Preparation of Fluorescein Isothiocyanate-Labeled \(\gamma\)-Globulin by Dialysis, Gel Filtration, and Ion-Exchange Chromatography in Combination

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ABSTRACT

DEDMON, ROBERT E. (Presbyterian-St. Luke's Hospital, Chicago, Ill.), ALBERT W. HOLMES, AND FRIEDRICH DEINHARDT. Preparation of fluorescein isothiocyanate-labeled \(\gamma\)-globulin by dialysis, gel filtration, and ion-exchange chromatography in combination. J. Bacteriol. 89:734–739. 1963.—Antiviral immune \(\gamma\)-globulins isolated from rabbit and guinea pig sera were labeled through dialysis membranes with fluorescein isothiocyanate and purified in several ways to eliminate nonspecific staining. Gel filtration of the conjugate with Sephadex G-25 coarse beads followed by column fractionation with diethylaminoethyl-Sephadex yielded consistently highly specific staining materials. Fluorescein-protein ratios varied between 1.0 and 4.0. This technique has proved to be simple and reliable, and is less time-consuming than previous techniques.

Nonspecific fluorescent staining has presented the greatest difficulty in immunofluorescent studies. Both unbound free fluorescent dye and labeled protein molecules with a high negative charge have shown to be responsible for this problem. Prolonged dialysis against phosphate-buffered saline, extraction with charcoal, or gel filtration with Sephadex have been used for the removal of unbound dye; these procedures were recently reviewed by Nairn (1962). Removal of labeled proteins with high negative charges has been attempted by various methods, i.e., repeated adsorption of labeled serum or \(\gamma\)-globulin with tissue powders or ion-exchange resins (Coons and Kaplan, 1950), diethylaminoethyl (DEAE)-cellulose ion-exchange chromatography (Riggs, Loh, and Eueland, 1960; Goldstein, Sliuzys, and Chase, 1961; Curtain, 1961; McDevitt et al., 1963), treatment with 2-ethoxy-6,9-di-aminoacridine-lactate (Rivanol, Winthrop Laboratories, Rensselaer, N.Y.) (Frommhen, and Martins, 1963), or 6,9-diamino-2-ethoxyacridine (Ethodin, Winthrop Laboratories) (Gordon, Edwards and Tompkins, 1962). Goldstein et al. (1961) used smaller amounts (6 to 8 mg of isothiocyanate per g of protein) for labeling of antibodies, and recently Clark and Shepard (1963) reported the preparation of labeled \(\gamma\)-globulins with less nonspecific staining by labeling through a dialysis bag.

The characteristics of Sephadex and DEAE-Sephadex suggested that these substances might be very efficient in removing both unbound dye and negatively charged protein molecules from labeled antibody solutions. The use of both Sephadex and DEAE-Sephadex in succession yielded antibody solutions giving a high degree of specific staining without nonspecific fluorescence. The method described in this report is simple and reliable, and has, at least in our hands, consistently yielded better results than previous techniques.

MATERIALS AND METHODS

Antisera. Experimental rabbit and guinea pig antisera specific for a given virus were used. The preparation of such antisera against mumps, Newcastle disease virus (NDV), herpes simplex, and Herpesvirus tamarinus has been fully described (Holmes et al., 1964). Individual sera were tested for complement-fixing and neutralizing antibodies; sera with titers of 64 or greater were pooled, and the pools were used in the preparation of fluorescent-antibody preparations.

Fluorescein isothiocyanate (FITC). Crystalline, chromatographically pure, isomer I FITC, obtained from BBL (list no. 04-580), was used in all studies.

Preparation of Sephadex and DEAE-Sephadex columns. Sephadex was obtained from Pharmacia

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Fine Chemicals, Uppsala, Sweden. In early experiments, Sephadex G-50 and Sephadex G-25 (medium grade) were employed. Later, Sephadex G-25 "coarse beads" were used because of the faster flow rates obtained with this preparation. DEAE-Sephadex A-50 (medium grade) was employed in the final purification step of all experiments.

