CORRELATIONS BETWEEN PLASMA PROTEIN FRACTIONS, ANTIBODY TITERS, AND THE PASSIVE TRANSFER OF DELAYED AND IMMEDIATE CUTANEOUS REACTIVITY TO TUBERCULIN PPD AND TUBERCULOPOLYSACCHARIDES*

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Delayed or tuberculin type sensitivity is characterized by the progressive development of an inflammatory reaction at the site of tuberculin deposits. This reaction reaches its peak after 24 hours and when intense is marked by tissue necrosis. The term, delayed, best applies to the gross appearance of the reaction since time lapse movies of this process (1) indicate that the reaction begins after a few minutes. Within several hours the gross reaction is marked by induration, erythema, and some edema. Histological studies of these changes, according to Dienes and Mallory (2), are characterized by a predominant round cell infiltration, vascular engorgement, and by variable amounts of polymorphonuclear infiltration. In contradistinction, Follis (3) found that polymorphonuclear leucocytes predominate.

Many efforts to show that this type response is due to a circulating antibody have been unsuccessful. Passive transfer of tuberculin type allergy by serum has been reported only once (4). Rich and Lewis found that tissue cultures of spleen, bone marrow, and peripheral blood cells from animals sensitized to tuberculin were injured by high concentrations of tuberculin (5). Moen and Swift (6) found that this cellular sensitivity is disease-specific. These *in vitro* observations have been the basis for the belief that delayed type allergy is cellular rather than humoral in the usual sense applied to immune responses.

There are certain features of tissue culture studies on tuberculin sensitivity which should be mentioned. Not all workers have been able to repeat these findings (7). Cultures of dermal cells (8) are quite insensitive to tuberculin in extraordinary amounts compared to those which cause brisk cutaneous tuberculin reactions. A similar lack of correlation between degrees of cutaneous sensitivity in the host and the noxious effect of tuberculin *in vitro* has been noted by others (9). An interesting approach to this problem has been offered by Miller and Favour who studied washed leucocytes from the peripheral blood of tuberculin-sensitive hosts (10). They found that lympho-

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cytes from the peripheral blood elaborate a humoral factor which, some of the time, can be found in the plasma. This factor is capable of causing cell lysis, by tuberculin, of a portion of cells in the system studied. Again a poor correlation between degrees of cutaneous tuberculin reactivity and *in vitro* cytolysis suggests that other factors condition tuberculin sensitivity in the living host. Waksman (11), who studied the tuberculin cytolysis phenomenon in sensitized rabbits, also found this same poor correlation.

Chase has supplied an important link in our knowledge of these phenomena (12). He demonstrated that cells from peritoneal exudates and lymph node creams will passively transfer tuberculin reactivity. In his hands these cellular transfers were unsuccessful if the cells were not viable. Lawrence, however, working with human peripheral blood leucocytes, has found that even lysed cells will transfer tuberculin reactivity (13). If confirmed, his work will be substantial evidence that at the cellular level tuberculin reactivity is mediated by a soluble factor elaborated by specialized cell types.

Delayed type tuberculin reactions also can be characterized by contrasting them with immediate or urticarial allergic responses. The gross lesions of immediate reactions begin within minutes after the local instillation of the appropriate antigen. Edema rather than induration accompanies an early rapidly appearing erythema. Microscopically these lesions show vascular engorgement, and little cellular infiltration. More intense reactions are accompanied by still greater vascular injury, secondary inflammation, and even cell necrosis. Polymorphonuclear cells are prominent in the tissue changes. Intense reactions may not reach their peak for 24 hours or longer (Arthus reaction). This reaction can be transferred with serum and is proportional to the antibody titer in the serum used. It can also be transferred with washed lymphnode cells (14). Following this type of transfer, circulating antibodies can be detected in the serum of recipients.

Tuberculin type allergy ordinarily refers to the delayed type of response just defined. It has long been known, however, that allergy in tuberculosis is dual in type. Baldwin (15), using crude extracts of tubercle bacilli, demonstrated a positive Schultz-Dale test and sometimes could produce anaphylaxis with tuberculin in sensitized guinea pigs. Zinsser and Parker (16) also described similar anaphylactic reactions to tuberculin. Immediate type reactivity appeared in animals infected with BCG after 3 weeks. Delayed type allergy appeared between 5 and 14 days. Some evidence has accumulated to suggest that this anaphylactic type response is elicited by tuberculopolysaccharide, whereas the delayed sensitivity pattern is caused by tuberculoprotein. Enders (17) found that, in guinea pigs actively or passively sensitized to tubercle bacilli, the tuberculopolysaccharide of Mueller (18) evoked positive Schultz-Dale reactions and anaphylaxis. On the other hand, a large body of evidence, well summarized by Drea and Andrejew (19), indicates that the delayed type of tuberculin reaction is in response to a protein material.

Certain evidence has suggested that the antibody response in tuberculosis is also of a dual nature. Keogh and his colleagues (20) described a method for the adsorption onto normal red blood cells, of polysaccharide from strains of *H. influenzae* and other bacteria. These sensitized cells were agglutinated by corresponding antisera. This technique has been applied to the study of tuberculosis by Middlebrook and Dubos

(21), who employed an extract from tubercle bacilli as a sensitizing agent, and demonstrated that tuberculopolysaccharide could inhibit the hemagglutination more effectively than could tuberculoprotein. Boyden (22) evolved a different type of hemagglutination technique, whereby diluted purified protein derivative (PPD) is adsorbed onto red blood cells pretreated with tannic acid. Grabar and his associates (23) found that tuberculoprotein inhibited this antibody more powerfully than did tuberculopolysaccharide, and confirmed the observation that the reverse was true of the Middlebrook-Dubos system. These observers also employed cross-adsorption studies to show that the two types of sensitized red blood cells measure antibodies to unrelated antigens. Meynell (24) has presented evidence to indicate that, in the preparation of Boyden antigen, the small amount of polysaccharide material present in PPD becomes so dilute as to be incapable of adsorbing onto the red blood cells in significant quantity.

The present paper attempts to explain and correlate some of the phenomena described in the brief preceding review. Large volumes of immune guinea pig plasma were fractionated by Method X of Cohn and his colleagues (25). Thereafter the separate fractions were injected intraperitoneally into normal guinea pigs. Following the passive transfer of these fractions, animals were skin-tested with a variety of derivatives from the tubercle bacillus. The fractions were also tested for their respective titers of hemagglutinating antibodies by the Middlebrook-Dubos method, and by a modified tanned cell method.

Two clear correlations between chemical and biological methods have been found.

1. Antibody to tuberculopolysaccharide can be demonstrated only in fraction II (gamma globulin). This fraction passively transfers anaphylaxis and urticarial type skin reactivity to tuberculopolysaccharide, and contains the entire Middlebrook-Dubos titer of the plasma.

2. Antibody to tuberculoprotein is found exclusively in a new plasma fraction which we have defined and named fraction IV-10. It is a subfraction of the alpha globulin, and represents approximately 0.6 per cent of the plasma protein. It will passively transfer a delayed type skin reactivity to tuberculin PPD which is maximal in 18 to 30 hours. It contains the entire Boyden titer of the plasma. When fractions II and IV-10 are combined, passive transfer of delayed type skin reactivity is completely inhibited. This combination does not affect the various hemagglutinating titers.

Materials and Methods

Animals.—Guinea pigs of both sexes, weighing 300 to 900 gm., were immunized by a series of subcutaneous injections of H37Rv tubercle bacilli. The organisms were grown on the mass culture medium described by Dubos and Middlebrook (26), autoclaved at 5 pounds' pressure for 20 minutes, washed 4 times in distilled water, and lyophilized. Loss of viability was demonstrated by culture of large numbers of this preparation, on standard laboratory media, for the growth of tubercle bacilli. Each injection contained 10 mg. of organisms suspended in 1 ml. light mineral oil. Animals received the first three injections at weekly intervals and

thereafter the injections were given every 3 to 5 weeks depending on the degree to which they reacted to skin test doses of 5 μg. PPD tuberculin. Skin reactivity to PPD appeared during the first 3 weeks of immunization and after 4 weeks, the skin reactions usually developed areas of central necrosis. Except when otherwise mentioned, a mixture of donors, immunized for 3 weeks to 5 months, was used in each experiment. Animals were skin-tested with PPD or OT 24 to 48 hours before being bled, and only those with skin reactions greater than 1 cm. diameter of induration were selected.

Recipient guinea pigs for passive transfer studies were albino animals of both sexes weighing from 250 to 300 gm. All recipients were shown to be tuberculin-negative within the 72 hours before injection of a plasma fraction.

Fractionation Procedure.—Method X (25) was followed closely but important minor changes necessitate a full description of the technique. In these experiments, it was found to be essential that all zinc be removed from the fractions and that they be prepared in the most physiological manner possible before injection into animals.

Groups of 16 to 20 guinea pigs were bled by cardiac puncture. To 1.75 ml. ACD solution,[1] was added 8.25 ml. of blood from a single animal. This was mixed and when all blood specimens had been collected, they were centrifuged at 1800 R.P.M., 4°C., for 45 minutes. The separate plasmas were then pooled and chilled for fractionation. The time interval from cardiac puncture of the first donor to commencement of the fractionation procedure was less than 3 hours.

The description of the method which follows is for 100 ml. ACD plasma. The procedure was carried out in a −5°C. cold bath[2] equipped with holders for reagents. The reagents were prepared at 25°C. and then cooled to −5°C. Centrifugings were done in 100 ml. tubes, in an International refrigerated centrifuge, model PR-1,[3] maintained at −5°C. Sterile glassware was used throughout.

A flask containing 400 ml. of solution A[4] (terminology of Lever and his colleagues (27)) was clamped in the cold bath. The 100 ml. ACD plasma was placed in a 1000 ml. flask, and held submerged in the cold bath with one hand. From this point to the end of the first step, the plasma was kept in constant motion by swirling rapidly enough to cause fairly vigorous movement without foaming. As 0°C. was approached, the solution A was slowly dripped in with a 5 ml. pipette during a period of 50 minutes, care being taken initially not to freeze the plasma. The material then stood for 40 minutes without stirring. The pH should be 5.9–6.0 (0.5 ml. diluted to 2 ml. with 0.02 N NaCl). At this point there was a thick precipitate.

IV-10.—The ultimate location of fraction IV-10 was determined when the plasma plus solution A had stood for 40 minutes. This was because it remained soluble in solution A for less than 3 hours. Thus, if the material was now centrifuged at 3,000 R.P.M. for 25 minutes, the fraction IV-10 was contained in the supernatant, which was poured into a flask. A fine precipitate was visible in 3 hours, and maximal after 18 hours, but did not settle to the bottom of the flask. It was then centrifuged at 3,000 R.P.M. for 45 minutes and the clear supernatant poured off. The precipitate formed a thin, very adherent layer coating the sides of the centrifuge bottles, as well as a small clump at their bottoms. The IV-10 was suspended in a minimal

[1] ACD solution: 2.67 gm. sodium citrate (5½ H_2O), 2.2 gm. dextrose, 0.8 gm. citric acid, distilled water to make 100 ml.

[2] A 50 gallon capacity cold bath supplied by the American Instrument Company, Silver Springs, Maryland, was used.

[3] International Equipment Company, Boston, Massachusetts.

