A Simple Fractionation Method for Preparation of Fluorescein-labeled Gamma Globulin.* (26207)

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To reduce the amount of non-specific staining in the fluorescent antibody technic, labeled gamma globulin has been used instead of whole serum. However, most methods for preparation of labeled gamma globulins in addition to being time-consuming, result in a loss in some of the antibody active fractions. The observations of Levy and Sober(1) using an anion exchange adsorbent, diethylamino-ethyl cellulose (DEAE) to prepare relatively pure gamma globulin, suggested that this procedure could be extended to preparation of fluorescein-labeled gamma globulins, since the high capacity of the cellulose ion-exchange adsorbent, together with the relatively mild conditions of salt and pH required for adsorption and elution resulted in almost quantitative recovery of purified antibody. The procedure to be described, therefore, represents a modified separation procedure employing the cellulose ion exchanger DEAE to recover labeled gamma globulin from previously labeled whole serum.

Materials and methods. Four different hyper-immune sera were conjugated with fluorescein isothiocyanate (FITC) and fractionated through DEAE columns as described below. These sera consisted of rabbit anti-*Hemophilus pertussis,* rabbit anti-*Salmonella typhosa,* monkey anti-polio virus type 3, and ferret anti-Asian influenza virus.

Conjugation procedure. Serum samples were conjugated in the usual manner(2,3) and were then dialyzed overnight against 0.0175 M PO_4 buffer, pH 6.3 in the cold. As a control, a sample of each serum was treated in the same manner, but with omission of the fluorescein isothiocyanate dye.

Ion exchange adsorbent. The anion cellulose exchanger, DEAE (40-100 mesh, 0.96 meq/g), was prepared by the method of Levy and Sober(1). After equilibration with the starting buffer, (0.0175 M phosphate buffer, pH 6.3), the exchanger was packed into glass columns under mild air pressure. For every 2 ml of undiluted conjugated serum a column of about 1 cm in diameter and 5 cm in height was prepared.

Fractionation procedure. A stepwise elution schedule with eluting solutions consisting of increasing concentrations of NaCl in 0.0175 M phosphate buffer, pH 6.3, was employed to fractionate both labeled and non-labeled sera. The fractionation process was carried out at 4°C with diluted serum, using relatively rapid flow rates of from 1 to 2 ml per minute. To each adsorbed column 8 ml of eluting fluid was added and the effluent collected as four 2 ml fractions, and their relative protein content determined by measurement of ultraviolet absorption at 280 mμ in the Beckman DU spectrophotometer.

Antibody assay and staining efficiency. A representative effluent fraction of each different eluent concentration was dialyzed against 0.01 M phosphate buffered saline, pH 7.0, and its antibody content and staining ability were determined. Antibody content of the fractions of the 2 anti-bacterial sera was determined by the tube agglutination technic, anti-influenza virus serum by the hemagglutination-inhibition procedure, and anti-poliovirus serum by the tube neutralization technic using monkey kidney cells. To determine the staining efficiency of the fractions obtained, smears of the bacterial antigens were made, heat fixed, and stained. Anti-poliovirus serum fractions were tested by scraping monkey kidney cells infected with type 3 poliovirus from the walls of the tube culture, suspending them in phosphate buffered sa-

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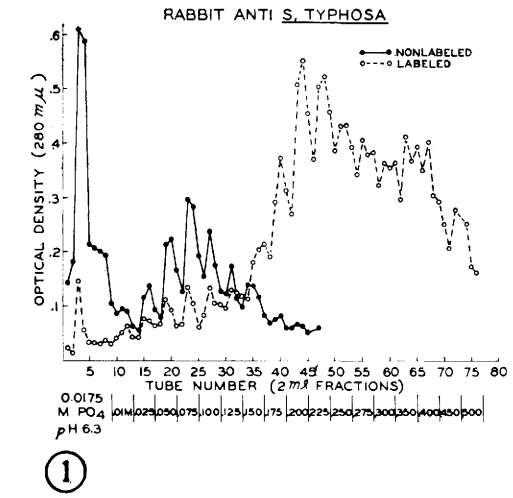