Sephadex was allowed to swell in an excess of distilled water for 24 hr with repeated deactants to remove the fines. The Sephadex was then washed with 0.1 NaOH, with repeated changes of distilled water until neutrality was reached, and finally with phosphate-buffered saline (PBS, 0.01 M PO₄, 0.14 M NaCl, pH 7.3). It was stored in an excess of buffer at 4°C.

Columns were prepared in both 4 by 40 cm and 2 by 25 cm size, depending upon the amount of material to be purified. The gel was poured as a suspension into glass chromatographic tubings partially filled with starting buffer, and the columns were equilibrated by passage of 3 to 4 column volumes of buffer through the gel bed.

Fractions were collected in a Vanguard fraction collector, and the column effluent was monitored at 280 mμ by a spectrophotometer (Beckman, Palo Alto, Calif.).

Zone electrophoresis. Sera and various concentrated fractions were evaluated by electrophoresis on filter paper (Block, Durrum, and Zweig, 1958) or cellulose acetate (Kohn, 1960).

Isolation of γ-globulin. Ammonium sulfate (40% saturation) precipitation (Kabat and Mayer, 1961) followed by dialysis against 0.15 M NaCl for 48 hr was used initially. DEAE-Sephadex column fractionation was used in all other experiments for the isolation of γ-globulin from whole serum. Whole serum (60 to 80 ml) was placed on top of a column (4 by 40 cm) equilibrated with 0.02 M phosphate buffer, pH 7.6. The γ-globulin passed through the column, and the remainder of the serum proteins were adsorbed in a fashion similar to that on DEAE-cellulose (Levy and Sober, 1960). The remaining serum proteins were then eluted with 2 M NaCl. All chromatographic and labeling procedures were performed in the cold room at 4°C.

The γ-globulin fraction was then concentrated by evaporation or dialysis against 15% polyvinyl pyrrolidone in PBS. Total protein was determined by the biuret method (Kabat and Mayer, 1961), and the protein concentration was adjusted to 1% by addition of carbonate-bicarbonate buffer, pH 9 (0.025 moles of NaHCO₃ and Na₂CO₃).

Conjugation of antisera. In early experiments, γ-globulin or whole serum was labeled by the method of Marshall, Eveland, and Smith (1958), except that 10 mg of FITC per g of protein were employed. The method finally adopted was that of Clark and Shepard (1963). The γ-globulin solution was placed in a dialysis bag in a graduated cylinder mounted on a magnetic stirrer in the cold room. A solution of carbonate-bicarbonate buffer (pH 9) containing 0.1 mg/ml of crystalline FITC (10 volumes per volume of γ-globulin solution) was placed in the cylinder, and labeling through the dialysis membrane was allowed to proceed for 16 to 24 hr. FITC concentrations greater than 0.2 mg/ml in the dialyzing buffer used for staining resulted in preparations exhibiting varying degrees of nonspecific staining even after Sephadex-DEAE-Sephadex fractionations. This is in accordance with the data reported by Clark and Shepard (1963).

Removal of unbound dye. At the end of the labeling period, the unbound dye was immediately removed by gel filtration through a column (4 by 40 cm) of Sephadex G-25 coarse beads equilibrated with PBS (0.01 M PO₄, 0.14 M NaCl, pH 7.3). The labeled protein solution was concentrated to a suitable volume and stored in the dark at 4°C. A portion of the material was saved for analysis of protein, antibody content, and staining characteristics. The remainder was purified by use of DEAE-Sephadex.

Removal of highly charged protein molecules with DEAE-Sephadex. Approximately 20 ml of conjugate containing 6 mg of total protein per ml were passed through a column (2 by 25 cm) of DEAE-Sephadex equilibrated with PBS (0.01 M PO₄, 0.14 M NaCl, pH 7.3). Prior dialysis was not necessary because of the previous passage of the conjugate through the G-25 column containing this buffer.

Stepwise elution with the use of 0.01 M phosphate buffer (pH 7.3) was used to remove the conjugate. In some instances, the sequence was 0.14 M NaCl followed by 2.0 M NaCl, but the final sequence was 0.14 M, 0.20 M, 0.3 M, and 1.0 or 2.0 M NaCl in 0.01 M phosphate buffer (pH 7.3). Prior to analysis, the various fractions were concentrated and dialyzed overnight against PBS.