[4] Solution A: 500 ml. 95 per cent ethanol, 3.7 ml. acetate buffer, and distilled water to 2000 ml. An aliquot tested against plasma should give pH 5.9–6.0 and may require addition of NaOH. For this procedure acetate buffer is: 400 ml. of 10 M acetic acid, 200 ml. 4 M sodium acetate, and distilled water to 1000 ml.

amount of 0.85 per cent NaCl, and dialyzed overnight in 3,000 ml. of 0.80 per cent NaCl in tap water. It was then a clear solution (Fig. 3).

If, after the first centrifugation described above, the zinc acetate step was taken, IV-10 precipitated with fractions IV + V and thence went mainly into fraction IV (Fig. 1). If, on the other hand, the ACD plasma–solution A mixture was not centrifuged at all until the next morning, IV-10 became exclusively a part of fraction II (Fig. 2).

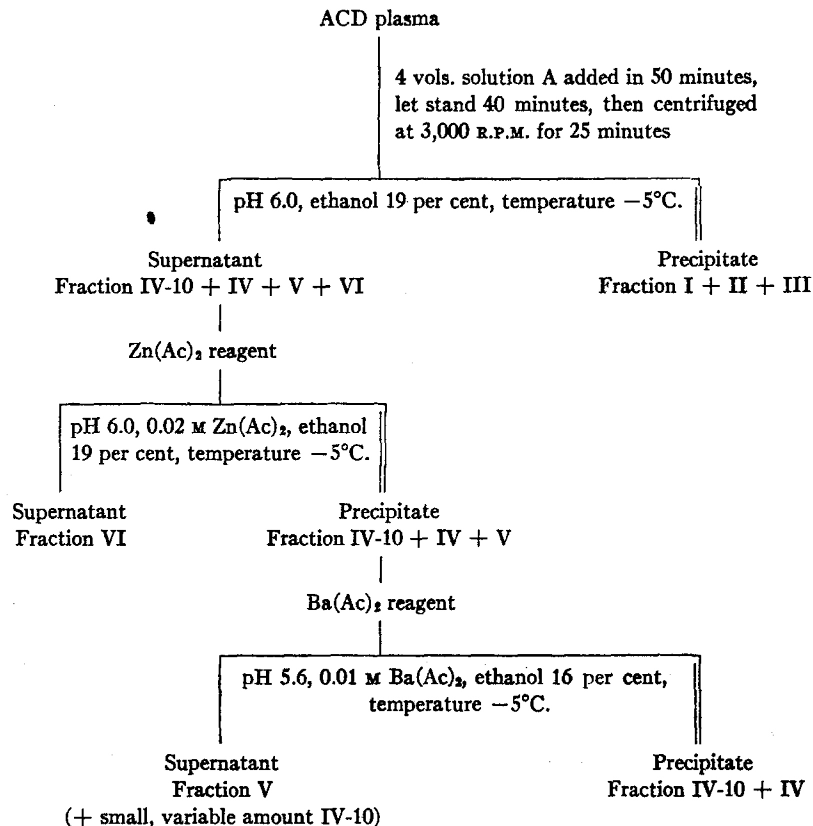
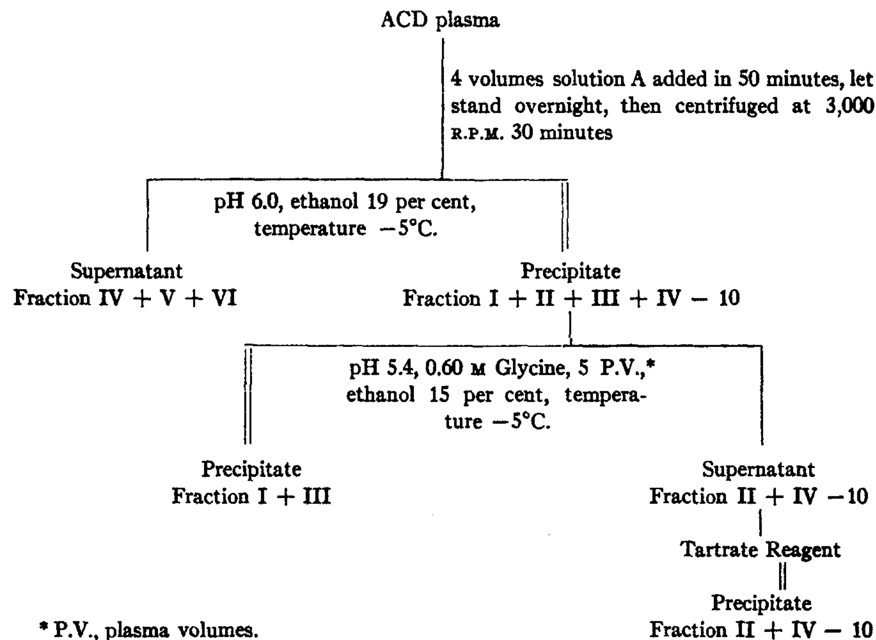


FIG. 1. Localization of fraction IV-10 in standard Method X.

IV + V (Alpha Globulin + Albumin).—The ACD plasma–solution A mixture was centrifuged, the supernatant poured into a chilled flask, and 40 ml. Zn $(Ac)_2$ reagent,[5] prepared the day of use and refrigerated immediately, was then added with stirring. After 45 minutes, this mixture was centrifuged at 3000 R.P.M. for 60 minutes and the supernatant poured into a second chilled flask. Most of the precipitated IV + V was aspirated directly into a pipette and transferred into a −5°C. dialysis bag. The remainder was suspended in a minimal volume of 0.5 M Na citrate and also transferred. The dialysis bag was tied compactly and 3 rubber

[5] 2.74 gm. $Zn(Ac)_2$, 10 ml. 95 per cent ethanol, and distilled water to 50 ml.

bands bound at intervals along its length to compress it. It was dialyzed at 0°C. for 48 hours against 3,000 ml. of 0.75 per cent NaCl, containing 10 gm. sodium ethylenediaminetetra-acetate (EDTA),[6] adjusted to pH 6.9–7.1 with a 0.1 M phosphate buffer. The dialysate was then changed to 3,000 ml. 0.75 per cent NaCl, pH 6.9–7.1, and dialyzed for an additional 18 hours. At this point the IV + V was completely dissolved, was free of Zn, and almost free of EDTA. A volume of approximately 40 ml. was obtained, which was ready for testing and injection.



* P.V., plasma volumes.

FIG. 2. Modification of Method X whereby fraction IV-10 falls into fraction II.

Sodium Ethylenediaminetetraacetate.—Disodium versenate,[7] analytical reagent grade was used. Because it is strongly acidic it was brought to the desired pH with 2 N NaOH. A 10 per cent solution, pH 7.3, was kept in either a siliconed flask or plasticene bottle. The latter was more satisfactory. EDTA removed Ca from glass at an alkaline pH.

Tests for Zn and EDTA.—A variation of the method described by Biedemann and Schwarzenbach (28) was used. Erio chrome black T,[8] in NH_4Cl-NH_4OH buffer pH 10.3, is blue but turns pink in the presence of trace amounts of Zn or Mg. A sensitive test for Zn can be done by adding 2 to 3 drops of the plasma fraction to the dye in buffered saline. For EDTA determinations, 0.15 ml. of 0.00028 M $MgCl_2$ was added to the dye solution and the unknown solution then added to the point when the pink color changed abruptly to blue. This color change indicated chelation of all the Mg. This is an equimolecular reaction. A safe EDTA level in plasma fractions for intraperitoneal injections was found to be 40 parts per million or less. The dye was prepared fresh for each use.

[6] EDTA, ethylenediaminetetraacetate.

[7] Kindly supplied by Bersworth Chemical Co., Framingham, Massachusetts.

[8] Allied Chemical and Dye Corporation, New York City.

IV and IV′ (*Alpha Globulin*).—To obtain fraction IV, the Zn-precipitated IV + V was extracted with 700 ml. of barium reagent,[9] for 1 hour. The degree to which V was removed depended upon the thoroughness of initial dispersion of the precipitate in the barium reagent. Fraction IV was then sedimented by centrifuging at 3000 R.P.M. for 30 minutes and freed of Zn as described under IV + V. When the Ba reagent with the dissolved albumin was held overnight at −5°C., a fine precipitate formed. Electrophoretic studies indicated that the precipitate contained alpha globulin and a proportionate trace of albumin. We have called this fraction IV′. Fraction IV′ was salvaged the day after fraction IV was harvested. It was injected into recipient pigs after removal of Zn. The IV′ came out of solution only after IV was first removed by centrifugation.

IV-1 (*Alpha-1 Lipoprotein*) *and IV-6,7* (*Alpha Glycoprotein*).—This subfractionation of IV was performed once according to the protocol in Method X (25). The IV-6,7 was recovered by repeating the conditions for precipitation with $Zn(Ac)_2$ reagent.

VI (*Glycoprotein, IV and V Contaminants*).—After precipitation of IV + V the supernatant was brought to pH 7.5 by the protocol of Schmid (29) and the resulting precipitate freed of Zn as described under IV + V.

I + II + III.—The initial precipitate, formed by solution A, was extracted with 75 ml. solution A′.[10] The supernatant after centrifuging at 3000 R.P.M. for 30 minutes was discarded to minimize possible contamination of IV + V + VI with small amounts of I + II + III.

II (*Gamma Globulin*).—The above precipitate was extracted with 5 plasma volumes of solution B[11] for at least 60 minutes. (Adequate dispersion of the precipitate was necessary. This was best done by adding small amounts of solution B to the precipitate, stirring, and pipetting it into a flask of solution B.) This was then centrifuged at 3000 R.P.M. for 30 minutes and the supernatant poured into an equal volume of sodium tartrate reagent.[12] The resulting precipitate was allowed to stand overnight before harvesting by centrifuging at 3000 R.P.M. for 30 minutes. Thereafter it was dialyzed against 3000 ml. 0.8 per cent NaCl in tap water (phosphate buffer caused a fine precipitate) to make it ready for tests and for injection.

III (*Beta Globulin*).—The precipitate I + III remaining after extraction with solution B was extracted with 75 ml. solution B′,[13] centrifuged at 3000 R.P.M. for 45 minutes, and the supernatant discarded. The precipitate was suspended in 20 ml. 0.85 per cent NaCl, buffered to 7.4 with phosphate buffer, and kept at 0 −4°C. overnight. After centrifuging at 2000 R.P.M., 0° for 40 minutes, the supernatant containing fraction III was ready for dialysis.[14] The precipitate, fraction I (fibrinogen), was discarded.

Volumes of the Isolated Plasma Fractions.—The final volumes of the plasma fractions were dependent upon the amount of saline adsorbed during dialysis. An increased volume was minimized by leaving no free potential space inside the dialysis membranes. The final volumes of the individual fractions were relatively constant as follows: II, 25 to 30 ml.; III, 25 to 30 ml.; IV-10, 6 to 8.5 ml.; IV, 10 to 15 ml.; IV′, 6 to 11 ml.; IV + V, 38 to 45 ml.; and VI, 12 to 19 ml.

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[9] 160 ml. of 95 per cent ethanol, 2.6 gm. $Ba(Ac)_2$, 20 ml. of M NaAc, and 7.3 ml. of M acetic acid per liter, pH 5.6.

[10] 200 ml. of 95 per cent ethanol, 40 ml. of M sodium acetate, 3.5 ml. of M acetic acid, and distilled water to 1000 ml., pH 5.8.

[11] 150 ml. of 95 per cent ethanol, 45 gm. glycine, 3.8 ml. of M NaAc, 2.6 ml. of M acetic acid, and distilled water to 1000 ml., pH 5.4.

