CHEMICAL, CLINICAL, AND IMMUNOLOGICAL STUDIES ON THE PRODUCTS OF HUMAN PLASMA FRACTIONATION.

X. THE CONCENTRATIONS OF CERTAIN ANTIBODIES IN GLOBULIN FRACTIONS DERIVED FROM HUMAN BLOOD PLASMA [1,2]

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INTRODUCTION

The separation of the albumin fraction from human blood plasma (1) made available, as by-products, the serum globulins which have been further separated and concentrated. For the sake of convenience, we again present here the list of these fractions, including the albumins, which have been derived from the plasma.

Fraction I	—Largely fibrinogen
Fraction II + III	—Largely β- and γ-globulin
Fraction IV	—Largely α- and β-globulin
Fraction V	—Albumin
Fraction VI	—Small amounts of protein in mother liquors, largely α-globulin and albumin

The so-called Fraction II + III, which contains most of the gamma globulins, has subsequently been broken into three fractions designated II, III-1, and III-2.

Since it is a matter of common knowledge that, in immune sera, antibodies against a variety of pathogenic agents and other antigens are associated with or actually consist of globulins and, since large quantities of globulins from human sera were made available as by-products in the preparation of albumin, it became of interest, both from a theoretical and a practical standpoint, to determine the concentration of various antibodies in these globulin fractions. Thus, for example, to the immunologist, precise knowledge would be of great value as to whether normal antibodies in human serum were all confined to a single species of globulin or whether those reacting with certain antigens (*e.g.*, proteins) were represented by one globulin and those with other antigens (*e.g.*, polysaccharide-protein complexes) by one or more different globulins. Furthermore, from the theoretical standpoint, it would be of interest to know whether the so-called normal antibodies of human origin in their chemical behavior closely resemble or are identical with immune antibody or whether they exhibit quite different properties. On the practical side, it is obvious that if antibodies directed against certain pathogenic agents were found to be present in sufficient concentration in one of the globulin fractions mentioned above, the material might have an immediate application to the prevention or therapy of the corresponding disease.

[1] This work has been carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

[2] This paper is Number 18 in the series "Studies on Plasma Proteins" from the Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross.

[3] The large number of the investigators who have carried out tests for certain antibodies rendered it impracticable to include as co-authors all the participants in the work to be described in this paper. Accordingly, grateful acknowledgment is here made for their insight and advice to Dr. Elliott Robinson of the Massachusetts Antitoxin and Vaccine Laboratory and Dr. A. R. Dochez of the Committee on Medical Research of the Office of Scientific Research and Development and to the following individuals for their share in providing certain of the data presented in this communication: Dr. W. C. Boyd, Boston University, Dr. W. L. Bradford, University of Rochester, Dr. M. D. Eaton, California Department of Public Health, Dr. G. Edsall, Massachusetts Antitoxin and Vaccine Laboratory, Dr. I. C. Hall, University of Colorado, Dr. G. K. Hirst, Rockefeller Institute, Dr. C. A. Janeway, Children's Hospital, Boston, Dr. S. D. Kramer, Michigan Department of Health, Dr. S. Mudd, University of Pennsylvania, Dr. M. F. Shaffer, Formerly of the Mass. Antitoxin and Vaccine Laboratory, Dr. J. Stokes, Jr., Children's Hospital, Philadelphia, and Dr. A. Wadsworth of the State of New York Health Department.

With these considerations in mind and with the collaboration of a number of investigators, an analysis for the following antigens of the antibody content of the various globulin fractions was undertaken.

Agglutinins against typhoid O antigen
Agglutinins against typhoid H antigen
Agglutinins against B. pertussis, phase I
Complement fixing antibody against influenza A virus, strain PR8
Complement fixing antibody against the virus of mumps
Inhibitor of the hemoagglutinin of influenza A virus
Mouse protective (neutralizing) antibody against influenza A virus
Mouse protective (neutralizing) antibody against swine influenza virus
Mouse protective (neutralizing) antibody against virus of poliomyelitis
Diphtheria antitoxin
Streptococcal antitoxin
Isoantibodies against cells of human blood groups A and B [4]

Tests for all of these antibodies were not carried out with specimens of all the fractions when it was found that certain of them, as will be subsequently shown, contain little or no antibody of any sort. The majority of determinations of antibody concentration have been confined to a large number of preparations of Fraction II + III and Fraction II. Even with these fractions, however, only a few tests have been made for the presence of certain of the antibodies mentioned above. However, tests for the antibody content of Fraction II, corresponding to 6 representative antigens, *i.e.*, the typhoid antigens, mumps virus, influenza A virus, diphtheria and streptococcal antitoxin, have been carried out as routine on a large number of preparations with the purpose of establishing a preliminary standard of potency. Accordingly, in the description of the technics employed in making these tests, which are given below, those procedures which have been invoked only occasionally are not included.

[4] As soon as it was determined that these antibodies were separated in Fraction II+III, their further investigation was undertaken first by Dr. W. C. Boyd and later by Lt. L. Pillemer, J. L. Oncley, M. Melin, Capt. J. Elliott and Lt. M. C. Hutchinson. Their results are considered in a separate paper in this series.

METHODS

We present the following description of the methods which have been used in the laboratory of the Department of Bacteriology and Immunology at the Harvard Medical School in testing for certain of the antibodies. In addition is included reference to the procedure adopted by Doctors A. Wadsworth and M. Kirkbride and Miss J. Hendry, at the State of New York Department of Health Laboratories, for determining the streptococcal antitoxin content of the fractions.

Determination of H and O agglutinins for E. typhosa

The fraction (or plasma) is diluted in saline from 1 to 4 to 1 to 256 by decrements of twofold, employing volumes of 2 ml. each.

H agglutinins. To 0.5 ml. of each dilution is added 0.5 ml. of a suspension of *E. typhosa* containing the H antigen.[5] As a control for the antigen, 0.5 ml. of saline is added to 0.5 ml. of the bacterial suspension. As additional controls, 0.5 ml. of saline is added to 0.5 ml. of each dilution of the fraction, since certain preparations of globulin when diluted may yield precipitates of protein resembling the H but not the O type of agglutination. The mixtures are incubated in the water bath at 48° to 52° C. for 2 hours, allowed to stand at 4° C. for 1 hour, and the degree of agglutination recorded. The dilution of the fraction (or plasma) causing a degree of agglutination denoted by 2+ is taken as the end-point (complete agglutination as shown by the presence of a clear supernate is denoted as 4+).

O agglutinins. To 0.5 ml. of each dilution is added 0.5 ml. of a suspension of *E. typhosa* containing the O antigen.[6] As a control for the antigen, 0.5 ml. of saline is added to 0.5 ml. of the bacterial suspension. The mixtures are incubated overnight (16 to 18 hours) at 48° to 52° C. and readings are made the following morning, after the tubes have stood for at least 1 hour at 4° C. The dilution of the fraction causing a degree of agglutination denoted by 2+ is taken as the end-point.

Titration of the substance inhibiting the agglutination of red blood cells by influenza A virus (2)

The fraction is diluted in saline from 1 to 8 to 1 to 256 by decrements of twofold. To 0.5 ml. of each dilution of the fraction are added 0.5 ml. of diluted virus [7] and 1 ml.

[5] Prepared according to the method described in Diagnostic Procedures and Reagents. Am. Public Health Assn., 1st Ed., N. Y., 1941, pp. 236–238.

[6] See footnote 5.

[7] The virus employed is the PR8 strain of influenza A. The chorioallantoic fluid of the developing hen's egg inoculated on the 10th day of incubation is collected on the 12th day and stored at −76° C. in a CO_2 ice cabinet until required. The virus dilution used is 4 times that required to cause 2+ agglutination of a 2 per cent suspension of chicken's red blood cells, according to the standards described by Hirst as indicated above.

of 2 per cent chicken red blood cells. The latter are washed four times using about 50 ml. of saline for each washing. The mixtures are allowed to stand at room temperature together with standards of comparison prepared in the following manner.

A 1 per cent red blood cells = 1 ml. 2 per cent susp. + 1 ml. saline
B 0.75 per cent red blood cells = 0.75 ml. 2 per cent susp. + 1.25 ml. saline
C 0.5 per cent red blood cells = 0.5 ml. 2 per cent susp. + 1.5 ml. saline
D 0.3 per cent red blood cells = 0.3 ml. 2 per cent susp. + 1.7 ml. saline

Readings are made after 1 hour by comparison with these standards:

Mixtures with a density of the supernatant equivalent to A = O
Mixtures with a density falling between A + B = 1+
Mixtures with a density falling between B + C = 2+
Mixtures with a density falling between C + D = 3+
Mixtures with a density less than D = 4+

We have taken the O reading as the end-point since we believe it is the most easily and accurately determined, although Hirst has employed the 2+ end-point. All end-points are expressed in terms of final dilutions of fraction or plasma in the mixture.

