1–5000 | 1–3300 | 1–5000 | 1–2500 | 1–2000 | 1–1600 | 1–1000 | 1–600 |
| Agglutinative titer | 1–10 | 1–10 | 1–100 | 1–200 | 1–200 | 1–200 | | | | 1–200 | | 1–200 |
| **Rabbit 2** | | | | | | | | | | | | |
| Weight in grams | 1970 | 1960 | 1790 | 1930 | 1800 | 1880 | 1945 | 1870 | 1960 | 1925 | | |
| Number of injection | 1 | 2 | 3 | 4 | | | 5 | 6 | 7 | | | |
| Hemolytic titer | | 1–100* | 1–3300 | 1–3300 | | 1–3300 | 1–1100 | | 1–2000 | 1–3300 | 1–1250 | 1–800 |
| Agglutinative titer | 1–10 | 1–10 | 1–100 | 1–200 | | 1–200 | 1–200 | | 1–200 | 1–600 | | –800 |

*Or less.

Rabbit 2 was desensitized on May 6 by injecting 1 cc. of the stroma suspension subcutaneously one and one-half hours before injecting the full dose intravenously. It was noted that the hemolytic titer in both cases declined rapidly after the end of the first week following the last injection, whereas the agglutinative titer remained constant or increased. In rabbit 2 the declining hemolytic titer was lifted to the original level by a second brief series of injections, and the agglutinative titer was increased four-fold. The hemolyte titer in each case signifies that 1 cc. of the dilution tabulated completely hemolyzes 0.5 cc. of standard 5 per cent cells, in the presence of 0.05 cc. of complement (the total volume being 2.5 cc.), in one hour at 37°C. The agglutinative titer does not represent ++++ agglutination, but merely a distinct macroscopic clumping, after one hour at 37°C. The tabulated agglutinating titer shows the final dilution of one part of diluted serum and one part of a 1 per cent suspension of cells. The serum was inactivated for both tests and in every case the necessary controls were set up.

Having shown that the antigenic properties of the red cells for the producton of hemolysins are connected with the stroma we attempted to ascertain whether any particular constituent of this material could be identified as antigen.

In these experiments being met with the difficulty of completely separating the stroma out of the suspension by centrifuging, we added small amounts of pure precipitated calcium carbonate to the stroma suspension. This substance, in settling, carried down with it the stroma and made a separation easily possible. Stroma so mixed with calcium carbonate was dried and extracted with alcohol and ether. A considerable amount of lipoidal material was obtained which, when suspended in salt solution in a manner similar to that used in preparing Wassermann antigen, was not able to bind amboceptor. This indicated to our minds that the antigenic properties of the red cells are not due to the lipoidal constituents of the stroma. We did not inject any of this lipoidal extract into animals. Other observers, however, among whom are Thiele and Embleton (7), have found that lipoids from sheep's red cells do not act as antigen.

Extractions of the stroma-calcium carbonate sediment with physiological salt solution and with 5 per cent salt solution at incubator temperature showed again that in neither case was a substance removed which was capable of binding amboceptor.

On treating the stroma with alkaline physiological salt solution (0.85 per cent NaCl containing 0.2 per cent NaOH), a substance is extracted which precipitates out on neutralization of the alkali, and does not redissolve on the addition of a slight excess of acetic acid. This material, if suspended in salt solution, is capable of binding amboceptor.

As prepared by us the extraction with alkali was allowed to continue at incubator temperature for three hours. After the first precipitation by addition of acetic acid, the material was purified by redissolving it in alkali and by reprecipitating it by neutralization. Hereafter, we shall speak of this precipitate as the alkaline extract.

In order to demonstrate the amboceptor-binding properties of this alkaline extract *in vitro*, we emulsified it in physiological salt solution and allowed it to come in contact over night in the ice-box with diluted hemolytic amboceptor made from whole sheep cells. The titer of this amboceptor, after the suspended precipitate had been carefully removed by centrifugation, was found in all cases to be much less than before. In table 4 the supernatant fluid consists of diluted amboceptor that has been in contact with the alkaline extract over night on ice, the sediment having been removed by centrifugation. Each tube contains 0.5 cc. of 5 per cent cells and 0.05 cc. of complement, the total volume being 2.5 cc.

After one hour at 37° the amount of hemolysis was as indicated in the table.

The untreated amboceptor was at the same time titrated with 0.05 cc. of complement and the unit found to be 0.05 cc. Ten times as much of the treated amboceptor, as of the untreated, is needed therefore for hemolysis. A control was carried out, also, to show that complement was not kept out of combination in the test by the anticomplementary nature of the fluid.

The lipoid-free alkaline extract secured by extracting a small amount of material with one part of absolute alcohol and four parts of ether three times for a period of ten minutes each, also binds amboceptor *in vitro* although not so vigorously as the alkaline extract plus lipoids. Under conditions as nearly paralleling those of table 4 as possible, 0.2 cc. of supernatant fluid from the lipoid-free alkaline extract sediment was sufficient to produce complete hemolysis, whereas in the case of whole stroma extract 0.5 cc. was necessary. One would suspect, therefore, that, upon injection into animals, this lipoid-free product might produce hemolysins. Thiele and Embleton (7) have, in fact, obtained such antbodies by injecting the lipoid-free red cells into rabbits. Our present experiments, however, do not include this further, desirable step.

TABLE 4

*Showing amboceptor-binding properties of "alkaline extract" in vitro*

| | SUPERNATANT FLUID USED AS AMBOCEPTOR (CC.) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0.1 | 0.15 | 0.2 | 0.3 | 0.4 | 0.5 | 0.8 | 1.0 | 1.5 |
| Degree of hemolysis | ++ | +++ | +++ | +++ | +++ | +++ | ++++ | ++++ | ++++ |

The amboceptor-binding property of the alkaline extract shows species specificity, for the titer of an antihuman red-cell amboceptor was unchanged after being treated with it.

In order to prove definitely that the hemolysin-producing property of red cells is referable to a substance contained in this alkaline extract, we injected a series of rabbits and were able to demonstrate hemolysins in every case. These are specific as was shown by the use of human cells in the controls, which were not affected. The amount injected each time represented, roughly, the precipitate from the stroma of 25 cc. of 100 per cent red cells. The intravenous route was used. The titer of the serum, as in table 3, was determined just prior to the injection tabulated in the same column. The results in three rabbits so treated are shown in table 5.

Tests were made for anaphylactic reactions as follows. Rabbit 1 on May 18 and rabbits 2, 3 and 4 on May 25 were injected intravenously with the amount of alkaline extract that had on previous occasions been used. Rabbits 1 and 3 showed partial

TABLE 5

*Showing production of hemolysins with the use of "alkaline extract" as antigen*

| | DATE | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | April 30 | May 3 | May 6 | May 9 | May 13 | May 16 | May 18 | May 22 | May 25 |
| **Rabbit 3** | | | | | | | | | |
| Weight in grams... | 1300 | 1415 | 1275 | 1290 | | 1375 | | | |
| Number of injection..... | 1 | 2 | 3 | 4 | | | | | |
| Hemolytic titer..... | | Trace | 1–100* | 1–300 | 1–500 | 1–1100 | 1–1250 | 1–400 | 1–300 |
| Agglutinative titer. | 1–2 | 1–2 | 1–2 | 1–2 | 1–2 | 1–2 | 1–2 | 1–2 | 1–2 |
| **Rabbit 4** | | | | | | | | | |
| Weight in grams.... | 1130 | 1120 | 1000 | 1030 | | 1035 | | | |
| Number of injection..... | 1 | 2 | 3 | 4 | | | | | |
| Hemolytic titer..... | | Trace | 1–100* | 1–500 | 1–400 | 1–2500 | 1–3300 | 1–400 | 1–250 |
| Agglutinative titer. | 1–10 | 1–10 | 1–10 | 1–10 | 1–10 | 1–10 | 1–10 | 1–10 | 1–10 |
| **Rabbit 5** | | | | | | | | | |
| Weight in grams.... | 1170 | 1270 | 1105 | 1220 | | 1310 | | | |
| Number of injection..... | 1 | 2 | 3 | 4 | | | | | |
| Hemolytic titer..... | | Trace | 1–100* | 1–500 | 1–1250 | 1–1600 | 1–1600 | 1–500 | 1–500 |
| Agglutinative titer. | 1–2 | 1–2 | 1–2 | 1–2 | 1–2 | 1–2 | 1–2 | 1–2 | 1–2 |

* Or less.

collapse, with involuntary bowel movements, but soon recovered; the other two apparently were unaffected. On May 28, rabbit 5 was given intravenously stroma suspension made from 15 cc. of 100 per cent cells, but showed no untoward symptoms. These tests, as may be noted from tables 3 and 5 were made from twelve to sixteen days after the last injections, and one would expect

severe anaphylactic reactions. This phenomenon, so far as the alkaline extracts are concerned, is in accord with what Lake (8) observed; viz., that even when nucleo-proteins have lost their power of causing anaphylactic reactions they may still be able to develop complement-fixing bodies.

## DISCUSSION

This leads us to a discussion of the nature of our alkaline extract. What, in fact, is our antigen? Is it a nucleo-protein? We have hesitated to call it by this name in view of the conclusions, based on careful work, of Pearce (9) and his associates and of Wells (10), who hold that nucleo-proteins play no important part in the course of production of cytotoxic immune sera. Wells (10) believes that the antigenic properties of nucleo-proteins lie in the simple proteins present and that these simple proteins are not a characteristic integral part of the conjugated protein, but rather an adventitious impurity, the character and the amount depending on the method of preparation. In view of the evidence cited by these and other workers, we cannot controvert their conclusions with such incomplete data as we have now at our disposal. At the same time our work is paralleled by that of Vannod (12) and others, cited by him, who succeeded in obtaining antigenic nucleo-proteins from gonococci and other bacteria.

Reasoning a priori, the amount of nucleo-protein being very slight in non-nucleated cells, this protein should figure insignificantly as an antigen for the development of hemolysins. That the amount is small is evidenced by the fact that from 150 cc. of 100 per cent cells we obtained, on one occasion, only 0.014 gram of the dried precipitate. Whether, upon injection, this precipitate exhibits its antigenic power as a simple or as a conjugated protein, we cannot say, but that it does contain nucleo-protein we are certain, for on hydrolysis for one hour with 5 per cent sulphuric acid, the filtrate gives positive tests for phosphorus and the purine bases.

The precipitate is insoluble in water and in solutions of alkali metal salts. It does not contain simple albumins nor globulins

as such. While lipoids are undoubtedly present, we are inclined to think, for reasons previously mentioned, that the lipoid-free product would be antigenic.

We can conclude from our experiments that lysins in the case of red cells are produced chiefly, if not wholly, by a substance contained in the stroma, and that an alkaline extract of the stroma, prepared as described above, likewise has similar antigenic properties. To produce hemolysis, it is necessary only to render the cell-covering permeable to hemoglobin in solution. Assuming that the cell membrane is composed of more than one kind of molecule, it probably would not be necessary to increase the permeability of more than one of the types of constituent molecules or molecular groups. Therefore a whole series of antigens might be prepared, and a corresponding number of antibodies produced, each one capable of reacting with the corresponding part of the cell membrane, but all producing the same apparent effect; viz., hemolysis. While the hemolytic amboceptor produced with our alkaline extract is identical with that produced with stroma, in its property of producing hemolysis, nevertheless it is impossible to assume that the two are absolutely the same.

By comparing the results in tables 3 and 5, it will be noted that while the injection of stroma is capable of producing agglutinins, as well as hemolysins, for the whole cell, the injection of our alkaline extract produces only hemolysins. This parallels the observation of Levene (11), who showed that the injection of a 0.5 per cent sodium carbonate extract of dog's erythrocytes into the rabbit caused the serum of the latter to acquire strong hemolytic but no agglutinating power. If these two antibodies are produced by two different substances then a difference between them in chemical composition may well be postulated.

## SUMMARY

1. *In vitro*, as hemolysis proceeds, the total amount of amboceptor is constant but the stroma of the laked red cells increasing in amount, they become less and less saturated with amboceptor, and so split off less of it by dissociation, thus causing the velocity of hemolysis to decrease.

2. *In vivo* the stroma produce specific hemolytic and agglutinative bodies of high titer. Since little protein is injected the toxicity seems to be nil.

3. Alcohol and ether extracts, as well as 0.85 and 5 per cent salt solution extracts, of the stroma do not bind amboceptor *in vitro*.

4. The extract of stroma with alkaline physiological salt solution, as described above, does bind amboceptor *in vitro* and on injection causes the development of specific hemolysins but not of agglutinins.

5. This alkaline extract contains nucleo-protein, but not simple albumin or globulin. It also contains lipoids but these probably are not essential to its antigenic function.

6. The presence or absence of the anaphylactic reaction is not a certain criterion for specificity when nucleo-protei nsare used as antigens. In our work, hemolysins, showing species specificity, were present in the absence of anaphylaxis.

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# A NOTE ON BLEEDING GUINEA-PIGS AND ON PRESERVING SHEEP'S ERYTHROCYTES

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## I. THE BLEEDING OF GUINEA-PIGS

In serological work where guinea-pig's blood is essential, investigators are often seriously handicapped by not being able to obtain this fluid at all, or in sufficient quantities. This handicap may be due to a scarcity of these animals or to the expense resulting from their use in large numbers.

Several methods may be employed for drawing the blood from the animal. If a very small quantity is desired, it is possible to bleed the animal from its marginal ear vein and collect the blood in a Wright tube. This is, however, a slow process and can be carried out successfully only on an adult animal. In a few laboratories blood is drawn directly from the animal's heart with a sterile syringe. By this method a considerable quantity of blood may be taken readily, without any serious injury to the animal; the technic involved is, however, so difficult that few investigators make use of it.

The most common method of obtaining guinea-pig's blood is to sever the large blood vessels of the neck, either on one or on both sides of the trachea, and collect the blood by allowing it to drain freely. Pain is avoided by anesthetizing the animal with ether, or by stunning it by a blow on the head before the operation. The quantity of blood obtained by this method varies, and often is much less than is expected, even though the animal is bled to death. The loss of a certain portion of the blood may be accounted for by the almost instantaneous formation of a clot at the seat of the blow in cases where the animal is stunned

by a blow on the scalp. Besides, a considerable quantity remains in the heart and blood vessels, regardless of the method used to deaden the pain, as may be shown by a dissection of the animal after the blood has been drained from the incision. While this is a quick method of obtaining guinea-pig's blood, it results in the loss of the animal at each bleeding, and the unsatisfactory quantity of blood often obtained necessitates the use of extra animals, thereby incurring an additional expense.

Recently, in connection with the manipulation of a series of complement fixation tests in which guinea-pig serum was used as complement, a method of bleeding was developed which is here described in detail. Guinea-pigs of any size may be used. The animal is suspended from a support by means of a cord fastened to its hind legs and at once held by its head with the left hand. In this way the animal is easily controlled by very slight pressure. The head is turned so as to be almost at right angles to the dorsal side of the animal's body. The hair is then clipped on the ventral side of the neck and the skin at this place washed with alcohol. A Petri dish or centrifuge tube for collecting the blood is placed on the table or some other support a few inches below the animal. With his right hand the operator, by means of a sharp pair of scissors, makes a transverse incision about 15 mm. long in the skin over the jugular vein; this vein is then easily located and partly severed with the same instrument, and the blood collected by allowing it to drain from the incision. The pain caused by the operation is very slight, and may be avoided by the injection of a few drops of a 0.1 per cent solution of cocaine, both intra- and subdermally, at the place where the incision is to be made.

When the desired amount of blood has been obtained, cotton is pressed into the wound and the animal placed on its back on the table. The flow of blood quickly stops, after which the cotton is removed, the wound treated with alcohol and closed by placing several stitches through the flaps of the skin. The animal may then at once be returned to its cage.

No ligature of the vein is necessary. The sewing up of the incision permits of the formation of a clot in the cavity of the

wound, thereby preventing further loss of blood. Care should be taken not to enlarge the incision more than is necessary to locate the vein. After the vein is located a transverse incision is made in it; if, by chance, the vein is completely severed, the animal need not be lost. In the writer's experience recovery in such cases occurred. The proper technic for this operation can be developed easily with a little practice. A pair of sharp blunt shears appears to be the most suitable instrument for making the incision both in the skin and in the vein.

After bleeding the animal is more or less weakened, depending on the amount of blood taken. It regains its normal strength in a few hours, however, and its blood supply is apparently readily replenished. The wound heals quickly, so that within a week only the scar of the incision is noticeable. At the same time circulation is restored, and the blood vessels assume an apparently normal condition. After repeated incisions at the same place at intervals of about two weeks, the blood vessels are as easily located as in the normal animal. In practice it is desirable to alternate the incisions in the two veins, thereby allowing for the bleeding of the same animal at weekly intervals.

The quantity of blood that may be taken from an animal without weakening it too much varies with the size of the animal. When adult guinea-pigs are employed, from 10 to 15 cc. may be taken at each bleeding. In cases where animals are bled repeatedly the intervals should be sufficient to allow for complete circulatory restoration.

This method of bleeding has been used successfully by the author for almost a year. Practically as large a quantity of blood may be obtained by it as when the animal is bled to death; furthermore, this quantity may be obtained repeatedly from the same animal. During the period of this investigation several animals were bled as often as ten times, alternating from one vein to the other, without any ill effects, thereby yielding from 100 to 120 cc. of blood. These animals are still in good condition and appear capable of being bled an indefinite number of times.

## II. THE PRESERVATION OF SHEEP'S ERYTHROCYTES BY THE USE OF FORMALIN

In conducting complement fixation work a ready supply of corpuscles is very desirable. In recognition of this fact, Bernstein and Kaliski (1) recommended the use of formalin in preserving sheep's corpuscles. In this way blood may be kept from three to four weeks without deterioration, and constantly used as a supply of erythrocytes, which are satisfactory for the complement fixation test and for the preparation of hemolytic ambocepter.

The following method of procedure is used in these laboratories.[1] The blood is collected in a sterile bottle containing shot or glass beads, and at once defibrinated. It is then filtered through sterile cotton into another sterile bottle and the quantity of fibrin-free blood estimated. This estimation is made readily in a previously graduated bottle; it is often more convenient to determine the quantity by measuring an equal amount of water placed in a similar bottle. Formalin is then added in the proportion of 1 part of 40 per cent formaldehyde to 800 parts of blood. This formalinized blood is kept in the ice box as a stock supply. Whenever erythrocytes are needed, the necessary amount of blood is removed and centrifuged, and the corpuscles washed three times with 0.85 per cent saline solution.

Bernstein and Kaliski also recommend a method for preserving washed erythrocytes. While by this method some of the routine of washing blood cells may be eliminated, the cells are not so well preserved as when the whole blood is used. In this treatment the following technic was employed by the writer. The freshly washed sheep erythrocytes were diluted with an equal quantity of saline solution. To this suspension formalin was added in the proportion of 1 part of formaldehyde to 500 parts of the suspension. The formalinized washed corpuscles were well shaken and placed in the ice box, where they were found to be

[1] This is essentially the method described by Bernstein and Kaliski, and is given here to renew the reader's interest in it.

well preserved for about two weeks, and to furnish a ready supply of erythrocytes for complement fixation work.

The writer wishes to express his indebtedness to Prof. L. F. Rettger for his helpful advice and criticism.

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# STUDIES IN PNEUMONIA. VIII

## A SKIN REACTION TO PNEUMOTOXIN[1]

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In previous communications from this laboratory (1, 2, 3) the nature and properties of the endocellular toxin of the pneumococcus (pneumotoxin) were thoroughly investigated and a tentative explanation of its rôle in the pathology of lobar pneumonia and of its bearing on the mechanism of the crisis was proposed. The present investigations deal with one phase of the experimental studies upon which our conclusions were based. This work was conducted at the suggestion and with the coöperation of Dr. S. Solis-Cohen of Philadelphia, to whom we are indebted for placing all of his clinical facilities at the Jefferson and Blockley Hospitals at our disposal.

Various authors, including Clough (4), Weil (5) and Steinfield and Kolmer (6) have studied allergic skin reactions in pneumonia during the course of their immunological investigations in this disease. Clough, working with the dried and ground residues of twenty-four to thirty-six hour old cultures of pneumococci washed and extracted at 37°C. for eighteen hours (before and after precipitation with alcohol) obtained negative results. Weil employed forty-eight hour old cultures grown on Loeffler's serum agar, emulsified in distilled water, shaken and autolyzed at 37°C. for two hours and heated at 60°C. for one hour. He reported no reaction during the pre-critical course of the disease while a considerable number of positive reactions were observed to occur after the crisis. Steinfield and Kolmer used three anaphylac-

[1] Aided by the Fels grant for research in pneumonia. Presented before the Annual Meeting of the American Association of Immunologists, Philadelphia, March 29, 1918. Published in abstract in the Proceedings of the Society for Experimental Biology and Medicine, 1918, **15**, 93–94.

togens, prepared from forty-eight hour old cultures of virulent pneumococci belonging to serologic types I, II and III by autolysing and heating the saline suspensions of the washed organisms at 60°C. for one hour. They observed skin hypersensitiveness to pneumococcus protein in 30 per cent of a series of 20 cases of lobar pneumonia *after* the *crisis*. No type specificity was demonstrated.

Since the extensive studies of Kolmer and his associates on anaphylactic skin reactions in relation to immunity (7) have indicated in general, that there is no correlation between hypersensitiveness to a bacterial *protein* and immunity to infection by the corresponding organism, it seemed logical to attack the problem after the method of Schick (8) and study skin reactions to the *endotoxin* of the pneumococcus.