[12] 9.0 gm. of tartaric acid, 4.75 gm. of NaOH, 475 ml. of 95 per cent ethanol, and distilled water to 1000 ml., pH 5.4–6.0.

[13] 150 ml. of 95 per cent ethanol, 45 gm. glycine, 5 ml. of M sodium acetate, 1.8 ml. of M acetic acid, and distilled water to 1000 ml., pH 5.4.

[14] This step was developed in our laboratory and has proved satisfactory.

Nitrogen Determination.—The method of Lanni, Dillon, and Beard (30) slightly modified, was employed.

Method of Passive Transfer and Testing of Plasma Fractions

Injection of Plasma Fractions.—Animals received the solutions of the plasma fractions intraperitoneally. The needle puncture site was sealed with flexible collodion. All fractions were well tolerated, though fractions IV + V, given in two doses on 2 days, caused transient edema of the abdomen. Fractions were injected immediately upon completion of dialysis.

Skin-Testing Procedure.—Skin test sites were prepared with a depilatory consisting of barium sulfide and flour in equal parts, made into a thin paste with tap water. Tuberculin tests were done with 5 μg. PPD freshly prepared from commercial tablets[15] or with dialyzed OT.[16] The test sites were measured for their greatest diameter of induration at regular intervals during the first 48 hours. These data are given in the tables. Brucellergen 0.1 ml., was used as a control antigen. In some instances it produced 2 to 3 mm. reactions at the time a delayed skin test to PPD was obtained. Other control antigens are mentioned in the text. Recipient animals were studied with skin tests for a minimum of 10 days.

Choucroun Polysaccharide.[17]—Skin test doses of 10 μg. were used. This soluble polysaccharide containing 1.05 per cent N, which is part of the "toxic fraction" of Choucroun (31), is obtained by hydrolysis of the fraction. It gave urticarial skin reactions in most of our donor guinea pigs. Repeated skin tests on normal guinea pigs failed to produce skin reactivity.

S4 and S6 Polysaccharides.[18]—Skin test doses of 10 μg. were used. Isolation of these polysaccharides is discussed by Seibert and Watson (32) and Tennent and Watson (33). S4 contains 0.85 per cent N and S6, 0.2 per cent N. Repeated skin tests on normal guinea pigs failed to produce skin reactivity. These polysaccharides gave minimal urticarial reactions in only a few of our donor guinea pigs.

Middlebrook-Dubos Test (*21*).—Plasma fractions did not give hemagglutination reactions unless 30 per cent normal guinea pig serum was present. This phenomenon seems well explained by Orlans (34). Also, fractions II, and IV + V, in high concentration lysed red cells in the absence of normal serum. To 0.4 ml. plasma fraction was added 0.2 ml. normal guinea pig plasma. The hemagglutination test was then done according to the protocol of Sherris (35), with the exception that Lederle 4X OT was used. No heat lability of this antibody was observed. The tubes were shaken, left standing for 30 minutes, shaken again, then kept at 4°C. overnight.

Boyden Test (*22*).—The modification described by Meynell (24) was followed with several important differences: our buffered saline was pH 7.3; tests were read after standing overnight at 4°C.; and the antigen employed was Parke-Davis PPD.[19] Most important in the protocol of Meynell is the use of 1.7 per cent instead of a 2.5 per cent (22) suspension of tanned sensitized, and unsensitized sheep cells, to increase the sensitivity of the test. Our red cell concentration was approximately 1.5 per cent due to cell losses into the supernatants following the tanning and sensitizing steps. Normal guinea pig serum (NGPS)[20] was used as a diluent

[15] Vials were kindly provided by Sharpe and Dohme Division of Merck and Company, Inc., Philadelphia.

[16] Kindly supplied by Lederle Laboratory Division, American Cyanamid Company, Pearl River, New York. OT five times international standard, lot 2725-28. Skin test doses were 0.1 ml. 1 to 1000 except where otherwise stated.

[17] Kindly given to us by Dr. Nine Choucroun.

[18] Kindly given to us by Dr. Dennis W. Watson, Department of Bacteriology, University of Minnesota, Minneapolis.

[19] Kindly donated by Parke, Davis and Company, Detroit. Lots 025194-G and 026269.

[20] NGPS, normal guinea pig serum.

instead of rabbit serum. Serum complement was inactivated by adding 10 per cent EDTA in a ratio of 0.4 to 10 ml. serum or plasma fraction (2½ times minimal estimated amount to chelate Ca and Mg in serum). We found it important to wash the sensitized cells twice with 0.4 per cent NGPS. The volume of the second washing was 5 ml. Tests were done in duplicate, one aliquot inactivated with EDTA and the other heated at 56°C. for 30 minutes (heat-inactivated NGPS diluent was used for the latter). This was done by adding 0.3 ml. of fraction to each of two tubes containing 0.1 ml. NGPS. The 56°C. aliquots were then heated. Two cell adsorptions with normal sheep cells for non-specific agglutinins were done, and then serial twofold dilutions 1:10 through 1:1280 were studied. After the test was set up, each tube received 0.015 ml. NGPS. Fractions were titered within 24 hours of completion of dialysis.

EXPERIMENTAL RESULTS

I. Method of Plasma Fractionation and Criteria for Skin Tests

Fractionation Method.—Method X (25) applied to guinea pig plasma gave results comparable to those obtained with human plasma. The large fractions were checked by an electrophoretic study[21] on two occasions, at protein concentration of 2 gm. per cent. Fraction II was electrophoretically "pure"; fraction IV + V had no visible I, II, or III; fraction IV and fraction IV′ each contained small amounts of V, but no visible I, II, or III.

Protein concentrations of the pooled plasma ranged from 4.8 to 6.0 gm. per cent. The protein concentrations of the individual fractions are shown in their respective tables. Their variations in concentration may be due to several factors: (*a*) duration of immunizations of the donors; (*b*) number of times donors were bled (always at least 12 days apart); (*c*) general health of donors. The amount of fraction V left with IV is variable at best.

Our orientation during the fraction procedure was directed largely toward the properties of fraction IV-10, which we cross-checked on the basis of two immunological characteristics: (*a*) its content of all the Boyden-reacting antibody and (*b*) its ability to passively transfer delayed type skin sensitivity. Chemically, this fraction is defined as being initially soluble in 19 per cent ethanol at pH 6.0, temperature −5°C., with precipitation on standing overnight.

We have been told by Dr. Oncley (36) that while Method X was being evolved with human plasma, a fine precipitate was found to occur consistently when supernatant IV + V + VI was allowed to stand for a number of hours. This precipitate was not extensively investigated. Dr. Oncley has agreed with our terminology.

The definition of IV-10 in this study applies to the conditions specifically outlined in this report. Other plasma volumes and time relations were not studied.

Passive Transfer of Cutaneous Reactivity.—Two types of reactivity were observed in these studies. The first was a typical "urticarial" or "immediate" reaction, which started immediately upon application of the antigen, with pseudopods emanating from the injection site. This type was maximal at 4 to 8 hours, disappearing by 24 hours. The reaction consisted of erythema and edema. In some instances, pale edema predominated over erythema; in other instances, erythema was marked and edema less prominent.

The second type of reactivity observed was a "delayed" type of reaction. Lesions

[21] American Instrument Company portable electrophoretic machine.

of this type started 3 to 5 hours after application of PPD, with the gradual onset of erythema and induration, and usually reached their maximum size between 18 and 30 hours. They were often greatly reduced in size at 48 hours. On one occasion, a slight papule of central necrosis was observed at 30 hours. The word "delayed" is used in this paper as a descriptive term only, and is not meant to imply necessarily that skin lesions of this type were actually tuberculin reactions.

II. Passive Transfer Results with Different Plasma Fractions

Each step of the fractionation procedure was studied by a series of passive transfer experiments, except when impractical for technical reasons. Every purified plasma fraction could thus be compared for antibody content with the step preceding its isolation. Since the immune plasma for fractionation was always obtained from a large number of donors, immunized for similar periods of time, such a comparison appeared reasonable. This technique of investigation was applied to each of the three fractionation procedures which are diagrammed in Figs. 1, 2, and 3. A rather large number of plasma protein preparations were therefore examined, and the total information obtained led to the chemical definition of the antibodies in fractions IV-10 and II. In the sections to follow, are presented the results of passive transfer with these various plasma fractions. Each may be interpreted through its content of fraction II or IV-10.

Passive Transfer Studies with Fraction II (without IV-10).—Twenty-nine guinea pigs received, intraperitoneally, immune gamma globulin prepared by the standard Method X, shown in Fig. 1. Passive transfer of "immediate" or "urticarial" skin reactivity to OT[22] and tuberculopolysaccharide was observed consistently.

Table I summarizes the 15 instances in which OT and PPD were used as test antigens. Reactions to OT were vigorous, whereas of 11 animals tested with PPD, 7 were negative, 2 had urticarial lesions less than 1 cm. diameter, and 2 had lesions greater than 1 cm. Maximal reactivity to OT occurred during the first 48 hours. After 4 to 6 skin tests, the reactivity disappeared, and this seemed to be a function of the number of tests rather than a function of time, as though the OT "used up" antibody. Brucellergen controls were negative. Guinea pigs 2-10, 2-31, and 2-35 were negative to skin test preparations of pneumococcus, staphylococcus, and streptococcus but did have weak immediate reactions to streptococcus antigens.[23] Animals 4-27 and 4-94 had relatively weaker and fewer positive skin reactions; their donors, however, had been immunized for less than 3 weeks. Nos. 2-35, 2-72, and 2-71 had anaphylaxis with death following the intracardiac injection of OT 1:10, 0.4 to 0.5 ml. This dosage was harmless in 3 normal animals tested. No. 8-8 received the gamma globulin from 152 ml. pooled plasma from normal animals (PPD skin-tested 24 hours before bleeding). This animal did not develop reactions to OT, brucellergen, and bacterial skin tests.

[22] OT is from 25 to 50 per cent polysaccharide by weight.

[23] These antigens were washed, heat-killed; whole bacterial suspensions in saline were adjusted to 900,000,000 organisms per ml. against a barium sulfate standard. Skin tests doses were 0.1 ml. of suspension. Some animals in our colony of guinea pigs harbored a low virulence, chronic streptococcus infection common in this species. This infection accounts for positive streptococcus reactions in some animals.