Complement fixation test for mumps and influenza A

Removal of anticomplementary activity of Fraction II. Since nearly all the globulin fractions have exhibited a greater or less degree of anticomplementary activity, in most of our work, they have been treated with trypsin which has been found frequently to remove this property without markedly reducing the concentration of antibody. "Bacto" trypsin has been employed, but since each lot has a different strength, it is necessary to run titrations to determine the dilution of trypsin that must be used. Most of the tests in which trypsinization has been employed have been carried out with one lot of trypsin which has been used in a quantity of 0.05 ml. of undiluted trypsin added to 1 ml. of the globulin fraction, so diluted in saline as to contain 4 per cent protein. The mixtures are incubated in the water bath at 37° C. for 30 minutes, then heated at 60° C. for 20 minutes. They are then diluted with saline *immediately*, using a volume of 1 ml., from 1 to 20 to 1 to 640 by decrements of two. The results, obtained by this method, both with influenza A and mumps antigen, cannot be accepted as accurately quantitative since the precise degree of antibody inactivation due to trypsinization cannot be precisely determined. The method has also not infrequently proved unsatisfactory, since even when anticomplementary activity is eliminated by trypsinization, certain globulin fractions fix complement in the presence of normal egg or parotid gland material.

Recently, a method of adding sufficient complement to satisfy the anticomplementary activity of Fractions II + III and II has been devised which has proved more satisfactory than that of trypsinization. The number of units of complement fixed by a 1 to 20 dilution of the fraction is determined by setting up a series of 10 tubes, each containing 0.125 ml. of a 1 to 20 dilution of the fraction. Complement is then titrated in the usual manner (see below) to determine the number of ml. of 1 to 60 dilution which contain 1 unit. The volume of a 1 to 10 dilution of complement which contains 1 unit is then calculated. Various amounts of 1 to 10 dilution of complement, containing from 1 to 10 units, are then added to each tube containing the 1 to 20 dilution of fraction.

Example

0.125 ml. of 1 to 20 fraction +1 unit comp. (0.02 ml. of 1 to 10)
0.125 ml. of 1 to 20 fraction +2 units comp. (0.04 ml. of 1 to 10)
0.125 ml. of 1 to 20 fraction +3 units comp. (0.06 ml. of 1 to 10)
0.125 ml. of 1 to 20 fraction +4 units comp. (0.08 ml. of 1 to 10)

0.125 ml. of 1 to 20 fraction +10 units comp. (0.20 ml. of 1 to 10)
Comp. titer: 0.12 ml. of 1 to 60 = 1 unit, therefore 0.02 ml. of 1 to 10 = 1 unit.

The volumes are adjusted with saline to equal the volume of the 10th tube. These mixtures are allowed to stand overnight at 4° C. and 0.25 ml. of sensitized cells are added the next morning. They are incubated for ½ hour at 37° C. in the water-bath and read. The end-point is taken as the number of units of complement that are almost completely fixed by the fraction.

Example

	Units of complement									
	1	2	3	4	5	6	7	8	9	10
0.125 ml. of a 1 to 20 dil. of fraction A66	4+	4+	4+	3+	3+	1+	tr.	0	0	0
0.125 ml. of 1 to 20 dil. of fraction MP 1,2,3,A	4+	4+	4+	3½+	3+	tr.	0	0	0	0

In the example given above, 0.125 ml. of 1 to 20 dilution of both fractions fixes about 5 units of complement. By titration, it was determined that 1 unit of complement was contained in 0.12 ml. of 1 to 60. Therefore, 3 ml. of 1 to 20 fraction fixed about 120 units of complement (24 × 5 units) or 14.4 ml. (120 × 0.12 ml.) of 1 to 60 dilution which was equivalent to 0.24 ml. of undiluted complement. Therefore, 0.24 ml. of undiluted complement was added to 3 ml. of 1 to 20 dilution of each of the fractions.

The mixtures were allowed to stand overnight at 4° C. and then heated at 55° C. for 15 minutes to inactivate any slight excess of complement. Dilutions were then prepared and the complement fixation test carried out as described below. The results were as follows:

Example

Fr. II MP 1, 2, 3, A

	Initial dilutions of fraction						
	1 to 20	1 to 40	1 to 80	1 to 160	1 to 320	1 to 640	1 to 1280
MP (mumps parotid susp.)	4	4	4	4	1	0	0
NP (normal parotid susp.)	0	0	0	0	0	0	0
PR8 (virus in egg fluid)	4	3	1	0	0	0	0
Normal egg (fluid)	0	0	0	0	0	0	0
Comp. control	sl. tr.	0	0	0	0	0	0
Hem. control	4	4	4	4	4	4	4
Fr. II-A66							
MP	3	1	tr.	0	0	0	0
NP	0	0	0	0	0	0	0
PR8	3	3	2	tr.	0	0	0
N. egg	0	0	0	0	0	0	0
Comp. cont.	0	0	0	0	0	0	0
Hem. cont.	4	4	4	4	4	4	4

4 = complete absence of hemolysis

Plasma. Pooled plasmas, from which the fractions are derived, have shown little anticomplementary activity. Accordingly, they are heated at 60° C. for 20 minutes and then tested in the usual manner. If they do exhibit some anticomplementary activity, they are heated for a second time at 60° C. for 20 minutes after they are diluted. This procedure has been shown not to reduce significantly the antibody content but is highly effective in removing the anticomplementary effect of plasma or serum but not that of the fractions.

Hemolytic system. To one volume of a 2 per cent suspension of thoroughly washed sheep's red blood cells, one volume of diluted anti-sheep red cell rabbit serum, containing 2 units of amboceptor, is added, 15 minutes before the sensitized cells are used in the test.

Complement. Sera from at least 6 guinea pigs are collected, pooled, and stored in the CO_2 cabinet (−76° C.). No diminution in complement titer has been observed under these conditions during a period of about 2 months. Titrations of complement, however, are performed each time the complement fixation test is done. Two titrations are carried out simultaneously, in one of which the volumes of complement are twice those employed in the other. This is done as a check on the titration utilizing the smaller volumes, since errors may be easily introduced in the latter due to the necessity of measuring such small quantities. The unit of complement is taken as the smallest amount that gives complete hemolysis in the titration which includes the smaller volumes.

Double volume titration. Complement is diluted 1 to 60. Of this dilution, 0.40 ml., 0.38 ml., 0.36 ml., 0.34 ml., 0.32 ml., 0.30 ml., 0.28 ml., 0.26 ml., 0.24 ml., and 0.22 ml. are pipetted into a series of tubes. These volumes are then brought up to 1 ml. with saline and 0.5 ml. of sensitized cells are added.

Standard complement titration. Complement is diluted 1 to 60. Of this dilution, 0.20 ml., 0.19 ml., 0.18 ml., 0.17 ml., 0.16 ml., 0.15 ml., 0.14 ml., 0.13 ml., 0.12 ml., and 0.11 ml. are pipetted into a series of tubes. These volumes are then brought up to 0.5 ml. with saline and 0.25 ml. sensitized cells are added. The contents of the tubes are mixed and incubated in the water bath at 37° C. for 30 minutes. For use in the test, the complement is diluted so that 0.15 ml. contains 2 units.

Antigens

Mumps parotid. One volume of a 20 per cent emulsion of infected monkey parotid [8] is diluted with 5 volumes of saline and rotated for 30 minutes at 3500 r.p.m. in an angle centrifuge. The supernatant fluid is then titrated with 3 known strongly positive mumps convalescent sera (monkey or man), in dilutions of 1 to 8. Three known mumps negative sera (monkey or man), diluted 1 to 8, are included as controls. For use in the complement fixation test, twice the concentration of antigen giving complete fixation (4+) in this titration is employed.

Normal parotid. Parotid gland from a normal monkey is prepared in the same manner as that employed in the case of the infected gland and used in a dilution equivalent to that of the mumps parotid.

Influenza A antigen. The PR8 strain of influenza A is used as antigen. The chorioallantoic fluid of the developing hen's egg, inoculated on the 10th day of incubation, is collected on the 12th day and stored at −76° C. in a CO_2 cabinet until required. It should be of such a strength as to give 2+ agglutination with 2 per cent hen cells in final dilution of 1 to 128 in the Hirst test. In the complement fixation test, the chorioallantoic fluid is employed in a dilution of 1 to 8.

Normal egg. Chorioallantoic fluid is collected from embryonated hen's eggs on the 10th to 12th day of incubation at 102° to 103° F. and stored at −76° C. in a CO_2 cabinet until required, when it is used in a dilution equivalent to that of the influenza A antigen.

The complement fixation test. To 0.125 ml. of each dilution of the fraction, 2 units of complement in a volume of 0.15 ml. and 0.1 ml. of antigen or corresponding control material are added.

Six sets of dilutions of the fraction in saline are prepared. To one set, mumps parotid suspension is added. To the second, normal monkey parotid is added, to the third row, the influenza A antigen, and to the fourth row, normal chorioallantoic fluid are added. The fifth row receives saline instead of any of these reagents, and thus represents a control on the anticomplementary activity of each dilution of the fraction. The sixth row receives neither complement nor antigen but contains only 0.125 ml. of each dilution of fraction and saline thus serving as hemolytic control.

The mixtures are placed overnight in the ice box at 4° C. and 0.25 ml. of sensitized sheep's red blood cells are added to each on the following morning. They are incubated ½ hour at 37° C. in the water-bath and read. The end-point is taken as the last dilution of the fraction (initial dilution) giving definite fixation of complement which is denoted as 2+. The following controls are included in which the volumes are all rendered equivalent to those employed in the test by the addition of the appropriate quantity of saline.

