Gel-Filtered Media for Purification of Extracellular Toxins.* (30017)

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The procedure described has facilitated production of several extracellular toxins. Media were fractionated on Sephadex or acrylamide gel columns, with subsequent culture of organisms on the low-molecular-weight media fractions. After removal of organisms, the media and small metabolic products were cleared from bacterial materials of high molecular weight (staphylocoagulase, diphtheria toxin, and streptococcal erythrogenic toxin) by passage of the culture through the same columns. In some instances chromatographic separation among the high-molecular-weight products was achieved simultaneously. Gel-filtered media can be prepared more rapidly than media dialyzates and more economically than the more complex chemically defined media.

Materials and methods. Columns. Sephadex columns (Pharmacia G-100 or G-75; 6 × 30 cm) and acrylamide gel columns (Bio-Rad P-100; 6 × 30 cm), preserved in 2% n-butanol or 1:10,000 Merthiolate, were washed exhaustively with 0.15 N NaCl prior to fractionation of media and subsequent separation of media from bacterial products. The gel columns were supported on glass wool and cotton and were operated at 3°C.

Media. Difco brain-heart infusion broth, Mueller's formula with casein hydrolyzate (1), and a modified peptone broth(2) were selected for production of staphylocoagulase, diphtheria toxin, and streptococcal erythrogenic toxin, respectively. These media were prepared in 5 times the conventional concentrations. During fractionation on the above columns the low-molecular-weight media fractions are diluted about 5-fold.

Organisms. Staphylocoagulase was produced by the Tager strain T104(3), diphtheria toxin by Park-Williams No. 8 strain 42112, and streptococcal erythrogenic toxin by Dochez N.Y. 5 strain 165(4).

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Analyses. Staphylocoagulase was quantitated according to Tager with citrated rabbit plasma as substrate and 2% peptone-saline as diluent(5). Diphtheria toxin was measured by flocculation tests(6) and by subcutaneous MLD in guinea pigs. Streptococcal erythrogenic toxin was determined by intradermal injection of varying saline dilutions in rabbits(4).

Nitrogen was determined by a micro-Kjeldahl technic. Ninhydrin reactions were according to Stein and Moore(7). Optical densities of column fractions were measured in a Beckman Model DU spectrophotometer at 280 mμ, or, in some instances, by continuous flow analysis with a Gilson Model UV-280IF flow analyzer.

Staphylocoagulase preparation. Portions (100 ml each) of concentrated brain-heart infusion broth were applied to the Sephadex G-100 columns and developed with 0.15 N NaCl. The high-molecular-weight fractions (volume effluent) were discarded. The lower molecular-weight fractions, approximating 500 ml each, were pooled and 1 ml of a trace element solution(5) was added per liter. This gel-filtered medium was autoclaved, 300 ml per 2-liter Roux bottle. It was inoculated with 1 ml of the second overnight culture of *Staphylococcus aureus* growing in 10 ml of the same gel-filtered medium. Coagulase production was maximum after 3 days at 37°C. Merthiolate was added to 1:10,000 and the organisms were removed by filtration through diatomaceous earth.

Samples (100 ml each) of the above filtrates were applied to the identical Sephadex G-100 columns. Columns were then developed with 0.15 N NaCl with automatic fraction collection of effluents. Fractions were analyzed for coagulase, absorbance at 280 mμ, and for reactivity with ninhydrin (Fig. 1). Recoveries among the fractions were compared with the amounts placed on the columns. Ten ml of culture were also chro-