TABLE I

Passive Transfer of Urticarial Type Skin Reactivity Via Fraction II Without IV-10. Studies with OT and PPD

Pig No. and date	Volume ACD plasma	Protein concentration of fraction in plasma	Amount protein injected	Antigen	Maximum diameter of edema* and day‡										Comments
					1	2	3	4	5	6	7	8	9	10	
	ml.	*gm. per cent*	*gm.*		*cm.*										
1-85	159.5	—	—	OT	3.7	3.0	0								OT 1:10 used
1-15-53															
2-7-53															
4-25	167	—	—	OT	2.7	1.7	1.7		1.5		0				OT 1:10 used
2-18-53				PPD			0		0						
2-22-53															
4-23	167	—	—	OT	3.0	3.5	1.7	1.7		1.7	0		0		OT 1:10 used
3-2-53				PPD	0.6			0.5							
3-4-53															
3-2	85	—	—	PPD	0	0	0			0	0				No OT skin test
3-16-53															
3-3	94	—	—	PPD	0	0	0			0	0				No OT skin test
3-10-53															
2-10	230	0.465	0.883	OT	3.0		3.0		2.5		0	0			Only strong PPD reactor
7-4-53				PPD	2.0		1.0								
2-31	200	0.618	1.120	OT	3.0	3.5		2.0						1.6	OT 1:10 used
7-21-53				PPD	0.6										
2-35	300	0.550	1.361	OT		3.6									0.5 ml. of OT 1:10 intracardiac on
8-4-53															5th day gave fatal anaphylaxis
2-72	103	0.465	0.349	OT		3.0		2.5							0.4 ml. of OT 1:10 intracardiac on
8-15-53				PPD		0		0							7th day gave fatal anaphylaxis
2-71	284	0.600	1.526	OT			2.0	1.5	1.5		1.5				As above
8-15-53				PPD			0.7	1.5							
4-02	91	0.700	0.525	OT	3.0	3.0	1.2			1.2	1.2	0		0	
11-21-53				PPD						0	0				
4-03	213	0.663	1.165	OT	2.0	2.0	1.4	1.2	1.2		1.0		0		
12-9-53															
4-27	201	0.410	0.677	OT	3.0			2.0		0	0				Donors immune 19 to 21 days. Only
1-27-54															2 positive tests
4-94	236	0.625	1.217	OT	0	2.0	2.0		0	0					Donors immune 10 to 13 days. Only
2-10-54				PPD		0	0		0	0					2 positive tests
4-96	227.25	0.600	1.126	OT	2.5	2.5	1.5	1.5	0	0					
3-12-54				PPD	0	0			0	0					
8-8	152	—	—	OT	0	0			0	0					Normal, unsensitized donors
4-17-53	Normal plasma														
3-52	II and III from pooled buffy coats	—	—	OT	0	0			0	0					Sensitized white blood cell control
11-21-53	of 11-21-53														experiment
3-53	II and III from pooled buffy coats	—	—	OT	0	0			0	0					Sensitized white blood cell control
12-9-53	of 12-9-53														experiment

* Maximum always between 6 to 8 hours after application of antigen.

‡ Fraction injected intraperitoneally on day 0.

In an attempt to rule out the possibility that the passive transfer antibody came from white blood cells which inadvertently had been taken into the plasma, the entire pooled buffy coats of two bleedings were harvested and subjected to the fractionation procedure. Animals 3-52 and 3-53 received this material (guinea pigs 4-02

and 4-03 were given the plasma fraction II from the same bleedings). No passive transfer resulted.

Tuberculopolysaccharide and PPD were studied as testing antigens in 14 instances, shown in Table I A. The polysaccharides consistently produced reactions identical with those obtained with OT. Reactions to the Choucroun polysaccharide were greater than those produced by S4 and S6. On several occasions, skin test doses of 1 μg. Choucroun polysaccharide were employed, and these produced reactions with diameters as large as those of 10 μg. doses in the same animals, though the intensity of the lesions was less. Of 14 guinea pigs tested with PPD, 2 had immediate reactions and the others gave no response. The polysaccharide reactions differed from those elicited by OT in that skin reactivity to them continued for 3 to 4 weeks, even when 100 to 150 μg. of antigen were added to the fraction or injected into the recipient pig. Maximal reactivity was present within the first 2 days.

Four adsorptions with a total of 3 ml. packed, Middlebrook-Dubos sensitized cells removed the immediate type passive transfer capacity from the fractions given to 3 animals, Nos. 1-51, 1-52, and 1-58. Guinea pig 2-13 was a normal control.

Vigorous Prausnitz-Kuestner reactions to OT, S4 and S6, and Choucroun test materials were obtained consistently *via* 0.1 ml. fraction II given intradermally.

Passive Transfer Studies with Fraction II + IV-10.—The fractionation procedure followed in this protocol is in Fig. 2. Results are shown in Table II. The 6 recipient animals displayed the same type of reactivity to Choucroun polysaccharide as that recorded in Table I A. One guinea pig only, No. 1-31, had a delayed type reaction to PPD. The other 5 animals showed no reactivity of any type to PPD. This failure to passively transfer delayed skin sensitivity was surprising, since fraction IV-10 was contained in the fraction II. It may be seen in Table V that the corresponding fractions IV + V (without IV-10), as expected, did not effect passive transfer. These observations appear to indicate that *in vivo*, fraction II from immunized animals, inhibits the manifestations of delayed type skin reactivity to PPD by fraction IV-10.

Passive Transfer Studies with Fraction III.—On 6 occasions fraction III from immunized donors was injected into recipient guinea pigs. Neither delayed nor immediate reactivity was transferred.

Passive Transfer Studies with Fraction IV + IV-10.—The fractionation procedure usually followed for this series is given in Fig. 1 and the results are recorded in table III. This fraction was the first one with which delayed type skin reactivity was passively transferred. It formed the focal point for the explorations which later led to the identification of fraction IV-10 as a separate entity. Of 25 recipient animals receiving fraction IV + IV-10 from immunized donors, 17 exhibited a delayed type skin reactivity to PPD. Skin reactivity was present from 3 to 6 days to 8 to 14 days after injection of the plasma fraction.

In this group of experiments varying degrees of reactivity were seen, possibly due to loss of irregular amounts of IV-10 into fraction V. The duration of skin reactivity of all animals was brief. Animal 1-90, which had the strongest skin reactivity, developed a 3 mm. area of central necrosis in the test applied on the 6th day. The plasma pool for this guinea pig, and that for No. 7-3, which also had strong reactivity, were each from donors immunized 20 to 21 days.

In four instances, recipient animals had unsustained reactions to PPD for from

TABLE I A

Passive Transfer of Urticarial Type Skin Reactivity via Fraction II without IV-10. Studies with Tuberculopolysaccharide and PPD

Pig No. and date	Volume ACD plasma	Protein concentration of fraction in plasma	Amount protein injected	Antigen	Maximum diameter of edema* and day‡ 1	2	3	4	5	6	7	8	9	10	Comments
	ml.	*gm. per cent*	*gm.*		*cm.*	*m.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	
1-40 4-13-54	104	0.656	0.564	Choucroun§	1.3	1.2		1.6			1.3	1.6		1.6	Choucroun 40 μg. added to fraction before passive transfer. Positive tests 3½ wks.
				S4‖					1.3						
				PPD	1.3	0.7		0.7		0	0				
1-41 4-15-54	120	0.600	0.594	Choucroun		1.0	1.0			2.8		1.5	1.5	1.5	150 μg. Choucroun given i.p. Positive skin tests 4 wks.
				PPD		0	0		0	0					
1-42 1-9-54	130	0.580	0.622	S4	2.6	1.5	1.0		2.5					1.5	S4 100 μg. given i.p. Positive skin tests 4 wks.
				S6¶	2.4										
				PPD		0		0		0					
1-43 4-23-54	128	0.641	0.677	S4			1.8	1.8	1.8		1.8			1.8	S4 100 μg. added to fraction before transfer and 110 μg. injected i.p. Positive skin tests 4 wks.
				PPD				0	0		0				
1-44 1-6-54	147	0.588	0.712	Choucroun	2.2	2.5								1.8	Reactivity lasted 3½ wks.
				PPD	2.0	1.0								1.2	
1-51 2-28-54	92	0.896	0.681	Choucroun	0	0	0	0	0	0				0	4 adsorptions of fraction with Middlebrook-sensitized sheep cells before injection.
				PPD	0	0	0	0	0	0				0	
1-52 4-7-54	112	0.681	0.627	Choucroun	0	0	0	0	0	0					As above
				PPD	0			0	0	0					
1-58 5-10-54	93	0.650	0.501	Choucroun	0	0	0	0	0	0					As above
				PPD	0			0	0	0					
1-97 6-2-54	100	0.623	0.514	Choucroun		2.5				2.0	2.0	2.0	1.5	1.5	Reactivity lasted 4 wks.
				PPD						0	0	0			
1-98 6-3-54	102	0.645	0.543	Choucroun		2.5				2.0	2.0	2.0	1.5	1.5	Reactivity lasted 4 wks.
				PPD						0	0	0			
1-96 6-5-54	106	0.450	0.397	Choucroun		2.5				2.0	2.0	2.0	1.5	1.5	Reactivity lasted 4 wks.
				PPD						0	0	0			
1-99 6-18-54	99	0.600	0.490	Choucroun		3.0				2.4	2.4	2.0	2.0	1.2	Reactivity lasted 4 wks.
				PPD						0	0	0			
2-13 8-13-54	105 Normal plasma	0.973	0.846	Choucroun	0	0		0		0	0			0	Normal, unsensitized donors
				PPD						0	0			0	
1-95 7-6-54	144	0.681	0.810	Choucroun	3.2	3.2				1.6		1.6		1.4	Choucroun reactivity lasted 3 wks.
				PPD				0	0	0		0		0	
2-07 8-9-54	104	0.844	0.720	Choucroun	2.6	3.0				2.5		2.5		1.5	Same as above
				PPD						0	0			0	

* Maximum always between 6 to 8 hours after application of antigen.
‡ Fraction injected intraperitoneally on day 0.
§ Choucroun polysaccharide 10 μg.
‖ S4 polysaccharide 10 μg.
¶ S6 polysaccharide 10 μg.

1 to 6 days before the appearance of lesions which were maximal at 24 hours. These unsustained reactions began as did the typical delayed type lesions, but suddenly faded away after about 12 hours.

There were eight passive transfer failures. In two of these, the donor animals were not skin-tested 24 hours before being bled. The skin testing was purposely omitted in these two experiments. The donors, in both instances, had received PPD skin tests 12 days before bleeding.

TABLE II

Passive Transfer of Urticarial Type Skin Reactivity via Fraction II + IV-10

Pig No. and date	Volume ACD plasma	Protein concentration of fraction in plasma	Amount protein injected	Antigen	Maximum diameter of edema* and day‡										Comments
					1	2	3	4	5	6	7	8	9	10	
	ml.	*gm. per cent*	*gm.*		*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	
1-85 6-22-54	168	0.400	0.530	Choucroun	3.0	3.2				2.5		2.5		2.0	Choucroun reactivity lasted 4 wks.
				PPD					0	0	0	0			
1-94 6-28-54	100	0.482	0.397	Choucroun	2.5	3.0				2.0		2.0		2.2	Same as above
				PPD				0		0	0	0			
1-84 7-5-54	44	0.725	0.269	Choucroun					1.5	1.5				1.5	Choucroun reactivity lasted 3 wks.
				PPD					0	0				0	
2-01 8-2-54	92	0.681	0.517	Choucroun	3.0	3.0				1.6		1.6		2.0	Same as above
				PPD					0	0	0			0	
2-00 8-3-54	120	0.475	0.413	Choucroun	2.2	3.0				2.0		2.0		1.5	Same as above
				PPD						0	0	0		0	
1-31 3-3-54 3-29-54	201			Choucroun	3.0	2.5				2.4		2.4		2.4	Reactivity to Choucroun lasted 3 wks. Choucroun 25 μg. intracardiac gave non-fatal anaphylaxis.
				PPD		0						1.0§	0.5§		

* Maximum always between 6 and 8 hours after application of antigen.
‡ Fraction injected intraperitoneally on day 0.
§ Delayed reaction. Diameter at 24 hours.