Mumps parotid gland + 2 units of complement
Normal parotid + 2 units of complement
PR8 egg fluid + 2 units of complement
Normal egg fluid + 2 units of complement
2 units complement
1 unit complement
Saline
Known + serum (mumps)
Known − serum (mumps)
Fraction A66 (standard for influenza antibody)

Titration of anticomplementary activity

The fraction is diluted in saline by decrements of 2 (1 to 4 to 1 to 256), employing volumes of 0.5 ml. With these dilutions, the test is carried out as follows. To 0.125 ml. of each dilution of the fraction, 2 units of complement prepared in the manner described above are added in a

[8] Enders, J. F., and Cohen, S., Proc. Soc. Exp. Biol. and Med., 1942, **50**, 180–184.

volume of 0.15 ml. To adjust the total volume of fluid to that employed in the complement fixation test, 0.1 ml. of saline is added.

The mixtures are allowed to stand overnight at about 4° C., when to each is added 0.25 ml. of a 2 per cent suspension of sensitized sheep's red blood cells. The tubes are then placed in a water bath at 37° C. for ½ hour and the fixation of complement then recorded as 0, tr., 1+, 2+, 3+, and 4+. Complete absence of hemolysis is denoted by a reading of 4+. The end-point of anticomplementary activity is indicated by that dilution (initial dilution) of the fraction showing a residue of non-hemolyzed cells read as "tr."

Mouse test for influenza A protective (neutralizing) antibody

The fractions are usually diluted in sterile infusion broth to 1 to 40, 1 to 80, 1 to 160, 1 to 320. Plasmas from which fractions have been prepared are diluted in broth to 1 to 2, 1 to 4, 1 to 8, 1 to 16. In preparing mixtures of virus and solution, either fraction or plasma, 0.5 ml. of dilution is mixed with 0.5 ml. of the virus [9] using *a clean pipette for each dilution.*

The mixtures are allowed to stand at room temperature for 20 minutes. All series of mixtures not being employed for inoculation are maintained at 4° C. while the mice are receiving injections. Under light ether anesthesia, 4 mice are each inoculated intranasally with 0.05 ml. of each mixture. The virulence of the virus is determined in each experiment, employing dilutions of 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}, prepared in infusion broth.

Specific deaths, confirmed by autopsy, are recorded over a period of 10 days. The 50 per cent mortality end-point is calculated according to the method of Reed and Muench (3).

Rabbit skin test for diphtheria antitoxin

Dilution of standard antitoxin. Diphtheria antitoxin standardized to contain 6 u in 1 ml. is employed. A key dilution of 1 to 30 is made and will be referred to as (A). According to the following procedure, dilutions of the standard antitoxin, ranging from 0.08 to 0.14 units of antitoxin per ml., are then prepared from the key dilution.

2.8 ml. (A) + 1.2 saline = 0.14 u/ml.
2.6 ml. (A) + 1.4 saline = 0.13 u/ml.
2.4 ml. (A) + 1.6 saline = 0.12 u/ml.
2.2 ml. (A) + 1.8 saline = 0.11 u/ml.
2.0 ml. (A) + 2.0 saline = 0.10 u/ml.
1.8 ml. (A) + 2.2 saline = 0.09 u/ml.
1.6 ml. (A) + 2.4 saline = 0.08 u/ml.

This series of dilutions is designated the L+/12 standard and is employed in determining the antitoxic potency of the fractions. An L+/120 standard prepared in a similar manner is used with the plasmas which includes an antitoxin range from 0.008 to 0.014 u/ml.

[9] The virus employed is the PR8 strain of influenza A propagated in the developing chicken embryo as described in footnote 7 and is usually diluted so that 0.05 ml. contains about 3000 50 per cent M. D.

Dilutions of fractions. The fractions are diluted with normal saline so as to contain the following amounts of the fraction in 1 ml.: 0.008 ml., 0.01 ml., 0.015 ml., 0.020 ml., 0.025 ml., 0.029 ml., 0.04 ml., 0.05 ml., 0.075 ml., 0.10 ml., 0.15 ml., and 0.20 ml. It is usually possible to find the end-point within these ranges, but occasionally a narrower range may be required.

Toxin. For the titration of the fractions, the diphtheria toxin is diluted to contain 1/12 L+ dose per ml. For plasmas, it is diluted so that 1/120 L+ is contained in 1 ml. It is rather unstable and should be mixed without bubbling just before using.

Procedure. To a series of tubes, each of which contains 1 ml. of the toxin dilution, are added 1 ml. each of the fraction or standard antitoxin dilutions. The toxin and fraction dilution or toxin and antitoxin dilution is mixed by inverting the tube three times. Of each mixture, 0.1 ml. is injected intradermally into the skin of a New Zealand white rabbit. Two rabbits should be injected with samples of the same mixtures in order to provide a check and, if the results do not agree, the test should be repeated. The reactions are read from 66 to 72 hours after injection.

Calculation of end-points. On each rabbit, the first injection site in each series which shows no reaction ("wipe-out") is recorded and compared with effect of the standard antitoxin.

Example. The smallest quantity of fraction which neutralized the test dose of toxin was found to be 0.029 ml. In the same experiment, the least amount of the standard antitoxin which neutralized the test dose of toxin was found to be 0.1 unit.
Let X = number of units of antitoxin per 1 ml. of the fraction, then $0.029:0.1::1.0:X$.
X = 3.4 units.

Determination of streptococcal antitoxin

The procedures employed in determining the content of streptococcal antitoxin of fractions and plasmas were in most instances those already published (4) and need not therefore be described in this place. These tests were carried out by Miss Jessie L. Hendry of the State of New York Department of Health. She has occasionally modified the technique. Thus, material of low antitoxic potency is tested by employing one skin test dose of toxin as the test dose instead of the standard 5 skin test doses.

RESULTS

The association of antibody with Fraction II + III

Much evidence is available which indicates that antibody consists of modified globulin and is usually, although not exclusively, associated with the gamma globulins. It was expected, therefore, that most of the antibody in human plasma would be found in Fraction II + III, since this material contains about 90 per cent of

TABLE I

Antibodies in globulin Fraction II + III derived from human plasma *

Antibody	Type of antibody	Investigator	Institution	Concentration comp. to plasma
Anti-diphtheria	Antitoxin neutralizing	Edsall	Mass. Antitoxin and Vaccine Laboratory	10
Anti-dysentery	Agglutinins	Mudd	Univ. of Pennsylvania	2–10
Anti-herpes simplex	Neutralizing	Stokes	Children's Hosp., Phila.	*
Anti-influenza (human PR8)	Hirst inhibition	Hirst	Rockefeller Institute	4–8
Anti-influenza (human PR8)	Hirst inhibition	Eaton	Calif. Dept. Public Health	4
Anti-influenza (human PR8)	Hirst inhibition	Enders	Harvard Med. School	10–15
Anti-influenza (human PR8)	Complement fixation	Enders	Harvard Med. School	10–15
Anti-influenza (human PR8)	Neutralizing	Stokes	Children's Hosp., Phila.	10
Anti-influenza (human PR8)	Neutralizing	Enders	Harvard Med. School	9
Anti-influenza (swine)	Neutralizing	Stokes	Children's Hosp., Phila.	10
Anti-influenza (swine)	Neutralizing	Shaffer	Mass. Antitoxin and Vaccine Laboratory	4
Anti-lymphocytic choriomeningitis	Neutralizing	Stokes	Children's Hosp., Phila.	*
Anti-measles	Protective (human)	Stokes	Children's Hosp., Phila.	
Anti-mumps	Complement fixation	Enders	Harvard Med. School	2–10
Anti-parapertussis	Agglutinins	Mudd	Univ. of Pennsylvania	64
Anti-pertussis	Agglutinins	Mudd	Univ. of Pennsylvania	4–10
Anti-pertussis	Agglutination	Enders	Harvard Med. School	10
Anti-pertussis	Mouse protection	Bradford	Univ. of Rochester	4–10
Anti-perfringens	Protective	Hall	Univ. of Colorado	*
Anti-poliomyelitis	Neutralizing	Kramer	Mich. Dept. Health	10
Anti-poliomyelitis	Neutralizing	Stokes	Children's Hosp., Phila.	16
Anti-poliomyelitis	Rat and mice protection	Kramer	Mich. Dept. Health	10
Anti-poliomyelitis	Rat and mice protection	Stokes	Children's Hosp., Phila.	10
Anti-scarlatina	Neutralizing	Bradford	Univ. of Rochester	*
Anti-scarlatina	Neutralizing	Wadsworth	New York Dept. Health	5–10
Anti-streptococcus	Antitoxin	Wadsworth	New York Dept. Health	4–10
Anti-typhoid	H agglutinin	Enders	Harvard Med. School	8–10
Anti-typhoid	O agglutinin	Enders	Harvard Med. School	8–10
Anti-vaccinia	Neutralizing	Janeway	Children's Hosp., Boston	*
Isoagglutinins	Agglutinins	Boyd	Harvard Med. School	8–10

* These assays were undertaken at the request of Dr. A. R. Dochez of the Committee on Medical Research of the Office of Scientific Research and Development who wrote to the investigators listed in March, 1942, "In the process of preparing human plasma used for transfusion purposes in the armed forces of the United States, by Dr. Edwin Cohn of Harvard University, a number of fractions of the original plasma result. Only one of these, the albumin fraction, is used for transfusion. It is the desire of the Government to ascertain to what useful purpose the remaining fractions can be put. Among these fractions is one containing the α-, β-, and γ-globulins. As you doubtless know, this fraction contains whatever immune bodies may have been present in the original plasma. In the process of purification approximately ten times concentration of the immune body fraction is effected. It is hoped that these immune bodies may be used practically either for the prophylaxis or treatment of certain infectious diseases. In order to test the validity of such a procedure it is first necessary to titrate the globulin fraction for its content of specific antibodies. . . . The first titrations would be with mixtures of the α-, β-, and γ-globulins. Later fractionation of the different globulins will be performed and the specific immune body containing globulin will be furnished for a similar titration." We are greatly indebted to Dr. W. C. Boyd for compiling this table.