Analysis of fractions. Absorption spectra were determined in a Beckman DK-2 or a Perkin-Elmer model 202 ratio recording spectrophotometer. Total protein determinations and electrophoresis were performed and labeling ratios were calculated as suggested by Nairn (1962). Complement-fixing and neutralizing antibody titers were determined by standard methods (Francis, 1956), except for mumps neutralizing antibody which was assayed by an interference method (Deinhardt and Shramek, 1965).

Preparation of cell cultures. Cell cultures were grown and infected with the different viruses on cover slips in Leighton tubes, as previously described (Holmes et al., 1964; Deinhardt and Henle, 1966). Control and infected cultures were harvested at appropriate times by washing in PBS, air-drying, and fixation with acetone. They were stored at 4°C prior to staining.

Staining of cover slip preparations. The cover slips were mounted, cells down, on a microscope slide to which had been fixed two Corning #2 cover glasses. These were attached to the slide with Canada balsam in xylene and served as a support for the cover slip. The fluorescent conjugate was added in a manner similar to that used.
TABLE 1. Recovery of protein and antibody from a guinea pig anti-mumps serum

<table>
<thead>
<tr>
<th>Material</th>
<th>Eluting buffer</th>
<th>Final vol of fraction</th>
<th>Total protein</th>
<th>Electrophoresis mobility</th>
<th>Neutralizing antibody titer</th>
<th>Complement-fixing antibody titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole serum</td>
<td></td>
<td></td>
<td>All components</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ-Globulin*</td>
<td>PO₄ (pH 7.6, 0.02 M)</td>
<td>90</td>
<td>6.3</td>
<td>γ-2</td>
<td>1:512</td>
<td>1:256</td>
</tr>
<tr>
<td>Sephadex G-25, labeled effluent</td>
<td>PBS (pH 7.3)</td>
<td>40</td>
<td>6.0</td>
<td>γ-2 (“fast”)</td>
<td>1:512</td>
<td>1:64</td>
</tr>
<tr>
<td>DEAE-Sephadex, labeled effluent</td>
<td>NaCl (0.14 M)</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>PBS (pH 7.3)</td>
<td>20</td>
<td>1.0</td>
<td>γ-2 (“fast”)</td>
<td>1:256</td>
<td>1:32</td>
</tr>
<tr>
<td></td>
<td>NaCl (2.0 M)</td>
<td>10</td>
<td>2.0</td>
<td>γ-2 (“fast”)</td>
<td></td>
<td>1:16</td>
</tr>
</tbody>
</table>

* Isolated on DEAE-Sephadex before labeling.

in filling a hemocytometer chamber. The cover slips were then incubated in a moist chamber at 37°C for 1 hr, washed with PBS and mounted in Eulanol (Rodriguez and Deinhardt, 1960).

Tests for specificity of staining. Staining of uninfected cells and cells infected with other viruses, and one and two stage inhibition tests, were performed to check specificity of staining (Cherry, Goldman, and Carski, 1961).

Fluorescence microscopy. A Zeiss model GFL fluorescent microscope and a Mercury vapor lamp (Osram HBO-200) as the exciting source were employed. The exciting filters were UG-2 and BG12 with a Schott KG1 heat-absorbing filter. Barrier filters were New Schott Designation 50 and 41.

RESULTS

Separation of γ-globulin and other serum proteins on DEAE-Sephadex A-50. Rabbit, guinea pig, horse, and human sera were studied. In all cases, γ-globulin came through the column as a single peak with 0.02 M phosphate buffer (pH 7.6). Other serum proteins could be eluted by lowering the pH and increasing the concentration of sodium chloride. Dialysis against starting buffer for 16 hr or passage of the serum through a Sephadex G-25 column was employed in early experiments, but this was found to be unnecessary and was eliminated.

Removal of unreacted fluorescein isothiocyanate. In some experiments, prolonged dialysis for 8 days against PBS was employed. Staining with this material, even though labeling had been performed through a dialysis membrane, resulted in marked nonspecific staining. In other experiments, Sephadex G-25 was used to remove the unbound dye, and the resultant material was used for staining. This treatment removed unbound dye very efficiently but not other non-specific staining material. The use of prolonged dialysis against PBS after Sephadex treatment also failed to reduce the remaining nonspecific staining.

