Hugli, T. E. (1978) *Adv. Immunol. 26*, 1.
Kolb, W. P., & Müller-Eberhard, H. J. (1975) *J. Exp. Med. 141*, 724.
Kolb, W. P. Haxby, J. A., Arroyave, C. M., & Müller-Eberhard, H. J. (1972) *J. Exp. Med. 135*, 549.
Kolb, W. P., Kolb, L. M., & Podack, E. R. (1979) *J. Immunol. 122*, 2103.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Laura, R., Robison, D. J., & Bing, D. H. (1980) *Biochemistry 19*, 4859.
Lay, W. H., & Nussenzweig, V. (1968) *J. Exp. Med. 128*, 991.
Lepow, I. H., Naff, G. B., Todd, E. W., Pensky, J., & Hinz, C. F. (1963) *J. Exp. Med. 117*, 983.
Loewus, F. A. (1952) *Anal. Chem. 24*, 219.
Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
March, S. C., Parikh, I., & Cuatrecasas, P. (1974) *Anal. Biochem. 60*, 149.
Müller-Eberhard, H. J. (1969) *Annu. Rev. Biochem. 38*, 390.
Müller-Eberhard, H. J. (1975) *Annu. Rev. Biochem. 44*, 697.
Müller-Eberhard, H. J., & Götze, O. (1972) *J. Exp. Med. 135*, 1003.
Müller-Eberhard, H. J., & Schreiber, R. D. (1980) *Adv. Immunol. 29*, 1.
Naff, G. B., & Ratnoff, O. D. (1968) *J. Exp. Med. 128*, 571.
Pangburn, M. K., Schreiber, R. D., & Müller-Eberhard, H. J. (1977) *J. Exp. Med. 146*, 257.
Park, J. T., & Johnson, M. J. (1949) *J. Biol. Chem. 181*, 149.
Pesez, M., & Bartos, J. (1974) in *Clinical and Biochemical Analysis* (Schwartz, M. K., Ed.) Vol. 1, p 439, Marcel Dekker, New York.
Podack, E. R., & Müller-Eberhard, H. J. (1979) *J. Biol. Chem. 254*, 9908.
Podack, E. R., Kolb, W. P., & Müller-Eberhard, H. J. (1976) *J. Immunol. 116*, 263.
Podack, E. R., Kolb, W. P., & Müller-Eberhard, H. J. (1978) *J. Immunol. 120*, 1841.
Podack, E. R., Kolb, W. P., Esser, A. F., & Müller-Eberhard, H. J. (1979) *J. Immunol. 123*, 1071.
Porter, R. R., & Reid, K. B. M. (1978) *Nature (London) 275*, 699.
Riordan, J. F., & Vallee, B. L. (1972) *Methods Enzymol. 25*, 449.
Rosenberg, R. D. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol. 36*, 10.
Tack, B. F., Morris, S. C., & Prahl, J. W. (1979) *Biochemistry 18*, 1490.
Ware, C. F., Wetsel, R. A., & Kolb, W. P. (1981) *Mol. Immunol. 18*, 521.
Wetsel, R. A., Jones, M. A., & Kolb, W. P. (1980) *J. Immunol. Methods 35*, 319.

# Stable, Soluble, Model Immune Complexes Made with a Versatile Multivalent Affinity-Labeling Antigen†

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ABSTRACT: We describe here the synthesis of a family of multivalent affinity-labeling antigens based on the soluble carbohydrate polymer Ficoll. Ficoll was derivatized successively with chloroacetate, ethylenediamine, and glutaric anhydride and finally esterified with 2,4-dinitrophenol. Prior to esterification, the polymer could also be derivatized with tyramine to allow trace iodination and with the monosaccharides galactose or mannose. The numbers of substituent groups could be controlled at several points in the synthesis. The resulting multiple dinitrophenyl esters on a Ficoll or glycosylated Ficoll polymer specifically cross-linked anti-dinitrophenyl antibodies to form covalently cross-linked antigen–antibody complexes. The glycosylated Ficolls were particularly made for studies of the influence of antigen structure on the behavior of immune complexes. The intermediates in the synthesis are suitable for other derivatizations as well. These model immune complexes are stable and soluble, they can be separated by size, and they overcome some of the limitations on the study of complexes imposed by previous techniques of preparing them.