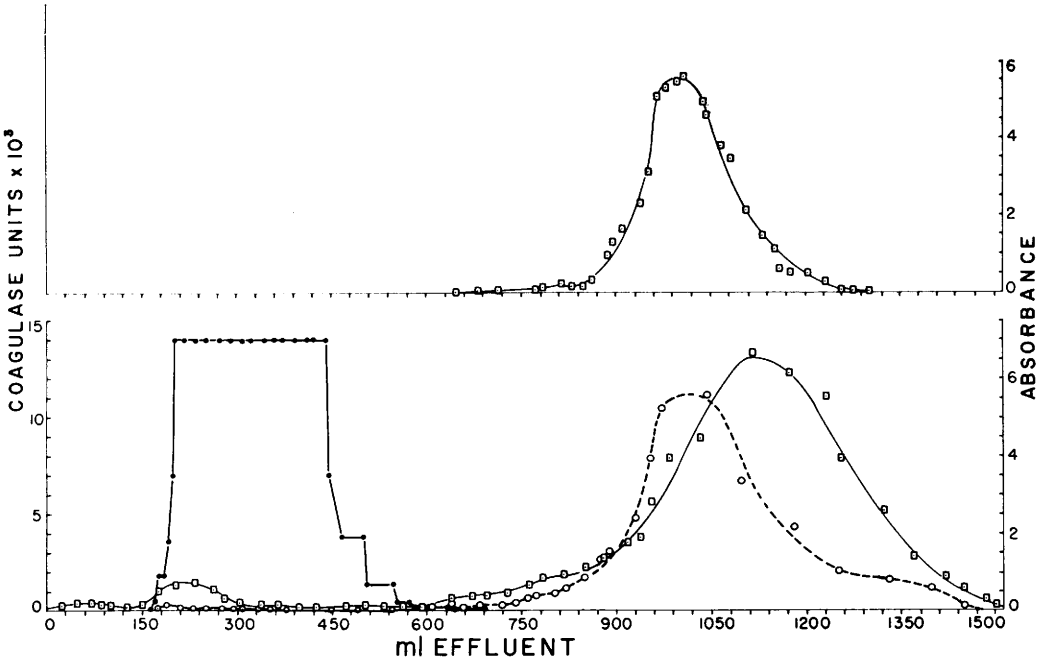

FIG. 1. Chromatography on Sephadex G-100 of the low-molecular-weight fraction of gel-filtered infusion broth. *Upper.* Before inoculation. *Lower.* Following culture of coagulase-positive *Staphylococcus aureus.* ●——● staphylocoagulase; □——□ absorbance at 280 mμ; ○- - -○ ninhydrin color.

matographed on a 2- × 120-cm column of Sephadex G-100.

One hundred ml of the uninoculated but previously gel-filtered medium were chromatographed on the identical Sephadex G-100 columns to prove absence of high-molecular-weight materials from the chromatographic region of the coagulase (Fig. 1).

Diphtheria toxin preparations. Samples (100 ml each) of the concentrated medium for diphtheria toxin production were fractionated on the identical Sephadex G-100 columns exactly as described in the staphylocoagulase section above. The high-molecular-weight material was discarded. The low-molecular-weight material, approximating 500 ml, was distributed, 200 ml per 2-liter Roux bottle, and autoclaved. Supplemental sterile glucose was added to a final 0.05% concentration in each bottle. The bottles were inoculated with a loopful of the second overnight culture of *Corynebacterium diphtheriae* in the same gel-filtered medium. Cultures were incubated without agitation for 6 days at 37°C. Pellicles were removed by filtration through cotton and residual organisms by a D-8 Hormann asbestos pad. Merthiolate was added to 1:10,000. The toxin content was measured by flocculation test and in guinea pigs.

Portions (100 ml each) of the cultures were chromatographed on the identical Sephadex G-100 columns used for preliminary fractionation of the medium and under identical conditions described for staphylocoagulase fractionation. Effluent fractions were measured by flocculation test and for absorbance at 280 mμ (Fig. 2c).

A 100-ml portion of the uninoculated gel-filtered medium was also analyzed on a column to prove absence of high-molecular-weight material in the toxin-containing area (Fig. 2b).

This method was compared with the conventional one for producing diphtheria toxin by culturing the organism on the unfractionated medium, adjusted to the concentration and glucose content of the above gel-filtered medium. After the toxin content was measured, 100 ml of the conventional culture were passed through the same G-100 columns (Fig. 2a).

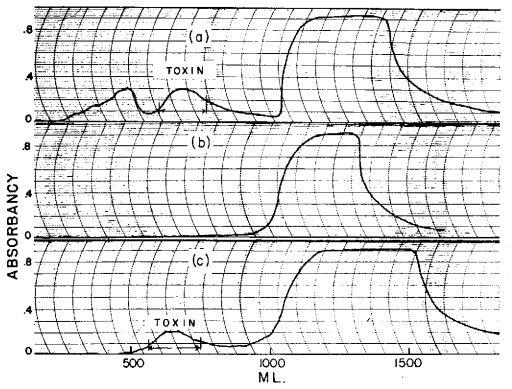

FIG. 2. Toxin location and 280 mμ absorbance among effluents from Sephadex G-100 columns loaded with (a) cell-free culture of *C. diphtheriae* on a conventional medium, (b) uninoculated low-molecular-weight fraction from the gel-filtered conventional medium, (c) cell-free culture of *C. diphtheriae* on (b).