## METHODS OF STUDY

### *1. Laboratory investigations*

The technic of preparation and the properties of pneumotoxin have been fully described by us in previous communications (1, 2). Briefly stated, the hemolytic endotoxin of the pneumococcus was produced as follows: 1000 to 1500 cc. of an eighteen hour broth culture of virulent type I pneumococci (Rockefeller Institute) were centrifugalized for one hour in a powerful electric centrifuge at very high speed. The bacterial sediment was washed once in isotonic salt solution, taken up in 5 cc. of saline and dissolved in 1 cc. of a 2 per cent solution of sodium chloeate. The total volume was made up to 30 or 40 cc.; the solution was again centrifugalized, to remove any undissolved pneumococci, and tricresol was added up to 0.25 per cent concentration. The "control fluid," containing no toxin was made similarly. The preparations were preserved in the refrigerator and they were never used when older than eighteen to twenty-four hours, since the toxin deteriorates very rapidly. All preparations were perfectly homogeneous and slightly opalescent.

The method of standardization has been described elsewhere (2). In these studies we found it impracticable to determine

both the minimum lethal dose and minimum hemolytic dose of each lot of pneumotoxin and we had to satisfy ourselves with testing only three of the five employed. The minimum lethal dose (tested after the total volume had been made up to 100 cc.) was about 6 to 7 cc. for a guinea-pig weighing 250 to 300 grams and the minimum hemolytic dose was 0.2 to 0.4 cc. when titrated in terms of a 1 per cent suspension of washed guinea-pig erythrocytes. The dose of toxin used in skin tests (0.1 cc.) represents therefore about $\frac{1}{20}$ minimum lethal dose.

In our preliminary experiments we studied the reaction to pneumotoxin under controlled conditions in guinea-pigs as follows: a series of animals weighing 250 to 300 grams were given intravenous injections of sublethal doses of the toxin. Four to six weeks later, the intracutaneous skin tests were made on the shaved abdomen, the dose of toxin being 0.1 cc.

The technic of the intracutaneous test and the necessary precautions to be followed in order to guard against misinterpreting *pseudo-reactions* are fully described by Zingher (9). We have kept these in mind and have included in our studies the following controls: First, a control on the irritant action of the tricresol and the sodium choleate was made by injecting 0.1 cc. of the "control fluid" containing proportionate amounts of the reagents dissolved in saline. Second, a control on the possible irritant action of any undissolved or adsorbed pneumococcic protein was made by injecting 0.1 cc. of a heavy suspension of washed pneumococci (type I) heated at 60°C. for thirty minutes and tricresolized as above. A third control suggested by Zingher was often included, to rule out the possible irritant action of any remaining nutrient broth, protein substance of pneumococci and especially of deteriorated and inactive pneumotoxic protein. This consisted in injecting 0.1 cc. of pneumotoxin that had been heated for five minutes at 70°C.—a process which destroys the free toxin but leaves the other protein constituents practically unaffected.

A second series of guinea-pigs were injected intraperitoneally or intravenously with freshly obtained blood serum or lung exudate of normal dogs or of dogs suffering with lobar pneumonia

as the result of the intrabronchial insufflation of virulent type I pneumococci, after the method of Lamar and Meltzer (10). A third series received foreign protein such as egg albumin or normal human serum. Others received injections of living, virulent type I pneumococci or of a single dose or repeated doses of type I pneumococcus vaccine. The latter was prepared from sedimented washed pneumococci by resuspending the organisms in saline, heating the emulsion at 56°C. for thirty minutes and preserving it with tricresol in the refrigerator. The dose, injected intraperitoneally was 1 cc. of the thick emulsion. The intracutaneous test in the former was done twenty-four hours, and in the latter forty-eight hours, after sensitization. A large number of normal, healthy guinea-pigs were also tested. A summary of the results of these preliminary experiments is shown in table 1.

An analysis of table 1 reveals the following points:

TABLE 1

*Summary of results of pneumotoxin and pneumococcus protein skin tests in guinea-pigs sensitized to various protein substances**

| SERIES | SENSITIZING SUBSTANCES | TOTAL NUMBER OF GUINEA-PIGS IN THE SERIES | PNEUMOCOCCUS SKIN TEST | | | PNEUMOCOCCUS PROTEIN SKIN TEST | | |
|---|---|---|---|---|---|---|---|---|
| | | | Positive | Negative | Doubtful | Positive | Negative | Doubtful |
| A | Pneumotoxin only | 11 | 11 | 0 | 0 | 3 | 8 | 0 |
| B | Pneumotoxin and other material † | 5 | 5 | 0 | 0 | 2 | 2 | 1 |
| C | Pneumonic exudate (dog) | 6 | 3 | 2 | 1 | 1 | 4 | 1 |
| D | Pnenumonic serum (dog) | 3 | 3 | 0 | 0 | 1 | 2 | 0 |
| E | One dose of living pneumococci ‡ | 7 | 0 | 7 | 0 | 0 | 7 | 0 |
| F | One dose pneumococcus vaccine | 7 | 3 | 3 | 1 | 1 | 4 | 2 |
| G | Three doses of pneumococcus vaccine | 3 | 3 | 0 | 0 | 0 | 3 | 0 |
| H | Control animals § | 0 | 0 | 20 | 0 | 0 | 18 | 0 |

* Only those results were accepted which were definitely positive (+++ to +) and persisted for at least seventy-two hours.

† The other material referred to consisted of serum or lung exudates of normal and pneumonic dogs.

‡ All these animals died within forty-eight hours after infection.

§ Control animals included normal guinea pigs, and animals injected with various proteins such as egg albumin, normal lung proteins, normal serum, etc. All other control injections referred to in the text produced no reaction.

1. Guinea-pigs sensitized by intravenous injection of sublethal doses of pneumotoxin regularly show hypersensitiveness to the toxin but only occasionally to the protein of the cell substance of the pneumococcus.

2. The fresh blood serum or lung exudate of dogs obtained forty-eight hours after intrafronchial insufflation of virulent type I pneumococci, if injected into guinea-pigs will sensitize them to pneumotoxin. This indicates that pneumotoxin is liberated *in vivo* during the course of an experimental lobar pneumonia in dogs and is in accord with our previous findings with the use of the anaphylactic method (3).

3. When guinea-pigs are infected with virulent pneumococci that produce in them a septicemia which is fatal in forty-eight hours, no cutaneous allergy to pneumotoxin is produced. It is probable that in this short interval insufficient time is allowed for sensitization to the toxin even though large quantities of it may be liberated *in vivo*. This is borne out by the fact that in a series of guinea-pigs, which we injected with large but non-fatal doses of pneumotoxin five hours previous to the intracutaneous toxin test, no reactions were demonstrable. These observations are worthy of further study.

4. Normal guinea-pigs or those sensitized with unrelated proteins such as egg albumin, serum, leucocytes, blood fibrin or lung exudate of normal dogs, or with normal human serum or with the reagents (sodium choleate, etc.) used in preparing the pneumotoxin, do not react to the intracutaneous injection of pneumotoxin.

5. Single or repeated doses of vaccine injected intraperitoneally into guinea-pigs seem to create a moderate degree of allergy to pneumotoxin in some animals. Pneumococci heated to 56°C. for one-half hour presumably still contain some active endotoxin which can be liberated by the digestive action of the body enzymes.

The studies of Rosenow (11) and of Cole (12) are of interest in this connection. The former held that pneumotoxin is a product of the autolysis of pneumococci and is liberated by the digestive action of their own enzymes. The latter believed that the toxin exists preformed in the bacterial cell, since when autol-

ysis of the pneumococci is prevented by dissolving large masses of them in solutions of bile salts, the endotoxin is liberated. Bile salts are known to act as zymo-excitors and zymo-accelerators. Their action is probably a similar one in Cole's method of preparing the toxin. We are therefore of the opinion that pneumococci contain a preformed endocellular hemolytic toxin the liberation of which is accomplished by an enzymic action in which both the normal proteases of the body (as shown by Jobling (13) and the bacterial ferments themselves take part. This liberation is accelerated and facilitated both *in vitro* and *in vivo* by the presence of bile. The increase in bilirubin and the occurrence of jaundice in many cases of lobar pneumonia which we have discussed elsewhere (2) are evidence of this adaptative power of the pneumococcus to the human body.

That pneumococcus vaccines should be inferior to free pneumotoxin as an anaphylactogen in these studies, even though they may contain some potent toxin is explained by the recent studies of Sexsmith and Petersen (14) which have indicated that the skin is often very poor in certain enzymes.

We have in a previous communication (2) advanced conclusive evidence to the effect that pneumotoxin is a definite and specific protein (distinct from the protein substance of the bacterial cell) possessing sensitizing powers as an anaphylactogen. The present findings confirm this conclusion. Attention is directed to the comparative constancy with which heat-killed pneumococci failed to elicit any reaction in the animals sensitized to pneumotoxin and pneumonic exudates. We now wish to present still stronger evidence: The animals reacting to the pneumotoxin showed a definite zone of edema and erythema around the point of injection. This tissue was excised and histological sections were made. The findings (see figure 1) are as follows: There is no change in the epidermis; immediately below it and including all of the submucosa to the first bands of muscle, there is an extensive edema and acute congestion and extravasation of erythrocytes. The latter are well preserved and widely distributed in the edematous area. Through this area are also to be found numerous lymphocytes and plasma cells with occasional

polymorphonuclear leucocytes. The muscle shows no involvement. The predominating change is the hemorrhagic edema in the submucosa. This picture is similar to the skin lesion produced by diphtheria toxin.

Contrasted with this is the pustular and nodular appearance of the area in guinea pigs reacting to the "pneumococcus protein"

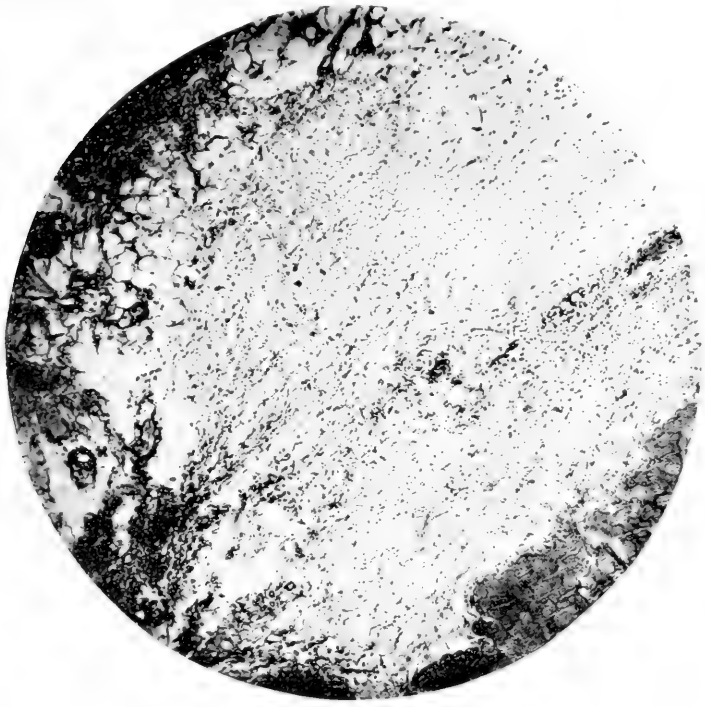

FIG. 1. SKIN REACTION TO PNEUMOTOXIN

Lesion in the abdominal skin of guinea pig forty-eight hours after the cutaneous injection of pneumotoxin; extensive area of hemorrhagic edema in the submucosa (B. & L.; obj. $\frac{2}{3}$ eyepiece No. 2).

skin test (fig. 2). Sections of these lesions show in some cases the epidermis intact, as likewise the submucosa subjacent and surrounding the hair follicles; others show marked destruction of the epidermis and adjacent submucosa through *suppurative* processes. In the deeper portions of the submucosa and the involved upper-

most bundle of muscle, marked changes are present characterized by acute hyperemia and dense collections of polymorphonuclear leucocytes. There is present a slight degree of edema and a occasional area of slight hemorrhagic extravasation. The predominating changes are the suppuration of the submucosa and muscle.

FIG. 2. SKIN REACTION TO PNEUMOCOCCUS VACCINE

Lesion in the abdominal skin of a guinea pig forty-eight hours after the intracutaneous injection of pneumococcus vaccine; extensive area of suppuration and necrosis in epidermis and submucosa (B. & L., obj. $\frac{2}{3}$ eyepiece No. 2).

There is thus a histological distinction between cutaneous reactions to pneumococcus protein and to pneumotoxin. We conclude that *skin reaction to pneumotoxin is a manifestation of a hypersensitiveness to a specific toxic protein which has been introduced parenterally into the tissues of the body.* We shall refer later to this phase of the work.

*2. Clinical investigations*

The desirability of discovering a method of studying the immunity to and the mechanism of recovery from pneumonia led us to apply the pneumotoxin skin test to patients suffering from lobar pneumonia in the various hospitals of Philadelphia during the winter of 1917–1918. All the precautions mentioned in connection with the laboratory studies were observed. Many control patients, normal adults and children and patients suffering from various chronic and acute infectious diseases of non-pneumococcic origin, were tested. The results of the tests done on children are of particular interest because these patients are less prone to be sensitized to various foreign proteins and above all the disease generally runs a more definite course not being complicated by alcoholism, tuberculosis, etc. Tables 2 and 3 summarize the results of these investigations.

Working as we did, on the hypothesis that pneumotoxin plays a distinct rôle in the pathology of lobar pneumonia and especially in the mechanism of the crisis, these results seemed very suggestive of a gradual disappearance of an excess of or hypersensitiveness to pneumotoxin after the crisis. An analysis of the results, shown in tables 4, 5 and 6, substantiates this belief. In children this phenomenon was found to be more marked. While no quantitative data can be given as to the relation between the severity of the disease and the degree of reaction it is our impression that the most violent reactions are to be observed in the most grave cases.

The appearance of the skin reaction to pneumotoxin is in all respects similar to that described for the Schick test with diphtheria toxin. Two or three cases tested just prior to crisis showed a strongly positive reaction marked by vesiculation of the surface layer of the epithelium. Other positive cases showed the usual definitely circumscribed area of edema and erythema persisting for 3 to 4 days and then gradually fading, leaving a definitely circumscribed scaling area of brownish pigmentation. After injection of the various control fluids in cases of lobar pneumonia (all were used excepting the vaccine), and of pneumotoxin in con-

trol cases, the skin remained normal or presented a slight scratch at the point of injection. Occasionally (in one or two cases of streptococcus infection) the *entire* arm presented a diffuse ery-

TABLE 2

*Summary of results of pneumotoxin skin tests among children* (Six months to ten years of age)*

| CLASSIFICATION | TOTAL NUMBER | POSITIVE | NEGATIVE |
|---|---|---|---|
| Acute lobar pneumonia (2d to 10th day) | 7† | 7 | 0 |
| Lobar pneumonia convalescents (10th to 20th day) | 7 | 0 | 7 |
| Bronchopneumonia | 3 | 0 | 3 |
| Intestinal colitis | 1 | 0 | 1 |
| Normal children | 10 | 0 | 10 |

* Only those cases with undoubtful diagnosis are listed.

† Six of these children reacted negatively one week later. They had passed their crisis and were running a normal temperature.

TABLE 3

*Summary of results of pneumotoxin skin tests among adults**

| CLASSIFICATION | TOTAL NUMBER | POSITIVE | NEGATIVE |
|---|---|---|---|
| Acute lobar pneumonia† | 31 | 31 | 0 |
| Convalescents (lobar pneumonia)‡ | 9 | 5 | 4 |
| Doubtful§ | 5 | 1 | 4 |
| Controls△ | 9 | 0 | 9 |

* Only those cases with undoubted diagnosis are listed.

† The test was repeated on two of these patients during their period of convalescence; the results were negative. These patients recovered by lysis.

‡ One of the convalescents originally reacting negatively gave a positive reaction after developing a pneumococcus empyema.

§ These cases included alcoholism and tuberculosis with possible superimposed pneumonia.

△ Controls included cases of gastric carcinoma, sexual neurasthenia, dysphagia, autonomic ataxia, tuberculosis, streptococcus arthritis, rheumatism, appendicitis and bronchitis.

thema without a *definite* area of edema. Such conditions never persisted longer than forty-eight hours and were considered pseudo-anaphylactic reactions.

We met with little or no success in negro patients, both children and adults owing to the difficulty of determining the degree of reaction.

Summarizing the results of the pneumotoxin skin test applied to adult cases of lobar pneumonia, we may say that a specific

TABLE 4

*Results of pneumotoxin skin tests in a group of cases of lobar pneumonia recovering by crisis*

| NAME OF PATIENT | AGE | DAYS OF DISEASE | DAYS BEFORE CRISIS | DAYS AFTER CRISIS | RESULTS OF TESTS |
|---|---|---|---|---|---|
| 1. Adults | | | | | |
| L. R. | 37 | 7 | 1 | 0 | + |
| F. M. | 26 | 19 | 0 | 11 | − |
| F. M.* | 26 | 35 | 0 | 27 | + |
| C. G. | 34 | 9 | 0 | 5 | + |
| W. C. | 21 | 5 | 2 | 0 | + |
| F. L. | 30 | 7 | Same day | | + |
| W. Y. | 40 | 10 | 0 | 2 | + |
| L. J. | 21 | 13 | 0 | 6 | + |
| H. F. | 21 | 7 | Same day | | + |
| T. F. | 28 | 7 | 1 | 0 | + |
| J. B. | 39 | 13 | 0 | 3 | + |
| D. T. | 34 | 12 | 0 | 3 | + |
| V. K. | 20 | 10 | 0 | 5 | + |
| J. A. | 33 | 6 | 0 | 2 | + |
| 2. Children | | | | | |
| M. K. | 2 | 7 | 7 | 0 | + |
| M. K† | 2 | 14 | 0 | 1 | − |
| E. C. | 2 | 10 | 0 | 2 | ± |
| I. G. | 1 | 7 | 1 | 0 | + |
| I. G.† | 1 | 14 | 0 | 6 | − |
| W. J. | 6 mo. | 6 | 1 | 0 | + |

* Patient developed pneumococcic empyema.

† Tests repeated after crisis.

reaction was elicited as early as the fifth and as late as the thirteenth day of the disease (two days before and six days after crisis, respectively). In children it was demonstrable about the same time, but was negative immediately or one or two days after the crisis. Patients recovering by lysis reacted as late as the thirty-

TABLE 5

*Results of pneumotoxin skin tests in a group of adult cases of lobar pneumonia recovering by lysis*

| NAME OF PATIENT | AGE | DAY OF DISEASE | RESULT OF TEST |
|---|---|---|---|
| J. J.* | 36 | 13 | + |
| W. B. H. | 19 | 7 | + |
| L. R. T.* | 36 | 5 | + |
| H. McG.* | 35 | 12 | + |
| F. K.* | 35 | 5 | + |
| J. D. | 50 | 10 | + |
| G. C. | 41 | 12 | + |
| C. B. | 32 | 11 | + |

* These patients received quinine treatment.

TABLE 6

*Results of pneumotoxin skin tests among patients convalescing from lobar pneumonia*

| NAME | AGE | DAY OF DISEASE (APPROXIMATE) | DAYS AFTER CRISIS | RESULT OF TEST |
|---|---|---|---|---|
| 1. Adults | | | | |
| F. M. | 26 | 18 | 11 | − |
| F. M. | 26 | 34 | 27 | +* |
| J. J. | 36 | 13 | Lysis | + |
| L. R. T. | 36 | 5 | Lysis | + |
| H. McG. | 35 | 32 | Lysis | + |
| F. K. | 35 | 21 | Lysis | + |
| H. K. | 37 | 17 | Lysis | −† |
| H. B. | 42 | 11 | 4 | −† |
| M. M. | 24 | 14 | 7 | −† |
| 2. Children | | | | |
| J. E. | 2 | 17 | 12 | − |
| C. B. | 11 | 14 | 8 | − |
| M. K. | 2 | 14 | 1 | − |
| L. P. | 11 mo. | 12 | Lysis | − |
| I. G. | 1 | 11 | 6 | − |
| J. V. | 8 mo. | 29 | 5 | − |
| W. J. | 6 mo. | 13 | 6 | − |

* Developed pneumococcic empyema.

† Patients were fully recovered and about to be discharged.

second day. In general the test was positive in all active cases, that is, throughout the toxemia. Cases earlier than the fifth day were not available. Control patients suffering with broncho-pneumonia or with acute or chronic infections not of pneumococcic origin, as well as healthy adults and children did not react.

It will be remembered that in the work of Steinfield and Kolmer (6) on "Allergic Skin Reaction in Pneumonia to Type Strains of Pneumococci" no constant relation was observed between reactions to the protein of various serological types of pneumococci and the types found in the sputum. In the present researches, as we have indicated, we confined ourselves to the use of an endotoxin derived from type I pneumococci. The thought of specificity at once suggested itself. For the purpose of determining this relationship between the type of organism found in the sputum and the type of toxin used, we made type diagnoses of the pneumococci found in the sputum of a series of ten cases which gave positive reactions to pneumotoxin skin test. Six of these were found to be type IV, the others type I. This skin test does not, therefore, seem to be of value as a method of serological type diagnosis. This conclusion is, however, based upon too little evidence. Pneumotoxin does produce specific precipitin reactions with immune sera. The problem is, therefore, worthy of further investigation.

We have found the pneumotoxin skin test to be of value as an *aid* in the differential diagnosis of doubtful cases of pneumonia, especially *central pneumonias*, pneumonias superimposed upon pulmonary tuberculosis, alcoholism, etc., *and especially in distinguishing between appendicitis or tuberculosis and pneumonia in children.* The technic of the preparation of the toxin is as yet, however, so fraught with difficulties as to make the test unavailable to the practitioner.

## DISCUSSION

The question as to the nature of the skin reaction described in this preliminary report, deserves first consideration. We had hoped, as stated above, to discover a method of studying the mechanism of immunity and especially of the crisis in lobar pneumonia.

