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On the Isolation of Antibodies from immunized Serum-globulin.

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Abstract

1. It is possible to isolate the immune bodies from the globulin fraction, which was obtained by the salting out with ammonium sulphate or electro dialysis, by the biologic method. The rate of isolation is almost the same as the rate of the isolation by the biologic method. 2. The isolated serum from the globulin fractions by the combination of the physical or the chemical with the biologic method, has less antigenic and nitrogen contents than the isolated serum by the biologic method alone; especially the isolated immune substance by the combination of the physical with the biologic method has the least antigenic contents. 3. The best results are obtained in the physiologic salt solution at 65°C, for the isolation of precipitin by means of the combination of the physical with the biologic method. It is a pleasure to express my indebtedness to Prof. Ogata for the encouragement and valuable suggestions which he has given. I am also indebted to Dr. Sunouti for various assistance he has offered in the preparation of this work.

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**On the Isolation of Antibodies from immunized
Serum-globulin.**

By

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The problem of purifying or isolating antibodies in antiserum from the immunized animals has a particular, direct and important significance for the practical medicine besides its theoretic importance. Consequently many attempts to isolate or purify the antibodies from the antisera have been made up to this time, and moreover the results of the chemical examinations of the immune substances have been reported.

The purification or isolation of an antibody may be accomplished, as it has been learned by experimentation, principally by three methods, namely the chemical, the physical and the biological method.

The chemical method: This method involves the precipitation of the antibody together with certain of the other serum constituents by the use of certain chemicals and dissolving of the precipitates. In 1891 *Tizzoni* and *Cattani*⁴⁷⁾ treated the tetanus antitoxin by half saturating it with ammonium sulphate and proved that the antitoxin is found in the globulin fraction of immune sera. By treating the immune serum with ammonium sulphate, *Widal* and *Sicard*⁴⁸⁾ found the bacterio-agglutinin in the serum-globulin. This phenomenon was tested again later by *Wintenberg*⁴⁹⁾. Thus, by treating certain immune sera with ammonium sulphate, *Pick*³⁶⁾ demonstrated that from the diphtheria antiserum produced in the horse, the antitoxin is precipitated with the pseudo-globulin, whereas from the antiserum of the goat it is precipitated with euglobulin. Using ammonium sulphate, *Brieger* and *Kraus*³⁾ state that they succeeded in eliminating 75% of the total nitrogen from diphtheria serum, while preserving the original power. By means of a half saturation with ammonium sulphate, and treatment of the precipitate with a saturated solution of sodium chloride containing acetic acid, *Gibson*¹²⁾ was able to concentrate the number of units of antitoxin per cubic centimeter from 200 to 500. He applied this method of concentration not only to diphtheria antitoxin but also to the concentration of agglutinin with his co-worker¹³⁾.

By these methods, the antibody is with certainty to some degree, concentrated,

but they do not satisfy our biological interest because we are far from the purification of antibodies in these cases.

The physical method: This method involves the use of some physical means, as the name indicates such as, electro dialysis, ultrafiltration etc., by which the antibody is concentrated and isolated. In this method, it depends upon the colloidal comprehension of immune problems, as it was first developed by *Landsteiner*²³⁾ then *Hirschfeld* and *Klinger*¹⁵⁾, *Pauli* and his co-worker³⁸⁾ and *Michaelis* and *Rona*²⁹⁾. Thus the nature of change which determines the specific action of immune serum depends upon the colloidal change of serum protein and first of all the globulin. On the supposition that the alteration of the dispersion of a colloid would always depends on the size of the molecule the acceptance of the colloidal property for immune sera led *Bechhold*⁴⁾ to examine and isolate many colloids by ultrafiltration. By this method, *Giemsa* and *Godoy*¹⁴⁾ were first to obtain a three fold efficacious fraction from antitoxin. *Henseval*¹⁶⁾ established that a little pseudoglobulin and antitoxin only pass through in the filtration with collodium membranes under pressure, and the larger part of the antitoxin remains on the filter. *M. and N. Stern*⁴²⁾ also were able to restrain the globulin on the filter.

In 1852 *Panum*³⁹⁾ reported that the colloidal serum proteins flocculate when a small amount of electrolytes present, and for their dispersion it is necessary to deplete them by dilution or dialysis. The precipitating fraction was shown by *Aronstein*²⁾ and *Schmidt*⁴³⁾ to be euglobulin. The application of this method to the purification of immune serum has not been made extensively because of the many practical difficulties and disadvantages in its use. *Dhéré*⁹⁾ showed that the use of the electrolytic process causes a much more rapid flocculation of the protein.

*Pauli*³⁷⁾ succeeded in isolating completely the hydrophilous albumins from hydrophobous globulins by electro dialysis. *Ruppel* and his collaborators⁴¹⁾ reported as to the general distribution of antibodies in both globulin fractions which are separated by electro dialysis. Using an apparatus of two dialysing thimbles, *A. Lock* and *F. Hirsch*²⁵⁾ accomplished the isolation of immune isoelectric pseudoglobulin (amboceptor) of high titre by means of an electro dialytic procedure from serum. *Adolf*¹⁾ observed that the antitoxin was in the globulin precipitate when the immune diphtheria serum was electro dialysed until the supernatant fluid had practically the same electric conductivity as distilled water. *Otto* and *Sukiennikowa*³¹⁾ demonstrated that the toxin combines only with the pseudoglobulin fraction and not with the albumin, in electro dialytic separation of antimuttonserum while the hemolytic amboceptor is found in both globulins. Then *Otto* and *Sirakawa*³²⁾ reported that the anaphylactic body of reaction and the precipitin were made to combine with several protein fractions by electro dialytic separation. *Ornstein*³⁴⁾ experimented and found that antitoxin is purified and concentrated by electro dialysis. *Otto* and *Iwanoff*³³⁾ established the fact that the immune fractions prepared by electro dialytic procedure show the specificity of fraction. *H. Schultze* and *H. Gross*⁴⁴⁾ reported that they fractionated and concentrated the hemolysin by the electro dialytic procedure. *Wernicke*⁵⁰⁾, and *Wernicke* and *Modern*⁵¹⁾, applying this method, succeeded in obtaining the concentrated diphtheria toxin.