Streptococcal erythrogenic toxin preparation. Portions (100 ml each) of the concentrated peptone broth were fractionated either on 6- × 30-cm columns of acrylamide gel (P-100) or Sephadex G-75. The low-molecular-weight fractions were autoclaved, 250 ml per 500 ml Erlenmeyer flask, followed by addition of glucose to 0.3%, phenol red to 0.075 mg/100 ml, and adjustment of the pH to 7.7. The inoculum consisted of 1 ml of an 8-hour culture of organism in the same gel-filtered medium. During 48 hours at 37°C additional glucose was added in 3 steps to give a final concentration of 2.0% and pH was maintained at 7.2. The culture was filtered through a D-8 Hormann asbestos pad and 100-ml samples of the product were chromatographed both on Sephadex G-100 and G-75. The erythrogenic toxin was located by skin tests in rabbits. Uninoculated media were tested for absence of material in the toxin area by chromatographing 100 ml on the G-100 and G-75 columns.

Results. Column operation. All materials applied to the 6- × 30-cm G-100 columns were completely removed in 8 hours, automatic fraction collection permitting second overnight cycles. Thus, this size of column produced about 1 liter of medium free of high-molecular-weight material per day or fractionated 200 ml of the subsequent toxin-containing culture. Column flow rates were only slightly reduced after 6 months' operation. Sephadex G-75 columns for fractionation of streptococcal media and toxin flowed more slowly, however.

Brain-heart infusion broth, gel-filtered on Sephadex G-100, supported production of staphylocoagulase titers to 1:4,096, the specific activity being 1,400 units per mg nitrogen. On subsequent gel-filtration this coagulase was recovered quantitatively in the medium-free area and partly overlapped a ninhydrin-negative bacterial product absorbing light at 280 mμ (Fig. 1).

Specific activities of the coagulase-containing effluents without *apparent* 280 mμ absorbance were 146,000 units per mg nitrogen. The Sephadex G-100 column (2 × 120 cm) completely resolved the coagulase from 280 mμ-absorbing material in the fractions where overlap occurred. Acrylamide gel electrophoresis of a 2% solution of this coagulase demonstrated 3 bands of protein-like material.

The gel-filtered culture medium was proved free of materials occurring in the region of the coagulase peak by chromatography on the same columns (Fig. 1).

Diphtheria toxin. Gel-filtered casein hydrolyzate medium supported production of diphtheria toxin titers to 60 Lf per ml while conventional, unfractionated casein hydrolyzate supported titers to 80 Lf per ml. From 75 to 90% of the activity was found in one peak after passage of the sterile filtrate through the same column used for fractionation of the medium (Fig. 2c). This toxin contained 1500-2000 Lf per mg total nitrogen.

The gel-filtered casein hydrolyzate medium was free of material behaving chromatographically like the toxin (Fig. 2b). Chromatography of diphtheria toxin produced on the unfractionated casein hydrolyzate revealed a second protein peak not encountered with gel-filtered media (Fig. 2a).

Streptococcal erythrogenic toxin. Erythrogenic toxin, chromatographed on G-100 columns, was eluted just prior to and overlapped the low-molecular-weight fractions that included the culture medium. On G-75 columns this toxin was adequately separated from the medium, indicating preference for the smaller pore-sized gel.

Discussion. The technic of dividing a culture medium into 2 fractions by gel filtration

has proven a workable method for general application. The proper culture medium and pore size of gel are selected, the medium is fractionated on the gel, and either the high- or low-molecular-weight fraction is inoculated depending on the molecular size of the microbial product to be purified. Preliminary gel filtration of the medium guarantees complete removal of those elements of medium that would otherwise contaminate the desired bacterial product during gel passage after culturing. Preliminary gel filtration of conventional media was found less expensive than preparation of complex chemically defined media and much quicker to accomplish than preparation of media dialyzates. Gel-filtered media supported production of the same staphylocoagulase titers as did the unfractionated media and nearly the same titers of diphtheria toxin. Quantitative recovery of staphylocoagulase after culture on the fractionated media was the rule while yields of diphtheria toxin were in the range 75-90% for this labile material.

Complete separation of media elements from the extracellular bacterial products by employing gel-filtered media resulted in diphtheria toxin and staphylocoagulase preparations of high purity. Specific activities of diphtheria toxin, 1500-2000 Lf per mg total nitrogen, are 50-66% of the maximum activities of reported toxin preparations, each of the latter resulting from combinations of fractionation procedures(8-10). Since yields are nearly quantitative, the toxin prepared by gel-filtered media technics would seem attractive starting material for subsequent fractionation. The effects on these preparations of using submerged culture technics with resultant higher titers and specific activities in culture(11) are not known.