Attention is again directed to the definite distinction between the skin reaction to pneumotoxin and the allergic skin reactions to pneumococcus protein. As was pointed out in reviewing the work of Clough, Weil, Steinfield and Kolmer, the latter reaction is generally negative during the course of the disease and occasionally positive after subsidence. The reverse is generally true in the case of the toxin test.

The mechanism of the pneumotoxin skin reaction is left for future investigation. Tentatively we regard the reaction as indicative of a state of allergy to pneumotoxin. As we have suggested elsewhere (2), sensitization takes place during its liberation (by the action of the normal body enzymes upon the pneumococci normally localized in the lung alveoli) at the time of the prolonged chilling of the body due to exposure. Viewed in this light, failure to elicit the reaction in any convalescing patient signifies the establishment of a temporary immunity or the disappearance of the toxin.

The object of these researches has been to indicate the importance and the rôle of the endotoxin of the pneumococcus in the pathology of lobar pneumonia. Auld (15) pointed out that the toxic albumose which he isolated from the tissues of rabbits dying of pneumococcus septicemia had "a selective affinity for the nerves of the upper extremities. We have isolated a similar albumose from lungs taken post-mortem from cases of lobar pneumonia and have suggested its *ultimate* derivation from the inhibitory action of the pneumotoxin on the autolytic processes (2, 3). The frequent occurrence in lobar pneumonia of hemiplegias (16), of delirium which is relieved by rachicentesis (17), of herpes (18), as well as the constant fatality of cases of pneumococcic meningitis, are suggestive of an injurious action of pneumotoxin on various nerve centers. Does it not seem logical, therefore, that treatment would be more effective with a specific serum which is both *antitoxic* and bactericidal? This suggestion is especially worthy of experimental investigation in type III and IV infections, where serum therapy has thus far been unsuccessful.

SUMMARY

The work reported is a part of the studies on the properties of pneumotoxin and its probable rôle in the pathology of lobar pneumonia. Previous investigators (Clough, Weil, Steinfield and Kolmer) working with dried, autolyzed or heat-killed pneumococci failed to elicit any constant reaction in cases of lobar pneumonia. The present authors used the endocellular hemolytic toxin of the pneumococcus freshly prepared for each test by dissolving the washed living organisms (type I) in solutions of sodium choleate. Guinea-pigs previously sensitized with sublethal doses of pneumotoxin or with the serum or lung exudate of dogs suffering from experimental lobar pneumonia, reacted to the intracutaneous injection of 0.1 cc. of the toxin by a local erythema and hemorrhagic edema in the subcutaneous tissue overlying the muscle. The skin reaction to heat-killed pneumococci was negative in most of these animals and when positive, was of a suppurative type, marked by less edema and more leucocytosis. Control animals gave uniformly negative results.

Among human adult cases of lobar pneumonia the reaction (which was characteristically that of a local edema and erythema) was elicited as early as the fifth and as late as the thirteenth day of the disease (two days before and six days after the crisis, respectively). In children it was demonstrable about the same time, but was negative immediately or one or two days after the crisis. Patients recovering by lysis reacted as late as the thirty-second day. In general, the test was positive in all active cases, that is, throughout the toxemia. Cases earlier than the fifth day of the disease were not available. Control patients, suffering with broncho-pneumonia or with acute or chronic infections not of pneumococcic origin, as well as healthy adults and children did not react.

The reaction is regarded as indicative of a state of allergy to pneumotoxin. Sensitization to the toxin presumably takes place with its liberation (by the action of normal body enzymes upon pneumococci normally localized in the lung alveoli) at the time of the prolonged chilling due to exposure. Failure to elicit the

reaction during convalescence indicates the establishment of a temporary immunity or the disappearance of the toxin. This skin test does not as yet seem to be of value as a method of serological type diagnosis but may aid in differential diagnosis between appendicitis or tuberculosis and pneumonia (especially in children). It is also of interest because of its bearing on the mechanism of the crisis.

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# A METHOD OF PREPARING BACTERIAL ANTIGENS[1]

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No method of preparing bacterial antigens for complement deviation can be regarded as wholly satisfactory until it offers a preparation in which the whole content of the complement deviating principles of the bacterial cell is rendered available and in which, at the same time, no substances occur that tend to interfere with the specific complement deviation reaction. The availability of the antigenic substances for the complement-deviation reaction depends; first, on their freedom in solution or suspension, and secondly, on the physical state in which they occur. While suspensions of whole bacteria have been used as antigen, it is most probable that their antigenic properties are to be attributed to a partial disintegration and extraction of the bacteria, for it is difficult to see how substances within the unbroken cell membrane could be available for reaction with colloidal substances in the serum, attributing, as we must by analogy, only semipermeable properties to the cell membrane. Disintegration and very thorough extraction of the bacterial cell would then appear to be the best means of rendering available in solution the greatest concentration of the complement-deviating principles.

Aqueous extraction of disintegrated bacteria yields a colloidal solution of an albuminous nature in which the partially extracted bacterial fragments appear in coarse suspension. Even with the most thorough extraction these fragments appear to attribute much to the antigenic properties of the suspension. The size of

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the suspended particles seems to influence the strength of the reaction. An extreme degree of fineness offers the greatest surface exposure and favors high antigenic properties. In the preparation of antigens the size of the particles can be controlled in a measure. The most minute fragmentation of the bacteria is then desirable both for the purpose of thorough extraction and for that of increasing the surface exposure of the partially extracted particles in the suspension that they may be the better available for the reaction.

The substances that interfere with the specific complement-deviation reaction may be grouped in two classes, (a) hemolytic substances and (b) anticomplementary substances. Bacterial hemolysins being quite labile do not appear in stock antigens prepared from bacteria killed and dried by heat. The hemolytic action of unsaturated fatty acids must be borne in mind. Hemolytic substances, however, are not of practical significance in the preparation of bacterial antigens.

Anticomplementary substances are of the greatest importance, since they are of frequent occurrence in bacterial antigens. It is difficult to define the nature of these substances, which may be quite varied. They appear to arise as the result of bacterial growth, either from the media in which growth occurs, or from the internal physiological processes of the bacterial cell, or from both sources. Anyone experienced in Wassermann work knows the frequency with which old sera or sera that are heavily contaminated with bacteria become highly anticomplementary. This property of the sera is not removed by filtering out the bacteria nor by heating the sera at 55°C. Such substances arise as well when bacteria are grown on artificial media and appear, in some measure, in the bacterial material collected for antigen preparation. Washing the moist bacteria with saline solution or extracting the dry bacteria with lipoid solvents eliminates these substances in large part.

It is well known that fat-like substances occur in the bacterial cell, in some cases indeed to a considerable percentage. A more or less general disregard of this fact has been the practice in the preparation of aqueous extracts of bacteria. The relative dis-

tribution of these fat-like substances, and the water soluble constituents of the cell concerns us only insofar as the presence of the former interferes with the aqueous extraction of the latter.

No better indication that the methods for extracting bacteria are not wholly satisfactory need be presented than that of the great variety of such methods employed. Of these, autolysis is the oldest. It is employed not so much because the products of autolysis are to be desired, but because partial disintegration of the bacterial cell allows a better extraction of the remainder. Of the physical methods, grinding of dry or moist bacteria material with or without the addition of sand; alternate freezing and thawing; bacteriolysis in distilled water; or combinations of these methods have been employed, all with the idea of breaking up the integrity of the individual organism preparatory to to its extraction. Undoubtedly each of these methods does, in part, what is expected of it, but uniformity of action can scarcely be claimed. If only the outer membrane of the bacterial cell is of a lipoid nature, or, what is more likely, if the fat and lipoid constituents are distributed throughout the cell, their removal must the better expose the remaining constituents for more ready extraction with aqueous solutions. So far as water penetration is concerned, the bacterial cell before their removal may be compared with a plug of non-absorbent cotton. After their removal it may be likened to a plug of absorbent cotton.

In view of these considerations, a chloroform-ether extraction was used to remove the fat-like substances from dried bacteria. The residue after thorough extraction was freed from traces of these solvents and suspended in sterile salt solution. Since the principles concerned in complement deviation are left behind in this extraction and since the extract contains substances that interfere with the complement-deviation reaction, this separation is, in itself, highly desirable. A further advantage is that the chloroform-ether extraction uniformly affects all cells by removing water-insoluble substances and so breaks up the integrity of the individual as to facilitate subsequent aqueous extraction. Complete extraction of the water-soluble[4] fraction cannot be

assured since the cellulose fraction left unchanged in the bacterial cell may be so distributed as to impair aqueous extraction. The use of the whole suspensions of this chloroform-ether extracted bacterial residue appears to supply any incompletely extracted bacterial particles to the complement deviation reaction, so that incomplete extraction does not entail a dead loss of the complement deviating substances.

## TECHNIQUE OF PREPARING ANTIGENS

All bacteria used in these preparations were grown on agar surfaces. Beef infusion agar was used for the typhoid and the dysentery groups. The pneumococci and meningococci were grown on beef-infusion blood agar impregnated with 0.5 per cent of soluble starch. Twenty-four hour growths were removed from these surfaces by means of a rubber-edged squeegee, great care being taken not to break the continuity of the surface or to remove any agar with the bacteria. This bacterial paste was collected in sterile petri dishes and dried at 53 to 56°C.

A razor-edge steel scraper was used to separate the dry adherent material from the bottom of the dish. The resulting granular and scaly mass was transferred to a mortar, moistened with chloroform and ground. As the chloroform evaporated small additions of ether were kept up from time to time during the grinding process. The final grinding was conducted after all the last addition of ether had evaporated and was continued until a very fine buff colored powder was obtained. This was now suspended in a mixture of equal parts of chloroform and ether and transferred to a bottle. The bottle provided with a tight fitting cork stopper was agitated on the shaking machine for from four to six hours. After removal from the shaking machine the bacteria residue for the most part settled out rapidly and the supernatant liquid was decanted on a filter. Three or four additions of ether were made and after thorough shaking the residue was allowed to settle out and the ether was decanted on the same filter. After the last decantation, the bottle with the bulk of the ether-moist residue was placed in an incubator at 56°C. to dispel traces of

ether. The filter after draining was subjected to the same treatment. After the drying the filter was punctured and the minor part of the bacterial residue collected on it was washed back into the bottle with sterile normal salt solution, a suspension of the whole being thus effected. The volume of the salt solution used for suspension was dependent upon the amount of bacterial residue employed in the particular preparation. In all of the cases a weighed amount of dried bacteria was used. Where 0.5 gram was taken at the start, the extracted residue was suspended in 25 cc. of salt solution. A half per cent phenol was added to these suspensions and they were again shaken for twelve to eighteen hours. This procedure yielded in all cases a suspension of the insoluble bacterial residue in a menstrum showing a great tendency to froth, so that the suspensions looked not unlike slightly opaque soap solutions. These products constituted the stock antigens ready for titration. In this manner antigens were prepared from the following organisms: typhoid, paratyphoid bacilli "A" and "B;" dysentery bacilli of the Shiga, Flexner and "Y" types; pneumococcus, types I, II and III; meningococcus of the normal, intermediate A, intermediate B and para types.

## TITRATIONS OF ANTIGENS

The antigoat hemolytic system was employed in these tests sensitized goat cells in 5 per cent suspension being used. Arbitrary dilutions of the antigen were made of a concentration stronger than that deemed necessary for routine use. The anti-complementary action of these dilutions was then tested. In cases where an anti-complementary action was noted, a unit of antigen of one-fifth the amount showing anti-complementary tendencies was chosen. In some cases where no anti-complementary action was noted in 0.5 cc. amounts of the antigen dilution, a 0.1 cc. unit was chosen. With these units thus roughly determined, their complement deviating powers were titrated in a series of immune serum dilutions. The highest serum dilution showing complete fixation of complement with this large unit of its specific

TABLE 1

*Antigen titrations*

| | CUBIC CENTIMETERS OF SERUM PER TUBE | | | | | | | | | | ANTIGEN, A.C. TEST | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.05 | 0.025 | 0.012 | 0.006 | 0.003 | 0.0015 | 0.00075 | 0.00037 | 0.00019 | 0.00009 | †0.1 | 0.2 | 0.3 | 0.4 | 0.5 |
| **Typhoid group** | | | | | | | | | | | | | | | |
| Typhoid serum, a.c. | − | − | − | − | | | | | | | | | | | |
| With typhoid* (1:12) | ++ | ++ | ++ | ++ | ++ | ++ | + | +− | − | − | − | − | − | − | − |
| With Para A (1:10) | ++ | ++ | ++ | ++ | + | +− | − | − | − | − | − | − | − | − | − |
| With Para B (1:10) | ++ | ++ | ++ | + | +− | − | − | − | − | − | − | − | − | − | − |
| Para A serum, a.c. | + | +− | − | − | | | | | | | | | | | |
| With Para A (1:10) | ++ | ++ | ++ | ++ | ++ | + | +− | − | − | − | | | | | |
| With Para B (1:10) | ++ | ++ | ++ | +− | − | − | − | − | − | − | | | | | |
| With Typhoid (1:12) | ++ | ++ | ++ | ++ | ++ | + | +− | − | − | − | | | | | |
| Para B serum, a.c. | +− | − | − | − | | | | | | | | | | | |
| With Para B (1:10) | ++ | ++ | ++ | ++ | ++ | +− | − | − | − | − | | | | | |
| With Para A (1:10) | ++ | ++ | ++ | + | +− | − | − | − | − | − | | | | | |
| With Typhoid (1:12) | ++ | ++ | ++ | ++ | ++ | + | − | − | − | − | | | | | |
| **Dysentery group** | | | | | | | | | | | | | | | |
| Shiga serum, a.c. | − | − | − | − | | | | | | | | | | | |
| With Shiga (1:3) | ++ | ++ | ++ | + | +− | − | − | − | − | − | − | − | − | − | − |
| With Flexner (1:5) | + | +− | − | − | − | − | − | − | − | − | − | − | − | − | − |
| With Y (1:10) | ++− | +− | − | − | − | − | − | − | − | − | − | − | − | − | − |
| Flexner serum, a.c. | − | − | − | − | | | | | | | | | | | |
| With Flexner (1:5) | ++ | ++ | ++ | ++ | ++ | +− | − | − | − | − | | | | | |
| With Shiga (1:3) | ++− | + | − | − | − | − | − | − | − | − | | | | | |
| With Y (1:10) | ++ | ++ | ++ | ++ | + | − | − | − | − | − | | | | | |
| Y serum, a.c. | − | − | − | − | | | | | | | | | | | |
| With Y (1:10) | ++ | ++ | ++ | ++ | ++ | +− | − | − | − | − | | | | | |
| With Flexner (1:5) | ++ | ++ | ++ | +− | − | − | − | − | − | − | | | | | |
| With Shiga (1:3) | ++ | ++ | + | − | − | − | − | − | − | − | | | | | |

Meningococcus group

| Serum Dilutions | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1280 | 1:2560 | 1:5120 | 1:10240 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| M1 serum, a.c. | − | − | − | − | − | − | − | − | | | | | | | |
| With M1 (1:6) | ++ | ++ | ++ | + | +− | − | − | − | | | − | − | − | − | − |
| With M10 (1:15) | ++ | ++ | ++ | ++− | + | − | − | − | | | − | − | − | − | − |
| With M30 (1:10) | ++ | ++ | ++ | + | +− | − | − | − | | | − | − | − | − | − |
| With M60 (1:15) | ++ | ++ | ++ | + | +− | − | − | − | | | − | − | − | − | − |
| M10 serum, a.c. | − | − | − | − | − | − | − | − | | | − | − | − | − | − |
| With M10 | ++ | ++ | +− | − | − | − | − | − | | | | | | | |
| With M30 | ++ | ++ | ++ | + | ++ | + | − | − | | | | | | | |
| With M60 | ++ | ++ | ++ | ++ | + | +− | − | − | | | | | | | |
| With M1 | ++ | ++ | ++ | ++ | + | +− | − | − | | | | | | | |
| M30 serum, a.c. | − | − | − | − | − | − | − | − | | | | | | | |
| With M30 | ++ | ++ | ++ | + | +− | − | − | − | | | | | | | |
| With M60 | ++ | ++ | ++ | ++− | + | +− | − | − | | | | | | | |
| With M1 | ++ | ++ | ++− | +− | − | − | − | − | | | | | | | |
| With M10 | ++ | ++ | + | +− | − | − | − | − | | | | | | | |
| M60 serum, a.c. | − | − | − | − | − | − | − | − | | | | | | | |
| With M60 | ++ | ++ | ++ | + | +− | − | − | − | | | | | | | |
| With M1 | ++ | ++ | + | +− | − | − | − | − | | | | | | | |
| With M10 | ++ | ++ | ++ | + | +− | − | − | − | | | | | | | |
| With M30 | ++ | ++ | ++ | ++ | + | +− | − | − | | | | | | | |

Pneumococcus group

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pneumo. I serum, | − | − | − | − | − | | | | | | | | | | |
| With PI (1:8) | ++ | ++ | +− | − | − | − | − | − | | | − | − | − | − | − |
| With PII (1:7) | ++ | ++ | ++ | ++ | + | +− | − | − | | | − | − | − | − | − |
| With PIII (1:30) | ++ | ++ | ++ | + | +− | − | − | − | | | − | − | − | − | − |
| P II serum, | − | − | − | − | − | | | | | | | | | | |
| With PII | ++ | ++ | ++ | ++ | +− | − | − | | | | | | | | |
| With PI | ++ | ++ | ++ | ++ | + | +− | − | − | | | | | | | |
| With PIII | ++ | ++ | ++ | ++ | + | +− | − | − | | | | | | | |
| PIII serum | − | − | − | − | − | | | | | | | | | | |
| With PIII | ++ | ++ | ++ | + | +− | − | − | − | | | | | | | |
| With PI | ++ | ++ | + | + | +− | − | − | − | | | | | | | |
| With PII | ++ | ++ | + | +− | − | − | − | − | | | | | | | |

* Indicates dilution of antigen suspension used. † Amounts of antigen dilution used in the anticomplementary test.

antigen was in this way determined. With this serum dilution as a constant in the next titration, the smallest amount of antigen giving complete fixation in this serum dilution was determined. This amount doubled became the antigen unit in subsequent comparative serum titrations.

The above tables show the results obtained with these antigen preparations in direct and cross titrations of immune rabbit sera.

### TECHNIQUE OF SERUM TITRATIONS

Here, as in determining the antigen unit, the anti-goat hemolytic system was used. The technique followed is more or less standard and will not be discussed in detail. The immune sera used were shown to contain natural amboceptor for goat's cells so that it became necessary to remove this. Sensitized goat cells were used throughout the titrations. These were prepared by adding the previously determined amboceptor unit to a 5 per cent suspension of cells and incubating the mixture at 37.5°C. for one hour. After the cells were thrown down by centrifugalization the supernatant liquid was drawn off and replaced with an equal amount of fresh salt solution in order to resuspend the cells. The complement used was a 40 per cent solution of pooled guinea-pigs' sera.

In order to obtain the serum dilutions as represented in the tables, a series of tubes was set up with 1 cc. of normal salt solution in each tube except the first, which contained 1.9 cc. To the first tube 0.1 cc. of immune serum was added. After being thoroughly mixed 1 cc. of the dilution was carried across to the second tube and this process was continued down through the series to the last tube where 1 cc. of the dilution was discarded.

Serum, complement, and antigen were added in order and the tubes were incubated for a half hour at 37.5°C. A 0.1 cc. unit of the sensitized cell suspension was then added and the mixtures were again incubated for an hour being thoroughly shaken at fifteen minute intervals. The results were read on removal from the water-bath at the end of this period.

## STANDARD DRY ANTIGEN PREPARATIONS

While stock antigen suspensions have been kept in the ice-box for as long as four months without appreciable loss of antigenic properties, it has been noted that some preparations would quite suddenly become anticomplementary. Cultures from these antigens invariably showed a profuse bacterial growth, while cultures from antigens continuing unchanged showed no such growth. Bacterial contamination seems to be in some way responsible for this sudden anticomplementary change. To obviate this difficulty dry antigen preparations were made. For these the bacteria were extracted in the manner described. After the last washing with ether the bacterial residue was dried and intimately mixed with 8.5 times its weight of sodium chloride. This bacteria salt mixture was reduced in a mortar to a very fine powder.

For use this powder is suspended in distilled water, 0.95 gm. per 100 cc. of water making the right proportions. This is shaken until, on solution of the salt, an evenly distributed slightly opalescent suspension of the bacteria is effected. Prepared in this manner it yields a 0.1 per cent suspension of bacteria in normal salt. It may be found on titration that further dilution is necessary to afford a convenient unit for routine work. In this case further dilution can be made with normal salt solution. Antigens once prepared in this way remain practically unchanged in the dry condition, so that they can be very readily standardized. Where a large number of tests are being made, enough antigen suspension can be prepared for one day's use only, with but little inconvenience. If the work is being conducted on a smaller scale enough for several weeks' work can be prepared and kept in the ice-box ready for immediate use without great danger of change in the antigenic or anticomplementary titer. Again the stock supply of the dry antigen is always in reserve and it can be made available in such quantities that experiments running over long periods can be conducted with the same stock antigen. These standardized dry antigens have been in use in the Army Medical School laboratories for about three months. The list

of preparations includes: Typhoid, paratyphoids "A" and "B," dysentery (Shiga, Flexner, and "Y" types); meningococcus of the Normal, intermediate A, intermediate B and Para types; pneumococcus, types I and II; and cholera.

## DISCUSSION OF RESULTS

The efficiency of the antigens appears evident from a study of the results obtained in the direct serum titrations. Subsequent work has demonstrated their usefulness in a comparative study of the antibodies developed in animals receiving a single dose of various vaccine preparations. The cross titrations indicate, for the most part, that the complement deviation reaction has a limited use in the type differentiation within the different bacterial groups studied. This failure to differentiate type is most pronounced in the pneumococcus and the meningococcus groups. In the typhoid group the striking thing is the degree of complement deviation by the typhoid antigen in cross titration with the paratyphoid bacilli "A" and "B" sera. Paratyphoid "B" is less efficient than paratyphoid "A" in fixing complement in cross titration with typhoid immune serum. In the dysentery group the results rather sharply differentiate the Shiga from the Flexner and "Y" types.