Removal of nonspecific staining material by use of DEAE-Sephadex A-50. Initially, a variety of buffers of varying NaCl concentration were used. In all cases, elution with PBS (0.01 M PO₄, 0.14 M NaCl, pH 7.3) yielded a fraction with good specific and negligible nonspecific staining. Higher concentrations of NaCl (0.2 M) likewise eluted occasionally similar materials, but the 0.3 M NaCl eluate usually showed marked nonspecific staining. However, in one experiment with NDV antiserum 0.3 M NaCl elution yielded two peaks, the first of which showed good specific and negligible nonspecific staining, and the second of which showed marked nonspecific staining and only slight specific staining.

Recovery of protein and antibodies is shown in Table 1 for a typical experiment. It is apparent that some loss of protein and antibody occurs with retention of the proteins, giving nonspecific staining on the column, as is the case with DEAE-cellulose (McDevitt et al., 1963). Nevertheless, the final product contains enough antibodies to produce excellent specific staining. Electrophoretic mobility of the labeled γ-globulin was slightly faster than that of the unlabeled protein.

Staining and labeling results. Table 2 summarizes the data from one guinea pig mumps antiserum experiment. Table 3 summarizes two experiments with rabbit NDV antiserum. In one of these, 0.3 M NaCl produced two peaks, one with specific and one with nonspecific staining. Although it is not reasonable to expect such splitting of fractions with 0.3 M NaCl consistently, recovery of increasing amounts of labeled material giving specific staining might be accom-
plished by the use of smaller step-wise increments of NaCl molarity.

Fluorescein-protein ratios of suitable conjugates ranged from 1.0 to 4.0. In contrast to the results of McDevitt et al. (1963), parallel increases in fluorescein-protein ratios and eluting molarity were not observed in all experiments. Results of labeling of several different antisera are summarized in Table 4.

Tests for specificity of staining. Noninfected cell cultures and cells infected with heterologous viruses showed no staining with the 0.14 M NaCl fractions. Fluorescent conjugates prepared against mumps and NDV, both myxoviruses, or against mumps and herpes were cross-stained with negative results.

Inhibition tests were used in checking the specificity of staining of some fluorescent conjugates. Both one- and two-step inhibition experiments were successful when reagents were used at appropriate dilutions.

Attempts to enhance staining by increasing pH. Pital and Janowitz (1963) reported increased intensity of staining of bacterial smears when stained preparations were washed and mounted in 0.5 M carbonate buffer (pH 9.0). Attempts to do this with stained mumps and NDV cover-slip preparations were unsuccessful.

Absorption spectra. Several of the conjugates were examined in the spectrophotometer in the range of 350 to 750 μ. Spectra were determined in PBS. The peak of maximal absorption of all samples, regardless of their point of elution from the columns, was constant at 495 μ.

Examination of the fluorescein isothiocyante solution surrounding the dialysis bag was also performed. Absorption spectra were checked on

| Table 2. Staining properties of a labeled anti-mumps guinea pig γ-globulin |
|-----------------------------|---------|-----------------|-----------------|
| Material                  | Fraction | Fluorescein-protein ratio | Staining properties |
| Crude conjugate           |          |                 |                  |
| Sephadex G-25 effluent    | PBS, pH 7.3 (0.14 M NaCl) | 3.7   | 4+             |
| DEAE-Sephadex effluent    | I        | PBS, pH 7.3 (0.14 M NaCl) | 3.6   | 4+             |
|                           | II       | PBS, pH 7.3 (2.0 M NaCl)  | 3.5   | 4+             |

| Table 3. Staining properties of a rabbit anti-NDV γ-globulin |
|-----------------------------|---------|-----------------|-----------------|
| Material                  | Fraction | Fluorescein-protein ratio | Staining properties |
| Sephadex G-25 effluent    | PBS, pH 7.3 (0.14 M NaCl) | 1.1   | 4+             |
| DEAE-Sephadex effluent    | I        | PBS, pH 7.3 (0.14 M NaCl) | 1.3   | 3+             |
|                           | II       | PBS, pH 7.3 (2.0 M NaCl)  | 2.4   | 3+             |