* Activity present but no quantitative data.

this species of protein originally included in the plasma.

In substantiation of this expectation, a considerable number of determinations of the antibody concentration of an 11 to 12 per cent solution of Fraction II + III and of the plasmas from which the various preparations in question were derived (Table I) have shown that in the majority of instances the yield of antibody in Fraction II + III was of the order to be expected if most of the antibody of the plasma were associated with the globulins recovered in this fraction, since the concentration of the latter over the plasma is 8 to 10 times.

Further indication of the almost complete association of antibody with Fraction II + III was

TABLE II

Antibodies in certain fractions of human plasma exclusive of Fraction II + III

Fraction	Antibody	Type of antibody	Investigator	Institution	Concentration comp. to plasma
I	Anti-typhoid	O agglutinin	Enders	Harvard Med. School	<1
	Anti-typhoid	H agglutinin	Enders	Harvard Med. School	1
	Anti-mumps	Complement fixation	Enders	Harvard Med. School	<1
	Anti-influenza (human PR8)	Complement fixation	Enders	Harvard Med. School	<1
	Anti-influenza (human PR8)	Neutralizing	Enders	Harvard Med. School	<1
	Anti-diphtheria	Antitoxin	Enders	Harvard Med. School	<1
IV	Anti-typhoid	O agglutinin	Enders	Harvard Med. School	2
	Anti-typhoid	H agglutinin	Enders	Harvard Med. School	<1
	Anti-influenza (human PR8)	Complement fixation	Enders	Harvard Med. School	<1
	Anti-diphtheria	Antitoxin	Edsall	Mass. Antitoxin and Vaccine Laboratory	1
	Anti-dysentery	Agglutinins	Mudd	University of Pennsylvania	1
	Isoagglutinins	Agglutinins	Boyd	Harvard Med. School	<1
Supernatant of II + III	Anti-typhoid	O agglutinin	Enders	Harvard Med. School	<1
	Anti-typhoid	H agglutinin	Enders	Harvard Med. School	1 or <1
	Anti-mumps	Complement fixation	Enders	Harvard Med. School	<1
	Anti-influenza (human PR8)	Complement fixation	Enders	Harvard Med. School	<1
	Anti-influenza (human PR8)	Neutralizing	Enders	Harvard Med. School	<1
	Anti-diphtheria	Antitoxin	Enders	Harvard Med. School	<1

obtained by testing a few specimens of Fractions I and IV and the supernatant fluids from Fraction II + III. These results are summarized in Table II. From them, it is clear that only small quantities of antibody have been found therein. The failure to detect significant quantities of antibody in the supernatant fluids of Fraction II + III is of especial significance, since it precludes the possibility that any considerable quantity of active material is carried over into Fractions IV, V, and VI.

We may conclude, then, that a very large proportion (it is impossible to give a precise estimate on the basis of the available data) of the various antibodies mentioned in the tables, and present in so-called normal human plasma, can be recovered in Fraction II + III. It cannot be assumed, however, from the evidence so far presented that the antibodies are associated only with the γ-globulin of Fraction II + III, since it will be recalled (1) that the fraction is comprised of about 12 per cent α- and 42 per cent β-globulin in addition to the γ-globulin which represents about 90 per cent of the total plasma γ-globulin. Upon the separation and immunologic analysis of Fraction II, some recent preparations of which contain 99 or more per cent γ-globulin, results have been obtained which strongly suggest the close association of certain antibodies with γ-globulin. The remainder of the experimental portion of this communication will be concerned mainly with a description of our analyses of the antibody content of Fraction II.

The antibodies of Fraction II

Fraction II + III was split into 3 subfractions (1,5) with the objective of concentrating and recovering (*1*) the thrombin in Fraction III-2 as a hemostatic agent, (*2*) the isohemagglutinins in Fraction III-1 for blood typing, and (*3*) a Fraction II containing practically nothing but γ-globulin. This chemical development has resulted in a further concentration in antibody activity. Moreover, with the elimination of α- and β-globulins, the purified γ-globulin exhibits greater stability. Finally, information concerning the association of antibody with the various species of globulin comprising Fraction II + III has resulted from the chemical fractionation of the γ-fraction from the α and β components.

A series of preparations have been prepared in the Plasma Fractionation Laboratory from Fraction II + III by slight alterations in the general method, all designated as Fraction II. These have been prepared and tested by immunologic technics. Those prepared first contained about

80 per cent γ-globulin, whereas the most recent products consist of 96 to 100 per cent γ-globulin.

Selection of a standard of comparison. Early in the course of this work one of these preparations, A66, was selected as a provisional standard and its antibody content determined at the same time as that of each newly prepared specimen of Fraction II. In this way, a considerable quantity of data (Table III) has been accumulated in respect to the standard which serves to indicate the range of variability one may expect when antibody concentrations of Fraction II are determined by the methods we have employed. In certain instances, titrations of the plasma from which A66 was derived have also been carried out simultaneously with those of the fraction itself. In Table IV are presented the results obtained in titrations of the plasma for 5 representative antibodies, *i.e.*, neutralizing antibody for influenza A, inhibitor of influenzal hemoagglutinin, diphtheria antitoxin, and H and O typhoid agglutinins. The plasma was preserved at the Harvard Plasma Fractionation Laboratory in the frozen and dried state. These figures give a conception of the variation of successive determinations for plasma which is, of course, very low in antibody content.

An examination of the data in Tables III and IV leads to several conclusions. First, the content of diphtheria antitoxin in both Fraction II and the plasma can be estimated in titrations done at different times with considerably greater accuracy than any of the other antibodies. Second, the rather extensive range in the values for the other antibodies, in particular the influenzal neutralizing substance, makes abundantly clear the necessity for including a standard of comparison, as we have done, in the assay of the

TABLE III

Determination of certain antibodies in Fraction A66 at various times

Determination	Titer of solution						Units diphtheria antitoxin per ml. solution
	Typhoid agglutinin		Mumps comp. fix.	Influenza A			
	O	H		Comp. fix.	Hirst test	Mouse prot.	
1	8	200			512	160	3.3
2	6	128			256		
3	4	256			512	106	3.9
4	6	200			256	106	3.9
5		128			256	160	3.4
6	12	250			256	100	3.8
7	8	200			256	160	3.3
8	8	256			256	120	3.8
9	8	256			256	130	3.5
10	12	200			512	250	3.4
11	12	200	{80	{320	128	200	3.8
12	8	200	{80	{320	256	140	3.8
13	6	200					4.2
14	16	64	{80	{320	256	80	4.0
15	16	64	{80	{160	256	160	3.8
Average	9.3	187	80*	280*	302	144	3.7

* Average of determinations 11, 12, 14, and 15 only, done by new method for removing anticomplementary activity.

The results of all titrations, with the exception of those for diphtheria antitoxin, are expressed as reciprocals of the dilution of the fraction. All dilutions are given as initial dilutions except those for hemoagglutinin inhibitor (Hirst test) which are final. The diphtheria antitoxin content of the fraction is given in units per ml. The various determinations were carried out during a period of 8 months and were fairly uniformly distributed during this period.

TABLE IV

Determination of influenza A neutralizing antibody, diphtheria antitoxin and typhoid agglutinins in plasma 65–66 at various times

Determination	Hemoagglutinin inhibition (Hirst test)	Neutralizing antibody Influenza A	Diphtheria antitoxin	Typhoid agglutinin	
				O	H
1	32	5	0.08	4	8
2	32	5	0.07	4	8
3	32	3	0.10	4	23
4	32	3	0.08	4	23
5	32	4	0.11	<4	8
6	32	4	0.12	4	<4
7	32	5	0.12	4	8
8		2.9	0.13		8
9		3.2	0.10		
10		2.5	0.14		
11		10			
12		12			
13		5.3			
14		3.2			
15		5.8			
Average	32	5	0.11	4	11

End-points of O and H agglutinins are expressed as reciprocals of the plasma dilution. The titers of neutralizing antibody are expressed as the reciprocal of the plasma dilution which protected 50 per cent of the mice against the test dose of virus. The diphtheria antitoxin content is expressed in units per ml. of plasma. The determinations were carried out over a period of 7 months. The distribution of the tests over this period was fairly uniform.

potency of each new and unknown product. In the third place, and most significantly for the purpose of obtaining a high concentration of antibody, a comparison of the average titers of fraction and plasma indicates that with most of the antibodies a concentration much greater than that of Fraction II + III has been attained. The data for diphtheria antitoxin and neutralizing antibody reveal a concentration factor of 34 for the former and 29 for the latter. The factor in the case of hemoagglutinin inhibitor (Hirst test) is lower (about 10). This probably is due to the presence of a non-specific inhibiting substance known to be present in normal plasma (2) which renders the plasma values higher than they should be. The average value for H agglutinin indicates a concentration of this antibody of about 17 times, in contrast to the O agglutinin which at best has been concentrated twofold. In Table IV, we have not included figures for the titrations of the remaining antibodies mentioned in Table III, since only one or two determinations were made. It may be stated, however, that on the basis of the most recent and most trustworthy data, complement fixing antibody for mumps antigen appeared to be concentrated about 20 times and complement fixing antibody for influenza A about 18 times.