Laubenheimer and *Vollmar*²⁶⁾ applied, for the isolation of hemolysins, an electro-filtration which was published by *Bechhold* and *Rosenberg*⁵⁾ as a method for the purification of colloids.

The biologic method: It was early recognized that an antibody would combine with its homologous antigen and in a case of the formed antigen, a combination could be washed free from most of the other serum constituents. This method involves the use of homologous antigen with which an antibody combines, and afterward the antibody is removed from its attachment.

The recovery of substances which become absorbed from immune sera by suspensions of homologous antigen was accomplished first by *Hahn and Tromsdorf*¹⁷⁾, in 1900. The suspension of bacteria was left in contact with homologous immune sera until the complete agglutination took place. The agglutinated sediment was then separated, thoroughly washed with isotonic salt solution, and resuspended in warm N/100 acid. A clear, agglutin-containing extract was obtained, which none of the accepted tests for the presence of protein showed positive result.

*Landsteiner*²³⁾, and *Landsteiner and Jagic*²⁴⁾ washed the blood cells, agglutinated by abrin in many changes of salt solution and then by treating with a salt solution at 42°C to 45°C, they were able to demonstrate that a certain amount of the abrin was split off from the combination and rendered reavailable. They also proved that the agglutinated erythrocytes washed with the physiological salt solution, gave off some of the agglutinin again (when they were brought into a physiological salt solution and that the quantity of liberated agglutinins is proportional to the temperature applied to the medium).

*Von Lieberman and Fenyvessy*²⁷⁾, employing the rabbit's immune serum against the pig's corpuscles, digested the sensitized antigen with N/100 HCl in a salt solution. These extracts were precipitated with alkali and the precipitate was dissolved and purified with ether. The final solution contained both agglutinin and hemolysin, but showed no albumin-reaction with the most delicate test.

*Spät*⁴⁵⁾, also working with cholera vibrio and cholera immune serum, digested the sensitized antigen in a salt solution at 42°C and demonstrated the splitting off of bactericidal antibody.

*Bail and Tuda*⁶⁾, working with cholera vibrio immune serum, digested the sensitized antigen in a salt solution at 56°C and were able to show that bactericidal antibody was reavailable, but not the agglutinin.

*Morgenroth*²⁸⁾ noticed that when the red blood cells sensitized with hemolytic amboceptor, were brought in contact with the unsensitized cells, some of the amboceptors were transferred to the fresh cells.

*Chickling*⁸⁾ showed that the extracts of the precipitates formed in the anti-pneumococcus-serum by the addition of bactericidal precipitinogen, not only exert an inhibiting influence on the growth of virulent pneumococci in vitro, but the extracts contain the agglutinins and precipitins so that they protect the susceptible animals as efficiently as the original antipneumococcus-serum.

Using the rabbit's serum containing the immune hemolytic amboceptor against sheep's red blood cells, *Kosakai*²²⁾ contributed an important paper to this problem of isolation. He found that the dissociation could be brought about in a solution of sugar containing no electrolyte, and attempted to work out the governing factors of the dissociation such as the influence of temperature and volume. *Huntoon* and his collaborator¹⁹⁾ have obtained the extracts containing the antipneumococcus immune substance in this way, approximating the antibody content in the best immune sera, and containing so little a serum protein that 5 cc of it sensitized the

guinea pig's only irregularly by a subsequent injection of the horse serum. His best preparations contain 860 times less nitrogen per immune unit than the immune serum from which they were derived. *Ottenberg* and *Stenbuck*³⁵⁾ obtained anti-typhoid immune extracts of a similar, remarkable degree of purity.

In the experiences of *A. Lock* and *F. Hirsch*²⁵⁾, the union of antigen-immune substance may be broken by a process of ether extraction, permitting the recovery of immune substance preparations which are almost entirely free from contamination with the dispersed antigen. The ether extraction causes a shift in the isoelectric range of lipoid containing the antigen from a value identical with that of the specific immune substance to a value sufficiently removed to permit the loosening the union of the anti-immune substance and the precipitin of the antigen residue. *Weinstein*⁵²⁾ found in the precipitates from typhus bacteria extract, the agglutinin and bactericidal antibody which are again removable.

*Huruhata*²⁰⁾ succeeded in isolating hemagglutinin and *Ogata*³⁰⁾ bacteri-agglutinin by the medium of saccharose solution and *Miwa*⁵³⁾ was able to dissociate typhus-agglutinin in distilled water. Later *Kageyama*²¹⁾ met with a success in the isolation of *Forssman's* antibody and *Sunouti*⁴⁶⁾, of serum precipitin of which the isolation was very difficult. Recently *Haiku*¹⁸⁾ isolated bacteri-precipitin.

In this paper the author reports the result of the isolation of several antibodies by the methods noted above and moreover studied the isolation by the combination of these two methods.