## CONCLUSIONS

The efficiency of a bacterial antigen is raised by the removal of the chloroform-ether soluble constituents of the dried bacteria.

Standardized dry antigen preparations can be made and rendered available for work over long periods.

The complement deviation reaction has limited uses in the differentiation of type within the bacterial groups studied.

# A STUDY OF SAPONIN HEMOLYSIS

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Hemolysis, either specific or nonspecific, is much influenced by various factors of environment. Eisler (1) found that in a medium of nonelectrolyte such as sugar, specific hemolysis is considerably inhibited, while the addition of various salts restores this function, though when the concentration of those salts is extended over a certain limit hemolysis is again checked. His explanation of this fact is that complement can not exercise its function in an environment free of salt.

Not only specific hemolysis, but also, he states, nonspecific hemolysis, such as that produced with aethyl alcohol or with corrosive sublimate, is influenced by conditions of environment. He points out many factors that play a rôle in this phenomenon—temperature, friction, velocity of diffusion, surface tension and solubility and also some other biological influences.

In the present study I have taken up the question whether the saponin hemolysis is affected by environmental conditions in the same way as specific hemolysis, according to the idea of Eisler.

Saponin hemolysis has been studied by numerous workers such as Ransom (2), Porges and Neubauer (3) and Port (4). It is generally stated that saponin has a great affinity for the lipoid substance, especially for the cholesterin of the red blood cells. This combination of saponin and cholesterin can result in the destruction of the blood corpuscles; that is, hemolysis.

As the material of my experiment I employed jegosaponin (straxsaponin) and sapotoxin Merck. Jegosaponin ($C_{55}H_{80}O_{25}$) was produced by Asahina and Momoya (5). This substance is a kind of acid and the hemolytic power is possessed by its salts

with cations. The calcium salt of jegosaponin has a greater hemolytic power than any of the other salts and it has a far greater hemolytic power than saponin Merck. I used, therefore, the calcium salt of jegosaponin, which is a white amorphous powder easily soluble in water.

Hemolytic tests were carried out with a 5 per cent suspension of red blood cells of the rabbit. As a nonelectrolyte medium I employed an isotonic solution of glucose or of saccharose. In these experiments the suspension of red blood cells was, of course, made with the respective sugar solutions and the solution of saponin or sapotoxin was also made with sugar solution. In the experiments with electrolyte medium, the suspension of blood cells and the solution of saponin were made with physiological salt solution.

In tables 1 and 2 is shown the influence of an electrolyte on the saponin and the sapotoxin hemolysis.

It is evident that both the saponin and the sapotoxin hemolysis are remarkably inhibited in the nonelectrolyte medium as compared with the electrolyte medium. It is seen that in the isotonic saccharose medium the hemolytic activity of 1/62,500 gram of saponin is almost completely inhibited, while 1/50,000 gram of the saponin is almost inactive in the glucose solution.

In the second experiment, the protocols of which are presented in tables 3 and 4, the influence of the various salts on the saponin hemolysis was studied. Increasing amounts of each salt were added to the nonelectrolyte medium containing a quantity of saponin that in the absence of electrolytes just failed to cause hemolysis. The usual amount of washed sheep's blood having been added to these mixtures, hemolysis occurred where the different salts were present in a certain concentration, this concentration varying with the different salts.

Similar experiments were made also with sapotoxin. The protocols of these experiments are shown in tables 5 and 6.

From these experiments, it is evident that the presence of the salts that were examined in this respect permitted, in most instances, the hemolytic effect of saponin and sapotoxin to be exerted. However, some salts, such as $MgCl_2$, $CaCl_2$ and $BaCl_2$

TABLE 1

*Showing the influence of an electrolyte on saponin hemolysis*

| JEGOSAPONIN (WITH SALT OR SUGAR) 1:5000 | WITH PHYSIOLOGICAL SALT SOLUTION (0.85 PER CENT) UP TO 1 CC. (HEMOLYSIS AT 37.6°C.) | | | | WITH 5.6 PER CENT SOLUTION OF GLUCOSE UP TO 1 CC. (HEMOLYSIS AT 37.6°C.) | | | | WITH 8 PER CENT SOLUTION OF SACCHAROSE UP TO 1 CC. (HEMOLYSIS AT 37.6°C.) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5 minutes | 30 minutes | 1 hour | 2 hours | 5 minutes | 30 minutes | 1 hour | 2 hours | 5 minutes | 30 minutes | 1 hour | 2 hours |
| 1.0 | c | c | c | c | alc | c | c | c | alc | c | c | c |
| 0.8 | c | c | c | c | alc | c | c | c | vst | c | c | c |
| 0.6 | c | c | c | c | w | c | c | c | w | c | c | c |
| 0.4 | c | c | c | c | tr | c | c | c | tr | alc | c | c |
| 0.2 | c | c | c | c | — | c | c | c | — | st | c | c |
| 0.1 | w | c | c | c | — | tr | tr | tr | — | w | st | alc |
| 0.08 | tr | c | c | c | — | — | — | tr | — | — | tr | w |
| 0.06 | — | alc | c | c | — | — | — | — | — | — | — | tr |
| 0.04 | — | vst | alc | c | — | — | — | — | — | — | — | — |
| 0.02 | — | w | st | c | — | — | — | — | — | — | — | — |

c = hemolysis complete; alc = hemolysis almost complete; vst = hemolysis very strong; st = hemolysis strong; w = hemolysis weak; tr = hemolysis trace.

TABLE 2

*Showing the influence of an electrolyte on sapotoxin hemolysis*

| SAPOTOXIN (WITH SALT OR SUGAR) 1:500 | WITH PHYSIOLOGICAL SALT SOLUHION (0.85 PER CENT) UP TO 1 CC. (HEMOLYSIS AT 37.6°C.) | | | | WITH 5.6 PER CENT SOLUTION OF GLUCOSE UP TO 1 CC. (HEMOLYSIS AT 37.6°C.) | | | | WITH 8 PER CENT SOLUTION OF SACCHAROSE UP TO 1 CC. (HEMOLYSIS AT 37.6°C.) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5 minutes | 30 minutes | 1 hour | 2 hours | 5 minutes | 30 minutes | 1 hour | 2 hours | 5 minutes | 30 minutes | 1 hour | 2 hours |
| 1.0 | c | c | c | c | w | c | c | c | tr | c | c | c |
| 0.8 | c | c | c | c | tr | c | c | c | — | vst | c | c |
| 0.6 | c | c | c | c | — | c | c | c | — | st | vst | c |
| 0.4 | alc | c | c | c | — | c | c | c | — | tr | st | c |
| 0.2 | st | c | c | c | — | tr | c | c | — | — | w | vst |
| 0.1 | w | alc | c | c | — | — | w | alc | — | — | tr | st |
| 0.08 | tr | alc | c | c | — | — | st | vst | — | — | tr | w |
| 0.06 | — | alc | c | c | — | — | tr | st | — | — | — | tr |
| 0.04 | — | vst | c | c | — | — | — | w | — | — | — | — |
| 9.02 | — | st | vst | c | — | — | — | tr | — | — | — | — |

TABLE 3

*Showing the influence of different salts on saponin hemolysis in a medium of isotonic saccharose solution*

| JEGOSAPONIN IN 8 PER CENT SOLUTION OF SACCHAROSE 1:62,500 | 8 PER CENT SOLUTION OF SACCHAROSE | NORMAL SOLUTION OF SALTS | NaCl | KCl | NaBr | KaBr | KI | $NaNO_2$ | $KNO_2$ | $NaNO_3$ | $KNO_3$ | KSCN | $KH_2PO_4$ | Na citric | K citric | $CH_3COONa$ | $CH_3COOK$ | $KC_2O_4H_2O$ | $Na_2SO_4$ | $(NH_4)_2SO_4$ | $MgSO_4$ | $MgCl_2$ | $Mg(NO_3)_2$ | $K_2CO_3$ | $Na_2CO_3$ | $CaCl_2$ | $BaCl_2$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1.0 | — | 2.0 | e | e | e | e | e | e | e | e | e | e | e | e | e | e | e | vst | e | | e | e | e | e | e | — | — |
| 1.0 | 0.2 | 1.8 | e | e | e | e | e | e | e | e | e | e | e | e | e | e | e | vst | e | | e | w | e | e | e | — | — |
| 1.0 | 0.4 | 1.6 | e | e | e | e | e | e | e | e | e | e | e | e | e | e | e | vst | e | | e | w | e | e | e | — | — |
| 1.0 | 0.6 | 1.4 | e | e | e | e | e | e | e | e | e | e | e | e | e | e | e | ale | e | | e | tr | e | e | e | — | — |
| 1.0 | 0.8 | 1.2 | e | e | e | e | e | e | e | e | e | e | e | e | e | e | e | e | e | | e | — | e | e | e | — | — |
| 1.0 | 1.0 | 1.0 | e | e | e | e | e | e | e | e | e | e | e | e | e | e | e | e | e | tr | e | — | e | e | e | — | — |
| 1.0 | 1.2 | 0.8 | e | e | e | e | e | e | e | e | e | e | e | e | e | e | e | e | e | st | e | — | e | e | e | — | — |
| 1.0 | 1.4 | 0.6 | ale | e | e | e | e | ale | e | e | e | e | e | e | e | e | e | e | ale | ale | ale | — | ale | e | e | — | — |
| 1.0 | 1.6 | 0.4 | vst | e | e | e | e | ale | vst | e | ale | e | e | e | ale | e | e | vst | st | ale | vst | — | ale | e | e | — | — |
| 1.0 | 1.8 | 0.2 | st | a e | ale | st | ale | st | st | ale | w | st | e | e | tr | vst | tr | st | st | ale | st | — | st | w | st | — | — |
| 1.0 | 1.9 | 0.1 | w | st | st | st | st | w | tr | st | tr | w | vst | — | — | vst | — | w | w | vst | st | — | st | tr | w | — | — |
| 1.0 | 1.92 | 0.08 | w | w | st | tr | st | tr | tr | w | tr | tr | vst | — | — | st | — | tr | w | vst | st | — | st | — | tr | — | — |
| 1.0 | 1.94 | 0.06 | tr | tr | w | tr | w | — | — | tr | — | tr | st | — | — | w | — | — | tr | st | tr | — | st | — | — | — | — |
| 1.0 | 1.96 | 0.04 | tr | tr | w | — | tr | — | — | — | — | — | w | — | — | tr | — | — | tr | w | tr | — | w | — | tr | — | — |
| 1.0 | 1.98 | 0.02 | — | — | tr | — | — | — | — | — | — | — | w | — | — | tr | — | — | tr | w | tr | — | w | — | tr | — | — |

TABLE 4

*Showing the influence of different salts on saponin hemolysis in a medium of isotonic glucose solution*

| JEGOSAPONIN IN 5.6 PER CENT SOLUTION OF GLUCOSE 1:50,000 | 5.6 PER CENT SOLUTION OF GLUCOSE | NORMAL SOLUTION OF SALTS | NaCl | KCl | NaBr | KBr | KI | $NaNO_2$ | $KNO_2$ | $NaNO_3$ | $KNO_3$ | KSCN | $KH_2PO_4$ | Na CITRIC | K CITRIC | $CH_3COONa$ | $CH_3COOK$ | $KC_2O_4H_2O$ | $Na_2SO_4$ | $(NH_4)_2SO_4$ | $MgSO_4$ | $MgCl_2$ | $Mg(NO_3)_2$ | $K_2CO_3$ | $Na_2CO_3$ | $CaCl_2$ | $BaCl_2$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1.0 | — | 2.0 | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | | c | tr | w | c | c | — | — |
| 1.0 | 0.2 | 1.8 | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | | c | tr | tr | c | c | — | — |
| 1.0 | 0.4 | 1.6 | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | | c | tr | tr | c | c | — | — |
| 1.0 | 0.6 | 1.4 | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | | c | — | — | c | c | — | — |
| 1.0 | 0.8 | 1.2 | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | | c | — | — | c | c | — | — |
| 1.0 | 1.0 | 1.0 | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | | c | — | — | c | c | — | — |
| 1.0 | 1.2 | 0.8 | c | c | c | c | c | c | c | c | c | alc | c | c | alc | c | c | c | c | | vst | — | — | c | c | — | — |
| 1.0 | 1.4 | 0.6 | c | c | c | c | c | c | c | c | st | c | c | c | alc | c | alc | c | c | alc | st | — | — | c | c | — | — |
| 1.0 | 1.6 | 0.4 | c | alc | c | alc | alc | c | alc | c | tr | c | alc | alc | tr | c | alc | alc | c | c | w | — | — | st | c | — | — |
| 1.0 | 1.8 | 0.2 | w | st | alc | alc | alc | alc | w | c | — | alc | alc | w | tr | e | st | alc | alc | c | tr | — | — | tr | c | — | — |
| 1.0 | 1.9 | 0.1 | w | w | st | st | alc | w | w | alc | — | st | st | tr | tr | alc | st | alc | alc | c | tr | — | — | tr | tr | — | — |
| 1.0 | 1.92 | 0.08 | tr | w | w | st | st | w | w | st | — | w | st | — | — | alc | w | st | st | st | tr | — | — | — | tr | — | — |
| 1.0 | 1.94 | 0.06 | — | w | w | w | w | w | w | w | — | w | w | — | — | alc | tr | w | w | w | tr | — | — | — | — | — | — |
| 1.0 | 1.96 | 0.04 | — | tr | — | w | w | tr | tr | tr | — | tr | w | — | — | w | tr | tr | tr | tr | tr | — | — | — | — | — | — |
| 1.0 | 1.98 | 0.02 | — | tr | — | tr | tr | tr | tr | — | — | tr | tr | — | — | tr | — | — | — | tr | — | — | — | — | — | — | — |

TABLE 5

*Showing the influence of different salts on sapotoxin hemolysis in a medium of isotonic saccharose solution*

| SAPOTOXIN IN 8 PER CENT SOLUTION OF SACCHAROSE 1:6,250 | 8 PER CENT SOLUTION OF SACCHAROSE | NORMAL SOLUTION OF SALTS | NaCl | KCl | NaBr | KBr | KI | $NaNO_2$ | $KNO_2$ | $KNO_3$ | $NaNO_3$ | KSCN | Na CITRIC | K CITRIC | $CH_3COONa$ | $CH_3COOK$ | $KH_2PO_4$ | $KC_2O_4H_2O$ | $Na_2SO_3$ | $Na_2SO_4$ | $NaHSO_3$ | $(NH_4)_2SO_4$ | $MgSO_4$ | $Mg(NO_3)_2$ | $MgCl_2$ | $Na_2CO_3$ | $K_2CO_3$ | $BaCl_2$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1.0 | — | 2.0 | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | | c | c | vst | c | c | tr |
| 1.0 | 0.2 | 1.8 | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | | c | c | vst | c | c | — |
| 1.0 | 0.4 | 1.6 | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | | alc | c | st | c | c | — |
| 1.0 | 0.6 | 1.4 | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | alc | c | alc | c | | vst | c | w | c | c | — |
| 1.0 | 0.8 | 1.2 | c | c | alc | alc | c | alc | alc | c | c | c | c | c | c | c | c | vst | c | alc | c | tr | st | c | tr | c | c | — |
| 1.0 | 1.0 | 1.0 | alc | vst | vst | vst | alc | vst | st | vst | vst | c | c | c | st | alc | c | st | c | vst | c | w | w | alc | tr | c | c | — |
| 1.0 | 1.2 | 0.8 | vst | st | w | w | vst | st | w | st | st | st | c | c | w | st | c | w | c | st | c | vst | tr | vst | tr | c | c | — |
| 1.0 | 1.4 | 0.6 | st | w | tr | tr | st | w | tr | w | w | st | c | c | w | w | c | tr | c | w | c | c | tr | st | — | c | c | — |
| 1.0 | 1.6 | 0.4 | w | tr | tr | tr | w | tr | tr | w | tr | w | c | c | tr | tr | alc | tr | c | tr | c | vst | tr | w | — | c | c | — |
| 1.0 | 1.8 | 0.2 | tr | tr | — | — | tr | tr | tr | w | tr | tr | alc | c | tr | tr | st | tr | c | tr | alc | st | — | tr | | c | c | — |
| 1.0 | 1.9 | 0.1 | — | — | — | — | — | — | tr | tr | tr | tr | w | st | — | tr | w | — | vst | tr | vst | w | — | tr | — | c | c | — |
| 1.0 | 1.92 | 0.08 | — | — | — | — | — | — | — | — | — | tr | tr | w | — | — | tr | — | st | — | w | tr | — | — | — | w | w | — |
| 1.0 | 1.94 | 0.06 | — | — | — | — | — | — | — | — | — | — | — | tr | — | — | tr | — | w | — | tr | — | — | — | — | tr | tr | — |
| 1.0 | 1.96 | 0.04 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | tr | — | — | — | — | — | — | — | — | — |
| 1.0 | 1.98 | 0.02 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |

TABLE 6

*Showing the influence of different salts on saponin hemolysis in a medium of isotonic glucose solution*

| SAPOTOXIN IN 5.6 PER CENT SOLUTION OF GLUCOSE 1:10,000 | 5.6 PER CENT SOLUTION OF GLUCOSE | NORMAL SOLUTION OF SALTS | NaCl | KCl | NaBr | KBr | KI | $NaXO_3$ | $KNO_3$ | $NaNO_3$ | $KNO_3$ | KSCN | Na citric | K citric | $CH_3COONa$ | $CH_3COOK$ | $Na_2SO_3$ | $Na_2SO_4$ | $(NH_4)_2SO_4$ | $MgSO_4$ | $Mg(NO_3)_2$ | $MgCl_2$ | $Na_2CO_3$ | $K_2CO_3$ | $BaCl_2$ | $KHPO_3$ | $Na_2HPO_3$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1.0 | — | 2.0 | c | c | c | c | c | c | c | — | c | c | c | c | c | c | c | c | | c | c | c | c | c | ale | c | c |
| 1.0 | 0.2 | 1.8 | c | c | c | c | c | c | c | — | c | c | c | c | c | c | c | c | | c | c | c | c | c | ale | c | c |
| 1.0 | 0.4 | 1.6 | ale | c | c | c | c | st | c | — | c | c | c | c | c | c | c | c | | ale | c | st | c | c | ale | c | c |
| 1.0 | 0.6 | 1.4 | st | ale | ale | ale | vst | w | vst | — | ale | c | c | c | c | c | c | ale | | vst | c | st | c | c | ale | c | c |
| 1.0 | 0.8 | 1.2 | w | st | st | st | st | tr | st | — | st | ale | c | c | ale | c | c | vst | tr | vst | c | w | c | w | c | c | c |
| 1.0 | 1.0 | 1.0 | tr | w | w | w | w | tr | st | — | st | ale | c | c | ale | c | c | st | w | w | c | tr | c | c | c | c | c |
| 1.0 | 1.2 | 0.8 | tr | tr | tr | tr | tr | — | w | — | st | st | c | c | st | c | c | w | tr | tr | c | tr | c | c | c | c | c |
| 1.0 | 1.4 | 0.6 | tr | tr | tr | tr | tr | — | tr | — | tr | w | c | c | w | c | c | tr | tr | ale | — | — | c | c | c | c | ale |
| 1.0 | 1.6 | 0.4 | tr | tr | tr | tr | — | — | tr | — | tr | tr | c | c | tr | tr | c | tr | tr | c | — | vst | c | c | c | c | vst |
| 1.0 | 1.8 | 0.2 | — | — | — | — | — | — | — | — | — | tr | c | c | tr | tr | e | — | e | — | — | — | c | c | c | c | st |
| 1.0 | 1.9 | 0.1 | — | — | — | — | — | — | — | — | — | — | e | e | — | — | e | — | st | — | w | — | e | e | vst | alc | w |
| 1.0 | 1.92 | 0.08 | — | — | — | — | — | — | — | — | — | — | st | e | — | — | ale | — | w | — | tr | — | e | e | st | st | tr |
| 1.0 | 1.94 | 0.06 | — | — | — | — | — | — | — | — | — | — | w | vst | — | — | st | — | tr | — | tr | — | e | alc | st | w | tr |
| 1.0 | 1.96 | 0.04 | — | — | — | — | — | — | — | — | — | — | tr | w | — | — | w | — | — | — | — | — | ale | st | w | tr | — |
| 1.0 | 1.98 | 0.02 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | st | w | w | — | — |

seem to be indifferent. Ammonium sulphate is peculiar in its action inasmuch as in the larger quantities it alters and precipitates the red blood cells and at the same time it inhibits the hemolysis by the saponin and sapotoxin. Furthermore, we see that salts of the same cation with different anions exert a different influence on saponin and sapotoxin hemolysis and that the same is true of the different cation salts with the same anion. This fact is interesting in view of the experiments of Hôber (6) and also of Port, who pointed out differences between anion and cation in the influence of salts upon hemolysis.

From the standpoint of the tonus of the medium, since the sugar solutions were isotonic, it is also important to study saponin hemolysis in isotonic solutions of the salts instead of in normal solutions. I, therefore, prepared the following isotonic solutions:

| | *per cent* |
|---|---|
| $NaNO_3$ | 1.30 |
| NaI | 2.35 |
| $Na_2SO_4$ | 1.63 |
| RbCl | 1.85 |
| NaBr | 1.58 |
| NaSCN | 1.24 |
| KCl | 1.14 |

In the medium of these solutions the saponin hemolysis was tested as in the previous experiment, and it was found that in all of them saponin can act more rapidly than in sugar solution.