In contrast to the large quantities of all other antibodies for which tests were made, those specific for the so-called O or somatic antigen of the typhoid bacillus were present only in low concentration. This observation is of considerable interest since it distinctly points to a qualitatively different antibody in respect to the species of protein of which it is composed. Indeed, if the fact determined by Jones (6) be recalled, *viz.*, that the antibody reacting with the H antigen of the hog-cholera bacillus is definitely more thermostabile than the O antibody, perhaps our finding may not be unexpected.

A few tests for the presence of isoagglutinins in A66 reveal concentration factors of 2 to 4 which are distinctly lower than the values found for Fraction II + III and suggest that the isoagglutinins either may be a different sort of γ-globulin or possibly included in the α- or β-globulin, along with the O antibody. This hypothesis is given additional support by the investigations of Pillemer, Oncley, Melin, Elliott and Hutchinson (7), who have determined conditions for the concentration of most of the isoagglutinin in a fraction corresponding to Fraction III-1.

Immunologic assay of various preparations of Fraction II. In Table V are summarized the various antibody titers obtained with 61 preparations of Fraction II. A brief survey of these data leaves the impression that the products prepared in slightly different ways and tested at rather widely different times are in general fairly uniform in respect to antibody content.

With a few exceptions, the quantity of diphtherial antitoxin is around 2 units per ml. of solution. It is apparent, in view of this fact, that the antitoxin of the standard (3.9 units) is exceptionally high. In the evaluation of the results of diphtheria antitoxin titrations, it should be borne in mind that the error inherent in the method may be as large as ± 28 per cent of the mean, as calculated on the basis of the data recorded in Table III. Consequently, in only a few instances, such as with preparations AS29, C70, C80, and A66 having definitely low or high values, is it possible to assert with any degree of certainty that these products are inferior or superior to the large majority we have tested. The same considerations apply to the results for streptococcal antitoxin. Here, the error of the method is probably about 25 to 30 per cent. In respect to the titers of influenza A mouse protective or neutralizing antibody, great caution must be exercised in drawing any conclusion as to significant differences based only on a comparison of titers between the various preparations, since it is clear from Table III that the error of the method may be as great as 150 per cent in tests carried out at different times. Again similar considerations apply to the results of complement fixation tests mainly because of the difficulty in removing the anticomplementary properties of the fractions. Indeed, under these headings we have included only a few results as the majority of determinations have given endpoints which we regard as untrustworthy from the standpoint of quantitative interpretation. The accuracy of the titrations of H and O agglutinins may be affected by the use of new batches of antigen and by other conditions im-

possible to control; accordingly, the end-point may vary within at least one dilution-interval.

In connection with the assays summarized in Table V, it should be pointed out that, although there was a decrease in α- and β-globulins in preparations made by methods 3A, 3C, 3D, and 3E, which yielded products that contained more than 95 per cent γ-globulin, as contrasted with methods 2 and 3, there is no indication of diminished antibody activity.

TABLE V

Immunologic assay of various preparations of Fraction II

Preparation Number	Titer of solution									Units antitoxin per ml. of solution	
	Isoagglutinin[a]		Anti-comp. act.	Typhoid Agglutinin		Mumps comp. fix.	Influenza A				
	Anti-A	Anti-B		O	H		Comp. fix.	Hirst test	Mouse prot.	Diphtheria[b]	Streptococcus[c]
Method No. 2											
A54R	4	16	640	0	128	(80[d])	(160[d])	512	96	1.9	40
A54K	32	8	32	4	90	(40[d])	(20[d])	256	100	1.5	35
A58	8	8	0	4	128	226	450	512	160	1.7	
Method No. 3											
A66 (stan.)	4	16	>128[e]	9.3[e]	187[e]	80[e]	280[e]	302[e]	144[e]	3.7[e]	50
A72	8	16	20	8	180	[g]	[g]	128	107	1.8	30
A29			16	4	64	[g]	[g]	256	90	1.2	
A35	16	8	>256	2	180	[g]	[g]	256	113	2.7	40
A80R			>256	6	64	[g]	[g]	256	169[h]	1.5	35
A84			>256	6	45	(160[d])	(160[d])	128	116	1.7	25
A74B		4	>256	24	>256	[g]	[g]	256	96	2.8	35
A109			128	16	64	80	320	256	80	4.6	35
C36	16	16		6	128	[g]	[g]	256	106	3.0	
C51	32	8	8	6	45	20	80	256	141[f]	2.4	50
C70	32	8	8	6	45	20	[g]	128	110	1.3	
C80	32	4	>128	4	64	[g]	[g]	256	120	4.0	
C97			>256	12	45	[g]	[g]	128	116	1.5	20
C102			>256	8	45	(320[d])	[g]	256	106	1.5	20
C103			>256	12	90	[g]	[g]	256	137	2.2	30
C104			128	8	45	[g]	[g]	512	137	2.4	20
C105			128	8	45	[g]	[g]	512	160	2.5	35
C106			128	8	45	[g]	[g]	256	60	2.4	30
C107			128	8	64	[g]	[g]	256	120	2.0	65
C108			>256	8	64	[g]	[g]	256	180	2.0	65
C109			16	6	64	[g]	[g]	256	175	2.3	35
D26	64	8	128	4	90	(40)	(40)	256	80	2.8	50
D36	64	16	64	6	90	[g]	[g]	256	98	2.8	40
Method No. 3A											
A74A	8	8	256	12	256	[g]	[g]	256	106	3.8	35
AS84			64	8	90	80	320	256	250	2.4	80
A97			128	8	45	80	640	256	160	1.6	30
B1			128	12	32	40	160	256	215	1.6	
B2			64	6	32	320	320	256	96	2.3	
B3			8	12	32	160	113	128	96	1.8	45
D5			4	22	128	113	450	256	120	3.0	55
D6			16	12	90	113	450	512	192	3.3	20
D8			16	12	64	56	450	512	266	7.5	
D10			128	12	64	56	450	512	267	2.8	
D16			256	12	90	80	450	512	240	3.2	
D20			256	12	64	56	320	512	240	3.1	
G13			>1024	6	32	[g]	[g]	256	267	3.3	
G14			>1024	6	23	[g]	[g]	256	213	2.8	
G15			256	6	16	113	450	512	266	3.3	

TABLE V—*Continued*

Preparation Number	Titer of solution									Units antitoxin per ml. of solution	
	Isoagglutinin[a]		Anti-comp. act.	Typhoid Agglutinin		Mumps comp. fix.	Influenza A				
	Anti-A	Anti-B		O	H		Comp. fix.	Hirst test	Mouse prot.	Diphtheria[b]	Streptococcus[c]
Method No. 3B											
A111			>256	12	64	80	160	128	240	2.8	45
A269K			256	8	64	80	640	128	181	2.8	35
A291K			>256	12	32	160	320	256	45	2.1	20
E1			32	8	64	80	113	256	96	2.2	40
E3			8	6	32	160	160	256	96	1.8	20
E4			512	23	64	113	450	256	267	2.6	
F1			128	12	64	[g]	[g]	256	120	3.7	
F2			16	6	90	56	80	256	69	3.0	
Method No. 3C											
A1–120			256	12	90	[g]	[g]	128		2.8	35
A2–120			256	22	64	[g]	[g]	256	48	1.8	
A1–122			128	12	16	[g]	[g]	256	103	1.8	30
A2–122			>256	12	32	[g]	[g]	32	89	2.0	30
A1–126			>1024	6	32	40	450	512	238	2.3	50
A1–371			256	6	45	40	450	512	200	2.2	35
A2–371			>1024	6	45	113	450	512	236	2.3	35
A1–388			>1024	6	16	40	450	512	160	4.1	40
A1–388B			256	8	32	40	320	512	320	3.5	80
A1–LY1			512	8	22	80	160	512	160	3.7	80
Method No. 3D											
A1–329			128	8	32	113	225	256	120	2.8	60
Method No. 3E											
A1–133			512	12	90	113	450	512	267	2.8	80
Averages	26	10		9	71	98	260	308	150	2.6	41

a. Carried out by Capt. J. Elliott and Lt. L. Pillemer. The titer represents the highest dilution which is found to give a 1+ reaction by test-tube centrifuge technic, using a 1.25 per cent fresh cell suspension.

b. Some of these titrations were carried out under the direction of Dr. G. Edsall at the Massachusetts Antitoxin and Vaccine Laboratory.

c. Carried out under the direction of Drs. A. Wadsworth and M. Kirkbride and Miss J. Hendry at the State of New York Department of Health.

d. After trypsin digestion to remove anticomplementary activity.

e. Average of values given in Table III.

f. Average of two values: 123 and 160.

g. Fixes normal parotid gland or normal egg, and test could not be carried out.

h. Average of 2 values: 125 and 213.

Figures represent reciprocals of end-point dilution of fraction except those for diphtheria and streptococcal antitoxins.