Five additional failures occurred in experiments in which solution A was added to the ACD plasma relatively rapidly during the initial precipitation of I + II + III. All 5 of these recipient animals, Nos. 3-31, 3-34, 3-45, 3-55, and 3-57, had immediate type or unsustained skin reactions to PPD. In one instance, not shown in Table III, the ACD plasma was fractionated by Method VI (37) and the resulting fraction IV also passively transferred immediate type reactivity to PPD. These immediate type reactions were essentially identical morphologically with the immediate type reactions to tubercular polysaccharide obtained in animals receiving fraction II, despite the fact that the two groups involved different antigen-antibody reactions. 4 of the 6 recipient guinea pigs were skin-tested with Choucroun polysaccharide and dis-

played no skin reactivity to this material. The transfer of immediate type skin reactivity to PPD by fractions separated by the rapid addition of alcohol to the plasma, or on the one occasion when 40 per cent alcohol was used (Method VI), suggests that these procedures partially denature this particular antibody, but do not change its specificity.

One instance, animal 1-37, was a failure for no apparent reason. This animal had unsustained reactions to PPD and was the only instance in which a positive reaction to Choucroun polysaccharide occurred in this group.

Our data suggest but are not extensive enough to demonstrate that fraction IV′ enhanced the positive transfer of delayed type reactivity. This fraction did not transfer skin reactivity when injected alone, however, as shown by guinea pigs 1-96 and 1-27.

On a single occasion fraction IV was subfractionated to IV-1 (alpha-1 lipoprotein) and fraction IV-6, 7 (alpha glycoprotein). Animal 1-34, which received the latter, developed delayed type sensitivity, whereas No. 1-33, receiving the IV-1, had only unsustained reactions.

Brucellergen control skin tests are mentioned under Methods and Materials. Several animals also were tested with preparations of staphylococcus, pneumococcus, and α- and β-streptococcus materials. No positive reactions were observed.

Guinea pig 1-04 was a normal plasma control. Animals 3-54 and 3-50 represented attempts to rule out the possibility that disintegration products of white blood cells were responsible for the successful passive transfer results. For these experiments the entire pooled buffy coats from 102 and 105 ml. whole blood, respectively, were subjected to the fractionation procedure. In 4 experiments not shown, the pooled buffy coats of entire bleedings were killed by freezing and thawing 3 times and then injected into normal animals. No skin reactivity was transferred.

Prausnitz-Kuestner reactions, attempted on several animals, were negative.

On one occasion, not shown in Table III, 100 ml. pooled ACD plasma were obtained from guinea pigs immunized to β-hemolytic streptococcus. These animals had received weekly injections of 0.1 ml. of a saline suspension of streptococcus antigen used in skin testing. They had been immunized for 2 to 3 months. Fraction IV + IV-10 effected passive transfer into a normal animal of delayed type skin sensitivity to β-hemolytic streptococcus skin test material[23]; PPD tests, employed as a control in this animal, remained negative. The lesions were from 1.0 to 1.2 cm. in diameter, and skin sensitivity lasted for 4 days.

Passive Transfer Studies with Fraction IV + V + IV-10.—This series was done according to the protocol in Fig. 1, and passive transfer results are shown in Table IV. Seven attempts at passive transfer of delayed type skin sensitivity with this fraction were all successful, and none of the recipients reacted to tuberculopolysaccharide. The recipients were consistent in developing maximal skin reactivity on the 5th to 7th day. Their skin reactions were slightly different from those in the recipients that received fractions IV + IV-10 or IV-10, however, in that the lesions had a bit more edema, and less erythema, and unsustained reactions were prone to appear before and after the development of sustained tests. Animal 1-50 was unusual in that the delayed responses to PPD were a little larger at 6 hours than at 24.

On two occasions, not described in Table IV, a trace of zinc remained in the frac-

TABLE III

Passive Transfer of Skin Reactivity to PPD via Fraction IV + IV-10

Pig No. and date	Fraction	Volume ACD plasma	Protein concentration of fraction in plasma		Amount protein injected		PPD tests: Diameter of induration and days*										Comments
							1	2	3	4	5	6	7	8	9	10	
		ml.	*gm. per cent*		*gm.*		*cm.*										
8-5	IV	67.5					1.1‡	1.4	0		0						Donors immune many
1-15-53							0.2‡	0.3	0		0						mos.
2-23	IV	92					0	1.4	1.0	0		0					Donors immune many
2-7-53							0	0.7	0.5	0		0					mos.
2-27	IV + IV′	85					0	2.1	0		0						
2-18-53							0	0	0		0						
1-90	IV + IV′	95						0	0	1.8		2.0			1.6	0	All donors immunized 20
3-2-53								0	0	0.8		1.4			0.4	0	days. Small central ne-
																	crosis on 6th day test.
4-43	IV + IV′	72						0	0	0	0						Donors skin tested 17 hrs.
3-4-53								0	0	0	0						before bleeding.
4-84	IV	94						0.8		0.4	0.4	0	0				4-84 and 1-96 same bleed-
								0.4		0	0	0	0				ing. Failure of passive
1-96	IV′							0		0	0	0	0				transfer with IV′.
3-10-53								0		0	0	0	0				
3-5	IV + IV′	85						0	0		0		0				Donors not skin-tested be-
3-16-53								0	0		0		0				fore bleeding.
7-3	IV + IV′	115					0			1.5		1.2	0.3	0	0		All donors immunized 21
6-17-53							0			0.8		0.6	0	0	0		days.
3-31	IV + IV′	103	IV	0.472	IV	0.397		U§	U	0		0					Solution A added to plasma
10-25-53			IV′	0.042	IV′	0.035		0	0	0		0					a 30 min. period.
3-34	IV + IV′	102	IV	0.499	IV	0.419	U	U		U		0					As above
10-29-53			IV′	0.042	IV′	0.035	0	0		0		0					
3-41	IV + IV′	102	IV	0.278	IV	0.234			0.4	0.6	0.4	0				0	Skin reactions were a little
11-5-53			IV′	0.056	IV′	0.047			0	0	0	0				0	larger at 6 hrs. than at 24
																	hrs.
3-45	IV + IV′	102	IV	0.221	IV	0.186				I‖c¶	Ic	0		0			Solution A added to plasma
11-11-53			IV′	0.065	IV′	0.055				0	0	0		0			over 30 min. period.
3-55	IV + IV′	91	IV	0.157	IV	0.118		I		I	Ic		0	0			As above
11-21-53			IV′	0.118	IV′	0.089		0		0	0		0	0			
3-57	IV + IV′	93	IV	0.341	IV	0.263			I	Ic	0	0					As above
12-1-53			IV′	0.034	IV′	0.027			0	0	0	0					
4-14	IV + IV′	120	IV	0.295	IV	0.292			0.5	0	0	0					
1-9-54			IV′	0.063	IV′	0.062			0.3	0	0	0					
4-35	IV + IV′	106	IV	0.295	IV	0.257		0	0	0.5	0	0					
2-10-54			IV′	0.066	IV′	0.058		0	0	0.3	0	0					
5-00	IV + IV′	105	IV	0.188	IV	0.164		1.8	2	0.5	0	0					
3-14-54			IV′	0.054	IV′	0.047		0.6	2	0	0	0					
1-27	IV′	124	IV′	0.042	IV′	0.043	0	0	0		0		0				Failure of passive transfer
3-19-54							0	0	0		0		0				with IV′.
1-28	IV + IV′	95	IV	0.205	IV	0.160	0		U				1.0	1.0	0.4	0	
3-22-54			IV′	0.023	IV′	0.018	0		0				0.3	0.3	0	0	
1-29	IV + IV′	90	IV	0.385	IV	0.289	0	0	0.7		0.9		0.4		0	0	Skin reactions were a little
3-26-54			IV′	0.039	IV′	0.029	0	0	0		0.4		0		0	0	larger at 6 hrs. than at 24
																	hrs.
1-30	IV + IV′	110	IV	0.233	IV	0.210	0	0		1.2	0.9	0	0				
3-29-54			IV′	0.042	IV′	0.038	0	0		0.5	0	0	0				
1-33	IV-1	112	IV-1	0.244	IV-1	0.224	0	Uc¶	Uc	Uc	0	0					
4-2-54							0	0	0	0	0	0					
1-34	IV-6,7		IV-6,7	0.042	IV-6,7	0.039	0	Uc	Uc	0.8c	0.8		0.3	0			
4-2-54							0	0	0	0.5	0.5		0	0			
1-35	IV + IV′	112	IV	0.277	IV	0.254	U	U		0.9		1			0.4	0	
4-7-54			IV′	—	IV′	—	0	0		0		0.5			0	0	

TABLE III—*Concluded*

Pig No. and date	Fraction	Volume ACD plasma	Protein concentration of fraction in plasma	Amount protein injected	PPD tests: Diameter of induration and days*										Comments
					1	2	3	4	5	6	7	8	9	10	
		ml.	*gm. per cent*	*gm.*	*cm.*										
1-36	IV + IV′	120	IV 0.298	IV 0.295					0.7	0	0	0			
4-9-54			IV′ —	IV′ —					0.3	0	0	0			
1-37	IV + IV′	104	IV 0.328	IV 0.282				U	U	0	0				Immediate reaction to Choucroun polysaccharide on days 4 and 5.
4-13-54			IV′ —	IV′ —				0	0	0	0				
1-39	IV + IV′	120	IV 0.280	IV 0.277	Uc	Uc	U				0.4	0	0		
4-15-54			IV′ —	IV′ —	0	0	0				0.3	0	0		
3-54	IV from pooled buffy coats of 11-5-53	—	—	—	0	0		0		0		0			White blood cell control experiment.
11-5-53					0	0		0		0		0			
3-50	IV from pooled buffy coats of 3-14-54	—	—	—	0	0		0		0		0			White blood cell control experiment.
3-14-54					0	0		0		0		0			
1-04 4-10-53	IV + IV′ Normal plasma	152	—	—	0	0		0		0		0			Unsensitized, normal guinea pig plasma experiment.

* Fraction injected intraperitoneally on day 0.
‡ Upper figure is 24 hour reading. Lower figure is 48 hour reading.
§ U, unsustained reaction.
‖ I, immediate reaction.
¶ c, no reaction to Choucroun polysaccharide.

tion at the completion of dialysis. The zinc was chelated soon afterward by addition of an appropriate amount of EDTA. However, the recipient animal on both occasions developed an urticarial type of skin reactivity to PPD which was vigorous, and had no reaction to tuberculopolysaccharide.

Animals 1-63 and 1-64 received respectively, 1.830 and 2.124 gm. of the fraction which had been adsorbed twice with a total of 0.25 ml. of PPD-sensitized, tannic acid–treated red cells prepared for use in the Boyden test. Each adsorption was for 60 minutes. All *in vitro* reactivity as measured by the Boyden test was removed during this procedure. Passive transfer was negative on both occasions. Animal 1-71 received fraction to which had been added 40 μg. PPD, and this preparation failed to passively transfer skin reactivity. One guinea pig received the fraction IV + V material from a concentrated white cell suspension and another the V from normal plasma. These data are shown in Table IV. Both animals remained PPD-negative.

Passive Transfer Studies with Fraction IV + V (without IV-10).—The fractionation procedure given in Fig. 2, or Fig. 3, was used. Results are summarized in Table V. This fraction, from immune plasma, was given 7 times with uniform failure to effect passive transfer of skin reactivity, as was the case with 1 animal receiving this fraction from normal plasma.

Passive Transfer Studies with Fraction IV-10.—This fraction was obtained according to the method outlined in Fig. 3. The results in recipients are shown in Table VI. Fraction IV-10, obtained from immune plasma, was injected into recipients on 5 occasions. Passive transfer of delayed type skin sensitivity to PPD occurred in each instance. Maximal skin reactivity was noted on the 5th and 6th days. None of the animals exhibited skin reactivity to Choucroun polysaccharide.