According to experiments of Poyarkoff (7), the action of spermatoxin, studied in an electrolyte and a nonelectrolyte medium is much influenced by the viscosity of the environment. Poyarkoff found that when the viscosity is very much increased or decreased by suitable mixtures of electrolyte and nonelectrolyte, the action of the spermatoxin, which was measured by the time taken to cause the motion of the spermatozoa to cease, is very much reduced. There is, accordingly, an optimal concentration of both substances. I have investigated the question whether this fact is also applicable to the action of saponin or sapotoxin. In this experiment the red blood cells of the rabbit were used. The results of the experiment are shown in table 7.

In table 7 the absolute quantity of saponin is the same in each mixture the variations affecting only the quantity of glucose and of salt. An increase in the quantity of sugar causes an increase of the viscosity of the medium. We see thus that as the viscosity of the medium increases there is an increased inhibition on of the hemolysis by saponin. I have not been able, however, to find any optimal viscosity for the saponin hemolysis such as that found by Poyarkoff for spermatoxin. The hemolytic power of saponin appears to be inversely proportional to the viscosity of the medium. The increase of viscosity may interfere with the velocity of diffusion of saponin into the red blood cells.

TABLE 7

| 5 PER CENT SUSPENSION OF RED BLOOD CELLS IN PHYSIOLOGICAL SALT SOLUTION | SAPONIN IN PHYSIOLOGICAL SALT SOLUTION | SAPONIN IN 5 PER CENT GLUCOSE SOLUTION | 5 PER CENT SUSPENSION OF RED BLOOD CELLS IN GLUCOSE SOLUTION | SALT: 5 PER CENT GLUCOSE | HEMOLYSIS (1 HOUR OF 37°C.) | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | 25,000:1 | 30,000:1 | 40,000:1 | 50,000:1 |
| 1.0 | 1.0 | 0 | 0 | 1:0 | c | c | c | c |
| 1.0 | 0.8 | 0.2 | 0 | 9:1 | c | c | c | alc |
| 1.0 | 0.6 | 0.4 | 0 | 8:2 | c | c | c | alc |
| 1.0 | 0.4 | 0.6 | 0 | 7:3 | c | c | alc | st |
| 1.0 | 0.2 | 0.8 | 0 | 6:4 | c | c | vst | tr |
| 1.0 | 0 | 1.0 | 0 | 5:5 | c | c | st | — |
| 0 | 0.8 | 0.2 | 1.0 | 4:6 | c | c | w | — |
| 0 | 0.6 | 0.4 | 1.0 | 3:7 | c | c | tr | — |
| 0 | 0.4 | 0.6 | 1.0 | 2:8 | c | alc | — | — |
| 0 | 0.2 | 0.8 | 1.0 | 1:9 | c | alc | — | — |

I have also investigated the question whether the relations described above with respect to rabbit's blood hold as well for the blood of other animals.

The results of these experiments are shown in tables 8, 9, 10 and 11.

With red blood cells of the pigeon and of the sparrow hemolysis could not be produced with even a 1:500 solution of jegosaponin.

In these experiments, with all of the species of blood corpuscles that are susceptible to the hemolytic action of saponin and sapotoxin the adjuvant effect of electrolytes is apparent. We see,

furthermore, differences in the resistance of the different species of corpuscles to saponin. Such differences have been noted by various workers. Thus, according to Meyer (8), the order of increasing resistance in corpuscles of the different species is:

TABLE 8

*Saponin and sapotoxin hemolysis with the red blood cells of the horse*

| 1: 5000 | HEMOLYSIS (AT 37°C) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Jegosaponin | | | | Sapotoxin | | | |
| | Salt medium | | Saccharose medium | | Salt medium | | Saccharose medium | |
| | 30 minutes | 1 hour | 30 minutes | 1 hour | 30 minutes | 1 hour | 30 minutes | 1 hour |
| 1.0 | c | c | c | c | c | c | tr | tr |
| 0.8 | c | c | c | c | c | c | — | — |
| 0.6 | c | c | c | c | c | c | — | — |
| 0.4 | c | c | c | c | c | c | — | — |
| 0.2 | c | c | c | c | tr | w | — | — |
| 0.1 | c | c | c | c | — | tr | — | — |
| 0.08 | c | c | c | c | — | tr | — | — |
| 0.06 | c | c | st | c | — | tr | — | — |
| 0.04 | c | c | tr | st | — | — | — | — |
| 0.02 | w | c | — | — | — | — | — | — |

TABLE 9

*Saponin and sapotoxin hemolysis with the red blood cells of the pig*

| 1: 5000 | (HEMOLYSIS (AT 37°C.) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Jegosaponin | | | | Sapotoxin | | | |
| | Salt medium | | Saccharose medium | | Salt medium | | Saccharose medium | |
| | 30 minutes | 1 hour | 30 minutes | 1 hour | 30 minutes | 1 hour | 30 minutes | 1 hour |
| 1.0 | c | c | c | c | c | c | c | c |
| 0.8 | c | c | c | c | c | c | c | c |
| 0.6 | c | c | c | c | c | c | c | c |
| 0.4 | c | c | c | c | c | c | st | st |
| 0.2 | c | c | c | c | st | vst | tr | tr |
| 0.1 | c | c | c | c | w | st | — | — |
| 0.08 | c | c | c | c | w | w | — | — |
| 0.06 | c | c | st | vst | tr | w | — | — |
| 0.04 | alc | c | — | — | tr | tr | — | — |
| 0.02 | st | alc | — | — | — | — | — | — |

horse < rabbit < pig < dog < sheep < ox. Meyer states, also, that this represents the order of the lecithin-cholesterin coefficients in the red blood cells of those species. However, according to Abderhalden, the order of the lecithin-cholesterin

TABLE 10

*Saponin and sapotoxin hemolysis with the red blood cells of the sheep.*

| 1:5000 | HEMOLYSIS (AT 37°C.) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Jegosaponin | | | | Sapotoxin | | | |
| | Salt medium | | Saccharose medium | | Salt medium | | Saccharose medium | |
| | 30 minutes | 1 hour | 30 minutes | 1 hour | 30 minutes | 1 hour | 30 minutes | 1 hour |
| 1.0 | c | c | c | c | c | c | c | c |
| 0.8 | c | c | c | c | c | c | c | c |
| 0.6 | c | c | c | c | alc | c | tr | w |
| 0.4 | c | c | c | c | st | c | — | — |
| 0.2 | c | c | c | c | w | alc | — | — |
| 0.1 | c | c | c | c | tr | w | — | — |
| 0.08 | c | c | alc | c | — | tr | — | — |
| 0.06 | c | c | tr | w | — | — | — | — |
| 0.04 | tr | w | — | — | — | — | — | — |
| 0.02 | tr | tr | — | — | — | — | — | — |

TABLE 11

*Saponin hemolysis with the red blood cells of the guinea-pig*

| 1:5000 | HEMOLYSIS (AT 37°C.) | | | |
|---|---|---|---|---|
| | Jegosaponin | | | |
| | Salt medium | | Saccharose medium | |
| | 30 minutes | 1 hour | 30 minutes | 1 hour |
| 1.0 | c | c | c | c |
| 0.8 | c | c | c | c |
| 0.6 | c | c | c | c |
| 0.4 | c | c | c | c |
| 0.2 | c | c | alc | c |
| 0.1 | c | c | st | alc |
| 0.08 | alc | c | tr | st |
| 0.06 | vst | c | — | tr |
| 0.04 | st | c | — | — |
| 0.02 | — | tr | — | — |

coefficients in the corpuscles is: horse < pig < rabbit < dog < sheep < ox.

The results obtained by other workers with respect to the resistance to the saponin hemolysis are as follows:

Rywosch (10): Guinea-pig < rabbit < dog < pig < cat < ox < goat < sheep.

Schauzenbach (11): Guinea-pig < man < horse < pig < ox < goat < sheep.

Port (4): Rabbit < man < dog < pig < ox < sheep.

According to my own experiments the order of resistance of the red blood cells against jegosaponin is as follows: Horse < guinea-pig < rabbit < pig < sheep < pigeon.

## SUMMARY

1. The hemolytic action of saponin or sapotoxin is, to a certain extent, inhibited in a nonelectrolyte medium. This phenomenon is, perhaps, attributable to the increase of viscosity of the medium, which makes the diffusion of saponin into red blood cells the more difficult.

2. Ions of various salts favor saponin hemolysis even in higher concentration, except $(NH_4)_2SO_4$, which can alter the red blood cells in higher concentration. $BaCl_2$ and $CaCl_2$ are indifferent.

3. The resistance of red blood cells against saponin is different in the different species of animal.

I desire to express my indebtedness to Professor S. Mita for his kind direction and encouragement during my experiments.

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# ACTIVE IMMUNITY IN EXPERIMENTAL POLIOMYELITIS[1]

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The search for a method of specific inoculation against acute poliomyelitis is not a new one. Shortly after the announcement by Landsteiner and Popper (1), in 1909, of the successful transmission of this disease to members of the monkey family, workers in this country and Europe began to study the immunity problems of this disease.

Flexner and Lewis (2), in 1910, demonstrated that monkeys could be immunized against poliomyelitis by repeated subcutaneous injections with increasing amounts of a saline suspension of the crude unmodified virus. They injected animals over a period of two and one-half months. About ten days after the completion of the course of inoculations, the animals were injected intracerebrally with 2 cc. of a filtrate of a very potent virus, of which 0.05 to 0.1 cc. would prove fatal. The animal tested presented no sign of infection, whereas the control died of poliomyelitis. In a later communication (3), they state that artificial active immunity either by the injection of a single large dose or by series of increasing small doses over a period of time is not uniformly successful. In the former method, some of the animals would develop poliomyelitis as result of the subcutaneous injection, and in both, some animals so inoculated would not resist the test intracerebral inoculations of rather large doses of a highly potent virus. In the blood serum of animals so immunized, the presence of neutralizing principles for the potent virus was demonstrated in good concentration.

[1] Read before the American Association of Immunologists at Philadelphia, March 29, 1918.

Landsteiner and Levaditi (4) attempted to devise a method for the prevention of poliomyelitis analogous to the Pasteur method for the prevention of rabies. They dried cords for as long a period as twenty-four days. However, while some animals so treated developed immunity without any ill effects as a result of the treatment, other animals similarly treated developed the clinical picture of experimental poliomyelitis.

Römer and Joseph (5) thought they had produced immunity in monkeys by the intracerebral injection of a mixture of virus and serum that contained neutralizing substances for the virus. They found that a monkey so inoculated resisted apparently a subsequent suitable intracerebral injection of straight virus. However, that this is not invariably true is evidenced by the experience of Flexner and Lewis and Landsteiner and Levaditi, who had no difficulty in infecting animals that had been previously intracerebrally injected with a neutralized mixture of serum and virus. In fact, Flexner and Lewis have had no difficulty in infecting an animal that had previously resisted a sufficient intracerebral dose of straight highly potent virus.

We can confirm these findings concerning neutralized and straight virus, and further than that we have re-infected, by suitable intracerebral inoculations, two animals that had been paralyzed over a year ago, and which presented residual palsies at the time of injection. These animals succumbed as promptly as control animals, which eceived a third of the dose. This fact will be pointed out later.

Flexner (6) stated in 1910 that while the results in artificial active immunity thus far achieved were encouraging, our knowledge at that time was not sufficient to render those results of practical value. This statement was the stimulus for the work which has engrossed our attention for the past year and a half.

The necessary requisites of any suitable method of artificial immunization are:

1. That the method shall protect against any reasonable exposure to the disease for which the individual is immunized.

2. That the inoculations in themselves shall be absolutely harmless.

3. That the method itself shall not be too cumbersome or prolonged, so as to render the production of immunity too slow to be of practical value.

These have been the conditions with which we have attempted to comply in our efforts to arrive at a suitable method.

After an extended and rather discouraging experience with young rabbits, we turned our attention to the use of monkeys of the rhesus variety in our efforts to devise a practical method.

The striking similarity in the characteristics of the virus of rabies and of that of poliomyelitis impressed us so as to cause us to work with the idea of attenuating a highly potent Rockefeller strain. Attempts were made to this end with two methods, one with chemical means and the other by subjecting the virus to the action of heat.

## MODIFICATION OF POLIO VIRUS BY CONTACT WITH 0.5 PER CENT FORMALDEHYDE

Cummings devised a method of anti-rabic treatment in which he put a 2 per cent emulsion of fixed rabic virus in contact with 0.5 per cent formaldehyde for four hours in the ice-box. At the end of this time, he dialyzed the formalin from the mixture through collodion sacs into distilled water until the cord emulsion failed to give test for formalin. The material was then inoculated daily into the rabbits to be protected in increasing doses.

We applied this method in our attempt to chemically modify polio virus. We used a 10 per cent emulsion of the cords and brains of monkeys dead of highly virulent poliomyelitis virus and made it up fresh for each injection. This 10 per cent emulsion was kept in contact with 0.5 per cent formaldehyde for four hours. It was assumed that this contact would kill the polio virus. However, our experience proved to us without any chance for doubt, that it did not kill the virus. The protocol follows:

*Experiment.* Macacus rhesi nos. 89 and 90 were injected subcutaneously as follows:

March 28, 1917, 10 cc.
March 30, 1917, 15 cc.
April 2, 1917, 15 cc.
April 5, 1917, 15 cc.

On April 7, 1917, no. 90 appeared ill; refused food; seemed to be tremulous; showed no sign of paralysis.

April 8, 1917, no. 90 died during the night. Postmortem examination showed nothing of note in the abdominal or thoracic viscera. Section of cord showed some swelling and reddening of the gray matter. Microscopic examination of the cord showed slight perivascular cell infiltration; moderate diffuse cell infiltration; considerable nerve cell degeneration and neurophagocytosis; only slight changes in the meninges; that is, all the classical pathologic findings of poliomyelitis.

Macacus rhesus 89 remained well until April 26, 1917, when it appeared to be ill. It would not feed well but it showed no paresis of any kind. The animal died during the night. Microscopic section of the cord showed the lesins of poliomyelitis.

These animals presented a rather unusual type of infection, one dying within ten days and the other within one month after the institution of the subcutaneous injections. The type resembles very much that described by Flexner as the "marantic" type of monkey poliomyelitis. In this type, animals may be sick over a longer period of time than the animals in the above experiment, yet they do not present flaccid paralysis.

Our results with this particular method were decidedly not encouraging, and as monkeys were very scarce, we turned our attention to the possibility of attenuating the virus by heat.

### MODIFICATION OF VIRUS BY HEAT

It has been demonstrated that poliomyelitis virus is rendered inert by exposing it to a temperature of 50° to 55°C. for one-half hour. With this fact as a basis, it was decided to expose a highly potent virus obtained from the laboratories of the Rockefeller Institute to heat in two ways. One method aims at a graded increase in the virulence of the material inoculated, paralleling after a fashion the use of increasingly more virulent cords in the production of immunity to rabies. This method consists of the

subcutaneous injection on four successive days of 5 cc. of a 10 per cent emulsion in saline of brains and cords of monkeys that have recently been paralyzed by intracerebral injections of highly virulent virus, heated as follows:

| | |
|---|---|
| First day | 55°C. for one-half hour |
| Second day | 55°C. for one-half hour |
| Third day | 45°C. for one-half hour |
| Fourth day | 37°C. for one-half hour |

On the fifth day, the animals received 5 cc. of 10 per cent emulsion of the virus unmodified by heat.

The other method of injection used consists in the subcutaneous injection on ten successive days of 5 cc. of 10 per cent emulsion in saline of brains and cords from monkeys recently paralyzed, which had been heated uniformly to 55°C. for one-half hour. This method corresponds to the use by Semple of subcutaneous injection of killed rabic virus in the Pasteur treatment. Such a method as this immediately removes the dangerous possibility of producing poliomyelitis in the treated animal, inasmuch as the heated material is unable to infect a monkey when injected in large amounts by the intracerebral route. In other words, the virus is apparently killed.

In testing the protective value of these two methods of vaccination, we wished to determine two facts:

1. Are the animals so treated capable of resisting a multiple lethal dose of the virus intracerebrally?

2. Does the blood of animals so treated contain neutralizing principles for a highly potent polio virus?

Three weeks after the completion of the series of subcutaneous injections, three animals of each of these series were bled from the heart. The serum obtained from these bleedings were put into contact with a 5 per cent emulsion of highly potent Rockefeller virus in the proportion of one to one for two hours at 37°C. and twenty-two hours in the ice-box. Then 0.6 cc. of each mixture were inoculated intracerebrally into each of six normal animals. At the time of these injections, the vaccinated animals were tested with the intracerebral injection of 0.15 to 0.3 cc. of

the same virus emulsion used in the neutralization test. Control animals were also inoculated intracerebrally with the same amount of the same emulsions used in testing the vaccinated animals and in the neutralization tests.

Charts 1, 2 and 3 give in detail the results obtained by these methods of modifying the virus. It will be noted that Macacus rhesus 81 of the 5-injection series had received a similar series of injections, and that Macacus rhesus 80 of the 10-injection series had received 5 injections of killed virus two and one-half months previously. At this time we were unable to obtain new monkeys for controlling our test inoculations and for our neutralizing experiments. We thought that if immunity in poliomyelitis lasts no longer than that of rabies in rabbits, it would be well to subject them to another series of injections.

Chart 1 shows that of five animals treated by the 5-injection method, three survived the test intracerebral injection of 0.3 cc. of an emulsion of a highly potent virus, 0.05 to 0.1 cc of the filtrate of which is fatal to monkeys. The two animals, nos. 91 and 97, that succumbed to the injection exhibited paralysis on the sixth day after the test injection. The normal control animals nos. 19 and 23 showed paralysis on the fourth and fifth day repectively.

Macacus rhesus 24, an animal that had survived an infection from virus of the 1916 epidemic, about one year ago, and which at this time presented a residual diplegia, was also injected intracerebrally with 1 cc. of the same emulsion. This animal exhibited paralysis of the arms six days after the inoculation and died on the seventh day. Animals that have survived an attack of the experimental disease are supposed to have a very high degree of immunity, yet here was such an animal that succumbed almost as readily as a control to only three and one-third times the dose used in the controls. This result argues well for the virulence of the material used in the test.

The virus neutralization table of this series shows that of the blood of the three animals bled, all show the presence of neutralization substances, but in varying degrees of concentration. The serum of no. 81 neutralized completely in the proportion of one

CHART 1

*Protection experiments. 5-Injection series. Virus heated one-half hour to 55°C., 55°C., 45°C., 37°C. and unheated. Material used, glycerolated brain and cord (Rockefeller virus in 5 per cent emulsion)*

| MACACUS RHESUS | PREVENTIVE INOCULATION COMPLETED | DATE OF TEST INOCULATION | TEST INOCULATION | PARALYSIS | OUTCOME | REMARKS |
|---|---|---|---|---|---|---|
| | | | cc. | | | |
| 81 | September 18, 1917 | September 27, 1917 | 0.3 | None | Alive—well | |
| 75 | | September 27, 1917 | 0.3 | October 3, 1917 | Died October 5, 1917 | Control for no. 81 |
| 90 | November 23, 1917 | December 14, 1917 | 0.3 | None | Alive—well | |
| 91 | November 23, 1917 | December 14, 1917 | 0.3 | December 20, 1917 | Died December 21, 1917 | |
| 96 | November 23, 1917 | December 14, 1917 | 0.3 | None | Alive—well | |
| 97 | November 23, 1917 | December 14, 1917 | 0.3 | December 20, 1917 | Died December 21, 1917 | |
| 19 | | December 14, 1917 | 0.3 | December 18, 1917 | Died December 21, 1917 | Control |
| 23 | | December 14, 1917 | 0.3 | December 19, 1917 | Died December 21, 1917 | Control |
| 24 | | December 14, 1917 | 1.00 | December 20, 1917 | Died December 21, 1917 | Recovered from previous infection with residual palsies. |

*Virus neutralization. By the serum of monkeys of the above series. Proportion, 1 part serum to 1 part 5 per cent Rockefeller virus*

| SERUM FROM MACACUS RHESUS | BLED | AMOUNT OF MIXTURE INOCULATED | MACACUS RHESUS INOCULATED | DATE OF INOCULATION | OUTCOME | REMARKS |
|---|---|---|---|---|---|---|
| | | cc. | | | | |
| 81 | September 26, 1917 | 0.6 | J— | September 27, 1917 | Alive—well. | Virus control no. 75 as above |
| 96 | December 13, 1917 | 0.6 | 98 | December 14, 1917 | Paralyzed January 4, 1918<br>Died January 10, 1918 | Virus controls no. 19 and 23 as above |
| 97 | December 13, 1917 | 0.6 | 92 | December 14, 1917 | Paralyzed January 8, 1918, Died January 10, 1918 | As above |

to one. The sera of nos. 96 and 97 extended the incubation period of four and five days of the controls nos. 19 and 23 to twenty-one and twenty-five days respectively. This prolongation of the incubation period of highly potent virus seems to us to be an indication of the presence of neutralizing substances. We have been compelled to apply a rather severe test on account of lack of animals. The proportion of 10 of serum to 1 of virus has been considered sufficient for the determination of neutralizing principles.

How great a quantity of these substances is developed by this process of injection, remains to be determined in experiments already planned, and which will be carried out as soon as monkeys are obtainable. No normal monkey serum plus virus control was used in these experiments, as it has been quite firmly established that the blood of normal monkeys does not contain neutralizing substances for poliomyelitis.

Another series of three monkeys were subjected to the 5-injection method, except that in these the inoculations were made up from glycerolated cords only, and that the intracerebral test inoculation was 0.15 cc. of the 5 per cent Rockefeller virus. This was done with the idea that possibly cord material might produce a higher degree of immunity. The test dose was reduced to one-half, because 0.3 cc. appeared to be much more than enough to bring down the controls.