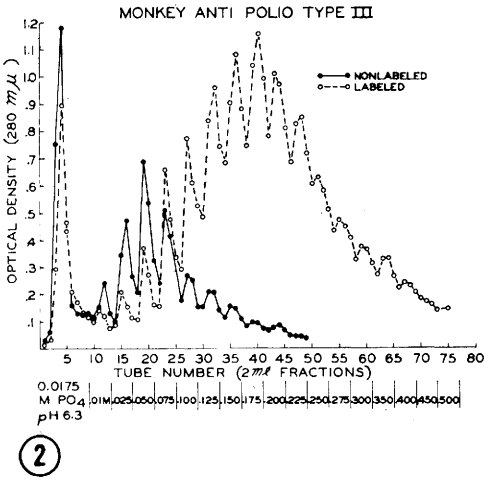

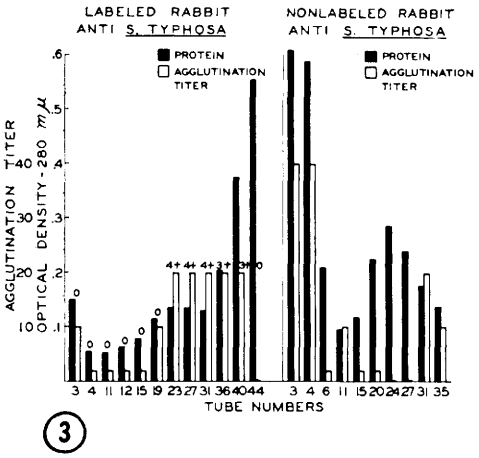

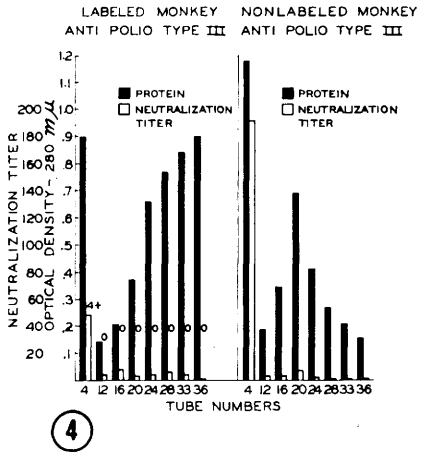

FIG. 1. Rapid stepwise anion exchange chromatography of fluorescein isothiocyanate-labeled and non-labeled rabbit anti-*S. typhosa* sera. Two ml of conjugated serum applied to a 1 × 5 cm column of DEAE cellulose. Fractions (4 × 2 ml) collected at a rate of 2 ml/min. Eluting buffer was 0.0175 M Na-phosphate, pH 6.3, with stepwise changes in NaCl concentration and constant pH. Temp. 4°C.

FIG. 2. Rapid stepwise anion exchange chromatography of fluorescein isothiocyanate-labeled and non-labeled monkey anti-poliovirus type 3 sera. Two ml of conjugated serum applied to a 1 × 5 cm column of DEAE cellulose. Fractions (4 × 2 ml) collected at a rate of 2 ml/min. Eluting buffer was 0.0175 M Na-phosphate, pH 6.3, with stepwise changes in NaCl concentration and constant pH. Temp. 4°C.

FIG. 3. Antibody activity in relation to staining efficiency and protein content of serum fractions obtained by anion exchange chromatography. The tube numbers correspond to those found in Fig. 1. Staining efficiency: 4+ excellent, 3+ good, 2+ fair, 1+ poor, and 0 none.

FIG. 4. Antibody activity in relation to staining efficiency and protein content of serum fractions obtained by anion exchange chromatography. The tube numbers correspond to those found in Fig. 2. Staining efficiency: 4+ excellent, 3+ good, 2+ fair, 1+ poor, and 0 none.

line, pH 7.0, and making smears of the suspended cells. After air drying, the smears were fixed in acetone for 10 minutes, air dried, and stained. The staining efficiency of anti-influenza virus serum fractions was carried out by Dr. A. Inglot of this laboratory by a method yet to be published. For both bacterial and viral antigens, the staining procedure was that essentially described by Riggs *et al.*(2).

Results. Typical fractionation patterns obtained by this method are shown in Fig. 1 and 2. Two definite peaks are obtained with non-labeled sera, which on further separation

with increasing concentrations of salt yielded no additional peaks. However, after labeling with FITC, additional protein peaks are obtained within the same range of salt concentrations (0.01 M-0.5 M NaCl). Whereas all antibody activity of the non-labeled sera appeared within the effluents obtained with the original phosphate buffer, that of the FITC-labeled rabbit sera tends to be more widely distributed (Fig. 3,4). This spread was not observed with monkey serum. Essentially all antibody activity of the labeled rabbit sera can be recovered with higher concentrations of salt (up to 0.175 M NaCl-Tube 40); however, as will be described below, staining with the antibody-containing higher salt concentration fractions (0.15 M NaCl), results in an increased amount of non-specific staining. When representative antibody-containing fractions of the 2 peaks obtained from non-labeled sera were examined by paper electrophoresis, it was found that they belonged to the gamma globulin component of serum. Fractions from labeled serum obtained at molarities corresponding to these peaks also showed electrophoretic mobility of gamma globulin. However, because of the labeling process the mobility is somewhat slower than with native serum.

The staining efficiency of the various antibody-containing fractions obtained was graded as follows: 4+ excellent, 3+ good, 2+ fair, 1+ poor, and 0 none. With monkey anti-poliovirus and ferret anti-influenza virus sera, only the fractions eluted with the starting buffer (0.0175 M phosphate buffer, pH 6.3) exhibited staining ability, while the rabbit anti-bacterial sera exhibited staining ability only with fractions eluted by higher salt concentrations, the strongest staining being observed in the 0.075 to 0.125 M NaCl fractions. With fractions above 0.15 M NaCl, a consistent increase in amount of non-specific staining was observed. Staining of the homologous antigens was well defined in that the background fluorescence indicating presence of unbound dye or dye bound to non-antibody proteins was not observed. Staining of impression smears made from *H. pertussis*-infected mouse brains, reveals bright specific staining of the bacterial antigen with little or no fluorescence in non-infected cells or other background material (Fig. 5). With the anti-poliovirus serum fractions, background fluorescence also was reduced, and positively stained cells could easily be distinguished from negative ones in the same microscopic field.