TABLE IV

Passive Transfer of Skin Reactivity to PPD via Fraction IV + V + IV-10

Pig No. and date	Fraction	Volume ACD Plasma	Protein concentration of fraction in plasma	Amount protein injected	PPD tests: Diameter of induration and day* 1	2	3	4	5	6	7	8	9	10	Comments
		ml.	*gm. per cent*	*gm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	
2-41 8-5-53	IV+V	105	—	—					1.0‡ 0.5‡	1.2 1.2	1.0 0.5	0.3 0	0 0	0 0	
2-42 8-6-53	IV+V	66	—	—					1.0 0.5	1.2 0.5	1.0 0.5	0.3 0	0 0	0 0	
1-47 5-10-54	IV+V	93	2.727	2.100		0 0		Uc§ 0	Uc‖ 0	2.0c 1.0	U 0	0 0		0 0	
1-50 5-13-54	IV+V	106	3.000	2.640			U 0		1.0c 0.5		1.0c 0.5	U 0		0 0	Skin reaction larger at 6 hrs. than at 24 hrs.
1-63 6-2-54	IV+V	100	2.800	1.830			0 0			0 0	0 0		0 0		Adsorbed twice with tanned, PPD-sensitized red cells. Titer 1:400 → 0.
1-64 6-3-54	IV+V	102	3.250	2.124			0 0			0 0	0 0		0 0		As above
1-48 6-5-54	IV+V	96	2.972	2.338			U 0		1.0 0.6	1.0c 0.6	U 0	0 0	0 0		
1-66 6-11-54	IV+V	102	2.440	2.050			0c 0			1.3 0.7	0.5 0		0 0		
1-69 6-17-54	IV+V	100	3.273	2.700				0 0		1.2c 0.2	0.3c 0		0 0		
1-71 6-18-54	IV+V (0.040 mg. PPD added)	99	2.970	2.435			0 0			0 0	0 0		0 0		40 μg. PPD added to fraction ½ hour before it was injected
3-50 8-5-53	IV+V from pooled buffy coats of 8-5-53	—	—	—			0 0			0 0	0 0		0 0		Sensitized white blood cell control experiment
1-02 4-17-53	V normal plasma	152	—	—		0 0	0 0		0 0	0 0			0 0		Unsensitized normal guinea pig plasma experiment

* Fraction injected intraperitoneally on day 0.
‡ Upper figure is 24 hour reading. Lower figure is 48 hour reading.
§ U, unsustained delayed reaction.
‖ C, no reaction to Choucroun polysaccharide.

The plasma protein concentration of IV-10 was from 0.031 to 0.040 gm. per cent or approximately 0.6 per cent of the total plasma protein. No attempt was made to further purify this material. Table VI shows that the plasma concentrations of the fraction IV-10 from the immune and normal sera were essentially equal.

TABLE V

Passive Transfer Attempts via Fraction IV + V without IV-10

Pig No. and date	Fraction	Volume ACD plasma	Protein concentration of fraction in plasma	Amount protein injected	PPD tests										Comments
					Diameter of induration and day*										
					1	2	3	4	5	6	7	8	9	10	
		ml.	*gm. per cent*	*gm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	
1-78	IV+V	168	2.841	3.938		0‡				0	0	0			
6-22-54						0‡				0	0	0			
1-79	IV+V	100	2.582	2.130		0				0	0	0			
6-28-54						0				0	0	0			
1-82	IV+V	44	4.000	0.759		0				0	0	0			
7-5-54						0				0	0	0			
1-83	IV+V	144	2.345	2.791					0c§	0	0				
7-6-54									0	0	0				
2-08	IV+V	92	3.927	2.991					0c	0	0			0	
8-2-54									0	0	0			0	
2-09	IV+V	120	3.900	3.863			0c		0c	0c	0				
8-3-54							0		0	0	0				
2-16	IV+V	104	3.336	2.869					0c	0	0			0	
8-9-54									0	0	0			0	
2-22	IV+V	105	3.900	3.359					0c	0c	0			0	Unsensitized normal
8-13-54	Normal plasma								0	0	0			0	guinea pig plasma experiment

* Fraction injected intraperitoneally on day 0.
‡ Upper figure is 24 hour reading. Lower figure is 48 hour reading.
§ c, no reaction to Choucroun polysaccharide.

The fractionation of 8-2-54, was altered to the extent that the I + II + III precipitate was centrifuged down only 15 minutes after the solution A had been added. As shown in Table VII, some of the IV-10 went into fraction II, indicating that the material had not reached equilibrium at this point. Passive transfer of delayed sensitivity was successful with the IV-10, but not with the II + IV-10 fraction.

The fraction IV-10 received by animals 2-23 and 2-24 was injected immediately upon recovery from the separation procedure, without being dialyzed. The antibody being labile, it was thought that stronger skin tests might ensue. These two animals did have more intense erythema and induration in their PPD reactions, though

the diameters of these reactions were not strikingly different from the other tests in this series.

Passive Transfer Studies with Fraction VI.—Six passive transfer experiments (not tabulated) with this fraction gave negative results to PPD and tuberculopolysaccharide testing materials.

Passive Transfer Studies with Fraction IV + IV-10 + V + VI.—Five passive transfer experiments (not tabulated) were attempted with this fraction. It was obtained, respectively, from 104, 108, 110, 118, and 120 ml. of immune ACD plasma.

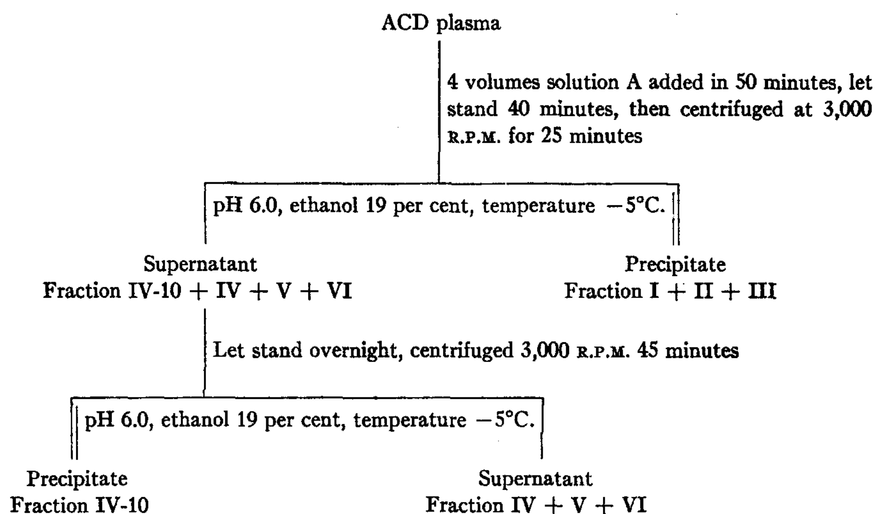

FIG. 3. Modification of Method X whereby fraction IV-10 is isolated.

This fraction was the supernatant after adding solution A and centrifuging, as seen in Fig. 1. It was harvested at that point and dialyzed in 3,000 ml. distilled water, 0°C., for 18 hours. In each instance the fraction almost doubled in volume during dialysis. It was then divided in two flasks, quick frozen at −70°C., and lyophilized. The material was brought back into clear solution with 20 ml. of distilled water for passive transfer.

Each of these five experiments resulted in failure to passively transfer skin reactivity to PPD or OT. It appeared that the process of freezing and lyophilizing had inactivated the antibody in fraction IV-10.

III. Correlation of Passive Transfer Results with in Vitro Reactivity

In the preceding sections on Results, evidence has been given to demonstrate that there exists in the plasma of sensitized guinea pigs two components associated with cutaneous reactivity to products of the tubercle bacillus. One, found in fraction II, will passively transfer typical anaphylactic reactivity to antigens containing tuberculopolysaccharide. Occasional reactions to PPD may be the result of the small amount of polysaccharide which does exist in this testing material. A second compo-

nent, present in a newly described fraction IV-10, has been found to transfer passively a delayed type of reactivity to PPD. It does not transfer any reactivity at all to tuberculopolysaccharide. The component in fraction IV-10 is easily denatured. The type of reactivity transferred by this subfraction of the alpha globulin appears in the recipient animal several days after transfer and is gone in 10 days. When plasma components which transfer each type of reactivity are present in the same

TABLE VI

Passive Transfer of Skin Reactivity to PPD via Fraction IV-10

Pig No. and date	Fraction	Volume ACD plasma	Protein concentration of fraction in plasma	Amount protein injected	PPD tests: Diameter of induration and days*										Comments
					1	2	3	4	5	6	7	8	9	10	
		ml.	*gm. per cent*	*gm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	
1-81 7-6-54	IV-10	144	0.034	0.038		0.3‡ 0‡		0.4c§ 0		2.0c —		0 0			Lesion biopsied at 24 hrs.
1-93 8-2-54	IV-10	92	0.033	0.025					0.5 0	1.0c 0.4c	1.0 0.4	0 0		0 0	Centrifuged 15 min. after A solution was added. Some IV-10 went with II. See Table VII.
2-02 8-9-54	IV-10	104	0.036	0.031						1.8c 0.8c	0.8 0.3	0 0	0 0		
2-14 8-13-54	IV-10 Normal plasma	105	0.041	0.035				0 0		0c 0c	0 0		0 0	0 0	Unsensitized normal guinea pig plasma fractionated.
2-23 8-26-54	IV-10	98‖	—	—						1.6c Biopsied		0.8 0.3			
2-24 8-27-54	IV-10	119‖	—	—					1.4 0	1.4c Biopsied		0.4 0.3			

* Fraction injected intraperitoneally on day 0.

‡ Upper figure is 24 hour reading. Lower figure is 48 hour reading.

§ c, no reaction to Choucroun polysaccharide.

‖ Fraction injected immediately following recovery from separation procedure.

material, factors related in some way to immediate sensitivity inhibit the development of the delayed reactions.

It has been suggested (24) that the Middlebrook-Dubos test measures antibody to tuberculopolysaccharide, whereas the Boyden test detects antibody to tuberculoprotein. These tests were done on the various fractions used in the foregoing passive transfer studies.