Immune complexes appear to be an important cause of tissue injury in many illnesses. Studying the mechanism of their effects has been limited by several factors, however. First, because the interactions between antigens and antibodies are noncovalent, the manipulations necessary to study them in vitro can alter the antigen–antibody complex. Second, since the antigen in the complex is usually not known, it is not possible to ask precise questions about the properties of the complex. Third, complexes found in the circulation may be pathologenetically irrelevant since they are representative of those very complexes which have not settled in the tissues to cause injury.

Several approaches to overcome these limitations have involved attempts to produce stable (covalently cross-linked) antigen–antibody complexes. Heat-aggregated γ-globulin has been used as a model of antibodies aggregated by antigen (Christian, 1960; Ishizaka et al., 1967; Knutson et al., 1977). Although some properties of such aggregates resemble the properties of antigen–antibody complexes, the aggregation induced by heat imperfectly resembles that induced by antigen in being nonspecific as well as difficult to control. Its failure to provide the proper orientation is demonstrated by the sometimes striking differences between the behavior of heat-aggregated immunoglobulin and immune complexes. Two other nonspecific methods of aggregation have yielded useful observations: cross-linking by dimethyl suberimidate of immunoglobulins in solution (Segal et al., 1977) and by

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dithiobis(succinimidylpropionate) in the presence of an antigen in order to preserve the relation of Fc regions (Wright et al., 1980).

A more specific method has been attempted by using bivalent affinity-labeling reagents. The antibody aggregates formed by these compounds resemble immune complexes in being specific antibodies linked through the antigen-binding regions of their antibodies, and some of their properties have been explored (Segal & Hurwitz, 1976; Plotz et al., 1979). All of the reagents of this class used so far, however, share a common limiting feature: the bridge between antibody molecules is very short. It is reasonable to infer that such tightly strung molecular aggregates resemble only complexes of antibodies crowded together on an antigen.

In order to prepare covalent model immune complexes free of this limitation and to allow modification of the antigen itself for other studies, we have prepared a large multivalent affinity-labeling antigen: Ficoll derivatized with multiple dinitrophenyl (DNP)[1] ester groups. These antigens can form covalent bonds specifically with anti-DNP antibodies, react rapidly under gentle conditions, are stable under storage, are readily synthesized and radiolabeled, and can be modified with various chemical groups without affecting their ability to interact with anti-DNP antibodies. In this paper, we describe the synthesis of this family of reagents and demonstrate their ability to form site-specific, covalently cross-linked, model antigen–antibody complexes.

## Materials and Methods

*Reagents.* Ficoll (Pharmacia, Piscataway, NJ), with an average molecular weight of 400 000 by light scattering (lot 0939), and Ficoll 70, with an average molecular weight of 70 000 (lot 9524), were used without further purification. Gel filtration showed them both to be heterogeneous in size, but in calculations, molecular weights of 400 000 and 70 000 were assumed. In other experiments, we have used repeated gel filtration of Ficoll 70 on Sephadex G200 (Laurent & Granath, 1967) and Sephadex G100 in water to obtain fractions of a more limited size range and fractions of smaller size, which can also be derivatized as described below.

The following reagents were purchased as the best available grade from the sources indicated: Thiophosgene, dimethylformamide, and glutaric anhydride, Aldrich, Milwaukee, WI; carrier-free $Na^{125}I$ and $Na^{131}I$, Amersham, Arlington Heights, IL; methanol and chloroform, Baker, Phillipsburg, PA; ethylenediamine dihydrochloride, dinitrophenol, and sodium methoxide (0.5 M) in methanol, Eastman, Rochester, NY; monochloroacetic acid, acetic anhydride, and mercuric acetate, Fisher, Fairlaw, NJ; *p*-aminophenyl β-D-mannopyranoside and tyramine, Calbiochem-Behring, La Jolla, CA; sodium borohydride and sodium borodeuteride, ICN, Irvine, CA; 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) and cyanomethyl thio-β-D-galactopyranoside, Sigma, St. Louis, MO. Dimethylformamide was freed of amines as completely as possible by passage over ethanol-washed and lyophilized AG-50 W-X8, 50–100 mesh (Bio-Rad, Richmond, CA) (Keim et al., 1973). It appeared that with finer mesh or less cross-linked exchanger a chemical reaction took place, leading to an acetone-like smell in the eluted material. The column-passaged material was twice distilled, discarding about the first and last 10% of the distillate each time. The material was protected from water by storage with molecular seives, type 4A (Fisher), in tightly stoppered glass bottles. These precautions appeared necessary since esterification (see below) did not occur when lots of the reagent less rigorously treated were used.