Experimental material.

Antibody : The rabbits were inoculated intravenously with ox serum, coli emulsion and red blood cells of the sheep and hen.

Precipitin : The rabbits were immunized intravenously three times with 0.5 cc of ox serum every 4 days and the highly immunized antioxserum was obtained by repeating this method several times at two week intervals. The titre of precipitin was determined on the 7th day after the last injection and the antiserum above 1 : 500 of diluting titre of immune serum by *Ogata's* method was used for experiments.

Agglutinin : An eighteen hour old agar slant culture of *B. coli* was emulsified in 10 cc of physiologic salt solution and killed by heating in a water bath at 60°C for two hours. The agglutinin was obtained by immunizing intravenously with an injection of 1 cc first and second with 2 cc, and third with 3 cc, of the coli-emulsion at three day intervals. The titre of the antiserum thus prepared was determined on the 7th day after the last injection, and the antiserum above 1 : 12,800 of titre was used as the immune serum.

Hemolysin : The rabbits were immunized intravenously three times with 5 cc of suspension of 10% washed sheep's corpuscles every 4 days. This method of immunization was repeated several times at two week intervals and the titre of the antiserum was determined on the 7th day after the last injection. The antiserum of above 1 : 1,000 was used for the use of isolation.

Hemagglutinin : The hemagglutinin was obtained by immunizing intravenously the rabbits many times with 5 cc of 10% washed chicken's corpuscles every 4 days

as it was done for obtaining the hemolysin. The antiserum of above 1 : 500 was used for the experiments.

Antitoxin : The diphtheria antitoxin which was supplied by the Infectious Diseases Research Institute in Tokyo was employed for the experiments.

Antigen : As the antigen, the ox serum was used for the isolation of precipitin ; the coli-emulsion, for the isolation of agglutinin and the stroma of blood cells of the sheep, for the isolation of hemolysin ; and the stroma of red blood cells of the chickin for the isolation of hemagglutinin.

Experimental procedure.

Precipitin test : There are two methods to titrate a precipitin titre of an immune serum. The one is *Uhlenhuth's* method and the other, *Ogata's* method³⁰⁾ of diluting the immune body. In this investigation, the *Ogata's* method was employed for the determination of precipitin titre. This method was devised first by *Ogata* and later together with his students proved that there is a definite quantitative relation of precipitin. This procedure is as follows : an antigen which is diluted with physiologic salt solution in descending manner, is stratified on the antiserum which in turn is diluted with 1% gum physiologic salt solution in the same manner. Then the reaction takes place between them and the strongest is found at a certain degree of the antigen dilution. This strongest part of the reaction of antigen dilution is called the 'binding zone' of the immune body, and at this binding zone, the reactive degree of the highest dilution of the immune body is called the 'diluting titre' of the immune serum by *Ogata*.

Agglutination test : Three platinum loopful (3 mm) of 18 hour old agar slant culture of *B. coli* were suspended in 10 cc of physiologic salt solution and killed by heating at 60°C in a water bath for two hours, were employed as agglutino-gen. Four drops of the coli-emulsion were added to 1 cc of the immune serum which was diluted with physiologic salt solution. This mixture was allowed to stand for two hours at 37°C in an incubator and left at the room temperature till the following morning when the results were read with an agglutinoscope.

Hemolysis test : Fresh sheep blood is defibrinated and centrifuged and the cell sediment is washed three times with the physiologic salt solution. Anti-sheep-corpuscle-serum was inactivated by heating in a water bath at 56°C for half an hour and then diluted with the physiologic salt solution. One cc of 2.5% sheep corpuscle solution and 1 cc of double doses of complement titre of a guinea pig's serum are added to each 1 cc of the antiserum. These mixtures were placed in an incubator at 37°C for two hours and then removed to an ice-chest and the reading was made the next morning.

Hemagglutination test : Four drops of 2% chickin's corpuscles suspension are added to 1 cc of antiserum which is diluted with the physiologic salt solution in the descending manner. This mixture was allowed to stand in an incubator at 37°C for two hours and set out at room temperature till the next morning when the determination of the titre was made with the naked eye.

Antitoxin test : In order to standardize a serum it is necessary to know the strength of the toxin which is so variable. The toxin is standardized against

a standard antitoxin which is supplied by the Infectious Diseases Research Institute at Tokyo. By mixing the varying quantities of toxin with one unit of this standard antitoxin and injecting these into a 250 g guinea pig, the L+ (limes death) dose is obtained, which is the dose of toxin required to kill a guinea pig in four days with one unit of antitoxin. The standard antitoxin which was supplied by the Institute and used in this work, possessed 600 units. I examined the L+ of toxin, which was also furnished by the same Institute, as follows.

One antitoxin unit +	0,2475 cc toxin	=	Edema, sometimes late paralysis.
"	0,2500	"	= Acute edema and sometimes death.
"	0,2525	"	= Always acute death about 4th day.
"	0,2550	"	= Death from second to third day.
"	0,2575	"	= Death about the second day.

Here the L+ dose is 0,2525 cc.

Having determined the L+ dose of the toxin, a series of 6 to 8 guinea pigs are injected with this constant dose of toxin and an increasing amount of the corresponding antitoxin serum: for example, No. 1 would receive 0,001 cc of serum; No. 2, 0,002; No. 3, 0,003; No. 4, 0,004; No. 5, 0,005; No. 6, 0,006. If at the end of the 4th day, No. 1, 2, 3 and 4 were dead and No. 5 and No. 6 were alive, the serum would contain 250 units of antitoxin in a cubic centimeter. The immunity unit is that quantity of antitoxin which will neutralize 100 times the minimal lethal dose for a 250 g guinea pig. Thus the strength of a purified antitoxin was determined by the same procedure.