Table VII indicates that the plasma fractionation procedure results in a clear cut chemical separation of the Boyden and Middlebrook-Dubos antibodies. It is readily evident that fraction II is the vehicle for the Middlebrook-Dubos antibody, whereas

TABLE VII

Correlation of Passive Transfer with in Vitro Reactivity

Date of fractionation	Fractions tested	Guinea pig No.	Middlebrook-Dubos titer	Boyden titer		Passive transfer of	
				EDTA	56°	Immediate type sensitivity	Delayed type sensitivity
6-2-54	II	1-97	64	0	0	Yes	No
	IV+V+IV-10	1-63	0	400	0	No	No*
	Plasma	—	128+	200	100	—	—
6-3-54	II	1-98	64	20	0	Yes	No
	IV+V+IV-10	1-64	0	400	0	No	No*
	Plasma	—	128+	200	100	—	—
6-5-54	II	1-96	64	0	0	Yes	No
	IV+V+IV-10	1-48	0	200	0	No	Yes
	Plasma	—	64	200	100	—	—
6-11-54	II‡	—	—	—	—	—	—
	IV+V+IV-10	1-66	0	400	0	No	Yes
6-17-54	II‡	—	—	—	—	—	—
	IV+V+IV-10	1-69	0	1280+	0	No	Yes
	Plasma	—	32	160	40	—	—
6-18-54	II	1-99	128+	40	0	Yes	No
	IV+V+IV-10	1-71	0	1280+	0	No	No§
	Plasma	—	32	40	0	—	—
6-22-54	II+IV-10	1-85	128+	1280+	640	Yes	No
	IV+V	1-78	0	0	0	No	No
	Plasma	—	64	640	40	—	—
6-28-54	II+IV-10	1-94	128+	1280+	1280+	Yes	No
	IV+V	1-79	0	0	0	No	No
	III	—	4	0	0	—	—
7-5-54	II+IV-10	1-84	128+	1280+	1280+	Yes	No
	IV+V	1-82	4	10	0	No	No
	III	—	0	0	0	—	—
7-6-54	II	1-95	128+	10	0	Yes	No
	IV+V	1-83	0	0	0	No	No
	IV-10	1-31	0	1280+	160	No	Yes
8-2-54	II‖	2-01	64	320	320	Yes	No
	IV+V	2-08	0	0	0	No	No
	IV-10	1-93	0	320	320	No	Yes
	III	—	0	0	0	—	—
	Plasma	—	64	160	40	—	—
8-3-54	II+IV-10	2-00	128+	1280+	320	Yes	No
	IV+V	2-09	0	0	0	No	No
	III	—	8	0	0	—	—
	Plasma	—	64	320	40	—	—
8-9-54	II	2-07	128+	0	0	Yes	No
	IV+V	2-16	4	0	0	No	No
	III	—	0	0	0	—	—
	IV-10	2-02	0	1280+	640	No	Yes
	Plasma	—	64	80	20	—	—
8-13-54¶	II	2-13	8	0	0	No	No
	IV+V	2-22	4	0	0	No	No
	III	—	0	0	0	No	No
	IV-10	2-14	0	0	0	No	No
	Plasma	—	0	0	0	—	—

* Antibody adsorbed out with sensitized cells before passive transfer.

‡ Lost through technical difficulty.

§ 40 μg. PPD added to fraction before passive transfer.

‖ Containing some IV-10 due to incomplete separation. See text.

¶ Plasma from unsensitized donors.

fraction IV-10 contains the entire Boyden antibody. Inhibition of IV-10 activity by fraction II occurs only *in vivo*.

The Boyden test was done in two aliquots, one heated at 56°C. for 30 minutes and the other unheated. The data in Table VII show that the antibody in fraction IV-10 is damaged by this amount of heat. It was also observed that the antibody lost its activity over a 4 to 5 day period when held at 4°C. in the refrigerator. The unheated aliquots received EDTA even though there was no evidence that those particular fractions contained complement.

IV. Other Characteristics of the Passive Transfer Phenomenon

Heat-Labile Property of the Fraction IV-10 Antibody in Plasma and Serum.—This study is shown in Table VIII. The Boyden technique, as modified in the present study,

TABLE VIII

Demonstration of Heat-Labile Property of Tuberculoprotein-Reacting Antibody

Date	Specimen	Boyden titer after inactivation of complement by	
		EDTA	56° 30 min.
6-2-54	Plasma	200	100
6-3-54	Plasma	200	100
6-5-54	Plasma	200	100
6-8-54	Serum	200	0
6-16-54	Serum	160	0
6-16-54	Serum	80	0
6-17-54	Plasma	160	40
6-22-54	Plasma	640	40
6-23-54	Serum	40	0
6-23-54	Serum	1280+	160
6-23-54	Serum	640	40
6-23-54	Serum	40	0
6-23-54	Serum	80	0
6-23-54	Serum	1280+	40
8-2-54	Plasma	640	40
8-3-54	Plasma	320	40
8-9-54	Plasma	80	20
8-16-54	Serum	160	20

was used to test 18 specimens of immune serum or plasma. The specimens were studied in duplicates, one heated at 56°C. for 30 minutes, and the other inactivated with EDTA (38). All 18 EDTA-inactivated aliquots had elevated titers. Of the 18 heated aliquots, 6 were negative, and the others had lower titers than their corresponding EDTA-inactivated portions. Heating appeared to produce more damage to the antibody in the serum specimens than in the plasma.

Passive Transfer Studies with Adsorbed Immune Serum.—The inhibition of IV-10 by II suggested that delayed type skin sensitivity might be transferred with serum, provided Middlebrook-Dubos antibody was first removed.

For each 20 ml. EDTA-inactivated serum, 3 ml. of packed Middlebrook-Dubos sensitized red cells were used. The sensitized cells were washed 9 times with buffered saline to remove as much tuberculin as possible. The cells remained well sensitized.

TABLE IX

Passive Transfer Attempts via Immune Serum Adsorbed with Middlebrook-Dubos Sensitized Red Cells

Pig No. and date	Volume serum	Middlebrook Dubos titer		Boyden titer		PPD tests: Diameter of induration and day*									Comments
		Before	*After*	*Before*	*After*	1	2	3	4	5	6	7	8	9	
	ml.					*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	
1-56	12	64	8	200	200	0‡			0	0	2.1	0.6	0	0	
5-24-54						0‡			0	0	0.5	0	0	0	
1-57	10	64	8	200	200		0			0	2.0	0		0	
5-24-54							0			0	0.4	0		0	
1-60	11	32	16	200	200				0	0c	0	0c			
5-25-54									0	0	0	0			
1-61	10	32	16	200	200				0c	0	0	0			
5-25-54									0	0	0	0			
1-65	18	128	16	200	200					0c	0	0	0		
6-5-54										0	0	0	0		
1-66	16	128	16	200	200				0	0	0	0	0		
6-8-54									0	0	0	0	0		
1-67 6-8-54	15	128	16	200	0				0	0	0	0	0		2 adsorptions with Boyden cells.
2-17 8-16-54	22	128+	128+	160	160				0	0C§	0C	0C	0C		Not adsorbed with Middlebrook cells.
2-18 8-16-54	22	128+	16	160	160				0	0	0c‖	0c	0c		
2-19 8-16-54	22	128+	16	160	160				0	0	0c	0c	0c		
2-20 8-16-54	22	128+	16	160	160				0	0	0c	0c	0c		
2-21 8-16-54	22	128+	128+	160	0				0	0	0c	0c	0c		2 adsorptions with Boyden cells, none with Middlebrook cells.

* Fraction injected intraperitoneally on day 0.

‡ Upper figure is 24 hour reading. Lower figure is 48 hour reading.

§ C, large reaction to Choucroun polysaccharide.

‖ c, small reaction to Choucroun polysaccharide.

Immune serum was adsorbed 10 times. Each adsorption was with 0.3 ml. cells, for ½ to 2 hours, with one adsorption going overnight. Serum was kept at 4°C. and the adsorption procedure was completed and the serum injected into the recipient within 24 hours.

Out of 9 attempts to transfer reactivity with immune serum, delayed type sensitivity was effected only twice, as shown in Table IX. It may be significant that these 2 instances were also the only times that the Middlebrook-Dubos titer could be brought down to 1:8. The Boyden titer on test aliquots, to the contrary, was easily removed with 2 adsorptions, each of 0.25 ml. Boyden-sensitized cells. Control animals, Nos. 1-67 and 2-21, received this material. Immediate type reactions to Choucroun polysaccharide of 1.5 cm. diameter were obtained in all the passive transfer failures but were not tested in the 2 successful instances. In the unadsorbed control guinea pig, No. 2-17, Choucroun polysaccharide produced a large cutaneous reaction.

V. Histology of the Delayed Type Skin Reactions

Comprehensive analysis of tissue changes in these passively transferred reactions is beyond the scope of this study. Limited observations on three biopsy groups were done as follows:—(*a*) Three PPD skin tests passively transferred *via* fraction IV-10; (*b*) Two PPD first strength lesions in H37Rv-sensitized and one in a BCG-infected guinea pig. These lesions were the same size as those in group (*a*); (*c*) two very large PPD reactions in H37Rv-sensitized animals and one in a BCG-infected guinea pig.

Biopsy of each entire skin test site was performed at 24 hours. The 3 groups presented the same histological characteristics. Group (*c*) specimens, of course, showed more extensive reactions. Groups (*a*) and (*b*) were microscopically indistinguishable. Cellular infiltration consisted of mononuclear and polymorphonuclear cells, with the latter predominating. A few eosinophiles were seen. There was some edema and vascular engorgement.

DISCUSSION

The foregoing study has demonstrated the presence, in immune guinea pig plasma, of two chemically distinct antibodies, each directed to a different antigen. The chemical characteristics of these antibodies were determined by the assay of a large number of different plasma fractions for their antibody content, using a passive transfer technique. The antibody in fraction II will transfer to normal guinea pigs immediate type reactivity to polysaccharides. It is not absolutely certain that this antibody is specific to tuberculopolysaccharide, since the purified preparations of the latter did contain from 0.2 to 1.05 per cent of nitrogen. However, recipient animals reacted in a vigorous manner to skin test doses of 1 μg. of Choucroun polysaccharide which contained only 0.01 μg. nitrogen, whereas they usually had no skin reactions at all to 5 μg. doses of PPD. Fraction II contains the entire demonstrable hemagglutinating antibody for Middlebrook-Dubos–sensitized sheep cells. Passive transfer of immediate type reactivity can readily be removed from fraction II by adsorption with these sensitized, red blood cells. Some support is thus offered for the suggestions (21, 23, 24) that the Middlebrook-Dubos test is a measure of antibody to tuberculopolysaccharide.

The second antibody is contained in a subfraction of the alpha globulin, which fraction we have named IV-10. A delayed type of cutaneous reactivity

to 5 μg. of PPD is passively transferred to normal guinea pigs by fraction IV-10. Since the recipient animals have no skin reactions to 10 μg. doses of three different tuberculopolysaccharides, it appears that the antibody in fraction IV-10 is directed specifically to tuberculoprotein. This specificity cannot be demonstrated conclusively, however, because of the 5 to 10 per cent of polysaccharide contained in PPD. Fraction IV-10 contains the entire demonstrable hemagglutinating antibody for Boyden-sensitized red cells. Passive transfer of delayed type reactivity can be removed from fraction IV-10 by adsorptions with Boyden-sensitized cells. This observation lends support to the suggestions (23, 24) that the Boyden test measures antibody to tuberculoprotein.

It is of interest that immune fraction II does not interfere with the *in vitro* reactivity of fraction IV-10, but does inhibit the passive transfer of skin reactivity to PPD by fraction IV-10. The mechanism of this inhibition could be elucidated more clearly if it were possible to work with a PPD preparation completely free of polysaccharide. A possible explanation for this ability of fraction II to block the passive transfer of delayed reactivity is the prompt leaching of tuberculin from the test site by coexistent immediate reactivity, of a degree too small to be apparent in the gross. There is, no doubt, sufficient polysaccharide in PPD to give rise to such a microscopic early type reaction. Pepys (39) observed that histamine depresses the tuberculin reaction by a similar mechanism. Studies reported in this paper are the first in which a delayed type reaction to tuberculin has been transferred passively with regularity. The occasional success in passive transfer studies with immune rabbit serum of Zinsser and Mueller (4) might be explained on the basis of this fraction II inhibition phenomenon. The sera used by these authors which did transfer delayed reactivity tended to be low in precipitins to the antigen they used. Wells and Wylie (40) have also described a tuberculin-neutralizing factor in the gamma globulin of serum of patients with sarcoidosis and kala-azar. They have suggested that this factor may account for tuberculin anergy in these diseases. Tuberculin anergy sometimes observed in Hodgkin's disease and certain infections (41) likewise may be related to an inhibiting factor in the serum gamma globulins.