*Synthesis of Derivatives of Ficoll.* Synthesis of DNP esters of Ficoll was carried out by limited carboxymethylation with chloroacetate, exhaustive aminoethylation with ethylenediamine, glutarylation with glutaric anhydride, and esterification with dinitrophenol (Figure 1). Limited tyramination was carried out on the carboxymethyl derivative and limited glycosylation on the aminoethyl derivative. Iodination was carried out either after aminoethylation (mannose derivatives) or just prior to esterification.

Carboxymethyl-Ficoll (step A) was prepared from Ficoll 70 (F70) and Ficoll 400 (F400) by using monochloroacetic acid as described (Inman, 1975). For the F70 preparation used in these experiments, 2 g of F70 was dissolved in 28 mL of 1.35 M chloroacetic acid. A volume of 5.4 mL of 10 N NaOH and 2 mL of $H_2O$ were added, and the reaction was allowed to proceed for 4 h at 40 °C. The reaction was terminated by the addition of 1.5 mL of 2 M $NaH_2PO_4$ and neutralization with 5.5 N HCl. The CM-F70 was dialyzed exhaustively against water in boiled dialysis tubing and lyophilized. It contained 51 mol of carboxyl groups per mol of F70 as determined by exhaustive dinitrophenylation of the aminoethylated derivative.

Tyramines for iodination were introduced by a carbodiimide coupling of tyramine to the carboxymethyl derivative (step B). CM-F70 (1 g) was dissolved in 75 mL of DMF and mixed with 12 mg of tyramine in 10 mL of DMF. Tyramine was coupled by addition of 625 mg of EDC in 30 mL of DMF and stirring at room temperature for 4 h. The reaction mixture was diluted with an equal volume of $H_2O$ and dialyzed exhaustively against water in the cold. Approximately 90% of the tyramine coupled under these conditions, leading to a product with about five tyramines per molecule of CM70 as determined by the absorbance at 275 nm, using $E_{1M} = 1405$ for the bound tyramine.

The tyraminated carboxymethyl-Ficoll 70 (CM-F70–tyr) was aminoethylated as described by Inman (1975) (step C). CM-F70–tyr (1 g) was dissolved in 50 mL of water and mixed with 18.6 g of ethylenediamine dihydrochloride; the pH was adjusted to 4.7 with dilute NaOH. EDC (1.34 g) was added with stirring, and the pH was kept at 4.7 with dilute HCl. After 3 h, the reaction mixture was dialyzed in the cold against several changes of water and lyophilized. Derivatization of Ficoll 400 was identical up to this point except that the tyramine step was bypassed.

Iodination (step E) was carried out at this stage on a portion of the material destined to be derivatized with mannose since it proved difficult to iodinate it at a later stage. $Na^{125}I$ (100 mCi/mL), at 0.2 mCi/mg CM-F70–tyr, was substituted by a modification of the iodine monochloride method as described (Helmkamp et al., 1960; Plotz et al., 1979). The iodinated material was dialyzed against 0.16 M sodium chloride–0.2 M sodium borate, pH 8.0 (BBS). A small amount of AG 1-X8 resin was added to the dialysate to trap free iodide. When iodination was carried out at other stages, the procedure was identical. Material iodinated in this fashion had a specific

[1] Abbreviations: DNP, 2,4-dinitrophenyl; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; BBS, 0.16 M sodium chloride–0.2 M sodium borate, pH 8.0; DMF, dimethylformamide; F70, Ficoll 70; F400, Ficoll 400; CM-F70, carboxymethyl-F70; AECM-F70, aminoethyl-CM-F70; GAECM-F70, glutarylated AECM-F70; GAECM-F70–tyr, GAECM-F70 coupled with tyramine; DNP-GAECM-F70, GAECM-F70 esterified with DNP; DNP-GAECM-F70–Gal, DNP-GAECM-F70 coupled with galactose; DNP-GAECM-F70–Man, DNP-GAECM-F70 coupled with mannose; EDTA, ethylenediaminetetraacetic acid; NaDodSO$_4$, sodium dodecyl sulfate; TNP, trinitrophenyl.

activity of about $3.5 \times 10^7$ cpm/mg.

