Refinement of Fluorescent Antibody by Gel Filtration.* (27114)

MORRIS A. GORDON, MERCEDES R. EDWARDS, AND VICTOR N. TOMPKINS
(Introduced by John F. Kent)
Division of Laboratories and Research, New York State Department of Health, Albany

Methods of preparation and refinement of fluorescein-labelled serum globulins have been improved and considerably simplified since the fundamental studies of Coons and Kaplan in 1950(1). Fluorescein amine and fluorescein isothiocyanate are widely available in stable, powdered form, and the conjugate can be prepared, following the method of Riggs *et al.*(2), simply by stirring a measured quantity of the isothiocyanate in buffered serum or globulin several hours, with elimination of unconjugated dye by dialysis. Technics have been developed for concentrating and purifying active fractions and minimizing nonspecific staining of heterologous organisms and tissues(3-6). Recently a new chromatographic tool, Sephadex(7), was introduced. Acting as a molecular sieve, this product is an insoluble polydextran consisting of cross-linked dextran molecules. Porath and Flodin(8) described its use in "gel filtration," for separating glucose from dextrans and in desalting serum proteins. This report concerns its adaptation to the purification of fluorescent conjugates, resulting in a saving of time and effort and in conservation of antibody proteins.

Methods. Fluorescein isothiocyanate was made from fluorescein amine (synthesized locally; method of Coons and Kaplan(1)) and thiophosgene (Matheson, Coleman, and Bell) by the method of Riggs(2). For some conjugates a commercially prepared isothiocyanate (Sylvana Chemical Co.) was used. Gamma-globulin was extracted from serum either by precipitation with half-saturated ammonium sulfate or by treatment with 6,9-diamino-2-ethoxyacridine (Ethodin, Winthrop Laboratories) as follows: 2.8 volumes of 0.5% Ethodin in 0.15 M NaCl solution buffered to pH 7.4 with 0.035 M phosphate were added to one volume of serum at room temperature and the precipitate was removed by centrifugation (1000 g, 20 min. at 4°C). The supernatant solution was combined with an equal volume of chilled acetone and the precipitate collected by centrifugation at 1000 g for 30 mintues. This precipitate was then either washed once with chilled 50% acetone and dissolved in normal (0.15 M) saline to original serum volume, or taken up in saline and subjected to repeated acetone precipitations to clear it of Ethodin. In the former case residual Ethodin was removed by the Sephadex column after conjugation.

Protein concentrations were determined either by the biuret method on a Beckman B spectrophotometer at 550 mμ or by absorbency of the solution at 287 mμ on a Beckman DU apparatus. Conjugates were prepared by addition of fluorescein isothiocyanate powder, at the rate of 0.05 mg/mg protein, to the globulin at pH 9.0 in a solution 0.15 M in NaCl and 0.5 M in Na_2CO_3-$NaHCO_3$, with stirring overnight in the cold. They were then either passed immediately through a Sephadex column or dialyzed in the usual manner against phosphate-buffered saline until fluorescence of the dialysate was less than that of a 1:40,000,000 aqueous solution of free fluorescein. Conjugates of the following antisera were evaluated after Sephadex passage: rabbit sera against *Candida albicans*, C-5 (6 runs under varying conditions); *Histoplasma capsulatum*, H-8; *Cryptococcus neoformans*, Cy-1; *Torulopsis glabrata*, T-1; *Listeria monocytogenes*, 1 and 4B and human gamma-globulin; also ferret sera, normal and anti-parainfluenzae type 3 virus; and horse diphtheria antitoxin. Following passage through the column, each of these was tested for specificity and intensity of fluorescent staining and direct comparison was made with these properties in dialyzed conjugates not passed through Sephadex. Some conjugates were both dialyzed and gel-filtered.

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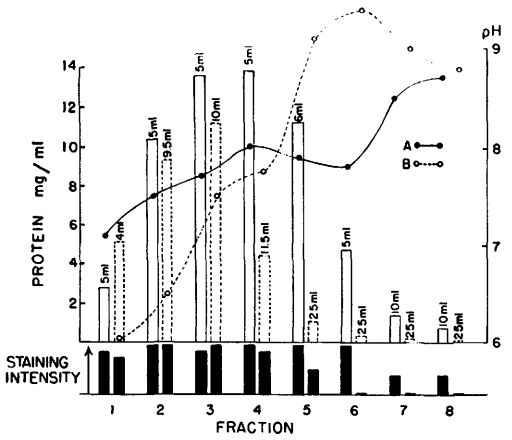

FIG. 1. Intensity of staining of *Candida albicans* with Sephadex fractions of labeled rabbit anti-*C. albicans* globulin, in relation to protein concentration and pH. A. (Exp. 6) and B. (Exp. 7) represent eluates of two 20 ml samples passed through columns equilibrated, respectively, with normal saline and distilled water. Collection of these fractions was begun upon the first appearance of fluorescence in the eluate. The staining intensities represented are those of a 1:2 dilution of each fraction in A. and of a 1:4 dilution in B.