Staphylocoagulase of specific activity 146,000 units per mg nitrogen represents a 104-fold purification from the gel-filtered culture medium. This activity is 21% of the highest activity reported by Tager in material prepared by isoelectric precipitation from conventional broth and repeated ammonium sulfate and ethanol fractionations(3). Further purification of this coagulase is under study. The apparent lack of ninhydrin-positive substance and 280 mμ absorbance in coagulase fractions suggests either that coagulase was present in minute amounts or that it contained only small amounts of peptide.

Recently this laboratory has used gel-filtered media for production of both low- and high-molecular-weight products of *Pseudomonas aeruginosa,* by culturing on fractionated (Sephadex G-100) brain-heart infusion broth, and by using the low-molecular-weight broth fraction for production of high-molecular-weight products and the high-molecular-weight broth fraction for production of low-molecular-weight bacterial products. The methods are being extended to purification of extracellular products from other than bacterial cells and to large-scale work. Acrylamide gels were too new to be investigated extensively in this work, but preliminary studies indicate they may serve with the cross linked dextrans for preparation of microbial products by gel-filtered media technics described herein.

Summary. Low-molecular-weight fractions of media obtained by Sephadex- or acrylamide-gel filtration successfully support production of staphylocoagulase, diphtheria toxin, and streptococcal erythrogenic toxin. These microbial products are purified by subsequent removal of the medium and undesired products on the identical gel columns used to prepare the media. Gel-filtered media are more cheaply and easily prepared than are some synthetic media, or medium dialyzates.

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A Selective Medium and Color Test for *Mycoplasma pneumoniae*.* (30018)

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Isolation and propagation of *Mycoplasma pneumoniae* depends upon a heart infusion-peptone base medium enriched with horse serum and yeast extract(1). While this formula permits luxuriant growth of *M. pneumoniae,* other mycoplasma (PPLO) are also supported. Once isolated the identity of *M. pneumoniae* is now established by a hemolytic plaque test(2,3) or serologically with specific rabbit antisera(4-9). This report will demonstrate that methylene blue chloride (MB) is an inhibitor for all mycoplasma species of human origin except *M. pneumoniae.* It will be shown that 2,3,5-triphenyltetrazolium chloride (TTC) can be used in a color test which is easily performed and specific for *M. pneumoniae.* Results from a study which applied MB inhibitor medium and TTC color tests will also be summarized.

Materials and methods. Mycoplasma, media, and cultural techniques. Six mycoplasma species of human origin and one saprophytic species were studied. Human species included *M. fermentans, M. salivarium, M. hominis* type I, *M. hominis* type II, *M. pneumoniae,* and *M. pharyngis*(10), while a type B strain of *M. laidlawii* was the saprophyte.† All were maintained at 36°C in fluid medium with serial transfers every 3-4 days. Growth in liquid cultures, including those containing MB‡ and TTC,§ was initiated from a 10% inoculum derived from 3-4-day-old stock cultures. To insure that inocula were viable when liquid to liquid transfers were made, the inoculum was plated, and then after 3, 6, or 7 days the subculture was plated to estimate growth. Plates for aerobic incubation were sealed with paraffin while anaerobic conditions in glass jars were created with 95% N_2, 5% CO_2 gas as recommended by Barile(11). The medium used was that of Chanock *et al*(1,8) with 3 modifications: horse serum was gamma globulin-free,‖ 2,000 U/ml penicillin were used, and amphotericin B was omitted from both cultures. The MB and TTC were prepared in 1.0% aqueous stock solutions sterilized by autoclave before adding to the medium.

MB procedures. Growth inhibition by MB was measured for each species in agar and broth. In agar the concentration tested ranged from 0.00001% to 0.02%. In broth the range was 0.00002% to 0.1% because resistance of PPLO to the dye was somewhat greater in broth. Plates of MB agar were

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† *M. fermentans, M. salivarium, M. hominis* type I, *M. hominis type* II, and the FH strain of *M. pneumoniae* were provided by Drs. N. L. Somerson and R. M. Chanock, Nat. Inst. Health, Bethesda, Md. *M. pharyngis* was received from Dr. W. A. Clyde, Jr., Univ. of North Carolina, Chapel Hill, who recently described this species. Dr. Ruth G. Wittler, Walter Reed Army Inst. of Research, Washington, D. C. furnished *M. laidlawii* type B.

‡ National Aniline Division, Allied Chemical and Dye Corp., New York.

§ Eastman Organic Chemicals, Distillation Products Industries, Rochester, N. Y.

‖ Agamma horse serum—Hyland Laboratories, Los Angeles, Calif.