Addition of galactose (step F) was carried out along the general lines described by Lee (Lee et al., 1976). For the Ficoll 70 described here, 22.4 mg of cyanomethyl 1-thio-β-D-galactopyranoside (0.095 mmol) was dissolved in 907 μL of absolute methanol, and 48 μL of 0.5 M sodium methoxide in methanol was added. After 48 h of stirring at room temperature in a tightly capped glass vial, the methanol was evaporated with a stream of nitrogen. To the dried residue was added 100 mg of AECM-F70–tyr in 10 mL of BBS. This was stirred at room temperature for 24 h when the reaction was stopped by adding 1 mL of 1 N acetic acid. The reaction mixture was dialyzed exhaustively against distilled water.

Addition of mannose (step G) was carried out along the general lines described by Smith (Smith et al., 1978). For the mannose derivative prepared here, 8.3 mg of *p*-aminophenyl α-D-mannopyranoside (0.03 mmol) was dissolved in 2 mL of 0.1 M sodium bicarbonate. Thiophosgene (6 μL) was dissolved in 2.5 mL of chloroform in a glass scintillation vial. The aqueous solution was added to the scintillation vial, and the resulting two-phase system was stirred vigorously in the tightly capped vial at room temperature for 1 h. The reaction mixture was transferred to a glass-stoppered conical 12-mL glass test tube, and the phases were allowed to separate. The lower (chloroform) layer was removed with a Pasteur pipet and discarded. The aqueous phase was twice extracted with 2 mL of chloroform. The aqueous phase was then bubbled with nitrogen to remove residual chloroform and was added to 20 mg of AECM-F70–tyr dissolved in 2 mL of 0.3 M sodium chloride–0.1 M sodium bicarbonate, pH 9.5. After 21 h of being mixed at room temperature, the reaction mixture was dialyzed exhaustively against phosphate-buffered saline, pH 7.4, followed by distilled water. For a galactose derivative of Ficoll 400, 30.5 mg of AECM-F400 was reacted with the imidate formed from 9.65 mg of cyanomethyl 1-thio-β-D-galactopyranoside in the presence of sodium methoxide as described above.

Glutarylation (step D) was carried out on AECM-F, AECM-F–tyr, AECM-F–[$^{125}$I]tyr, and monosaccharide derivatives of all those molecules in the same fashion. The polymer was dissolved in water (or was available as a dialyzed solution after the previous step) at about 10–20 mg/mL. Glutaric anhydride was added in excess, never less than 3 mg/mL but usually at 9 mg/mL, in several steps over 5 min with stirring at room temperature. The pH was continually adjusted with 0.1 N NaOH to keep the pH at 9, but it was not possible to avoid substantial swings just after each addition of solid. Nevertheless, glutarylation proceeded to completion as judged by the disappearance of amino groups available to bind trinitrobenzenesulfonate. The resulting GAECM-Ficolls were dialyzed against water and then lyophilized and stored in a desiccator at room temperature.