Chart 2 tabulates the results of this series. This shows that two of three animals survived the intracerebral test dose of 0.15 cc. The third, no. 27, showed the first symptoms on the sixth day after the test inoculation. Both controls nos. 73 and 45 showed the first symptoms on the fourth day after the test inoculation.

An additional indication of the potency of the testing virus is shown in the fact that monkey 10, which had survived an experimental infection from the virus of the 1916 epidemic, with a residual paralysis of the right lower, was reinfected fatally with 0.5 cc. of 5 per cent Rockefeller virus. Despite the high degree of immunity conferred on the animal by recovery from the disease, this animal succumbed readily to re-infection with only three times the dose of the control animals in this series, and to less than twice the dose used on the controls in the first series.

CHART 2

*Protection experiments. 5-injection series. Virus heated one-half hour to 55°C., 55°C., 45°C., 37°C. and unheated. Material used, glycerolated cord (Rockefeller virus in 5 per cent emulsion)*

| MACACUS RHESUS | PREVENTIVE INOCULATION COMPLETED | DATE OF TEST INOCULATION | TEST INOCULATION | PARALYSIS | OUTCOME | REMARKS |
|---|---|---|---|---|---|---|
| | | | *cc.* | | | |
| 36 | January 29, 1918 | February 19, 1918 | 0.15 | None | Alive and well | |
| 27 | January 29, 1918 | February 19, 1918 | 0.15 | February 25, 1918 | Etherized February 26, 1918 | |
| 66 | January 29, 1918 | February 19, 1918 | 0.15 | None | Alive and well | |
| 73 | | February 19, 1918 | 0.15 | February 23, 1918 | Died February 26, 1918 | Control |
| 45 | | February 19, 1918 | 0.15 | February 23, 1918 | Died February 26, 1918 | Control |
| 10 | | March 11, 1918 | 0.5 | March 18, 1918 | Died March 20, 1918 | Recovered from previous infection |

*Virus neutralization. By the sera of monkeys of the above series. Proportion, 1 part serum to 1 part 5 per cent Rockefeller virus*

| SERUM FROM MACACUS RHESUS | BLED | AMOUNT OF MIXTURE INOCULATED | MACACUS RHESUS INOCULATED | DATE OF INOCULATION | OUTCOME | REMARKS |
|---|---|---|---|---|---|---|
| | | *cc.* | | | | |
| 36 | February 18, 1918 | 0.5 | 4 | February 19, 1918 | Alive and well | Virus controls no. 73 and 45 |
| 27 | February 18, 1918 | 0.5 | 71 | February 19, 1918 | Paralyzed March 3, 1918. Died March 4, 1918 | Virus controls nos. 73 and 45 |
| 66 | February 18, 1918 | 0.5 | 16 | February 19, 1918 | Paralyzed March 1, 1918. Died March 2, 1918 | Virus controls nos. 73 and 45 |

Of the sera of the three animals of this series, one, no. 36, completely neutralized the virus in the proportion of one of serum to one of virus. The sera from nos. 27 and 66 prolonged the four day incubation period of the controls nos. 73 and 45 to fifteen and twelve days respectively. As in the first series, the sera from the treated animals contain neutralizing substances, but in varying degrees of concentration.

The controls, nos. 73 and 45, showed the first symptoms of the fatal disease in four days, though the intracerebral inoculation was only 0.15 cc. of a 5 per cent emulsion, or one-half the dose used in the first series.

Three other monkeys were subjected to the injection of increasingly virulent material. In these, the tests were not completed, and therefore they were not included in the tables. The protocols follow:

*Experiment 1.* Macacus rhesus 57 received subcutaneously injections of 5 per cent Rockefeller virus as follows:

February 13, 1917, 10 cc. heated to 55°C. for one-half hour.

February 16, 1917, 10 cc. heated to 45°C. for one-half hour.

February 20, 1917, 5 cc. heated to 37°C. for one-half hour.

February 24, 1917, 5 cc. unheated 5 per cent virus.

This animal was not bled for the determination of neutralizing substances, and remained well till time of intracerebral test injection.

March 20, 1917. Injected intracerebrally with 0.6 cc. of a 5 per cent emulsion of the Rockefeller virus. This was twice the dose used in the series shown on chart 2.

March 26, 1917. Left hind leg is weak. Does not attempt to get up. Has tremors.

March 27, 1917. Completely paralyzed. Abdominal respiration.

Etherized to death. Postmortem examination shows typical changes of poliomyelitis in the cord.

Comment: This animal was not protected against a very large dose of a virus of high potency. However, the vaccination in itself had no harmful effects.

*Experiment 2.* Macacus rhesus 94, an animal that had some months previously been injected with an emulsion of rat fleas obtained from houses in which had occurred cases of poliomyelitis and that had shown no symptoms as result of such injection, was inoculated with a 10 per cent emulsion of Rockefeller virus as follows:

April 20, 1917, 5 cc. heated to 55°C. for one-half hour.
April 23, 1917, 5 cc. heated to 45°C. for one-half hour.
April 25, 1917, 5 cc. heated to 37°C. for one-half hour.
April 30, 1917, 5 cc. unheated.

This animal had not been well for some time. Had been coughing and was somewhat emaciated.

On May 6, 1917, animal died.

Postmortem showed generalized pulmonary tuberculosis.

Microscopic examination of cord showed no changes indicative of poliomyelitis.

*Experiment 3.* Macacus rhesus 100 was injected subcutaneously with a 10 per cent Rockefeller virus as follows:

May 10, 1917, 5 cc. heated to 55°C. for one-half hour.
May 11, 1917, 5 cc. heated to 55°C. for one-half hour.
May 12, 1917, 5 cc. heated to 45°C. for one-half hour.
May 14, 1917, 5 cc. heated to 37°C. for one-half hour.
May 15, 1917, 5 cc. unheated.

May 29, 1917. Animal is well. As result of cardiac puncture to obtain blood for the purpose of testing for the presence of neutralizing substances, the animal died. Postmortem showed hemopericardium. Microscopic examination of cord showed no lesions of poliomyelitis.

June 1, 1917. A mixture of 0.2 cc. of the serum of Macacus rhesus 100 and 0.2 cc. of 5 per cent Rockefeller virus that had been in contact for two hours at 37°C. and twenty-two hours in the ice box, was injected intracerebrally into Macacus rhesus 51. This animal survived the inoculation and is alive and well.

Comment: The vaccination in itself was harmless and the serum of vaccinated animal contained neutralizing substances.

## SUMMARY OF RESULTS OF 5-INJECTION METHOD

Eleven animals were subjected to injections of the highly potent Rockefeller strain modified by heat as follows:

First heated to 55°C. for one-half hour; second heated to 55°C. for one-half hour; third heated to 45°C. for one-half hour; fourth heated to 37°C. for one-half hour. The fifth injection was made up from glycerolated material from recently paralyzed animals and injected without previously being subjected to heat. These injections were administered subcutaneously on successive days.

Not one of these animals exhibited any untoward symptoms that were recognizable by careful observation as a result of the course of injections.

Five out of eight of these animals tested intracerebrally resisted successfully the very reasonable test injection of from three to six fatal doses. The potency of the testing virus in 1910 was such that 0.05 to 0.1 cc. of a Berkfeld filtrate was sufficient to produce the fatal disease in monkeys. It has been passed through many additional generations since and it is safe to assume that the virulence has mounted considerably in the past eight years.

The sera of seven of these animals tested in the proportion of 1 part serum to 1 part 5 per cent virus contained neutralizing substances. Three completely neutralized the virus in this proportion and four prolonged the short four to five day incubation period as shown in the controls to from twelve to twenty-five days.

### 10-injection series with virus killed by heat

Chart 3 shows that of three animals vaccinated subcutaneously by daily injections of 5 cc. of 10 per cent emulsion of Rockefeller virus heated to 55°C. for one-half hour, on ten successive days, only one Macacus rhesus 1 survived the intracerebral test injection. This animal received 0.15 cc. of 5 per cent Rockefeller virus intracerebrally three weeks after the completion of the process of vaccination.

On the fourteenth day after the test inoculation, this animal began to show weakness in the legs, which progressed slowly, so that on January 5, 1918, both arms and legs were completely paralyzed. After this, progress of paralysis ceased. On January 10, animal was as lively as he could be under the circumstances. The eyes were bright and he eagerly ate fruit that was held up to his jaws. The animal, however, became so infected with body lice that he was etherized to death on January 20, 1918.

Two others, Macacus rhesus 80, died one week after the test intracerebral inoculation of 0.3 cc. of 5 per cent Rockefeller

CHART 3

*Protection experiments. Ten injections on successive days with 10 per cent virus heated to 55°C. for one-half hour. Material used, glycerolated brain and cord (Rockefeller virus in 5 per cent emulsion)*

| MACACUS RHESUS | PREVENTIVE INOCULATION COMPLETED | DATE OF TEST INOCULATION | TEST INOCULATION | PARALYSIS | OUTCOME | REMARKS |
|---|---|---|---|---|---|---|
| | | | cc. | | | |
| ‡80 | September 14, 1917 | October 7, 1917 | 0.3 | October 13, 1917 | Died October 14 | |
| 84 | | October 7, 1917 | 0.3 | October 12, 1917 | Died October 14 | Control for No. 80 |
| 1 | November 30, 1917 | December 21, 1917 | 0.15 | January 3, 1918 | Paralysis of arms and legs. | Recovered; etherized January 20, 1918 |
| 2 | November 30, 1917 | December 21, 1917 | 0.15 | December 27, 1917 | Died December 28, 1917 | |
| 25 | | December 21, 1917 | 0.15 | December 27, 1917 | Died December 28, 1917 | Control for nos. 1 and 2 |

‡ Had received series of five injections of 10 per cent virus heated to 55°C. for one-half hour. June 26–June 30, 1917, inclusive.

*Virus neutralization. By serum of monkeys of above series. Proportion, 1 part serum to 1 part 5 per cent Rockefeller virus*

| SERUM FROM MACACUS RHESUS | BLED | AMOUNT OF MIXTURE INOCULATED | MACACUS RHESUS INOCULATED | DATE OF INOCULATION | OUTCOME | REMARKS |
|---|---|---|---|---|---|---|
| | | cc. | | | | |
| 80 | October 6, 1917 | 0.6 | 99 | October 7, 1917 | Alive and well | Virus control Macacus rhesus 8 |
| 1 | December 21, 1917 | 0.6 | 100 | December 22, 1917 | Paralyzed January 9, 1918; alive with residual lowers | Virus control Macacus rhesus 25 |
| 2 | December 20, 1917 | 0.6 | 83 | December 21, 1917 | Paralyzed December 31, 1917; died December 31, 1917 | Virus control Macacus rhesus 25 |

virus and Macacus rhesus 2 died one week after an intracerebral inoculation of 0.15 cc. of 5 per cent Rockefeller virus. Macacus rhesus 84, the control for no. 80 also died 1 week after the intracerebral injection of 0.3 cc. of 5 per cent Rockefeller virus. Macacus rhesus 25, the control for nos. 1 and 2, died one week after the intracerebral inoculation of 0.15 cc. of 5 per cent Rockefeller virus. The blood of all three of the animals vaccinated with killed virus contained neutralizing substances but in varying degree of concentration as virus neutralization table on chart 3 indicates.

Macacus rhesi 99, 100 and 83 were each injected intracerebrally with 0.6 cc. of a mixture of equal parts of 5 per cent emulsion of Rockefeller virus and serum obtained from Macacus rhesi 80, 1 and 2 respectively. Rhesus 99 is alive and well. Rhesus 100 began to show first symptoms on the eighteenth day after the inoculation. The next day complete paralysis of both legs. This did not progress. The animal is alive with residual palsies of both lowers. Rhesus 83 showed first symptoms on the tenth day after the inoculation and died the same day of respiratory paralysis.

The control animals in this series, Rhesi 84 and 25, which received 0.3 cc and 0.15 cc. of 5 per cent Rockefeller virus intracerebrally respectively, died of the infection with an incubation of five and six days respectively.

### SUMMARY OF RESULTS OF 10-INJECTION SERIES

Of three animals so treated, two succumbed to the intracerebral test dose as promptly as the controls. The third exhibited paralysis after an incubation of fourteen days. This animal, Rhesus 1, recovered, but with complete paralysis of arms and legs. The infection of the control animal in this case had an incubation period of six days.

Of the three sera tested, one, that of Rhesus 80, neutralized completely in the proportion of 1 part serum to 1 part 5 per cent Rockefeller virus. Another, that from Rhesus 1, delayed the incubation period to eighteen days with recovery, but residual paralysis. The serum from Rhesus 2 prolonged the incubation

period to ten days. None of these animals showed any ill effects from the method of injection.

It would seem from the above results that the animals subjected to ten subcutaneous injections of virus killed by heating at 55°C. for one-half hour, did not offer as great a resistance to the intracerebral test injection of from three to six fatal doses, as those that received the 5-injection series. We, therefore, endeavored to increase this resistance by injecting virus heated to 50°C. for one-half hour for seven injections and finishing off with three injections of virus heated to 45°C. for one-half hour. Two animals were so injected. The protocols follow:

*Macacus rhesus 46.* January 18, 1918. Received daily subcutaneous injections of 5 cc. of 10 per cent Rockefeller virus in saline heated to 50°C. for one-half hour for seven days.

January 26, 1918. Received daily subcutaneous injections of 5 cc. of 10 per cent Rockefeller virus of saline, heated to 45°C. for one-half hour for three days.

January 29, 1918. Series of injections completed.

January 30, 1918. Animal is quiet. Does not feed well. Seems tremulous on moving.

January 31, 1918. Completely paralyzed.

February 1, 1918. Autopsied. Sections show poliomyelitis.

*Macacus rhesus 7.* January 18, 1918. Received a course of injections similar to that of Rhesus 46.

January 29, 1918. Course of injections completed.

February 6, 1918. Animal appeared sick. Did not eat his food. Was quiet. No sign of muscular weakness.

February 7, 1918. Was found dead in the cage. No sign of paralysis had been observed. Autopsy showed pneumonic consolidation of lower right lobe. Section of cord showed slight but unmistakable signs of poliomyelitis.

Comment: From this experiment, it is apparent that heating virus to 50°C. for one-half hour does not sufficiently attenuate to exclude the dangerous possibility of infection as a direct result of the injections per se. One animal developed frank symptoms of paralysis on the eleventh day after the beginning of the series of injections. The other died 19 days after the first injection of lobar pneumonia with lesions of poliomyelitis in the cord.

## DISCUSSION

We feel that this work opens up a field for the practical application of specific preventive measures in poliomyelitis. We have subjected eleven monkeys to the 5-injection series without any ill effects to the animals as a result of the injections. The possibility of harmful effects from this method, if applied to human beings, is, of necessity, less than when applied to monkeys, inasmuch as this virus has been adapted to monkeys for the past nine years and by continuous passage through this animal, has become highly virulent for the monkey.

The period of time required for this series of injections is a short one, only five days, which, of course, would render it highly practicable in time of epidemic, when a rapid method is to be desired.

This method confers a substantial degree of immunity as shown by the resistance to multiple intracerebral doses of this highly potent virus and by the presence of neutralizing substances in the serum.

It is a well established fact that persons who recover from poliomyelitis have in their blood neutralizing substances for the virus of poliomyelitis and that in all probability, the presence of these substances is an indication of immunity to re-infection. The same can be said of monkeys that recover from the experimental disease. If this is so, then why should not animals that have been artificially made to produce such substances and some of which have been made to resist multiple intracerebral injections of most virulent material, be considered immune? The natural disease is far less serious than is the intracerebral infection of experimental poliomyelitis. In the former condition, the body fluids have an opportunity to combat the virus of the infection at the portal of invasion. In the experimental disease, the defensive forces of the body are circumvented. It would, therefore, be true that an animal body, which contained a large reservoir of these anti-poliomyelitis substances artificially produced, would be able easily to take care of the comparatively mild infection of human poliomyelitis.

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# EXPERIMENTAL POLLINOSIS[1]

## PRELIMINARY REPORT

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That hay fever is a form of hypersensibility to pollen proteins is conceded by everyone. The mechanism of this form of hypersensibility, likewise the phenomenon of its desensitization with pollen extract (phylactically or prophylactically administered) are still mooted questions.

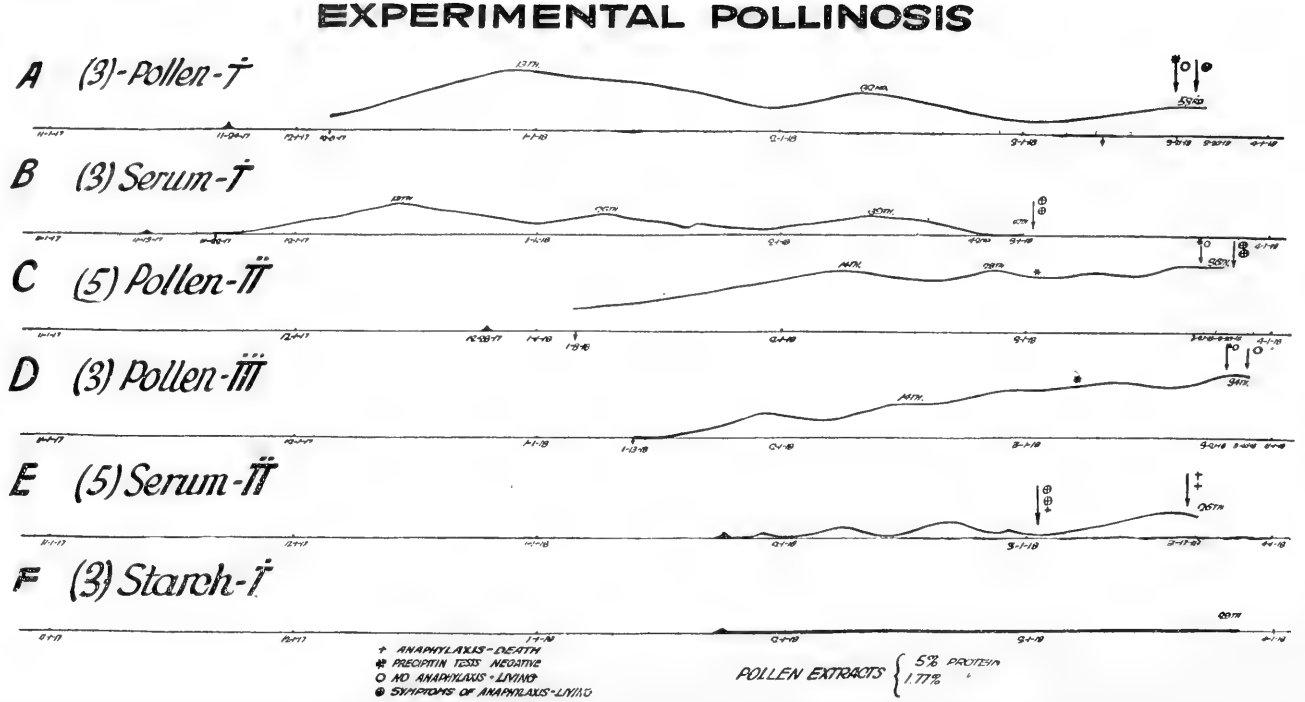
Cooke, Flood, Coca's (1) definition of hay fever as a "clinical symptomatic expression of local hypersensitiveness" cannot hold. The skin and conjunctional reactions in hay fever subjects are certainly extranasal. Sewall's (2) criticism that no local manifestation of hypersensibility can occur without the background of a general hypersensibility is amply justified.

Up to now animal experiments to produce clinical hay fever have not been successful. Koessler (3) reports passive anaphylaxis in guinea-pigs. Cooke, Flood and Coca (4) and myself[2] have failed to produce active or passive anaphylaxis with pollen extracts. My experiments to produce a passive anaphylaxis were made with patients' blood out of season. It is possible that Koessler's experiments were made in season. At any rate the assumption (5) that there are no circulating antibodies in hay fever patients should be made with some reservation.

In an effort to produce hay fever in animals, an entirely new method was used which proved satisfactory enough to justify its publication. A group of animals were sensitized to pollen pro-

[1] Read before the annual meeting of the American Association of Immunologists, Philadelphia, March 29, 1918.

[2] Unpublished experiments.


EXPERIMENTAL POLLINOSIS
A (3)-Pollen-I
B (3) Serum-I
C (5) Pollen-II
D (3) Pollen-III
E (5) Serum-II
F (3) Starch-I
+ ANAPHYLAXIS-DEATH
# PRECIPITIN TESTS NEGATIVE
O NO ANAPHYLAXIS-LIVING
⊕ SYMPTOMS OF ANAPHYLAXIS-LIVING
POLLEN EXTRACTS { 5% PROTEIN 1.77%

tein by the injection of a suspension of pollen in salt solution, intraperitoneally. One series received 1 mgm. of pollen in 1.0 cc. of salt solution respectively; the other series received 25 mgm. suspended in 1.0 cc. of salt solution respectively. Ten to twelve days later insufflation of pollen in the nares was begun. This was repeated every other day for a long period of days. After each insufflation notes were made of the clinical behavior of the animals. The animals were watched usually for one hour after the insufflation. Notes made were on each individual animal. The curves on the chart portray the indices of reactions on successive days of the groups. The curves, therefore, represent cage reaction and are merely charts of clinical symptoms measured graphically. The pollen used in these experiments was collected during the 1917 season. It was cleansed and washed in pure acetone, dried and stored in sterile vials of two grams capacity. Another group of animals were sensitized to horse serum—1 cc. intraperitoneally. Ten days later instillation of horse serum in the nares was begun and repeated every other day for a long period. This group virtually was a control to the pollen series. Some experimental data with this method had already been published by Sewall (6).