Experiment.

The isolation of an immune body by salting out with ammonium sulphate.

This method is the chemical one, as previously mentioned, namely by half saturation with ammonium sulphate, the immune body in serum is precipitated with some kind of globulin. Four cc of the antiserum were half saturated with ammonium sulphate and then the flocculation soon occurred. The milkwhite fine precipitate of the serum protein was filtered off on a small *Buchner's* funnel and the precipitate on the funnel was dissolved in physiologic salt solution to the original volume. This solution of isolated immune body was titrated by the methods above mentioned.

The isolation of an immune body by electro dialysis.

The removal of electrolyte from a sol by means of an electric current has been termed by *Dhéré*⁹⁾ and *Ruppel*⁴¹⁾ as electro-osmose and by *Pauli*³⁷⁾ as electro dialysis. The utilization of the electric current in dialysis causes a speeding up of the migration of the diffusible ions through membranes.

For electro dialysis of antiserum *Pauli's* apparatus was used. It consists of three compartments of glass. The outside compartment are filled with distilled water in which the positive and negative platinum net-electrodes are placed. The parchment membranes are inserted between the electrodes and the middle compartment. The middle compartment of parchment membranes and glass receives antiserum, which is collected in the morning before the breakfast and centrifuged and diluted to 1/10 with distilled water.

The electrodes are connected with the terminal of 100 volt direct current. Two 10 watt lamps and a miliamperemeter are placed in the circuit to avoid strong degeneration of the immune body by heating. The apparatus was always kept under 30°C and 10 ma. pro quadrat centimeter. pH was determined electrometric with quinhydrone electrode and the value of pH was 3.9 to 4.9.

The water is introduced through two tubes and the outflow takes place through other two tubes. The rapid flow of water through each electrode compartment not only cools the apparatus but carries away the products of electrode reaction.

The precipitate by electro dialysis was dissolved to the original volume in physiologic salt solution and its titration was determined.

The Table 1 shows an experiment demonstrating the relations of ratio among many purified immune bodies (agglutinin, hemolysin, hemagglutinin, antitoxin and precipitin), isolated by the above-mentioned two methods, against their corresponding original serums. As Table 1 shows, the immune bodies salted out with ammonium sulphate, are commonly 50% hemagglutinin, sometimes 100% precipitin, 20% agglutinin or 25% hemolysin, compared with the original serum. The relations of the isolations of the immune bodies precipitated by electro dialysis are almost the same by the chemical method. The ratios of isolated immune bodies are commonly 50% precipitin and hemagglutinin, sometimes 25% agglutinin and hemolysin, or 10% diphtheria antitoxin, compared with the original serum.

Table 1. A. The isolation of immune bodies by salting out with ammonium sulphate.

Antiserum.	Original serum.	Antigen.	Dilution of fraction.	Rate of isolation.
Precipitin.	D. T. 1 : 500 B. Z. 1 : 250	Ox serum.	D. T. 1 : 500 B. Z. 1 : 250	100%
Agglutinin.	1 : 25,000	B. coli.	1 : 5,000	20%
Hemolysin.	1 : 1,000	Sheep's red cell.	1 : 250	25%
Hemagglutinin.	1 : 500	Hen's red cell.	1 : 250	50%

Table 1. B. The isolation of immune bodies by electro dialysis.

Antiserum.	Original serum.	Antigen.	Dilution of fraction.	Rate of isolation.
Precipitin.	D. T. 1 : 1,000 B. Z. 1 : 500	Ox serum.	D. T. 1 : 500 B. Z. 1 : 500	50%
Agglutinin.	1 : 12,800	B. coli.	1 : 3,200	25%
Hemolysin.	1 : 1,000	Sheep's red cell.	1 : 250	25%
Hemagglutinin.	1 : 2,500	Hen's red cell.	1 : 1,000	50%
Diphtheria antitoxin.	600 units.	Diphtheria toxin.	60 units.	10%

Notes: D. T. = Diluting titre of immune serum.

B. Z. = Binding zone.

The ratios of the isolations of the immune bodies depends not on the kind of antiserum, but on other conditions.

The isolation of the immune bodies by the combination of the chemical or the physical method with the biologic methods.

In the former chapter the isolation of the immune bodies by both the chemical and physical methods were tried, and further the purification of the antibodies by the combination of the chemical or the physical with the biologic methods were carried out.

The isolation of the antibodies by the combination of the chemical with the biologic method.

Precipitin.

Antigen: Ox serum was employed as the antigen of sensitization.

Sensitization: Eight thousandth cc of ox serum was mixed with 4 cc of antitoxin serum which was salted out with ammonium sulphate and the precipitin power of the serum was 1:500 after salted out by *Ogata's* method. This mixture was shaken and digested in an incubator at 37°C for two hours. During the incubation it was frequently shaken and then placed in an ice-box. On the following day it was centrifuged and the supernatant fluid was pipetted off for testing under the designation, 'supernatant fluid'.

Washing: The sediment was washed three times with the physiologic salt solution by centrifugation to remove all trace of free antibody and the last supernatant fluid was saved for testing under the designation 'washed fluid'.

Dissociation: The washed sediment was emulsified in 4 cc of the salt solution, and was placed in a water bath at 65°C for half an hour, during these hours it was shaken vigorously at a five minutes interval. After the centrifugation the supernatant fluid was removed for testing under the designation 'isolated serum'. The results obtained are expressed as precipitating units in the following table.