Esterification (step H) with 2,4-dinitrophenol (and, in earlier experiments, with 4-nitrophenol) proved difficult until the extreme lability of the ester bonds to hydrolysis was recognized. Thereafter, great care was taken to exclude water and to prevent water condensation in all handling. The lyophilized GAECM-Ficolls were dissolved in dry, amine-free DMF (see above) at 10 mg/mL in a small, dry glass beaker. In a usual preparation, 20 mg of a GAECM-F would be esterified. Dinitrophenol (8.5 mg) was added, followed by EDC (8.8 mg). The EDC, though stored at −20 °C, was allowed to reach room temperature before it was removed from the desiccator for weighing in order to prevent condensation. The beaker was capped with parafilm and covered with aluminum foil, and the mixture was stirred magnetically at room temperature for 2 h. All subsequent operations were carried out at 4 °C. The reaction mixture was applied, using a precooled Pasteur pipet, to a 1.25 × 51 cm glass column of Sephadex LH-20 (Pharmacia) equilibrated with DMF. Because of DMF's properties as a solvent, it proved important to minimize contact with plastics. Polyethylene tubing (Clay-Adams, Parsippany, NJ), Pharmaceal K75 three-way stopcocks (Pharmaseal, Toa Alta, Puerto Rico), and very short stretches (<1 cm) of silicon rubber tubing (LKB, Bromma, Sweden) were used for connections between the reservior for DMF (from which the moisture was excluded with a trap containing Drierite) (W. J. Hammond, Xenia, OH) and between the column and the fraction collector. Flow was controlled by gravity and a no. 27 stainless-steel needle (Becton-Dickinson, Parsippany, NJ). Fractions of 40 drops were collected at about 2.5–3-min intervals. Aliquots (10 μL) of each fraction were tested for carbohydrate and for optical density at 360 nm released after the addition of 0.1 N $NH_4OH$. When esterification was successful, a carbohydrate-containing peak, which was faintly yellow, emerged clearly separated from the unreacted dinitrophenol. Upon addition of the alkali, a strong yellow peak coinciding with the carbohydrate was brought out. The tubes from the peak, usually five tubes, were pooled, and the material was distributed into precooled 0.4- or 1.5-mL microfuge tubes (Beckman, Palo Alto, CA) and carefully capped. The microfuge tubes were stored at −80 °C until immediately prior to use.

Stable DNP-Ficolls (step I) were made by reacting 2,4-dinitrobenzenesulfonate or 2,4-dinitrofluorobenzene with AECM-Ficoll, with or without tyramine and monosaccharides, as described (Inman, 1975).

*Analytical Methods.* Carbohydrates were determined by the phenol–sulfuric acid method scaled down for quantities of 5–100 μg. When Ficoll or a glycosylated derivative of Ficoll was assayed for carbohydrate, a standard solution of Ficoll 70 was used. When the carbohydrate analysis was done on a glycosylated derivative, a correction for the contribution of the monosaccharide was not usually made since it rarely exceeded 10% by weight.

Galactose bound to Ficoll was determined by gas–liquid chromatography of the sugar liberated from the galactose derivative as described (Krantz & Lee, 1976). An approximately 10-mg portion of GAECM-F70–tyr–Gal or AECM-F70 was weighed accurately and dissolved in water. Xylose (50 μg) from an analytically prepared standard solution was added, followed by 900 μL of water, 100 μL of 1 M acetic acid, and 63.7 mg of mercuric acetate. After 2 h at 55 °C in a water bath, the reaction mixture was applied to 5 mL of AG-50 XW in a plastic disposable pipet, and the column was eluted with 5 mL of water. The eluate was brought to pH 8.0 with dilute sodium hydroxide, and 15 mg of sodium borohydride was added. After 90 min at room temperature, glacial acetic acid was added dropwise until bubbling stopped. The resulting solution was evaporated to dryness on a rotary evaporator. Absolute methanol (5 mL) was added and the evaporation repeated twice. The residue was dissolved in 6 mL of pyridine–acetic anhydride (2:1) and heated in a boiling water bath for 30 min. After being cooled in an ice bath, 4 mL of absolute methanol was added. When this reaction mixture had cooled, it was evaporated and reevaporated 3 times after the addition of toluene. The residue as dissolved in 1 mL of chloroform and 5 mL of water. The chlorform layer was reextracted twice with water. Gas–liquid chromatography was kindly performed on the chloroform layer by Drs. David Zopf and Bo Nilsson

of the Division of Pathology of the National Cancer Institute. A Perkin-Elmer 900 gas chromatography equipped with a flame ionization detector was used for gas–liquid chromatography. Separation was done on a glass column (1 m × 2 mm) packed with SP-2340 100/120 at 190–240 °C. Gas–liquid chromatography/mass spectroscopy was carried out on a Hewlett-Packard 5992A quadrupole instrument fitted with a glass column (1 m × 2 mm) packed with SP-2340. The spectra were recorded at 70 eV and an ion source temperature at 130 °C. The amount of galactose was determined from the ratio of galactose to xylose, and the molar ratio of galactose to Ficoll was calculated from a carbohydrate analysis of the sample.