Sephadex was also employed to desalt normal and immune sulfate-precipitated globulins, the products being tested for antibody activity by the indirect fluorescent stain method.

Sephadex G-25 medium grade was used throughout. Other grades providing more rapid filtration and presumably equally sharp separation for our purpose are available. Columns varying in capacity from 2 ml to 50 ml were employed, but we found those greater than 20 ml to be less efficient. A glass wool pad supported the gel, which was both equilibrated and eluted with either distilled water or normal saline solution. Filtration was done in a cold room, the column being chilled for at least 15 minutes (for a 7 ml column) before use. Eluant level was adjusted to the surface of the gel and the sample added by pipette. When the last of the sample had entered the gel, the surface was washed with a small quantity of eluant, followed by a larger quantity to start the elution. Progress of the conjugate through the column was followed visually by its yellow color and fluorescence under the Wood's lamp. After collection of desired fractions, the gel was regenerated by continued passage of eluant. As judged by complete disappearance of fluorescence, this process in the cold took 2 to 3 hours depending upon size of column. It was completed more rapidly at room temperature.

Results. Each of the conjugates processed through Sephadex yielded material at least the equal of the dialyzed product. Data from a pair of representative Sephadex runs are summarized in Fig. 1, parts A and B denoting 20 ml portions of the same conjugate passed through a saline and a water-equilibrated column, respectively. Total protein recovery was 100% for A and 94% for B. Following collection of an initial "holdup" volume characteristic of a given column (roughly equivalent to its elution volume) all of the protein was contained in the first yellow band of eluate, the volume of which was approximately twice that of the original specimen. These relationships were consistent in all of the Sephadex trials. In water-equilibrated columns the second band, colorless or pale yellow and practically or entirely devoid of protein (fractions 5 through 8 of Exp. 7), apparently comprised most of the high-pH buffer. Immediately behind this a second yellow front contained unconjugated fluorescein.

Use of distilled water rather than saline resulted in an acid pH, accompanied by less intense staining, in the first portion of eluted conjugate (fractions 1 and 2 in Exp. 7 and similarly in other conjugates). In Exp. 7 staining intensities recorded are those of the respective fractions diluted 1:4 with buffered saline, with resulting pH higher than those indicated in the figure. Adjustment to pH 7.2 increased staining intensity of undiluted eluates from 2+ or 3+ to 4+. The necessity for adjusting pH after column passage was avoided in Exp. 6 by use of normal saline. In this case fractions 1 through 8 were ready for use immediately upon emergence from the column.

Volumes of conjugate from 4 to 20 ml were successfully processed through appropriate Sephadex columns. Fluorescent staining activity of the column fractions of each conjugate corresponded to their respective protein content, and compared favorably with that of dialyzed conjugates. Globulins prepared by either ammonium sulfate precipitation or the Ethodin-acetone method gave equally satis-

factory results, the latter procedure often resulting in a saving of time by elimination of prolonged dialysis required of sulfate globulin. Although complete removal of Ethodin from the extracted globulin proved difficult, requiring repeated acetone precipitation, residual Ethodin following a single acetone precipitation did not interfere with conjugation and was readily removed during passage of conjugate through the Sephadex column. Regeneration of the column in this case, however, required flushing with weak hydrochloric acid solution to elute the Ethodin. Removal of sulfate from globulin by Sephadex passage instead of dialysis, as reported by Porath and Flodin(8), resulted in considerable saving of time, with protein recoveries of 92 to 99%. However, some sulfate appeared in the final small fractions of protein.

Ferret anti-parainfluenza Ethodin-globulin conjugate, processed through Sephadex, stained tissue cultures as intensely and as specifically as a routinely dialyzed product but both demonstrated nonspecific staining of host cells. Most of this nonspecific reaction was removed by a single acetone precipitation of the Sephadex eluate. Quantitative studies on the bacterial conjugates were not undertaken, the column here being applied as a preparative rather than analytic tool. The entire first yellow band, minus a small discarded portion at each end, was collected as a single fraction, which was employed successfully in specific staining.