Studies with fraction IV + IV-10 demonstrated that, in six instances, strenuous application of ethanol during the fractionation procedure did not alter the specificity of the antibody for PPD. However, the type of skin reaction thereupon passively transferred was entirely different from that customarily seen to PPD. It was an immediate type reaction, always of a vigorous nature. In two other instances, zinc was not entirely removed from the purified fraction IV + V + IV-10 during the dialysis procedure. The zinc was detected, and chelated by addition of EDTA, shortly after the material reached room temperature. It is known that proteins may be denatured in the presence

of zinc when the temperature is increased above 0–4°C. (42). On both occasions, the fraction effected passive transfer of specific urticarial type skin reactivity to PPD. It appears likely that both the ethanol and zinc caused chemical alterations of the antibody. The type of specific skin reactivity to PPD thus seems to have been governed by the actual physical constitution of the antibody involved. Structural alteration of the antibody, related to fractionation methods, might explain the slight difference in appearance between the delayed type reaction passively transferred by fraction IV-10, and by fraction IV + V + IV-10.

The antibody to tuberculoprotein appears to be labile in several respects. It is damaged at 56°C. for 30 minutes or at 4°C. for 4 to 5 days. The process of freezing and lyophilizing apparently destroys its ability to effect passive transfer.

It is of interest that donor animals must be skin-tested with PPD within 48 hours of being bled. This phenomenon might be related to the rapid lysis of certain cells, in response to PPD, with liberation of antibody into the plasma.

Zinsser and Mueller (4) noted that their most successful passive transfer resulted from an immune rabbit which had been skin-tested 24 hours previously. Favour and his associates (43) observed that the cytolysis test was enhanced 24 hours after the application of a PPD skin test. Boyden and Suter (44) on the other hand, found that a skin test augmented the Middlebrook-Dubos test in guinea pigs, but this rise in titer usually did not start until 4 days later and was maximal at 10 days.

It is probable that the tanned cell technique, described by Boyden, does not detect the same antibody to tuberculoprotein as that found in fraction IV-10 in the present study. Boyden obtained high titers after heat-inactivating his sera. Moreover, his technique was successful only when Weybridge PPD was used whereas he had unsatisfactory results when PPD, prepared by the method of Seibert (45) was used as a sensitizing agent. In our study, Parke-Davis PPD prepared by the method of Seibert was an adequate antigen for the demonstration of the antibody in fraction IV-10.

The lag period of 3 to 6 days between the intraperitoneal injection of plasma fraction and the onset of delayed type skin sensitivity appears to be characteristic for large volumes of injected antibody, as well as for this route of passive transfer. Zinsser and Mueller (4) and Dienes (46), described a similar lag in passive transfer studies.

It seems unlikely that the anergic interval signifies active immunization in the present study because: (*a*) Addition of PPD to the fraction, or adsorption of the fraction with PPD-sensitized cells, causes failure of transfer; (*b*) antibody to PPD is present in the plasma fraction; (*c*) the duration of skin reactivity is brief; and (*d*) occasionally, positive delayed type skin tests are obtained 24 hours after passive transfer. This is too short a time interval for active sensitization.

Fraction II behaved quite differently than did IV-10. It regularly transferred

vigorous urticarial type reactivity to tuberculopolysaccharide, except in two instances in which the donors had been sensitized for only brief periods. Most of the PPD tests in these transfer experiments were negative. Occasional mild urticarial reactions occurred, probably caused by the small amount of polysaccharide present in this PPD preparation.

Further investigations will be required to determine whether the delayed type skin sensitivity to PPD of this study is a tuberculin reaction of the classic type, or is an Arthus phenomenon or, indeed, is something else. Tissue culture studies (5) employing the immune fraction IV-10 would be of interest. It would be pertinent to test recipient animals with tuberculin injected into the anterior chamber of the eye as described by Holley (47).

Arthus reactions to PPD have not been described before. It is well known that guinea pigs sensitized with antigenic tuberculoprotein develop Arthus reactions when skin-tested with the tuberculoprotein, but they do not react at all to PPD (48, 49). This fact is also mentioned by Rich and Follis (50). The limited extent of histological examination of the lesions in this study was not a decisive factor in helping to determine their nature. New information on the nature of immune bodies in the plasmas of animals immunized with tubercle bacilli, made available by fractionation techniques, suggests that the relation between cellular changes and the time sequence of events in the tuberculin reaction needs further study. This problem in definitions of reaction types is beyond the scope of the present report.

SUMMARY

Guinea pigs sensitized with tubercle bacilli demonstrate a dual allergic response mediated by two chemically distinct plasma fractions:

1. Antibody to tuberculopolysaccharide is located exclusively in fraction II (gamma globulin). This fraction will passively transfer systemic anaphylaxis and urticarial type skin reactivity to tuberculopolysaccharide, and contains the Middlebrook-Dubos antibody.

2. Antibody to tuberculoprotein is contained exclusively in a new plasma fraction called fraction IV-10. By Cohn's Method X, fraction IV-10 is a part of fraction IV (alpha globulin) and to a lesser extent V (albumin). This fraction will passively transfer to normal guinea pigs a delayed type skin sensitivity to tuberculin PPD which is maximal between 18 and 30 hours, and it contains the Boyden antibody.

When fractions II and IV-10 are combined, the antibody to tuberculopolysaccharide inhibits the passive transfer of delayed type reactivity. Combination of these two fractions does not alter their separate *in vitro* hemagglutinating properties. Adsorption of IV-10 with Boyden sensitized cells removes its ability to transfer delayed type tuberculin sensitivity. Adsorption of II with

Middlebrook-Dubos–sensitized cells removes its capacity to effect passive transfer of immediate type reactivity to tuberculopolysaccharides.

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BIBLIOGRAPHY

1. Ebert, R. H., *Am. Rev. Tuberc.*, 1952, **65,** 64.
2. Dienes, L., and Mallory, T. B., *Am. J. Path.*, 1932, **8,** 689.
3. Follis, R. H., Jr., *Bull. Johns Hopkins Hosp.*, 1940, **66,** 245.
4. Zinsser, H., and Mueller, J. H., *J. Exp. Med.*, 1925, **41,** 159.
5. Rich, A. R., and Lewis, M. R., *Bull. Johns Hopkins Hosp.*, 1932, **50,** 115.
6. Moen, J. K., and Swift, H. F., *J. Exp. Med.*, 1936, **64,** 339.
7. Baldridge, G. D., and Klegman, A. M., *Am. Rev. Tuberc.*, 1951, **63,** 674. Jacobi, F., and Marks, J., *J. Hyg.*, 1953, **51,** 541.
8. Cruikshank, C. N. D., *Nature*, 1951, **168,** 206.
9. Marks, J., and James, D. M., *J. Hyg.*, 1953, **51,** 340.
10. Miller, J. M., and Favour, C. B., *J. Exp. Med.*, 1951, **93,** 1.
11. Waksman, B. H., *Am. Rev. Tuberc.*, 1953, **68,** 746.
12. Chase, M. W., *Proc. Soc. Exp. Biol. and Med.*, 1945, **59,** 134.
13. Lawrence, S. H., *J. Clin. Inv.*, 1954, **33,** 951.
14. Chase, M. W., *Fed. Proc.*, 1951, **10,** 404.
15. Baldwin, E. R., *J. Med. Research*, 1910, **22,** 189.
16. Zinsser, H., and Parker, J. T., *J. Exp. Med.*, 1923, **37,** 275.
17. Enders, J. F., *J. Exp. Med.*, 1929, **50,** 777.
18. Mueller, J. H., *J. Exp. Med.*, 1926, **43,** 9.
19. Drea, W. F., and Andrejew, A., The Metabolism of the Tubercle Bacillus, Springfield, Illinois, Charles C. Thomas, 1953, 280.
20. Keogh, E. V., North, E. A., and Warburton, M. F., *Nature*, 1948, **161,** 687.
21. Middlebrook, G., and Dubos, R. J., *J. Exp. Med.*, 1948, **88,** 521.
22. Boyden, S. V., *J. Exp. Med.*, 1951, **93,** 107.
23. Grabar, P., Boyden, S. V., Taquet, A., and Borduas, A., *Compt. rend. Acad. Sc.*, 1952, **234,** 899.
24. Meynell, G. G., *J. Path. and Bact.*, 1954, **67,** 137.
25. Cohn, E. J., Gurd, F. R. N., Surgenor, D. M., Barnes, B. A., Brown, R. K., Derouaux, G., Gillespie, J. M., Kahnt, F. W., Lever, W. F., Liu, C. H., Mittelman, D., Moulton, R. F., Schmid, K., and Uroma, E., *J. Am. Chem. Soc.*, 1950, **72,** 465.
26. Dubos, R. J., and Middlebrook, G., *Am. Rev. Tuberc.*, 1947, **56,** 334.
27. Lever, W. F., Gurd, F. R. N., Uroma, E., Brown, R. K., Barnes, B. A., Schmid, K., and Schultz, E. L., *J. Clin. Inv.*, 1951, **30,** 99.
28. Biedemann, W., and Schwarzenbach, G., *Chimia*, 1948, **2,** 56.

29. Schmid, K., *J. Am. Chem. Soc.*, 1953, **75**, 60.
30. Lanni, F., Dillon, M. L., and Beard, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1950, **74**, 4.
31. Choucroun, N., *Am. Rev. Tuberc.*, 1947, **56**, 203.
32. Seibert, F. B., and Watson, D. W., *J. Biol. Chem.*, 1941, **140**, 55.
33. Tennent, D. M., and Watson, D. W., *J. Immunol.*, 1942, **45**, 179.
34. Orlans, E. S., *Brit. J. Exp. Path.*, 1952, **33**, 451.
35. Sherris, J. C., *J. Clin. Path.*, 1953, **6**, 64.
36. Oncley, J. L., personal communication.
37. Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, 1946, **68**, 459.
38. Levine, J., Osler, A. G., and Mayer, M. M., *J. Immunol.*, 1953, **71**, 374.
39. Pepys, J., *Acta Allerg.*, 1953, **6**, 265.
40. Wells, A. Q., and Wylie, J. A. H., *Lancet*, 1949, **256**, 439.
41. Schier, W. W., *New England J. Med.*, 1954, **250**, 353.
42. Surgenor, D. M., personal communication.
43. Favour, C. B., Harrison, B. A., and Osgood, C. K., *Proc. Soc. Exp. Biol. and Med.*, 1950, **73**, 122.
44. Boyden, S. V., and Suter, W. E., *J. Immunol.*, 1952, **68**, 577.
45. Seibert, F. B., *Am. Rev. Tuberc.*, 1934, **30**, 713. Seibert, F. B., and Glenn, J. T., *Am. Rev. Tuberc.*, 1941, **44**, 9.
46. Dienes, L., *J. Immunol.*, 1927, **14**, 43.
47. Holley, S. W., *Am. J. Path.*, 1935, **11**, 937.
48. Sabin, F. R., and Joyner, A. L., *J. Exp. Med.*, 1938, **68**, 659.
49. Seibert, F. B., *J. Infect. Dis.*, 1932, **51**, 383.
50. Rich, A. R., and Follis, R. H., Jr., *Bull. Johns Hopkins Hosp.*, 1940, **66**, 106.