For determination of the amount of mannose coupled to Ficoll, 500 μL of a solution of GAECM-F400–Man or of GAECM-F400 containing approximately 1 mg of Ficoll was mixed with 40 μg of xylose from a standard solution. The mixture was dried by vacuum on a rotary evaporator, and 2 mL of a solution of 9 parts 88% formic acid and 1 part water was added. The vessel was tightly stoppered and then heated at 100 °C for 5 h in a heating block before the solution was evaporated to dryness. Evaporation was repeated 3 times after the addition of 2 mL of methanol. The residue was dissolved in 1 mL of 0.25 N sulfuric acid and transferred to a serum vial, and another 1 mL of 0.25 N sulfuric acid was used to wash over sample to the vial. The vial was stoppered and sealed and then heated for 18 h at 100 °C. After the vessel was cooled, a few grams of AG-3 X-4A (Bio-Rad), hydroxyl form, were added, and then the gel was removed by passing the slurry over glass wool. The solution was then reduced with sodium borodeuteride and acetylated as described above. Neither galactose nor mannose was found on unglycosylated Ficoll.

The extent of dinitrophenylation was determined by liberating esterified dinitrophenol with 0.1 M ammonium hydroxide and measuring the absorbance at 360 nm, using an extinction coefficient of 13 250 for 2,4-DNP in this buffer.

*Antibodies.* Mouse anti-DNP antibody was raised in ascitic fluid (Tung et al., 1976). Dinitrophenyl conjugated keyhole limplet hemocyanin (1.1 mL) at 4.5 mg/mL was emulsified with 10 mL of complete Freund's adjuvant (Difco, Detroit, MI). Three-month-old male A/Jax mice were injected intraperitoneally with 0.2 mL of this mixture on days 1, 14, 21, and 28. Ascitic fluid was removed with a 19-gauge needle before the boost on days 21 and 28. The ascites from several mice were pooled, centrifuged at high speed to remove the fibrin clot, and stored at −20 °C until further use.

Purification of mouse anti-DNP antibody was achieved by applying 20 mL of ascitic fluid, diluted with an equal volume of 0.01 M sodium acetate–0.15 M NaCl–0.01 M EDTA, pH 6.0, to a 40-mL column of TNP-L-lysine–Sepharose 4B equilibrated with the same acetate buffer, pH 6.0. Antibody was eluted with 20 mL of 0.01 M 2,4-DNP-glycine, pH 8.0. The eluted protein was passed over a 3-mL column of Dowex 1x-8 and dialyzed against BBS. After concentration in a collodion bag (Schleicher & Schuell), the antibody solution was applied to paired columns of AcA22 and AcA34 as described (Segal & Hurwitz, 1976). The material eluted in the region expected for IgG was pooled and concentrated. Mouse γ-globulin (Miles, Kankakee, IL) was passed over the TNP-Sepharose column prior to use. Protein solutions were radiolabeled with carrier-free $Na^{125}I$ as previously described (Plotz et al., 1979).

*Preparation of Model Immune Complexes.* Specifically cross-linked immune complexes were prepared by mixing radiolabeled purified mouse anti-DNP in BBS (pH 8) at a concentration of 2–2.5 mg/mL with the desired cross-linking antigen in 20% of the reaction volume. After the mixture was incubated in an ice bath for 5 min, the reaction was terminated by adding 0.1 volume of 0.2 M ethanolamine. The solution of cross-linked immune complexes and monomer IgG was assayed by $NaDodSO_4$–polyacrylamide gel electrophoresis or fractionated by gel filtration on sequential (1.6 × 100 cm) columns of AcA-22 and AcA-34 equilibrated with BBS at room temperature.