| Table 4. Summary of antisera labeled with FITC and purified by G-25 and DEAE-Sephadex* |
|-----------------------------|---------|-----------------|-----------------|
| Serum source               | Virus antigen | Staining properties | Fluorescein-protein ratio | Absorption peak in PBS |
| Rabbit                     | Herpes-M    | 4+              | 0               | 500 |
| Rabbit                     | Herpes simplex | 3-4+       | 0               |          |
| Guinea pig                 | Mumps       | 4+              | 0               |          |
| Rabbit                     | Mumps       | 4+              | 0               | 495  |
| Rabbit                     | NDV         | 3-4+            | 0               | 495  |
| Guinea pig                 | Mumps       | 4+              | 0               | 495  |

* In all cases, the fraction labeled was γ-globulin.

the fluid, on a portion which was kept in a separate container on a magnetic stirrer during the dialysis period, and on the unbound dye separated from the conjugated γ-globulin by Sephadex G-25 coarse beads. No changes in the spectrum were found. Paper chromatography of the
solutions revealed no alterations in mobility with 0.2 M Na₂HPO₄ used as the developing solution (Clark and Shepard, 1960). The same result was obtained with cellulose acetate electrophoresis.

**Discussion**

DEAE-Sephadex proved to be at least as efficient as DEAE-cellulose (Riggs et al., 1960; Goldstein et al., 1961; Curtain, 1961; McDevitt et al., 1963) in the purification of FITC-labeled antibody solutions. In some experiments, single-step purification of the conjugate with DEAE-Sephadex yielded satisfactory results. But uniformly good results, i.e., preparations giving a high degree of specific staining without nonspecific fluorescence, were only obtained when the preparation was passed through Sephadex G-25 coarse beads prior to DEAE-Sephadex fractionation. The Sephadex filtration requires only about 30 min for 100 ml of cold conjugate and, therefore, adds little time to the procedure.

Although labeling through a dialysis membrane was of little help in eliminating nonspecific staining, it was retained as part of the routine procedure because an occasional conjugate was satisfactory after labeling in this fashion followed by gel filtration on Sephadex G-25 only. Also, on some occasions conjugate labeled with crystalline FITC in a flask could only be eluted at high NaCl molarity (1.0 or 2.0 M) with resultant nonspecific staining. No such problem occurred with the dialysis technique.

With this method, the unbound dye is removed by Sephadex G-25 gel filtration. For the further purification by DEAE-Sephadex, the same principle applies as for DEAE-cellulose. (Goldstein et al., 1961; McDevitt et al., 1963). Step-wise increments of NaCl in phosphate buffer are necessary to separate highly charged nonspecific portions on DEAE-Sephadex. When using this material, it is probably advisable to employ step-wise elution (0.01 M phosphate buffer) within the range of 0.14 to 0.30 M NaCl.

One area in which DEAE-Sephadex may be used with advantage is the purification of 19S fluorescein-labeled antibodies. McDevitt et al. (1963) pointed out that with DEAE-cellulose ion-exchange chromatography a high molarity of NaCl is required for elution of macroglobulins. Consequently, this procedure would probably make it impossible to purify such a conjugate. Hogman and Killander (1962) recently reported the elution of 19S isohemagglutinins with 0.14 M phosphate buffer on DEAE-Sephadex. If other 19S proteins can be eluted in this fashion, the purification of labeled 19S antibodies on DEAE-Sephadex might be feasible. This, of course, remains to be demonstrated.

The present results are consistent with those of previous investigations (Goldstein et al., 1961; McDevitt et al., 1963) regarding column retention of proteins with nonspecific staining properties. Unlabeled 7S γ-globulin is not retained by the column, and strongest binding affinity is present for the γ-globulin molecules causing the most nonspecific staining. Conjugated γ-globulin could not be eluted from the column at 0.02 M phosphate without NaCl added or with the same buffer with 0.07, 0.10, or 0.125 M NaCl.

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**Literature Cited**