Table 2. The dissociation of immune bodies by the combination of the chemical and the biologic method.

Original Antiserum.	Dilution of fraction.	Antigen.	Super-natant fluid.	Rate of absorption.	Isolated serum.	Rate of isolation.
Precipitin. D.T. 1:500 B.Z. 1:250	D.T. 1:500 B.Z. 1:250	Ox serum.	D.T. 1:160 B.Z. 1:250	3/5	D.T. 1:32 B.Z. 1:250	1/10
Agglutinin. 1:25,000	1:5,000	B. coli.	1:1,000	4/5	1:500	1/8
Hemolysin. 1:1,000	1:250	Sheep's red cell.	1:100	2/3	1:16	1/6
Hemagglutinin. 1:500	1:250	Hen's red cell.	1:100	2/3	1:32	1/5

As Table 2 shows, it is seen that the antibody of precipitin which was united with ox serum after salting out with ammonium sulphate, is separated in the physiologic salt solution.

In the table, the rate of absorption means the precipitin units which are combined with ox serum during the sensitization, that is, the number present in the solution of fraction minus the number present in the absorbed serum divided by the precipitin units in the solution of fraction. The rate of isolation means the ratio of isolated antibody to combined antibody units.

The immune serum which had a precipitin titre of 1:500, was used for isolation. After salting out with ammonium sulphate the titre is also the same as the original one (1:500). The supernatant fluid shows a titre of 1:160 that means that 340 units of precipitin combine with ox serum after the sensitization. The third washed fluid contains no trace of precipitin and the isolated serum yields a titre of 1:32. This shows that 1/10 of the absorbed precipitin is separated from the sensitized serum during the digestion in the physiologic salt solution.

Agglutinin.

Antigen: Five agar slant cultures of *B. coli* which were cultured for 18 hours were suspended in the physiologic salt solution and killed by heating at 60°C in a water bath for two hours. They were then centrifuged and washed three times with the physiologic salt solution.

Sensitization: The washed *B. coli* sediment was added to 5 cc of anticoli serum which was precipitated with ammonium sulphate and the agglutinin titre which was reduced from 1:25,000 to 1:5,000 after salting out. This mixture was placed in an incubator at 37°C for two hours after shaking, and during this incubation it was frequently shaken. They were then placed in an ice-box for 18 hours. These mixtures were centrifuged and the supernatant fluid was saved for testing under the designation 'supernatant fluid'.

Washing: The sediment was washed three times with the physiologic salt solution to remove all trace of free antibody by centrifugation.

Dissociation: The washed sediment was emulsified in the physiologic salt solution of the original volume. The emulsion was placed in a water bath at 65°C for 30 minutes, during this period it was vigorously shaken every five minutes. The precipitates were sedimented by centrifugation and the supernatant fluid saved for testing under the designation 'isolated serum'.

As Table 2 shows, the first antiserum gives a titre of 1:25,000 and the titre was reduced to 1:5,000 after precipitating with ammonium sulphate. The washed fluid contains no trace of the antibody and the supernatant fluid yields a titre of 1:1,000 and the isolated serum, a titre of 1:500, so the rate of absorption is 4/5 and the rate of isolation 1/8.

Hemolysin.

Antigen: The red blood cells are hemolytic at 65°C, so I used stroma instead of the red blood cells as the antigen of sensitization. The stromas were prepared as follows; the defibrinated, centrifuged sheep's corpuscles were laked by distilled water and washed three times with the physiologic salt solution by centrifugation and then the washed ash-coloured stromas were removed.

Sensitization: Two cc of the prepared stromas were added to 4 cc of anti-sheep's-corpuscle-serum which was salted out with ammonium sulphate, and of which the hemolytic power was reduced from 1:1,000 to 1:250 after the procedure. This mixture was placed in an incubator at 37°C for two hours, during these hours it was

shaken and then put in an ice-box till the next morning. It was centrifuged and the supernatant fluid removed for testing under the designation 'supernatant fluid'.

Washing: The sediment was washed three times with the physiologic salt solution to remove all free antibodies by centrifugation and the last supernatant fluid was removed for testing under the designation 'washed fluid'.

Dissociation: The washed sediment was emulsified in physiologic salt solution and heated in a water bath at 65°C for half an hour, during this period it was vigorously shaken every 5 minutes. The emulsion was soon recentrifuged and the supernatant fluid was pipetted off for testing under the designation 'isolated serum'.

As Table 2 shows, the antiserum employed in this experiment yielded a titre of 1:1,000. The titre was reduced by salting out with ammonium sulphate to 1:250 and the isolated serum to 1:16 and the supernatant fluid to 1:100; so the rate of absorption is $\frac{2}{3}$ and the rate of isolation $\frac{1}{6}$.

Hemagglutinin.

Antigen: The stromas of a hen's red blood cells prepared in the same way as the sheep's stromas, were employed as the antigen of sensitization.

Sensitization: Four cc of the antiserum of hemagglutinin, the titre of which was reduced from 1:500 to 1:250 after salting out with ammonium sulphate, was sensitized with 2 cc of the prepared stroma. The mixture was digested in an incubator at 37°C for two hours, during which period it was shaken. Then it was placed in an ice-box till the following morning and centrifuged and the supernatant fluid was pipetted off for testing under the designation 'supernatant fluid'.

Washing: The centrifuged sediment was emulsified in the physiologic salt solution and washed three times with the same and the last supernatant fluid was saved for testing under the designation 'washed fluid'.