Further evidence is included in Table VI for the close association with γ-globulin of all the antibodies investigated except the O typhoid agglutinin and isoagglutinins. In this table are given the quotients obtained by dividing the value of the end-point of each preparation mentioned in Table V by that of the standard, A66. With the exception of the figures for streptococcal

TABLE VI

Immunologic assay of various preparations of Fraction II

Antibody Concentration Referred to Prep. A66

Preparation Number	Concentration Ratio*										
	Isoagglutinin		Anti-comp. act.	Typhoid Agglutinin		Mumps comp. fix.	Influenza A			Diph-theria Antitoxin	Strepto-coccus Antitoxin
	Anti-A	Anti-B		O	H		Comp. fix.	Hirst test	Mouse prot.		
Method No. 2											
A48										0.5	1.0
A54R	1.0	1.0								0.5	0.8
A54K									0.6	0.4	0.7
A58	2.0	0.5								0.4	
Method No. 3											
A66 (stan.)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
A72	2.0	1.0		0.7	0.7			0.5	1.1	0.5	0.6
A29			0.5	1.0	1.0	1.0	0.5	1.0	0.6	0.4	
A35				1.0	0.7			1.0	0.8	0.7	0.8
A80R				0.8					1.3	0.4	0.7
A84			0.2	0.5	0.2			0.5	1.2	0.4	0.5
A74B			1.0	2.0	1.0	*	*	1.0	0.8	0.7	0.7
A109			2.0	1.0	1.0	1.0	2.0	1.0	0.5	1.2	0.7
C36									0.7	0.8	
C51				1.0	0.2	1.0	1.0		1.1	0.6	1.0
C70				1.0	0.2	1.0			1.1	0.3	
C80				0.7	0.4			1.0	1.3	1.3	
C97			>2.0	1.0	0.2			0.5	1.2	0.4	0.4
C102			>2.0	0.7	0.2	0.5		1.0	1.1	0.4	0.4
C103			>2.0	1.0	0.4			1.0	1.4	0.6	0.6
C104			1.0	1.0	0.3	*		2.0	0.9	0.7	0.4
C105			1.0	1.0	0.3	*	*	2.0	1.0	0.7	0.7
C106			1.0	1.0	0.3	*	*	1.0	0.4	0.7	0.6
C107			0.5	0.7	0.3	*	*	1.0	0.9	0.6	1.3
C108			1.0	1.0	0.3	*	*	1.0	1.4	0.6	1.3
C109			0.1	0.5	0.3	*	*	1.0	1.3	0.6	0.7
D26								1.0	0.8	0.8	1.0
D36				1.0	0.5	*	*	1.0	1.0	0.7	0.8
Method No. 3A											
A74A			>1.0	1.5	1.0	*		1.0	0.9	1.0	0.7
AS84			8.	0.7	0.4	1.0	1.0	2.0	1.3	0.6	1.6
A97			1.0	1.0	0.2	1.0	2.0	1.0	1.1	0.4	0.6
B1			1.0	0.8	0.5	0.5	1.0	1.0	2.7	0.4	
B2			2.0	0.4	0.3	2.7	2.0	1.0	1.0	0.5	
B3			0.5	1.0	0.3	1.0	0.7	0.5	1.0	0.4	0.9
D5			0.3	1.8	1.0	0.7	2.8	1.0	1.2	0.7	1.1
D6			0.5	1.0	1.0	0.7	4.0	2.0	2.0	0.7	0.4
D8			0.1	1.0	0.7	1.0	2.8	2.0	2.0	1.7	
D10			1.0	1.0	0.7	0.7	3.9	2.0	1.4	0.6	
D16			1.0	1.0	0.7	0.7	2.8	2.0	1.3	0.7	
D20			1.0	0.8	0.7	1.0	2.8	2.0	1.5	0.8	
G13			16.	0.5	0.3			2.0	1.5	0.8	
G14			>16.	0.5	0.2			1.0	2.2	0.7	
G15			4.	0.5	0.2	2.0	2.8	2.0	2.0	0.7	

TABLE VI—*Continued*

Preparation Number	Concentration Ratio*										
	Isoagglutinin		Anti-comp. act.	Typhoid Agglutinin		Mumps comp. fix.	Influenza A			Diph-theria Antitoxin	Strepto-coccus Antitoxin
	Anti-A	Anti-B		O	H		Comp. fix.	Hirst test	Mouse prot.		
Method No. 3B											
A111			>2.0	0.8	0.5	0.5	0.5	1.0	1.5	0.6	0.9
A269K			2.0	1.5	0.3	1.4	a	1.0	0.9	0.6	0.7
A291K			8.	1.0	0.3	1.0	2.0	2.0	0.7	0.5	0.4
E1			1.0	1.0	0.5	0.7	0.7	1.0	1.0	0.5	0.8
E3			0.3	0.5	0.5	1.4	1.0	1.0	1.0	0.4	0.4
E4			2.0	1.0	2.0	0.7	4.0	1.0	1.7	0.6	
F1			8.	1.0	0.5			1.0	1.2	0.8	
F2			1.0	0.5	0.7	0.3	0.5	1.0	0.7	0.7	
Method No. 3C											
A1–120			16.	0.7	0.7			0.5		0.6	0.7
A2–120			>8.	1.4	0.5			1.0	0.4	0.4	
A1–122			4.	0.7	0.1			1.0	1.1	0.4	0.6
A2–122			>8.	0.7	0.3			0.1	0.9	0.5	0.6
A1–126			>16.	0.5	0.5			2.0	1.2	0.6	1.0
A1–371			0.5	0.8	0.5	1.4	2.8	2.0	1.4	0.6	0.7
A2–371			>4.	0.5	0.7			2.0	1.3	0.6	0.7
A1–388			>8.	0.5	0.2			2.0	0.8	0.7	1.6
A1–388B			2.0	0.7	0.5	0.7	2.8	2.0	1.5	0.8	2.0
A1–LY1			>8.	0.7	0.2	0.7	1.0	2.0	0.8	0.9	2.0
Method No. 3D											
A1–329			1.0	0.7	0.3	0.7	1.3	1.0	0.6	0.7	1.5
Method No. 3E											
A1–133			4.	1.0	1.4	0.7	2.8	2.0	1.2	0.7	2.0
Averages	(1.5)	(0.9)		0.9	0.5	1.0	1.9	1.3	1.2	0.7	0.9

* Ratio = $\frac{\text{end-point of fraction}}{\text{end-point of standard (A66)}}$.

a. Fixes normal parotid gland or normal egg, and test could not be carried out.

antitoxin and the isoagglutinins, the standard was titrated simultaneously with each of the preparations.

The figures serve again to emphasize the general uniformity of the majority of the preparations in respect to antibody content. It is necessary, however, to consider the significance of certain outstanding differences. It might be inferred that the pools of plasmas from which the preparations exhibiting high ratios were prepared contained an originally higher concentration of a particular antibody. In most instances, a correlation of this sort has been noted between the level of plasma antitoxin and that of the fraction. Thus, preparation A109, was found to contain about 4.6 units of diphtheria antitoxin. Its plasma was assayed at 0.25 units. In contrast, preparations A80 and A84 (Tables V, VI, and VII) were assayed at only 1.8 and 1.7 units, respectively, and the corresponding plasma values were determined as about 0.08 and about 0.09 units. The plasma value for diphtheria antitoxin of the

TABLE VII

Immunologic assay of various preparations of Fraction II

Antibody Concentration Referred to Plasma

Preparation number	Concentration referred to plasma									
	Isoagglutinin		Typhoid agglutinin		Mumps comp. fix.	Influenza A			Diphtheria antitoxin	Streptococcus antitoxin
	Anti-A	Anti-B	O	H		Comp. fix.	Hirst test	Mouse prot.		
Method No. 2										
A48	4	4								20
A54R	4	4	1	32			16	16	20	27
A58	1	1	2	32			16	25	39	
Method No. 3										
A66	2	4	2	17	20	18	10	29	34	20
A72	3	4	2	30			16	68	41	20
A80R			<2	16			4	27	23	25
A84			2	<10			4	24	18	
A109			4	16			8	18	18	
Method No. 3A										
A97			2	12			8	31	25	
Method No. 3B										
A111			>3	>16			4	25	20	
Method No. 3C										
A1–120			2	22			4	18	20	
A2–120			3	16			8	5	21	
A1–122			1	8			8	10	30	
A2–122			1	16			1	9	30	
A1–126			2	11			16	21	24	
Method No. 3D										
A1–113								17	27	
A2–113								50	24	
Method No. 3E										
A1–133			2	14			16	10	20	
A2–133			5	11			16	9	17	
Averages	(3)	(4)	2	19	(20)	(18)	10	23	25	22

pool from which the standard (A66) was prepared was not unusually high, and it is therefore impossible on this basis to account for the large amount of antitoxin found in the standard. Possibly the plasma antitoxin was originally higher than indicated by the titration values, since it was allowed to stand at ice-box temperature for some time before it was preserved in the

frozen and dried state and titrations were carried out. One can also think that the processing of A66 differed in some unknown respect from that of other preparations. This possibility, however, seems unlikely.