Another group of animals was not previously sensitized but was subjected merely to pollen insufflation at two days intervals. This group again was controlled by a horse serum group, the serum being instilled in the nares at two day intervals without previous sensitization by the intraperitoneal route. Lastly, a group of animals was subjected to insufflation of pure starch. This group was to act as a control to the pollen group in order to throw out the presumption that sneezing was caused by the local irritation of dust particles (7). Microscopically, the surface of starch granules and pollen granules are not at all alike. The question of irritation may be settled at this point by the fact that in these experiments, the pollen animals, which reacted by sneezing, always exhibited a latent period after insufflation. In the starch animals there were no evidences of sneezing or disagreeable sensations at any time.

It may be also added, that a similar series of experiments were started at the same time with rabbits. The rabbit groups were conducted for six weeks. At the end of that time the experiments were stopped because at no time was there any evidence of hypersensibility obtained by the methods employed. In other words the nasal route as a method to demonstrate clinical hay fever (hypersensibility) in rabbits is not possible.

The symptoms in guinea-pigs obtained by these methods were those closely resembling clinical hay fever in man. After a latent period of five to twenty minutes following exposure to pollen, sneezing, lachrymation, itching of the nares and the body were obtained, this being often followed by excitability, or depression, increased respiratory rate, deefication and urination. As the days progressed, nasal and bronchial stenosis were observed with rales in the chest, moist and rhonchus in type, but at no time was there prolonged expiratory breathing, so typical of asthmatic attacks. In the serum animals, similar clinical symptoms were observed but never so severe nor so consistent as in the pollen groups. During the period of the experiments, the animals thrived and grew in size and weight. No controls in growth or weight were made. Several of the females became pregnant and gave birth to healthy young.

*Experiment 1, chart A, pollen I.* On November 24, 1917, three guinea-pigs average weight (575 grams) received 1 mgm. of pollen suspended in 1 cc. of salt solution, respectively. December 6, 1917, each animal received a nasal insufflation of pollen by means of a medicine dropper. The animal was held gently on its back by an assistant. One nostril[3] was held closed, the other mostril received the tip of the dropper, which contained a column of dried pollen. The animal thus inhaled the dust aided by a gentle compression of the head of the dropper. The other nostril was treated in the same way. This method was repeated every other day. Fifty-three exposures to pollen were made, covering a period of one hundred and six days. Including the period of sensitization the whole time involved was one hundred and sixteen days. The first insufflation called forth reactions resembling mild hay fever, sneezing,

[3] Guinea-pigs will not breathe through their mouths except in the last gasps of anaphylactic shock.

itching of the nares and the body, and lachrymation occurred. A latent period varying from five to twenty minutes was noticed after each insufflation. After twenty minutes the symptoms usually were at their height. Spasmodic sneezing, besides the itching, lachrymation and nasal stenosis were most striking. At no time were true asthmatic symptoms obtained. Rales in the chest were made out at the height of some of the attacks. Usually after an hour's time all evidence of clinical manifestations had ceased. Occasional sneezing was heard some hours after the exposure. A definite rise and fall of nasal irritability was noticed during these fifty-three exposures. The height of the first rise occurred approximately at the thirteenth exposure and depth of the fall at about the twenty-eighth exposure. Another rise reached its height at the thirty-second exposure and dropped to its lowest at about the forty-third exposure. Another high tide was at the close of the experiments March 26, 1918. At each successive rise and fall there was a definite impression of waning sensibility (see chart). A refractory stage was impending but was never entirely established (?). On March 23, the blood of one animal was tested for precitins with negative results. Another animal on the same day received 1 cc. of pollen extract (0.5 per cent protein, refractometer method) intracardially with negative results. The third animal on March 20 received a stronger extract (1.77 per cent of protein, refractometer method). This animal reacted with symptoms of acute anaphylaxis with recovery.

*Experiment 2, chart B, serum I.* In the meanwhile a control group of animals was under observation. On November 13, 1917, three guinea-pigs had received 1 cc. of Lederle's normal horse serum intraperitoneally. This was repeated at two day intervals until forty-seven instillations had been given. The serum was dropped into nares off the needle that usually accompanies the Lederle's package. No definite amount was given. Sometimes one, sometimes two or three drops were used in each nostril. Enough was instilled to insure bathing of the turbinates. The characteristic welling up of salivary secretion was noticed in these animals after each instillation. It was not until the third or fourth instillation that any semblance of hay fever symptoms were manifested. On the whole these animals exhibited the same complex of symptoms that were obtained in the pollen animals, but never so marked. At no time was asthma obtained. The group also showed the curves of increasing and decreasing reactivity. In fact at the forty-second instillation complete refractivity was obtained. This continued thereafter and therefore after the forty-seventh instillation the animals were

no longer subjected to horse serum by the nasal route. On March 6, one hundred and sixty-five days after the initial intraperitoneal exposure, two[4] of the animals received 1 cc. of horse serum intracardially. Acute anaphylaxis occurred with recovery in both animals. This group supported or illustrated more than any others the possible hypothesis put forth by several observers (8) that there may be a protective mechanism as a part of the function of the nasal mucosa.

*Experiment 3, chart C, pollen II.* Five animals, average weight 418 grams, were subjected intrapertioneally to 25 mgm. of pollen, suspended in 1 cc. of salt solution respectively. On January 8, 1918, insufflation of pollen in nares was begun and continued at two days intervals. This experiment was a repetition of experiment 1, with younger animals, a larger sensitizing dose and a larger number of animals. The first insufflation provoked the characteristic symptoms. The reaction in these animals was more intense than those in experiment 1. Nasal and bronchial stenosis was obtained as early as the seventh insufflation. These animals also differed from the first in that no curves of refractivity were obtained. On the other hand, a steady rise of nasal susceptibility was noticed. On March 1, 1918, one of the highly susceptible animals was bled. The blood was tested for precipitin with negative results. March 31, 1918, one of the animals was injected intracardially with 1 cc. pollen extract (0.5 per cent protein by refractometer method). There were no symptoms of anaphylaxis. The blood serum of this animal previous to the intracardiac reaction was tested for precipitins with negative results. On March 20, 1918, two animals in this series were injected by the cardiac route with 1 cc. of a 1.77 per cent protein pollen extract. Both animals reacted with acute stormy anaphylactic symptoms with recovery.

*Experiment 4, chart D, pollen III.* January 13, 1918, three animals with an average weight of 604 grams were subjected to insufflation of pollen without previous sensitization of any kind. The insufflation was conducted at two day intervals just as in the other experiments. January 19, 1918, after the fourth insufflation mild symptoms of sneezing, itching, and lachrymation was obtained. These animals were subjected to thirty-four treatments. No curves of refractivity were obtained but a steady rise of susceptibility was noticed. Precipitin tests were made on one animal on March 1, 1918, likewise another on March 21, 1918, and

[4] The third animal had disappeared from its cage on February 15, and was not found again.

on March 26, 1918; intracardiac injections of 1 cc. of pollen extract in one animal for each date resulted in no symptoms.

*Experiment 5, chart E, serum II.* These animals, five in number of an average weight of 400 grams, were used as controls for experiment 4. Horse serum was instilled intranasally without previous sensitization. The instillation was begun on January 23, 1918. No effect was noticed until after the fourth instillation. For nearly one month indefinite, individual reactions occurred, resembling the symptoms of the pollen animals but not so uniformly or clearly defined. During the next month, however, more definite symptoms were obtained. On January 6, 1918, three of the animals received 1 cc. of horse serum intracardially. Two responded with marked anaphylaxis with recovery, the third died in typical anaphylactic shock. On January 17, 1918, when the curve of sensibility was most marked up to this time, the two remaining animals were subjected to 1 cc. of horse serum intracardially and both died in typical anaphylactic shock. The series conclusively proved that hypersensibility can be produced by the nasal route.

*Experiment 6, chart F, starch I.* January 25, 1918; three animals, of an average weight of 390 grams, were subjected to insufflation similar to the method used in the pollen animals. The animals were used as a control to the pollen group in order to answer the question which might be raised whether the nasal irritation and symptoms were due to mere mechanical irritation of dust. The animals were exposed to 29 insulations on the same days as the pollen animals and at similar intervals. At no time did the animals manifest any symptoms of discomfort or annoyance by the treatments.

## CONCLUSIONS

1. Clinical manifestations of hay fever, pollinosis, can be produced in laboratory animals.

2. Horse serum produces similar manifestations in the guinea pig but not as clearly nor as uniformly. At no time were true asthmatic symptoms obtained.

3. Evidence of refractive phenomena was obtained by rhythmic exposure of the nasal mucous membrane to foreign proteid.

4. Sensitization by the nasal route was established; first, by the manifestation of clinical signs of pollinosis after the fourth exposure; secondly, by the anaphylactic phenomena in the serum animals when serum subsequently was injected into the blood.

5. Non-protein dust gave no symptoms of hypersensibility.

## DISCUSSION

That we can sensitize the guinea-pig to pollen by mere exposure of the nasal mucous membrane is of great importance to those who are interested in the membrane from a physiological point of view. Cooke and Van der Veer (9) have shown the influence of heredity on protein sensitization. Their cases were those occurring entirely "spontaneously," from exposure either to pollen or to food. It would be well to consider, in reflecting on the mechanism of hay fever, whether we are not dealing with a congenital or acquired defect of function of the nasal mucosa. It may be possible that the difference between the person not sensitive to pollen and the one sensitive lies in the rate of the digestion of the pollen proteids by the respective nasal mucosa. The sensitive membrane may have lost the faculty of rapid conversion of the proteins to innocous amino acids or may never have had it. This may be the explanation of the food types of sensitization through an intact mucosa. The rabbit is insensitive to nasal exposure to pollen. The guinea-pig is quite sensitive. The difference in reactions of the mucous membrane in the two species is suggestive of a difference in function phylogenetically.

The animals in chart D, pollen 3, which had not been previously sensitized, at no time showed precipitin reactions with the blood, nor anaphylactic symptoms after pollen protein had been introduced into the blood. This experiment approaches in some details what has been found in the human sufferer. Clowes (10) claims he has found precipitin and Koessler (11) reports passive transfer of immune bodies from the patient to the guinea. pig. Cooke, Flood and Coca (12) and I myself have never been able to reaffirm these claims. It would be of interest to attempt passive transference during the height of the hay fever season. Apropos of this: one animal highly sensitive, in chart C, pollen 2, was bled and its serum was injected peritoneally into a normal pig. Forty-eight hours later pollen extract introduced intracardially elicited no response. Again a group of (3) animals were injected with 3 cc. of human serum respectively, from a hay fever sufferer. Two days later these animals were subjected to insuf-

flation of pollen into the nares. Symptoms such as sneezing, itching and lachrymation occurred. The experiment was repeated in another group (3), with another patient's blood with entirely negative results. These observations will be repeated and elaborated at some future time. The possibility of demonstrating a passive transfer of immune bodies by a reaction such as that in the skin or mucous membrane, is, as far as I know, a new method of approaching this problem. Lastly, the fact that we can inject pollen grains intraperitoneally in the guinea-pig without untoward results suggests the idea of the possible use of pollen grains direct as a phylactic or prophylactic measure in the treatment of the disease.

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# PROMPT MACROSCOPIC AGGLUTINATION IN THE DIAGNOSIS OF GLANDERS

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The agglutination test for the diagnosis of acute cases of glanders is conceded by all workers to be of great value. Miessner (1) and others have shown that agglutinins in glanders are generally at their height between the fifth and eleventh day of the infection, after which time they begin to decline. At the end of two months, they may fall even below 1: 500. This test, if used alone, would fail to detect chronic cases and it should, therefore, be used as an adjunct to the complement fixation[1] and ophthlamic mallein[2] tests. No one test can be depended on alone as each one has its peculiar value in certain stages of the disease. If all these tests are combined, very few cases of glander can escape detection. Even though a case may appear to be negative by one or two of the methods, it may be found positive by the third.

The great objection to the employment of the macroscopic agglutination method used heretofore in the routine diagnosis of glanders, is the length of time required for the appearance of a reaction. While some tests can be read at the end of twenty-four hours, others do not show definite agglutination within forty-eight or seventy-two hours or even longer. This drawback led to the centrifuge method for which Muller (2) claims priority.

[1] Specific amboceptors for the complement fixation test may be demonstrated seven to ten days after infection and they remain during the course of the disease (6).

[2] The ophthalmic mallein test is reliable twenty-one days after infection while the subcutaneous mallein test may be relied upon for diagnosis fifteen days after infection (6).

By it the process of the agglutination reaction is shortened considerably. Miessner (1) also Pfeiler (3) employed this method claiming it to be a great success.

According to this method, the test fluid is prepared in the same way as in the old method—namely, a forty-eight hour growth of a suitable strain of *B. mallei* is suspended in salt solution and heated at 60°C. for two hours. It is then diluted to a certain density with 0.5 per cent carbolic acid. The tubes are set up in the ordinary way, each tube containing 2 to 3 cc. of the titrated test fluid with varying quantities of serum to make the final dilution, 1: 400, 1: 500, 1: 800, 1: 1000, etc. After one hour incubation at 37°C. the tubes are centrifuged for ten minutes, after which they are allowed to stand at room temperature for one and one-half hours. The tests are read at the end of that time. The appearance of an irregular veil like clumping at the bottom of the tube with clearing of the upper portion is considered an agglutination, while a dense white precipitate with a cloudiness of the upper part is considered a negative reaction.

Though the centrifuge method possesses great advantages over the old method in point of time, it is difficult always to separate an agglutination from a sedimentation and the result is not always readable. Thus, Anthony and Grund (4) found this test unreadable in 12 per cent of their cases tested, even after an incubation of twenty-four hours.

In order to eliminate the shortcomings of the former methods, I have worked out a modification by which an agglutination reaction, clear cut in appearance, can be obtained in less than two hours with positive sera in dilutions up to 1: 2000 or higher.

The strain of *B. mallei* used was one called B. M. 5, isolated at this laboratory about five years ago from a case of human glanders. Fourteen other strains from various sources (chiefly horses) were tested also, but none have given invariably the prompt and clear cut reaction that B. M. 5 does. The specificity of this strain was ascertained by testing it with a number of syphilitic, typhoid, streptococcus and pneumococcus sera; also with a number of sera from horses with equisepticus. One of the latter sera gave a positive reaction but the possibility of

glanders could not be ruled out. When passed through guinea-pigs, B. M. 5 became more virulent but, after successive passages, did not agglutinate so well; in fact, I found that it was not advisable to pass this strain through a guinea-pig, as most writers advocate to keep the strain agglutinable. Other strains, however, have not proved so constant as this one.

Next in importance to the native agglutinability and constancy of the strain, is the medium on which it is grown. The medium which has given the most satisfactory results is potato-glycerin-veal agar that is 2.5 per cent acid to phenolphthalein. It is prepared as follows:

| | |
|---|---|
| Veal infusion* (unadjusted) | 1000 cc. |
| Agar | 30 grams |
| NaCl | 5 grams |
| Pepton (Fairchild) | 10 grams |

* Veal infusion: Chopped lean beef 10 pounds, water 10 liters. Soak over night. Heat for one hour at 45°C. then boil for one-half hour. Strain through cheesecloth. This infusion may be sterilized and stored for stock or used at once.

Put in autoclave or Arnold to melt agar.

Titrate at room temperature and lower the *natural* acidity, if necessary, to 2.5 acid (phenolphthalein) by adding normal sodium hydroxid. *Do not add acid* under any circumstances.

Clear with egg and filter. Titrate again and if necessary, adjust to 2.5 acid. Add glycerin (C. P.) 5 per cent and potato juice 5 per cent. Sterilize in autoclave for one-half hour at 15 pounds pressure. .

The potato juice used in this medium is prepared by adding one pound of unpealed potatoes, sliced thin, to 1 liter of distilled water. Autoclave one-half hour at 15 pounds, strain through cheese-cloth and a thin layer of cotton. Bottle for stock and sterilize in autoclave one half hour at 15 pounds. This stock must be filtered each time before adding to medium.

That too much stress cannot be laid on the careful preparation of the medium is shown by the following experience. At one time in the beginning of my work, the suspension of B. M. 5 grown on a freshly prepared medium did not give the proper reactions with the control sera. Keeping in mind that the strain

was old and in need of rejuvenation, I passed it through a guinea-pig and recovered the organism from the heart's blood. Meanwhile, a second fresh medium was made upon which the newly recovered organism was planted. This suspension reacted very successfully and I should have believed that the guinea-pig passage was responsible for the success had I not planted the old organism on the second medium also. The suspension of this growth worked as well as the one just isolated from the guinea-pig. Therefore, the old culture is still used.

Careful attention should be given to all the glassware used in connection with cultures as well as the tests. It should always be neutralized. Bichlorid of mercury should be avoided as a disinfectant for the pipettes, bottles, tubes, etc.

The stock culture of *B. mallei* should be transplanted every ten to twelve days and incubated at 37°C. for two or three days. It should then be kept in the ice-box at 10°C.

The suspension for the tests is prepared in the following manner: Forty-eight hour cultures are used to inoculate agar slants of the potato-glycerin-veal agar described above. A good growth should be insured by inoculating the surface generously. After forty-eight hours incubation at 37°C. the growth is washed off with 0.85 per cent salt solution and killed by heating at 60°C. for two hours. No carbolic acid is added to this stock suspension which is of considerable density. It can be kept in the ice-box for two months or more in 100 cc. bottles, corked and capped, if handled with aseptic precautions. The tests are carried out with a fresh dilution of the stock suspension made by adding 0.85 per cent saline solution.

A sample dilution, which has been tested with known negative and positive sera, should always be kept in the ice-box and the fresh dilutions of the suspension compared with it on printed matter. The fresh suspension (dilution) is not filtered as the filtering seems to hold back something essential to the reaction; moreover, this is not necessary as it can be shaken up to a perfectly homogeneous fluid.

The tests are carried out in the following manner: A primary dilution of the serum 1 : 40 is made. Each tube receives varying

quantities of this dilution to which 3 cc. of the bacterial suspension (prepared as directed above) is added to make a final serum dilution of 1:500, 1:800, 1:1000, 1:1200, 1:1600, 1:2000. A known positive and a known negative serum are always used as controls with each test; also a control tube of the bacterial suspension without serum. The tubes, in copper racks are placed in a water-bath (37° to 42°C.) for two hours.

With this technic a reaction up to 1000 or more may be obtained in ten to twenty minutes; a positive reaction always appears in two hours. The tests may be kept over night in the ice-box and read again in the morning. The reaction, if complete is designated by two plus signs, incomplete by one plus, slight by plus minus, negative by a dash. The reaction is so clear cut there is no difficulty in interpreting it. One can watch the bacilli clump through the tube, then fall to the bottom in a white granular mass, leaving the supernatant fluid crystal clear in a complete reaction. There is no necessity to look for "buttons," "veils" or "films." It is either an agglutination as we see it with other organisms or it is not considered a reaction.

In reading the tests, a reaction is considered as *positive* which has double plus through 1:1600; as *suspicious* with double plus in 1:1200; as *doubtful* with double plus in 1:1000. Any reactions below 1:1000 are considered as negative.

It is essential to titrate the dilute bacterial suspension often with known positive and negative sera to be sure it is working. Tests should never be one with a suspension that has not been titrated the previous day.

The Department of Health of the City of New York has been employing the agglutination test for the diagnosis of glanders together with the complement fixation and ophthalmic mallein tests. During the last year, over 2000 sera were examined, which might have given us very rich material for arriving at definite conclusions as to the comparative value of the serodiagnostic and ophthalmic tests, checked by autopsy findings, had there been closer coöperation between the city and state veterinarians and the laboratory. Such coöperation should be based on a systematic method of recording full data in connection

with each horse in a central file. For a thorough study of glanders in any region, each horse should be marked and registered under an identifying number, as are automobiles, only in this case the number should be a permanent one which should pass with the horse when it is sold. The traditional methods of horse dealers might make this a difficult matter, yet, for economic reasons a law to that effect rigidly enforced, would be thoroughly worth while.

On only 180 horses was it possible to obtain complete data which I have endeavored to analyze. These horses fall into three groups as shown in tables 1, 2, 3.

| | |
|---|---|
| Street railway horses | 111 |
| Horses from an infected stable | 14 |
| Horses for slaughter | 55 |
| Total | 180 |

The street railway horses and those from a badly infected stable (125 in all) are considered together as at autopsy they all proved to have glanders with the exception of two. These two were in the infected stable and they were killed with the others because of their marked exposure to the disease. One of these horses, number 562, table 2, had been given vaccine; thus the number of 125 is reduced to 123 on which the percentages are based.

The 55 horses for slaughter were among those that received careful tests to determine their fitness to be used for food. These horses came under the supervision of Dr. L. Price, veterinarian of the Department of Health and the data are the most satisfactory of all.

Lately, we have been trying to confirm autopsy findings by pathological sections and this promises to be of great advantage. Many lesions which are suspicious of glanders do not prove so microscopically.

The bacteriological inoculation of suspicious material into guinea-pigs for the Strauss reaction does not prove of much aid, as, according to Miessner (3), it is successful in only 25 per cent of the cases. My own experience is in accordance with this

estimate. It was only in acute cases when *B. mallei* was isolated from the blood (human) or in horses with an acute infection of glanders that the Strauss reaction was obtained in the guinea-pig after inoculation with emulsified material from the lesions. All other inoculation tests resulted negatively.

In considering the horses, in tables 1 and 2, the necessity of applying all three tests stands out prominently. Of the 123 horses which proved at autopsy to have glanders, 122 were shown to be positive, suspicious or doubtful by one, two or three of the tests. Only one case (horse 123) was negative with all three tests—a failure of about 0.8 per cent.

The *agglutination* test showed 31 horses to be positive, 15 suspicious and 32 doubtful, making a total of 78 horses or 64.2 per cent. Thus it failed in 35.8 per cent of the cases, probably because most of the horses in this series were chronic cases. Three horses[3] were picked out by this test alone—the complement fixation and eye mallein tests being negative. These may have been acute cases.