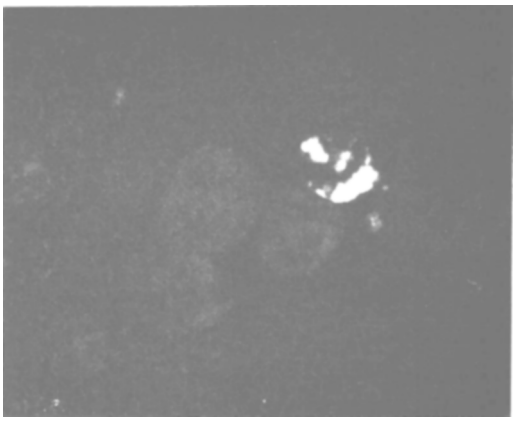

FIG. 5. Impression smear of mouse brain infected with *H. pertussis* and stained with labeled gamma globulin (0.125 M NaCl eluate) fractionated through the DEAE cellulose column.

As a routine procedure for preparation of labeled gamma globulin, it is now suggested that immune serum after appropriate dilution with physiological saline and conjugation with FITC, be dialyzed overnight at 4°C, against 0.125 M NaCl in 0.0175 M phosphate buffer, pH 6.3. After dialysis the conjugate is put through the DEAE-cellulose column. For every 2 ml of undiluted starting serum, a column of about 1 cm diameter and 5 cm in height is prepared. Size of the column can be adjusted to amount of starting serum available. The non-absorbed gamma globulin fractions are collected and the column washed through with more of the eluent (0.125 M NaCl in 0.0175 M phosphate buffer, pH 6.3). After determination of protein and FITC content of each aliquot by their absorption at 280 mμ and 490 mμ respectively, aliquots showing the highest protein and dye content are combined, and dialyzed overnight at 4°C against phosphate buffered saline, pH 7.0. Concentration of the fractions can be carried out by pervaporation, ultra-filtration, or dialysis against PVP (polyvinylpyrrolidone), although if a serum

of high titer is used initially, the dilution factor may be ignored.

By using whole labeled immune serum, advantage is taken of the fact that the gamma globulins are isolated at the same time as unbound dye is removed from the conjugate. The alternative of first fractionating the serum and then labeling the gamma globulins has also been carried out although the extended periods of dialysis to remove unbound dye from the conjugate are not curtailed by this procedure. One may, however, refractionate on the DEAE column after labeling. thereby further purify the antibody and remove unbound dye.

The procedure described can be used readily in any laboratory, and presents several advantages over the usual chemical or physical methods for isolation of labeled antibody. Aside from the relative ease and expediency of the method, mildness of the conditions thus required for isolation of FITC-labeled gamma globulin with specific antibody activity, should be appreciated. Also important is the marked reduction of non-specific fluorescence by elimination of non-antibody containing serum components. The additional steps of absorption with tissue powders necessary in other procedures to reduce non-specific staining could often be eliminated.

Discussion. The change in fractionation behavior of serum onto DEAE cellulose column after labeling with FITC is of considerable interest. Our investigation has once again reaffirmed the heterogeneity of gamma globulin(5). Only 2 components, both electrophoretically identified as gamma globulin, are obtained by elution under these conditions; the first component contains the majority of the specific antibody. Labeling of the serum with FITC, however, brought about a change in both the fractionation behavior and physiological activity of the gamma globulin. In addition to a depression in protein content of both the early peaks noted in native serum, further fractionation with increasing salt concentrations revealed that additional serum components could be eluted. Preliminary investigations suggest that these additional proteins are composed of α and β globulins and albumin. An explanation for this change in chromatographic behavior is being pursued. With regard to antibody behavior it is of interest that the shift in antibody character requiring a higher concentration of salt for elution is found only with labeled rabbit sera. Antibody activity in ferret anti-influenza virus and monkey anti-poliovirus sera was essentially unaltered. Whether this difference in labeled antibody behavior is due to a species difference in sera, or to the different antigens used in the immunization process is yet to be determined. However, the heterogeneity in antibody activity of immune sera against different antigens when subjected to fractionation on cellulose columns has also been described by Fahey and Horbett(4) and Sober and Peterson(5).

Summary. A relatively simple method for separation of fluorescein isothiocyanate-labeled gamma globulin from serum has been presented. The procedure has been applied to a number of labeled anti-bacterial and anti-viral sera, and the labeled gamma globulin obtained gave definite specific fluorescence when used in the staining procedure with markedly low background fluorescence.

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