Dissociation: The washed sediment was emulsified in the physiologic salt solution and heated in a water bath at 65°C for half an hour, during which period it was shaken frequently at intervals of every five minutes. Then it was soon centrifuged and the supernatant fluid was saved for testing under the designation 'isolated serum'.

As Table 2 shows, the first antiserum which had a titre of 1:500, was used for isolation. The titre was reduced to 1:250 after the salting out and the supernatant fluid to 1:100 and the isolated serum

to 1:32, so the rate of absorption is $\frac{2}{3}$ and the rate of isolation $\frac{1}{5}$.

The isolation of precipitin by the combination
of the physical method with
the biologic method.

The isolation of many immune bodies by the combination of the chemical and the biologic method was successful. Then further it was tried to ascertain whether the isolation of precipitin by the combination of the physical and biologic methods is possible or not.

Antigen: Ox serum was used as the antigen of sensitization.

Sensitization: Eight thousandth cc of ox serum was added to 4 cc of the antiserum, the titre of which was reduced from 1:1,000 to 1:500 after electro dialysis. The mixture was placed in an incubator at 37°C for two hours, during which it was shaken and then placed in an ice-box over night. It was centrifuged and the supernatant fluid was removed for testing under the designation 'supernatant fluid'.

Washing: The centrifuged sediment was washed three times with the physiologic salt solution to remove all free antibodies.

Dissociation: The washed sediment was emulsified in the physiologic salt solution and placed in a water bath at 65°C for half an hour, during which it was shaken every five minutes. The mixture was soon centrifuged and the supernatant fluid was saved for testing under the designation 'isolated serum'.

Table 3. The dissociation of precipitin by the combination
of the physical and the biologic method.

Original antiserum.	Dilution of fraction.	Antigen.	Supernatant fluid.	Rate of absorption.	Isolated serum.	Rate of isolation.
D.T. 1:1,000 B.Z. 1:500	D.T. 1:500 B.Z. 1:500	Ox serum.	D.T. 1:100 B.Z. 1:500	4/5	D.T. 1:64 B.Z. 1:500	3/6

As Table 3 shows, the antibody of precipitin which was united with ox serum after electro dialysis, was separated again in the physiologic salt solution.

The rate of absorption and that of isolation by this method was almost the same as the rates observed by the combination of the chemical with the biologic method. The immune serum which had a titre of 1:500, was used for isolation. After electro dialysis the titre was reduced from 1:1,000 to 1:500. The supernatant fluid showed

a titre of 1:100, the washed fluid contained no precipitin and the isolated serum yielded a titre of 1:64; so that the rate of absorption is $\frac{4}{5}$ and the rate of isolation, $\frac{1}{5}$.

In accordance with these results, the isolation of the other antiserum by this method will probably be possible, though the isolation of the other antiserum was not attempted.

The relation of antigenic and nitrogen contents
among the isolated sera investigated
by the three methods.

It was possible to isolate several immune bodies by the above mentioned three methods, whereupon further test to find the relation of antigenic and nitrogen contents in the isolated sera was investigated by the three methods. For both the antigenic and nitrogen contents have great significance in the purification of the isolated sera.

Table 4. Antigenic and nitrogen contents
among the isolated precipitin sera
by the three methods.

Original antiserum.	Antigen.	Method of isolation.	Isolated antiserum.	Antigenic contents.	Nitrogen contents per 100 cc.
Precipitin. D. T. 1:500 B. Z. 1:250	Ox serum	Biolog.	D. T. 1:64 B. Z. 1:259	4/1,000	0.02
		Comb. of ammon. sul.	D. T. 1:32 B. Z. 1:250	2/1,000	0.01
		Comb. of electr.	D. T. 1:32 B. Z. 1:250	1/1,000	0.01

Notes: Biolog. means the biologic method.

Comb. of ammon. sul. means the combination of the chemical and the biologic method.

Comb. of electr. means the combination of the physical and the biologic method.

From the results in Table 4, it may be said that the quantity of recovered antigenic and nitrogen contents vary in amount and percentage with the methods employed for isolation. As to the antigenic contents, as Table 4 shows, the serum isolated by the combination of the chemical, or the physical with the biologic methods, contains commonly less antigen than the serum isolated

by the biologic method alone ; the serum dissociated by the combination of the physical with the biologic methods has the least antigen.

Next, as to the nitrogen contents, the serum isolated by the combination method contains generally less nitrogen than that isolated by the biologic method. The nitrogen contents of the antibody solution after isolation was determined by the macro *Kjeldahl's* method.

The influence of temperature and medium on the isolation of precipitin.

As above mentioned, the isolation of precipitin by the combination of the physical and biologic methods was successful. Then further the influence of temperature and medium on the isolation of precipitin was investigated.

*Landsteiner*²³⁾ states that the quantity of agglutinin separated from the sensitized bacteria is proportional to the temperature of the medium, but *Pietro Rondoni*⁴⁰⁾, who dissociated the acute immune hemolysin from sensitized red blood cells by means of diluted NaOH solution at 0°C and 37°C, maintains that the quantity of separated antibody does not vary with the temperature. *Kosakai*²²⁾ demonstrated that high temperature greatly favour the dissociation of the corpuscle-hemolysin combination. *Sunouti*⁴⁶⁾ demonstrated that, with the physiologic salt solution, the best result is observed at 65°C and with saccharose solution and distilled water, at 55°C.

Antigen: Ox serum was used as antigen of sensitization.