Discussion. The Sephadex column is characterized by extreme simplicity of operation. Samples are fractionated on the basis of molecular weight, and continuous passage of either distilled water or a salt solution serves both to elute and to regenerate the column. Denaturation and loss of protein are minimized and peptides or other protein fragments that may contribute to nonspecific staining are eliminated. With normal saline as eluant the product emerges at precisely the desired pH although the conjugate when admitted is at pH 8.5-9.0. Exposure of the protein to this destructive alkalinity is thus minimized. The desired eluate fraction can be sharply separated since it is readily detected by its yellow color, which is followed by a colorless band preceding the unconjugated dye. If the 1:2 dilution of active material inherent in the column must be compensated, this can be done either by the use of more concentrated globulin in the conjugation reaction mixture or by subsequent concentration with Sephadex in a batch process(9). The latter method is reported to effect concentration of high molecular weight solutes while preserving unaltered the ionic strength and pH of the solution. Another possible benefit, when sterility of the product is important, is the fact as reported by Bill *et al.*(10) that the gel may be sterilized by boiling or by autoclaving at 110°C.

Use of Ethodin for fractionation of serum, with subsequent elimination of the reagent during passage of the conjugate through the Sephadex column, results in a purer gamma-globulin product as well as additional saving of time. Recently fluorochromes dispersed in Celite, a diatomaceous powder, have been put on the market (California Corp. for Biochemical Research, Los Angeles). It is claimed that use of Celite reduces conjugation time to 5 minutes. We have not tested the product, but if this is so then the entire procedure from collection of serum to elution of 20 ml of finished conjugate from Sephadex need take no longer than 3 hours.

As this report was being prepared, an article(11) appeared in which the use of Sephadex G-50 for filtration of sheep anti-rabbit globulin conjugate is mentioned. A dialyzed sulfate globulin coupled with fluorescein isothiocyanate was processed in a column equilibrated with buffered saline solution at pH 7.2. Protein recovery was 90% or more, contained in a volume 2 to 3 times the original. Staining of tissue impression films was good, but neither Sephadex treatment nor DEAE fractionation eliminated nonspecific staining of tissue cells.

Summary. "Gel filtration" through a Sephadex column permits a significantly simpler and more rapid purification of fluorescent conjugates than either anion-exchange or electrophoresis. Dialysis was eliminated and unconjugated dye as well as other low molecular weight fractions were separated with minimum loss of protein or titer. De-

tailed data on purification of some anti-fungal conjugates are presented. Ethodin extraction of serum antibody preparatory to conjugation and gel filtration further expedites the process.

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Effect of Vitamin B_6 And Magnesium on Renal Deposition of Calcium Oxalate Induced by Ethylene Glycol Administration.* (27115)

S. N. GERSHOFF AND S. B. ANDRUS (Introduced by F. J. Stare)

Department of Nutrition, Harvard School of Public Health and Department of Pathology, Harvard Medical School, Boston, Mass.

It has recently been estimated(1) that 40-60 deaths occur each year in the United States from ingestion of ethylene glycol, commonly used as anti-freeze and an industrial solvent. Acute ethylene glycol toxicity is characterized by central nervous system depression, hemolysis and renal tubular necrosis. In chronic ethylene glycol poisoning kidney damage is caused by deposition of calcium oxalate since ingested ethylene glycol is partially oxidized to oxalic acid. Recent studies from this laboratory(2,3,4) have associated the endogenous production of oxalic acid accompanied by renal calcium oxalate deposition with the state of Vit. B_6 nutriture. Diets high in magnesium have been found to protect against deposition of calcium oxalate in the kidneys of Vit. B_6 deficient rats even though urinary oxalate levels were not decreased(5). In the present study the effect of Vit. B_6 and magnesium on the metabolism of ethylene glycol and renal deposition of calcium oxalate derived from ingested ethylene glycol have been studied.

Materials. In these experiments male Charles River rats were used. The basal diet fed contained (expressed as %): casein 15, sucrose 74.45, glycine 3, Jones and Foster Salts(6) minus its magnesium and calcium salts 2.25, corn oil 4, cod liver oil 1, and choline 0.3. Each 100 g of diet was supplemented with the following (in mg): thiamine 0.4, riboflavin 0.8, niacin 4, Ca pantothenate 2, folic acid 0.1, menadione 0.1, biotin 0.02, and Vit. B_{12} 0.005. Pyridoxine HCl, MgO, and $CaCO_3$ were added to the basal diets as required.

Anatomic study—methods. In this experiment 4 groups of 6 weanling rats were fed *ad libitum* the basal diet supplemented with 1.5 g $CaCO_3$ per 100 g. Two-tenths or 20 mg of pyridoxine HCl and 40 or 400 mg of Mg as MgO were also added per 100 g of diet. Water was provided by a 0.25% ethyl-

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