On the basis of our results and observations of many tests in mice, we are inclined to regard a ratio of 2 in measurements of influenza A neutralizing antibody as signifying that the preparation contains definitely more antibody than the standard. Similarly, ratios of 0.5 or less suggest that the fraction is not equivalent to the standard. We have no definite indication that the plasmas employed in the preparation of certain of the fractions and which have been tested for influenza neutralizing antibody differed significantly in the amount of this antibody and therefore are unable, as was the case in certain instances with the diphtheria antitoxin, to attribute large differences between ratios to this cause. It is possible, however, that repeated testing of plasmas, as well as analysis of a larger number, collected from different geographical areas than we have examined, might in some instances reveal significant differences in the antibody content of successive pools. Certainly, one would expect that pooled blood, collected after the cessation of an epidemic of influenza A, such as now exists in the United States, should exhibit a marked increase in antibody compared with the pools mentioned in this report which were studied before the onset of the epidemic. Indeed, this expectation has been confirmed by the results of titrations on the most recently prepared fractions which are derived from plasma collected subsequent to this epidemic of influenza (see Tables V and VI).

In Table VII, the results of a few direct comparisons of antibody content of plasmas and their corresponding fractions are presented. It is evident that the factors of concentration for the antitoxins, influenzal neutralizing antibody and typhoid H agglutinin, in most instances, are between 20 and 30. Such concentrations are comparable to those obtained for the standard, A66. Data for mumps and influenza complement fixing antibody are not included (except in the case of the standard) as they were regarded as not quantitatively accurate in these instances. The results from titrations of the hemoagglutinin inhibitor give what are probably erroneously low values because of the presence of the non-specific factor which has already been mentioned.

The stability of antibody in Fraction II

From the practical standpoint it was important to ascertain whether antibody associated with Fraction II remained unchanged in amount during (*1*) prolonged storage in the dried state and (*2*) after preservation at elevated temperatures. In Table VIII are included the results of titrations on 3 preparations, 2 of which had been kept at room temperature during the course of 9 months, the third having been preserved in the ice-box at 0° C. The titrational data are also presented which were recorded on the basis of tests carried out before storage was inaugurated and shortly after the processing was completed. It is apparent that storage under either condition did not significantly reduce the titer of any of the antibodies mentioned in the table. The results for complement fixing antibodies are not given, since again they were regarded as quantitatively unreliable, but they do not suggest that loss of activity has occurred. The figures recorded in Table IX, which were obtained from tests on preparations which were heated for 2, 4, and 14 days, respectively, at 50° C., indicate that little or no loss of antibody activity took place under these conditions.

TABLE VIII

Immunologic assay of Fraction II after storage as dried powder

Preparation number	Time (months) and temp. of storage	Titer of solution				Units diphtheria antitoxin per ml. solution
		Typhoid agglutinin		Influenza A		
		O	H	Hirst test	Mouse prot.	
A29	0	4	64	256	90	1.2 a
A29	9 (RT)	8	128	256	64	2.0
D26	0	4	90	256	80	2.8
D26	9 (RT)	<4	64	256	106	2.3
D36	0	6	90	256	98	2.8
D36	9 (0°)	6	64	512	193	2.7

a. Early titration value uncertain.

Although the data are few, it is clear from these experiments on the effect of storage and

TABLE IX

Immunologic assay of immune serum globulin solutions after heating at 50° C.

Preparation number	Time and temp. of storage	Titer of solution				Units diphtheria antitoxin per ml. solution
		Typhoid agglutinin		Influenza A		
		O	H	Hirst test	Mouse prot.	
A54R	0	0	128	512		1.9
A54R	44 hours	4	128	256		1.2 a
A35	0	12	180	256	113 b	2.7
A35	96 hours	4	180	256	80	2.7
A35	14 days	6	64	512		2.6

a. Value may be too low because of titration interval.
b. Average 3 values—67, 60, and 213.

heating that the antibodies of Fraction II, for which tests have been made, are stable under conditions to which they would be subjected if employed in the civil or military practice of medicine.

The antibody content of Fraction III-1

It will be recalled that Fraction II was developed by subfractionation of Fraction II + III. A 12 per cent solution of the latter will occupy only one-tenth the volume of the plasma from which it was prepared, and hence would show a maximum concentration of about 10 times. Fraction III, obtained by the separation of Fraction II, has been separated into 2 subfractions designated III-1 and III-2. On the basis of its volume compared to the plasma from which it was obtained, a 20 per cent solution of Fraction II would show a maximum antibody concentration of about 40 times that of the plasma and Fraction III-1 about 25 times. Fraction III-2 containing the prothrombin and complement component $c'1$ (1), consists of a small fraction of the proteins composing Fraction II + III.

Although the 15- to 30-fold concentrations of antibody over plasma obtained by the isolation of Fraction II are remarkably high, as judged by a comparison of the results obtained by a variety of methods which have previously been employed in the concentration of immune sera, it was apparent from the factors of protein concentration just mentioned that considerable quantities of antibody were either inactivated during the process or were recovered in Fractions III-1 or III-2. That no significant amount of antibody was carried over into Fraction IV will be recalled from the findings previously set forth. It became of interest, therefore, to assay immunologically a few specimens of Fraction III-1. The results obtained with 2 preparations and recorded in Table X indicate that much of the

TABLE X

Immunologic assay of 2 preparations of Fraction III-1

Preparation number	Antibody concentration referred to plasma				
	Typhoid agglutinin		Influenza A		Diphtheria antitoxin
	H	O	Hirst test	Mouse prot.	
III-1 A80	12	21	5	12	5
II A80	16	1	4	27	20
III-1 A84	> 6	6	5	15	0.7
II A84	>16	2	4	24	30

antibody not accounted for in Fraction II is concentrated in Fraction III-1. Thus, on the basis of the protein concentration factors mentioned above, it can be calculated that considerable amounts of the influenza mouse protective antibody were found in Fraction III-1 of A80 and A84.[10] The distribution of influenza hemoagglutinin is similar to that of the mouse protective antibody. Again in this case, the relatively low values are to be attributed to the presence of the non-specific factor, we believe. About 20 per cent or less of the available diphtheria antitoxin may go into the III-1 moiety and somewhat over half the H typhoid agglutinin. As was expected, because of the low value of Fraction II for O agglutinin, nearly all of this antibody was shown to be associated with III-1. The fact that a marked separation of O agglutinin was secured, in contrast to the behavior of the other antibodies, affords further evidence that it is probably not associated with the same species of protein as the latter.

[10] The yields of gamma globulin should decrease in Fraction III-1, and increase in Fraction II, as improvements are made in the theory and practice of this separation.

DISCUSSION

The plasma fractionation method being employed in the processing of Red Cross blood has yielded Fraction II, a concentrate of the normal human γ-globulins in which a fairly representative group of antibodies has been separated from the other proteins of human plasma.

In the case of neutralizing antibody for influenza A virus, the potency of Fraction II appears to approximate that of the average of sera taken during early convalescence from the disease. It will be recalled that the mean titer of the 29 preparations of Fraction II mentioned in Table V was 1:129. Seven preparations, however, have exhibited titers of 1:160 or over, and 11, of 1:130 or over. Horsfall (8), using essentially the same technic as ourselves, obtained a mean titer of 1:209 for convalescent sera, drawn on the average 30 days after the onset of the disease. This figure is perhaps somewhat too high to be representative of the true mean of convalescent sera studied by him since it is derived from the data obtained in 4 epidemics, in 3 of which the mean titers were 1:61, 1:130 and 1:194 respectively, whereas, in the fourth, the mean titer was 1:424.

When the titers of complement fixing antibody in sera taken from individuals recently convalescent from influenza A are compared with the end-points obtained with Fraction II, it is found that the latter again approach the average convalescent serum in concentration of this antibody. For example, in a recent epidemic of influenza A in Boston, the mean titer of sera drawn from 20 individuals on the average of 18 days following the onset of symptoms was 1:363.[11] This figure does not widely differ from the average titer of complement fixing (1:280) antibody, determined in the case of the standard globulin preparation (Table III).

In contrast to influenza A, the concentration in Fraction II of the complement fixing antibody of mumps is definitely less than that occurring in most convalescent sera. The mean final titer [12] of mumps complement fixing antibody in 33 sera drawn during the third and fourth weeks of convalescence has been determined as 1 : 1300.[13] The average final titer of this antibody in the standard (Table III) was 1 : 240 (*i.e.*, 3 times the initial titer given in the table). The most probable explanation for this difference between the amounts of influenzal and mumps complement fixing antibody in the fraction may lie in the fact that the quantity of mumps antibody in plasma is on the average less than that of influenzal antibody. As indicated by the results from comparative determinations on 6 pools of plasma, the mean titer of the influenzal antibody is about 4 times greater than that of the mumps antibody.

Very little information is available on the antitoxin content of the serum in those convalescent from untreated diphtheria, since the administration of antitoxin has been generally employed for many years. The largest number of such determinations which we have been able to find recorded are those of Schürer (9) who examined the sera of 18 convalescents, drawn between the 25th and 60th day after onset. In this group, the average was 0.13 unit per ml. Individual sera varied from 0.02 unit to somewhat greater than 1 unit in content of antitoxin. It is evident, then, that the preparations of Fraction II (Table V) contain on the average about 10 times the quantity of antitoxin which is usually present in convalescent sera. But in this connection it should be pointed out that the low antitoxin content of the diphtheria sera is exceptional. In most other acute infectious diseases, the serum of the recent convalescent generally contains many times the quantity of antibody which is characteristic of the normal serum (10).