Sensitization: Each 0,004 cc of ox serum was added to each 4 cc of antiserum which was electro dialysed and the titre reduced from 1:1,000 to 1:500 after electro dialysis. These mixtures were placed in an incubator at 37°C for two hours and in an ice-box over night. Then they were centrifuged and the supernatant fluid was saved for testing under the designation 'supernatant fluid'.

Washing: The centrifuged sediments were washed three times with the physiologic salt solution to remove all free precipitins and the last supernatant fluids were saved for testing under the designation 'washed fluid'.

Dissociation: The washed sediments were divided into three groups, of which the first was emulsified in the physiologic salt solution, the second in saccharose solution and the third in distilled water. They were all placed in a water bath at 35°C, 45°C, 55°C, 65°C, 75°C, for half an hour and centrifuged soon, when the supernatant fluids were removed for testing under the designation 'isolated serum'.

Table 5. Influence of temperature and mediums on the isolation of precipitin.

Original Antiserum.	Supernatant fluid.	Rate of absorption.	Medium of isolation.	Temp. °C.	Isolated serum. D. T.	Rate of isolation.
D. T. 1 : 1,000 B. Z. 1 : 1,000	D. T. 1 : 200 B. Z. 1 : 1,000	3/5	Physiologic salt solution.	45	16	1/19
Dilution of fraction.				55	32	1/9
				65	64	1/5
D. T. 1 : 500 B. Z. 1 : 1,000				75	8	1/37
			10% saccharose solution.	35	4	1/75
				45	8	1/37
				55	16	1/19
				65	8	1/37
			Distilled water.	35	4	1/75
				45	4	1/75
				55	16	1/19
				65	8	1/37

As Table 5 shows, the best result is observed at 65°C in the physiologic salt solution, but at 75°C the amount of recoverable antibody is somewhat less. At this high temperature, the antibody would be more dissociated, but would undergo destruction. The precipitin is more dissociable from the antigen-antibody complex in the physiologic salt solution than in the other mediums. The most favourable temperature to dissociation of precipitin is observed at 55°C in the non-electrolyte mediums.

Discussions.

There are two principal objects sought in the isolation and purification of immune substances by the several methods. The one object is therapeutic which will make it possible to supply the purified antibodies to practical medicine and the other is theoretic which will enable us to understand the nature of purified immune substances and antigen-antibody reaction.

In the foregoing chapter, the isolation and the purification of immune bodies by the several methods were demonstrated, and now the theoretical discussions will be given below :

The chemical examinations of the tissues and the tissue fluids of immune and non-immune animals were made and the results indicated that the immune substances do exist as the definite entities; that they are neither dialysable nor extractable by fat solvents; that they are readily destroyed by agents which denature or hydrolyse proteins; and that they are associated in the serum with some protein fraction, usually pseudoglobulin or a closely bordering euglobulin fraction.

However, this purely analytical method of approach revealed little as regards the true nature of the immune substances. It was apparent that an absorption of immune substances in which globulin might resist dialysis and the fat extraction regardless of its nature, and its inactivation by heat and enzymic digestion, might be due simply to changes included in the protein adsorbent.

Contemporary studies of the physico-chemical relation of antigen and antibody undertaken by *Landsteiner*²³⁾ and by *Morgenroth*²⁸⁾ and later by *Bordet*⁷⁾, were more productive. They demonstrated the existence of a property which completely differentiates the immune principle of an immune serum from the associated serum protein, namely, a capacity to become selectively adsorbed by the homologous antigen. Sheep erythrocytes, for example, added to chilled, homologous immune rabbit serum, not only were agglutinated and flocculated, but they also removed nearly all of the antisheep immune substances from the serum. The adsorption of the immune substances was demonstrated by centrifuging the agglutinated cells, washing them free of adherent serum, resuspending them in the physiologic salt solution, and testing their capacity to agglutinate the untreated cell suspensions. The agglutination capacity of the artificial suspension resembled in every way a solution of the corresponding whole, immune serum. Moreover, a part of the adsorbed immune substances could be recovered from the sediments cells by extraction with warm, isotonic salt solution.

Later, *Huntoon* and his co-workers¹⁹⁾ demonstrated that the antibody molecules are of large size, not being dialysable, indicating the colloidal nature of the substance, and that antibodies are not affected by trypsin over a considerable period, indicating that they are not protein in nature, or have been racemized by the dilute alkali used, or belong to the peptide group having carboxyl-amino linkage.

As for the so-called protein-free agglutinin solution, *H. Eagle*¹⁰⁾ states as follows. The solution has been prepared containing a minute, almost undetectable quantity of protein, yet causing definite agglutination. The following calculation illustrates the fallacy involved.

The surface area of a cylindrical bacterium measuring, e. g., $1 \times 5 \mu$ will be approximately 2×10^{-7} sq. cm. Assuming the value for the diameter of the globulin molecule as given by *Freundlich*¹¹⁾ to be of the correct order of magnitude, it would require $2 \times 10^{-7} \times 5 \times 10^{-7} = 10^{-13}$ cc of globulin to cover a cell completely. One cubic centimeter of a suspension containing 10^8 bacteria would be completely covered by 10^{-5} g of globulin. Even this quantity is many times too large because of the following indeterminate correction: (1) the water of hydration in the globulin micelle (2) a flatter of globulin micelle on the surface of the antigenic particle (3) the intermolecular space in a surface film (4) only a very small portion of the cell surface need be covered in order to cause the agglutination.