The *complement fixation* test showed 74 horses as positive, 4 suspicious and 17 doubtful making a total of 93 horses or 75.6 per cent. This test failed wholly in 24.4 per cent of the cases. Four horses[4] were picked out by this test alone.

On the other hand the *mallein* test (ophthalmic except for 9 horses in table 2 on which subcutaneous mallein was used) gave 39 horses as positive, 65 suspicious and 2 doubtful making a total of 106 horses or 87.8 per cent. It failed in 12.2 per cent, but 15 horses[5] were picked out only by this test.

The grouping of suspicious and doubtful reactions with the positive ones is based on the interpretation that any serodiagnostic reaction not negative, should place an animal in the suspicious category to be subjected to close observation and frequent retests. That such a method is of great value is fully shown in tables 1 and 2.

[3] Table 1, horses 76, 1218, 1504.

[4] Table 1, horses 570, 1281, 1389, 2047.

[5] Table 1, horses 19, 449, 462, 494, 612, 621, 1143, 1146, 1310, 1374 and 1390. Table 2, horses 730, 737, 751, 763.

*Further analysis*

| | |
|---|---|
| Positive by all three tests | 9 horses* |
| Positive by agglutination and negative complement fixation | 6 horses† |
| Positive by complement fixation and negative by agglutination | 23 horses |
| Positive by mallein and negative by complement fixation and agglutination | 15 horses‡ |

* Table 1, horses 418, 506, 1776; table 2, 645, 683, 738, 746, 750, 757.

† Table 1, horses 163, 181, 605, 1218, 1360, 1504.

‡ Table 1, horses 19, 449, 462, 494, 612, 621, 1143, 1146, 1310, 1374, 1390; table 2, horses 730, 737, 751, 763.

Of the 55 horses tested before slaughter for food purposes (table 3), 4 horses[6] proved to have glanders. The complement fixation test failed to detect this fact in all 4 cases while the agglutination test failed in 3 of them and the ophthalmic mallein in only 1 (horse 2001) which the agglutination reaction picked out. The agglutination test gave 3 false "suspicious" reactions,[7] the complement fixation none, and the eye mallein 9.

In a set of horses such as are listed in tables 1 and 2, a large number of chronic cases may be expected and the agglutination tests positive in the least number of instances. On the other hand in a large number of sera examined during a whole year where different stables are tested there may be a great many cases of beginning infection or cases in the acute stages of the disease.

There were tested 1890 sera from various sources and among these the agglutination test gave positive and suspicious reactions in a larger proportion than the complement fixation. Unfortunately the eye mallein and final outcome of the cases could not be obtained.

Thus, with the *complement fixation*, about 14.5 per cent of the cases were either positive or suspicious while the *agglutination* registered 28.2 per cent. The doubtful reactions occurring with two tests were almost the same in number (complement fixation 300 and agglutination 291).

[6] Table 3, horses 2001, 2006, 3044, 3064.

[7] Table 3, horses 3027, 3040, 3053.

*With complement fixation test*

| | |
|---|---|
| Positive | 254 |
| Suspicious | 21 |
| Doubtful | 300 |
| Negative | 1315 |

*With agglutination test*

| | |
|---|---|
| Positive | 262 |
| Suspicious | 171 |
| Doubtful | 291 |
| Negative | 1166 |

*With both tests*

| | |
|---|---|
| Positive | 138 |
| Suspicious | 5 |
| Doubtful | 66 |
| Negative | 800 |

*With either one of the two tests*

| | |
|---|---|
| Positive | 378 |
| Suspicious | 187 |
| Doubtful | 525 |
| Negative | 800 |

These 1890 sera did not represent 1890 horses for about 15 per cent were retests. If it were not for these retests, a greater percentage of agglutination reactions would have been obtained for, after a lapse of time, the agglutinins disappeared in the horses retested, while the complement fixation antibodies remained, or appeared for the first time.

Another factor which influences the sero-diagnostic tests is the subcutaneous injection of mallein. It is generally held that six to eight weeks should elapse before the blood is taken for tests after mallein has been injected subcutaneously. Recently, in a large number of horses that had been given subcutaneous mallein, many showed four plus complement fixation and positive agglutination reactions, when retested about six weeks later. At the time of the first test these same horses had shown either doubtful or negative results. These horses are being kept under observation and time will show whether the high titers in both tests were due to the subcutaneous injection of mallein or to a fresh infection.

SUMMARY

1. A method has been devised whereby a prompt clear cut macroscopic agglutination for the diagnosis of glanders can be obtained in two hours.

2. Fifteen strains of *B. mallei* were tested and one obtained which is constant in its agglutinability with positive sera.

3. The best culture medium is glycerin-potato-agar (2.5 acid to phenolphthalein) carefully prepared (see text).

4. Great care must be used in the preparation of the stock suspension of *B. mallei* (see text). For the tests, *fresh dilutions* in 0.85 per cent salt solution are made from the stock suspension. *No carbolic acid is added* to the stock nor to the dilutions.

5. The agglutination test is valuable in the routine examination of horses. With this test nearly all early and acute cases can be detected and thus the spread of the disease can be prevented.

6. A negative reaction by a single agglutination test if not confirmed by the ophthalmic and the complement fixation tests does not prove the case negative; nor does a single negative result either by the complement fixation or ophthalmic tests. A positive reaction by any of the three methods is more significant than a negative one. All three tests must be performed in order to pronounce the horse negative.

7. In my experience with the present series of cases a reaction of less than 1: 1000 by agglutination (if the other two tests are negative and if there are no clinical symptoms) is not indicative of glanders in the horse. If the reaction is above a 1: 1000 the horse should be kept under observation; even if he does not show active glanders, the possibility of a carrier condition should be thought of. Isolation of such horses would be a very safe measure for preventing the infection among the other horses.

8. The bacteriological method of inoculating the guinea-pig with glanders material for the "Strauss" reaction is successful in only 25 per cent of cases (active lesions).

9. Autopsy findings, if possible, should be confirmed by pathological sections. A great many cases are pronounced glan-

ders on autopsy, which do not show typical lesions under the microscope.

10. All stables should be under the supervision of the City Health Department and glanders in horses should be put in the same category as typhoid, meningitis, etc. in man, if glanders in New York City is to be eradicated. Sera that come to the Health Department for diagnosis should be accompanied by a complete history of each horse. Follow up work should be possible.

11. Records of the three tests and autopsy findings should be accessible and some mark of identification for every horse should be devised by which all records of a horse can be compared. Only in some such way can a really scientific study of the disease be made and the right conclusions drawn.

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TABLE 1

*Street railways horses*

| HORSE NO. | COMPLEMENT FIXATION | AGGLUTINATION | OPHTHALMIC MALLEIN | REMARKS AND AUTOPSY |
|---|---|---|---|---|
| 36 | Pos. | Doubt. | Susp. | Glanders; lesions in lung |
| 155 | Pos. | Neg. | Susp. | Glanders; slight lesions in lungs and pleura |
| 164 | Pos. | Neg. | Pos. | Glanders; slight lesions in lungs |
| 285 | Pos. | Doubt. | Susp. | Glanders; lesions in lungs, pulmonary node and liver |
| 392 | Pos. | Susp. | Susp. | Glanders; slight lesions in lungs and pleura |
| 540 | Pos. | Pos. | Susp. | Glanders; lesions in lungs, pulmonary node and liver |
| 626 | Pos. | Susp. | Susp. | Glanders; lesions in lungs, pulmonary node and liver |
| 649 | Pos. | Pos. | Susp. | Glanders; lesions in lungs, pulmonary node and liver |
| 660 | Pos. | Pos. | Susp. | Glanders; lesions in lung |
| 791 | Pos. | Neg. | Susp. | Glanders; slight lesions in lung |
| 855 | Pos. | Pos. | Susp. | Glanders; lesions in lung and pleura |
| 969 | Pos. | Neg. | Susp. | Glanders; lesions in pulmonary node, lungs and pleura |
| 981 | Pos. | Pos. | Susp. | Glanders; lesions in lungs |
| 1034 | Pos. | Susp. | Susp. | Glanders; lesions in lungs |
| 1120 | Pos. | Neg. | Susp. | Glanders; slight lesions in lungs |
| 1186 | Pos. | Doubt. | Susp. | Glanders; lesions in lungs, pulmonary node and liver |
| 1204 | Pos. | Susp. | Susp. | Glanders; lesions in lungs, pulmonary node and liver |
| 1232 | Pos. | Doubt. | Susp. | Glanders; slight lesions in lungs |
| 1368 | Pos. | Neg. | Susp. | Glanders; lesions in lungs |
| 1370 | Pos. | Pos. | Susp. | Glanders; slight lesions in lungs and pleura |
| 1571 | Pos. | Neg. | Susp. | Glanders; slight lesions in lung, liver and pulmonary node |
| 19 | Neg. | Neg. | Pos. | Destroyed; glanders lesions found |
| 42 | Pos. | Neg. | Pos. | |
| 63 | Doubt. | Neg. | Susp. | |
| 76 | Neg. | Doubt. | Neg. | |
| 123 | Neg. | Neg. | Neg. | |
| 152 | Pos. | Susp. | Neg. | |
| 163 | Neg. | Pos. | Susp. | |
| 166 | Pos. | Neg. | ? | |
| 180 | Doubt. | Susp. | Pos. | |
| 181 | Neg. | Pos. | Susp. | |
| 192 | Pos. | Susp. | Pos. | |
| 275 | Pos. | Susp. | Neg. | |
| 296 | Pos. | Susp. | Susp. | |

TABLE 1—*Continued*

| HORSE NO. | COMPLEMENT FIXATION | AGGLUTINATION | OPHTHALMIC MALLEIN | REMARKS AND AUTOPSY |
|---|---|---|---|---|
| 341 | Pos. | Pos. | Susp. | |
| 351 | Neg. | Doubt. | Pos. | |
| 418 | Pos. | Pos. | Pos. | |
| 443 | Doubt. | Doubt. | Susp. | |
| 449 | Neg. | Neg. | Pos. | |
| 462 | Neg. | Neg. | Susp. | |
| 471 | Pos. | Doubt. | Susp. | |
| 484 | Doubt. | Susp. | Susp. | |
| 493 | Pos. | Doubt. | Pos. | |
| 494 | Neg. | Neg. | Susp. | |
| 506 | Pos. | Pos. | Pos. | |
| 541 | Doubt. | Doubt. | Pos. | |
| 570 | Doubt. | Neg. | Neg. | |
| 579 | Neg. | Doubt. | Susp. | |
| 583 | Pos. | Doubt. | Susp. | |
| 586 | Doubt. | Doubt. | Pos. | |
| 605 | Neg. | Pos. | Susp. | |
| 612 | Neg. | Neg. | Susp. | |
| 614 | Pos. | Susp. | Pos. | |
| 621 | Neg. | Neg. | Susp. | |
| 654 | Pos. | Doubt. | Neg. | Destroyed; glanders lesions found |
| 670 | Susp. | Doubt. | Susp. | |
| 679 | Pos. | Doubt. | Susp. | |
| 699 | Pos. | Pos. | Susp. | |
| 759 | Pos. | Doubt. | Susp. | |
| 818 | Doubt. | Pos. | Susp. | |
| 820 | Doubt. | Doubt. | Susp. | |
| 836 | Pos. | Neg. | Susp. | |
| 850 | Pos. | Neg. | Susp. | |
| 867 | Pos. | Doubt. | Susp. | |
| 871 | Neg. | Doubt. | Pos. | |
| 878 | Doubt. | Doubt. | Susp. | |
| 913 | Pos. | Susp. | Neg. | |
| 922 | Pos. | Neg. | Pos. | |
| 925 | Pos. | Doubt. | Pos. | |
| 962 | Pos. | Neg. | Pos. | |
| 963 | Pos. | Doubt. | Neg. | |
| 973 | Pos. | Doubt. | Neg. | |
| 1001 | Pos. | Neg. | Pos. | |
| 1018 | Neg. | Doubt. | Susp. | |
| 1055 | Pos. | Neg. | Pos. | |
| 1066 | Pos. | Neg. | Susp. | |

TABLE 1—*Continued*

| HORSE NO. | COMPLEMENT FIXATION | AGGLUTINATION | OPHTHALMIC MALLEIN | REMARKS AND AUTOPSY |
|---|---|---|---|---|
| 1072 | Pos. | Doubt. | Neg. | |
| 1074 | Pos. | Neg. | Susp. | |
| 1082 | Pos. | Doubt. | Pos. | |
| 1088 | Pos. | Pos. | Neg. | |
| 1121 | Susp. | Pos. | Pos. | |
| 1139 | Pos. | Pos. | Susp. | |
| 1143 | Neg. | Neg. | Susp. | |
| 1146 | Neg. | Neg. | ? | |
| 1161 | Doubt. | Susp. | Susp. | |
| 1163 | Pos. | Pos. | Susp. | |
| 1164 | Pos. | Neg. | Susp. | |
| 1178 | Pos. | Pos. | Susp. | |
| 1199 | Pos. | Doubt. | Pos. | |
| 1207 | Pos. | Susp. | Susp. | |
| 1215 | Susp. | Neg. | Susp. | |
| 1218 | Neg. | Pos. | Neg. | |
| 1219 | Doubt. | Doubt. | Susp. | |
| 1281 | Pos. | Neg. | Neg. | Destroyed; glanders lesions found |
| 1305 | Neg. | Doubt. | Susp. | |
| 1310 | Neg. | Neg. | Susp. | |
| 1360 | Neg. | Pos. | Susp. | |
| 1374 | Neg. | Neg. | Susp. | |
| 1389 | Pos. | Neg. | Neg. | |
| 1390 | Neg. | Neg. | Susp. | |
| 1393 | Doubt. | Doubt. | Susp. | |
| 1395 | Doubt. | Susp. | Pos. | |
| 1423 | Pos. | Neg. | Susp. | |
| 1504 | Neg. | Pos. | Neg. | |
| 1776 | Pos. | Pos. | Pos. | |
| 1784 | Doubt. | Neg. | Pos. | |
| 1880 | Pos. | Neg. | Pos. | |
| 1906 | Pos. | Pos. | Neg. | |
| 2031 | Pos. | Neg. | Pos. | |
| 2047 | Doubt. | Neg. | Neg. | |
| 2048 | Pos. | Pos. | Susp. | |

*Complement fixation:* Positive = 4+ and 3+; suspicious = 2+; doubtful = 1+; negative = ± and −.

*Agglutination:* Positive = 2+ in 1: 1600 or over; suspicious = 2+ in 1: 1200; doubtful = 2+ in 1: 1000; negative = any reactions below 1: 1000.

TABLE 2

*Horses from infected stable*

| HORSE NO. | COMPLEMENT FIXATION | AGGLUTINATION | OPHTHALMIC MALLEIN | SUBCUTANEOUS MALLEIN | REMARKS AND AUTOPSY |
|---|---|---|---|---|---|
| 645 | Pos. | Pos. | | Pos. | Glanders; lesion in pulmonary node |
| 683 | Pos. | Pos. | Pos. | | Glanders; lesion in pulmonary node |
| 730 | Neg. | Neg. | | Pos. | Glanders; lesion in pulmonary node |
| 737 | Neg. | Neg. | | Pos. | Glanders; lesion in pulmonary node |
| 738 | Pos. | Pos. | Pos. | | Glanders; lesions in pulmonary node |
| 745 | Susp. | Neg. | | Pos. | Glanders; lesion in pulmonary node |
| 746 | Pos. | Pos. | | Pos. | Glanders; lesions in pulmonary node |
| 747 | Doubt. | Doubt. | | Pos. | Glanders; pulmonary and nasal lesions |
| 750 | Pos. | Pos. | Pos. | | Glanders; lesion in pulmonary node |
| 751 | Neg. | Neg. | | Pos. | Glanders; nasal lesion only |
| 757 | Pos. | Pos. | | Pos. | Glanders; lesions in pulmonary gland |
| 763 | Neg. | Neg. | | Pos. | Glanders; pulmonary and nasal lesions |
| 762 | Susp. | Neg. | Neg. | Neg. | No glanders lesions; caseous material in one bronchus |
| 562 | Neg. | Pos. | | Pos. | Had been given vaccine. No glanders lesions found |

TABLE 3

*Horses for slaughter*

| HORSE NO. | COMPLEMENT FIXATION | AGGLUTINATION | OPHTHALMIC MALLEIN | INTRAPALPEBRAL MALLEIN | REMARKS AND AUTOPSY |
|---|---|---|---|---|---|
| 2001 | Neg. | Pos. | Neg. | | Lesions in lung. Pathological sections showed *glanders* nodules |
| 2002 | Neg. | Neg. | Neg. | | Cyst in liver |
| 2003 | Neg. | Doubt. | Neg. | | Heated pneumonic lesions |
| 2004 | Neg. | Doubt. | Neg. | | Emphysema; multiple cysts in liver |
| 2005 | Neg. | Neg. | Neg. | | No autopsy |
| 2006 | Neg. | Neg. | Pos. | Pos. | *Glanders:* encapsulated lesions with gelatinous infiltrated pneumonic areas |
| 2007* | A.C. | Doubt. | Neg. | | No glanders lesions |
| 3008 | Neg. | Doubt. | Neg. | | Few calcified parasitic lesions in liver |
| 3009 | Neg. | Neg. | Neg. | | Extensive parasitic lesions in liver |
| 3010 | Neg. | Neg. | Neg. | | Lungs—alveolar emphysema |
| 3011 | Neg. | Neg. | Neg. | | No glanders lesions |
| 3012 | Neg. | Neg. | Neg. | | No glanders lesions |
| 3013 | Neg. | Neg. | Susp. | Neg. | No autopsy. Submaxillary gland tubercular? |
| 3014 | Neg. | Neg. | Neg. | | No glanders lesions |
| 3015 | Neg. | Neg. | Neg. | | Multiple cysts in liver; adhesions lung |
| 3016* | A. C. | Neg. | Neg. | | Encapsulated area of parasites |
| 3017 | Neg. | Neg. | Neg. | | Parasitic lesions in liver |
| 3018 | Neg. | Neg. | Susp. | | Parasitic lesions in lungs |
| 3019 | Neg. | Doubt. | Neg. | | No glanders lesions |
| 3020 | Neg. | Neg. | Neg. | | Calcified brown area in lung; parasitic liver |
| 3021 | Neg. | Neg. | Neg. | | Liver parasitic; organized haematoma in coecum |
| 3022 | Neg. | Neg. | Neg. | | Fibrous nodule in lung |
| 3023 | Neg. | Neg. | Neg. | | Parasitic liver |
| 3024 | Neg. | Neg. | Neg. | | No glanders lesions |
| 3025 | Neg. | Neg. | Neg. | | Parasitic liver |
| 3026 | Neg. | Neg. | Susp. | | Chronic pleuritis |
| 3027 | Neg. | Susp. | Susp. | | Cirrhosis of liver; endocarditis |
| 3028 | Neg. | Neg. | Neg. | | No lesions of glanders |
| 3029 | Neg. | Doubt. | Neg. | | Liver cystic. No glanders lesions |
| 3030 | Neg. | Neg. | Susp. | | Suspicious lesions in lung. Pathological sections showed no glanders lesions microscopically |
| 3031 | Neg. | Neg. | Neg. | | No lesions of glanders |
| 3032 | Neg. | Neg. | Neg. | | No glanders lesions |

* Mules.

TABLE 3—*Continued*

| HORSE NO. | COMPLEMENT FIXATION | AGGLUTINATION | OPHTHALMIC MALLEIN | INTRAPALPEBRAL MALLEIN | REMARKS AND AUTOPSY |
|---|---|---|---|---|---|
| 3033 | Neg. | Neg. | Neg. | | No glanders lesions |
| 3034 | Neg. | Doubt. | Neg. | | Parasitic lesions in lungs and liver |
| 3035 | Neg. | Neg. | Neg. | | No glanders lesion |
| 3036 | Neg. | Neg. | Neg. | | No glanders lesions |
| 3037 | A. C.† | Neg.† | Susp. | | Purulent broncho-pneumonia |
| 3038 | Neg. | Neg. | Susp. | | Lymphatic nodes—calcified lesions; liver—parasitic lesions. Microscopic sections negative |
| 3039 | Neg. | Neg. | Neg. | | Cirrhotic liver |
| 3040 | Neg. | Susp. | Neg. | | Liver—calcified lesions |
| 3041 | Neg. | Neg. | Neg. | | Liver—parasitic lesions |
| 3042 | Neg. | Neg. | Neg. | | Liver—parasitic lesions |
| 3043 | Neg. | Neg. | Neg. | | No glanders lesions |
| 3044 | Neg. | Pos. | Pos. | | Typical *glanders* lesions |
| 3045 | Neg. | Neg. | Neg. | | Pigmented deposits in lungs |
| 3046 | Neg. | Neg. | Neg. | | Parasites in liver |
| 3047 | Neg. | Neg. | Neg. | | Parasites in liver |
| 3048 | Doubt. | Doubt. | Susp. | | Lungs, spleen, liver and nodes—suspicious lesions. Pathological sections show no glanders lesions |
| 3049 | Neg. | Neg. | Neg. | | Parasitic nodules in liver |
| 3050 | Neg. | Neg. | Neg. | | Parasitic nodules in liver |
| 3051 | Neg. | Pos. | Neg. | | Suspicious lesions in lungs. Pathological sections show no glanders lesions |
| 3052 | Neg. | Doubt. | Neg. | | Slight adhesions of diaphragm |
| 3053 | Doubt. | Susp. | Susp. | | Slightly suspicious lesions in lungs. Gelatinous infiltration of abdominal wall. Pathological sections show no glanders lesions |
| 3055 | Neg. | Neg. | Neg. | | Parasitic liver and lung |
| 3064 | Neg. | Neg. | Pos. | | *Glanders* lesions in lung |

† Two tests.

# INDEX TO VOLUME III



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