As with the data for levels of diphtheria antitoxin in the sera of recent convalescents, so the

[11] The titrations on which this figure is based were done partly in the laboratory of the Department of Bacteriology and Immunology at the Harvard Medical School and partly in the laboratory of the Thorndike Memorial, Boston City Hospital, by Dr. Maxwell Finland and his associates to whom we are indebted for permission to use their data.

[12] We have employed final dilutions in computing the average for convalescent sera because the volumes employed in our earliest tests on convalescent sera were not the same as those subsequently used.

[13] This relatively high value is the average of unpublished data obtained in the Department of Bacteriology and Immunology, Harvard Medical School. On the other hand, two pools of mumps convalescent sera collected for fractionation at the Harvard Plasma Fractionation Laboratory from about 400 donors have final titers of 1 : 240 and 1 : 480.

recorded information concerning the amount of streptococcal antitoxin in those just recovered from scarlet fever is scanty. Rhoads and Gasul (11), in 12 pools of convalescent sera, found the average titer of the pools to be about 10 units per ml. These authors do not mention the number of individual sera of which each pool was composed. For purposes of comparison, they also determined the average titer of 3 pools of normal adult sera and found it to be somewhat less than 5 units per ml. Moore and Thalhimer (12), in a study of 51 convalescent sera, found the average titer to be 3.3 units per ml. This figure is similar to the results obtained earlier by Henry and Lewis (13). Thus, a comparison of the titrational values of Fraction II for streptococcal antitoxin (Table V) with these results which have been recorded in the literature shows that the globulin concentrates contain on the average more than 3 times the quantity of antitoxin. Even the preparations exhibiting the lowest titers are at least twice as potent as the average convalescent serum.

The concentration of the H typhoid antibacterial antibody in Fraction II is, on the average, definitely less than that ordinarily encountered during the latter stages of typhoid fever and convalescence therefrom. Thus, Gardner and Stubington (14), in a series of 40 typhoid patients, obtained titers of over 1:1000 in 14, and over 1:128 in 23. Only 3 cases exhibited titers of less than 1:128. The average was 1:2130. The mean of our preparations of Fraction II was 94 (Table V). In 6 instances, however, titers were recorded comparable to those found for certain of the sera studied by Gardner and Stubington.

The O antibody level in Fraction II, as we have pointed out, scarcely exceeds that of the pooled plasma, since this antibody is separated from the others and recovered in Fraction III-1. There the amount of O antibody in the few specimens which have been titrated is considerably less than that found by Gardner and Stubington in their 40 sera, the average titer of which was 1 : 906.

A few determinations of the titer of Fraction II in protective antibody against infection of mice with *E. typhosa* have been carried out by Major G. S. Luippold at the Army Medical School. The results revealed antibody levels comparable to those characteristic of individuals who recently have received the standard course of typhoid vaccines.

Since, in a number of infectious diseases, convalescent serum has been successfully employed as a therapeutic or prophylactic agent, we have carried out the foregoing comparisons with the purpose of securing some indication as to whether Fraction II on *a priori* grounds might prove as effective in such applications as the serum. It is clear that no general conclusion in this respect can be drawn. In certain instances, the fraction may exceed or equal the potency of convalescent sera; in others, it falls definitely below the average level of convalescent antibody. In no case, however, where the data are adequate, save in that of the O typhoid antibody, is the factor of difference in potency of the average preparation of Fraction II and the convalescent serum much over 4 or 5.

These comparisons also serve to emphasize the expediency of titrating each newly prepared lot of Fraction II along with a standard preparation, in order to ascertain whether or not it bears the same general relation in respect to a variety of antibodies to convalescent sera as the standard. Since it has not yet been demonstrated that Fraction II is of value in any disease save measles, and since we are unable to measure the concentration of measles protective substance directly, the reader may be at a loss to appreciate the necessity for this recommendation which involves an assay of entirely unrelated antibodies. It would seem, however, to be the only manner by which we can, in our present state of knowledge, obtain at least presumptive evidence that the measles antibody (or any other antibody for which laboratory tests cannot be made) is present in adequate amount. We make this statement because of the following considerations. The standard has been shown to be effective by clinical test in the prevention and modification of measles (15, 16). The levels of the various antibodies in pooled adult plasma obtained in the northeastern, midwestern, and Pacific sections of the United States appear on the basis of our findings to show relatively little variation. Consequently, it seems logical to think that the quantity of antibody for measles virus should

not vary greatly in the plasma pools and should be concentrated in Fraction II to the same degree as those reacting with the antigens for which we have tested, in particular the viruses of influenza A and mumps.

If this hypothesis be correct, it is clear that a given preparation of Fraction II which is shown to be markedly inferior in potency to the standard should not be employed. It is, of course, possible that as experience is gained from trial in the field that the procedure adopted in the present investigation for estimating the value of preparations of Fraction II can be modified in the direction of simplification by eliminating tests for certain antibodies.

In respect to the theoretical problems raised at the beginning of this communication, it must be frankly stated that we cannot as yet give definitive answers. Thus, the evidence presented does not warrant the statement that the antibodies of human plasma are exclusively associated with the γ-globulin. Our findings, however, strongly suggest that the antibodies reacting with 2 viruses (or their products), diphtheria and streptococcal antitoxin, and the H antibody for *E. typhosa* are γ-globulins. This is indicated by the fact that 50 per cent or more of these antibodies can be recovered in certain preparations of Fraction II which contain 95 to 100 per cent γ-globulin. Furthermore, no decrease was observed in the concentration of these antibodies in Fraction II as the percentage of γ-globulin was increased by appropriate procedures from 80 to 100 per cent. The observed antibody concentrations over plasma are also very near that calculated for the concentration of γ-globulin in these solutions.[14]

The nature of the protein associated with the O typhoid antibody and isoagglutinins is less certain since these antibodies could be almost quantitatively recovered in Fraction III-1 which contains approximately 3 per cent α- and 70 per cent β-globulin in addition to 25 per cent γ-globulin. Because of the large amount of γ-globulin present in III-1, it is entirely possible to believe that these antibodies are included therein. But if this be so, it would seem that the γ-globulin with which they are bound is probably different from that associated with antibody in Fraction II because of the separation of these antibodies from the others. There is no evidence, however, to eliminate the possibility that the O antibody and the isoagglutinins are included in either the α- or β-globulins or indeed in both of them.

Because of the nature of the antigens involved, the difference in chemical behavior of these two antibodies naturally gives rise to speculation. Our knowledge of the nature of the antigens homologous for the various antibodies with which we have dealt indicates or suggests that both the toxins, the two viruses, and the H typhoid antigen are composed largely or entirely of protein and that the O typhoid and the isoagglutinins are complex substances consisting, in part, of polysaccharides. Accordingly, it is perhaps not illogical to consider the possibility that antigens of the latter type react with antibodies which in turn can be distinguished by differences in their physicochemical properties from those which are specific for simple protein antigens. Before any correlation of this sort can be definitely asserted, it is clear that many more antigenic species and their corresponding antibodies must be investigated.

In concluding this discussion, we would again emphasize the following facts of practical value which the data have revealed. A concentration of about 15 to 30 times of certain of the normal antibodies of human plasma has been obtained by the separation and purification of the γ-globulins. A large number of preparations of Fraction II, produced both at Harvard and by manufacturers elsewhere, from pools of plasma obtained in several areas throughout the United States, have been tested for their content of 6 different antibodies and a satisfactory degree of uniformity between these various preparations has been demonstrated.

CONCLUSIONS

1. The fractionation of normal human plasma collected by the American Red Cross yields a

[14] Plasma contains about 6.6 grams of γ-globulin per liter. If we standardize on a γ-globulin concentration of 165 grams per liter in our final Fraction II solution, this yields a concentration factor of 25 times. Such a solution will contain 16.5% protein if Fraction II is 100% γ-globulin, but must be more concentrated if the purity is lower, a 20% solution of Fraction II containing 82% γ-globulin yielding about the same concentration factor.

gross fraction comprised of α-, β- and γ-globulins designated Fraction II + III which contains a large proportion of the antibodies reacting with a variety of pathogenic bacteria and their products, viruses, and the iso-antigens of the human blood groups concentrated from 4 to 10 times.

2. The normal human γ-globulins have been further separated and concentrated in Fraction II. This has been found on immunologic assay to contain antibodies reacting with diphtheria toxin, streptococcal erythrogenic toxin, influenza A virus, mumps virus, and the H antigen of *E. typhosa*. These antibodies were concentrated from 15 to 30 times as compared to pooled plasma.

3. In solutions concentrated to at least 25 times the plasma pool Fraction II gives titers of certain antibodies comparable with or greater than those of the corresponding convalescent sera. In other cases, the potency of Fraction II is somewhat lower than the convalescent serum, but not by a factor of more than 4 or 5.

4. Antibody reacting with the O typhoid antigen was present only in low titer in Fraction II but was recovered in large amounts in Fraction III-1,—another derivative of Fraction II + III.

5. Immunologic assay of the antibodies in 62 preparations of Fraction II, derived from plasma collected in various sections of the United States, has revealed a general uniformity of potency. There has, however, been an increase in influenza A antibodies following a recent epidemic of the disease.

6. The titer of the antibodies of Fraction II after prolonged storage and exposure to moderately elevated temperatures is not significantly reduced under the experimental conditions described.

7. The implications of these findings to the standardization of Fraction II as an agent in the prophylaxis and therapy of disease are discussed, together with their significance in respect to the nature of normal human antibodies.

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