An excessively minute quantity of protein would therefore suffice to coat a suspension of antigenic cells, and not even almost N-free solution can be adduced as proof of the non-protein nature of agglutinin. A solution might contain many hundred times the calculated quantity of protein necessary to cover a bactericidal suspension and yet be completely negative to the usual test for protein, and sensitive to utmost one part in 10^{-4} g per cubic centimeter.

Hirsch and *Lock*²⁵⁾ have obtained antibody solution so active that even a quantity of $1-2 \times 10^{-7}$ g of protein would hemolyse 0.5 cc of a 5% suspension of cells. This quantity is negative to the usual protein test, yet as they have shown, the N-content corresponds in all probability to a trace of protein. The number of cells used would have a surface of about 5×10^{-7} sq. cm and would be completely covered by 10^{-6} g of globulin.

As to the mechanism of antigen-antibody reaction, *Eagle* further states as follows; one may assume the globulin molecule to be polar, containing both hydrophilic and hydrophobic group. In ordinary non-specific adsorption of serum protein by the red blood cells or bacteria, the molecules would orient themselves at the adsorbing surface so that the hydrophilic groups faced the water. Normal serum globulin should then act as a protective hydrophilic colloid, making even the normal zone of acid agglutination of bacteria. In antibody globulin, a certain number of the hydrophilic groups are so altered that they have a enormous affinity for the specific antigen, exceeding their affinity to the aqueous phase. In the antigen-antibody complex, these groups would therefore face the antigen, the hydrophobic groups, now facing the water, would accordingly determine the surface properties of the complex. The stability would be due not to the hydrophilic properties of the surface, as it is in the case of the original antigen, or of adsorbed normal serum protein, but solely due to the charge on the adsorbed

denatured (hydrophobic) antibody protein. In an electrolyte free medium, this charge suffices to keep the particles dispersed. But when it is depressed by electrolytes, there is aggregation due to the cohesion of the films of antibody globulin, since the cell surface is no longer hydrophilic.

In the case of the cellular antigen, this antibody is presented as an invisible film of specifically adsorbed protein, while in the precipitation reaction it may constitute the bulk of the material formed. In both cases, the originally hydrophilic globulin becomes water-insoluble upon combination with water. This change in properties is not a phenomenon peculiar to the immune reaction, but is commonly observed and as yet unexplained property of adsorbed proteins, responsible for their sensitizing effect upon otherwise stable colloidal suspensions. It may be suggested that in the case of the immune reaction, this denaturation of the antibody globulin is due to the fact that the specificity is determined by hydrophilic groups. When these combine with antigen, hydrophobic groups necessarily face the water phase, determining the surface properties of the antigen-antibody complex. But when normal serum protein is adsorbed, and since there are no groups with a specific affinity to antigen, the molecules naturally orient themselves at the interface so that the hydrophilic groups face the water, and the adsorbed protein acts as a protective film away from its isoelectric point.

There are therefore three facts which determine the specific flocculation: (1) the hydrophilic antigen is covered; with (2) a film of immune globulin, denatured by its combination with antigen. In the absence of electrolyte the charge due to the ionization of this protein suffices to prevent aggregation; minute concentration of electrolyte (3), however, depress this surface charge below the critical value necessary for stability. The resultant aggregation is therefore primarily of the immune globulin surface, and only incidentally of the associated antigen.

With an insufficient amount of immune serum only a very small portion of the cell surface is covered with antibody globulin; most of the impacts are between hydrophilic antigen surface, ineffective in producing the cohesion. The more immune serum the greater is the proportion of antigen surface covered with the sensitizing denatured protein, and the correspondingly greater the proportion of effective impacts.

The optimum hydrogen ion concentration for the flocculation is intermediate between that of the original cell and that of the antibody globulin shifting toward the latter as the degree of sensitization is increased. At the optimum reaction, the ionization and the surface

charge are minimal. An addition of electrolytes are not necessary to produce the aggregation. In more acid or more basic reaction the surface charge due to the ionization of the adsorbed protein causes a mutual repulsion of the particles; but traces of electrolyte depress this charge and allow the cohesion of the denatured antibody films. The flocculating ion is always the one opposite in charge to the ionized protein, and its flocculating efficiency increases enormously with increasing valence. The further from the isoelectric zone, the greater is the degree of ionization and the more electrolytes are necessary to depress the surface charge below the critical value.

According to the theory by *Eagle*¹⁰⁾, the fact may be explained that it is impossible or very hard to completely dissociate an immune body from an antigen-antibody complex by isolation, for in this complex a certain number of the hydrophilic groups are so altered that they have an enormous affinity for the specific antigen, exceeding their affinity to the aqueous phase, and these groups would therefore face the antigen and the hydrophobic groups now facing the water would accordingly determine the surface properties of the complex. But an interpretation of the influence of salt on the isolation of immune substances from the complex can not be given by the theory, though the influence of salt on the aggregation of antigen and antibody is explained by it. However, it is our hope that the theory will be more completely established in the near future.

Summary.

1. It is possible to isolate the immune bodies from the globulin fraction, which was obtained by the salting out with ammonium sulphate or electro-dialysis, by the biologic method. The rate of isolation is almost the same as the rate of the isolation by the biologic method.

2. The isolated serum from the globulin fractions by the combination of the physical or the chemical with the biologic method, has less antigenic and nitrogen contents than the isolated serum by the biologic method alone; especially the isolated immune substance by the combination of the physical with the biologic method has the least antigenic contents.

3. The best results are obtained in the physiologic salt solution at 65°C, for the isolation of precipitin by means of the combination of the physical with the biologic method.

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