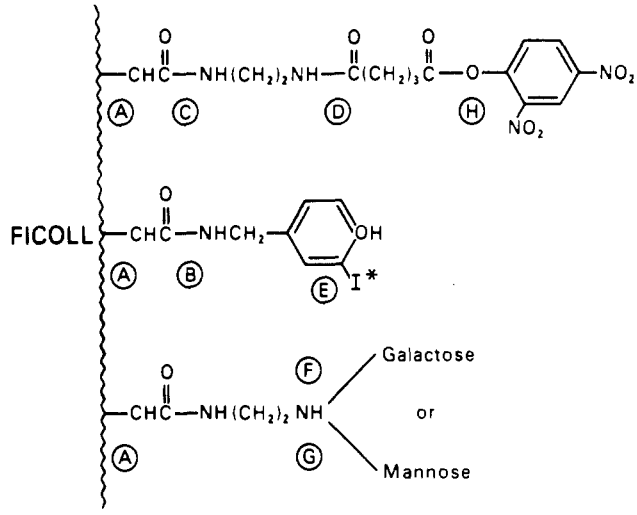


FIGURE 1: Scheme for the synthesis of derivatized Ficolls.

*Polyacrylamide Gel Electrophoresis.* Polyacrylamide gradient slab gels (PAA 2/16, Pharmacia) were equilibrated with $NaDodSO_4$ buffer (0.04 M Tris, 0.02 M EDTA, and 0.2% w/v $NaDodSO_4$, pH 7.4) at 70 V for 1 h in a vertical electrophoresis apparatus (Pharmacia). Samples were mixed with a denaturing solution, 0.1 M Tris and 0.001 M EDTA in 20% (v/v) glycerol containing 2% (w/v) $NaDodSO_4$ and 0.1 mg/mL bromophenol blue, pH 7.5, and boiled for 2 min. Aliquots (10 μL) were loaded onto the gel, and electrophoresis was carried out for 15–20 min at 70 V and 3–16 h at 100 V. Gels were stained for at least 8 h in 0.05% Coomassie blue in 10% acetic acid–25% isopropyl alcohol and then destained in Gel Destainer 4 (Pharmacia) with 8% acetic acid. Destained gels were washed 1–2 h in 10% glycerol solution prior to drying on filter paper backing (Bio-Rad) using Slab Gel Dryer 224 (Bio-Rad) with high vacuum. Dried gels with radioactivity were marked and sliced into 2-mm pieces, and radioactivity was determined.

## Results

*Synthesis (Figure 1). Step A.* It proved most convenient to limit the extent of substitution on the Ficoll by controlling the first step of synthesis as described (Inman, 1975). This is easily done by adjusting the time and/or temperature of the reaction between Ficoll and chloroacetate. With this approach, subsequent additions to the bridge (step C, aminoethylation; step D, glutarylation; and steps H and I, addition of DNP) are carried out under conditions favoring complete derivatization. We have made derivatives with a wide range of substitution. The preparation described here was chosen to allow an adequate number of each of the other groups—tyramine, galactose, and mannose—to be added to the bridge while still leaving groups free for esterification with DNP. Ficoll 70, assuming a molecular weight of 70 000, has about 200 sucrose or 400 glucose units per molecule. Thus, about 12% of the glucoses in $CM_{51}$-F70 have a carboxymethyl stump.

*Step B.* Tyramine addition proceeded poorly or not at all in aqueous media by several approaches but efficiently in DMF. As judged by the absorbance at 275 nm of the product, which probably overestimates the extent of derivatization, essentially all added tyramine coupled to CM-F within an hour provided a large excess of carboxymethyl groups and the carbodiimide reagent were present.

*Step C.* Aminoethylation was carried out in a great excess of ethylenediamine in order to assure that most or all free carboxyl groups were aminoethylated and that the diamine would couple monogamously rather than cross-link carboxyls of the same or another molecule. Under these conditions, close to 90% of carboxymethyl groups will be derivatized (Inman, 1975). The extent of aminoethylation was monitored by exhaustive dinitrophenylation with 2,4-dinitrobenzenesulfonate (Inman, 1974) and measurement of the absorbance at 360 nm.

*Step D.* The amino groups at this stage or those remaining after monosaccharide coupling could be completely converted to carboxyl groups by reacting with excess glutaric anhydride, as demonstrated by the loss of groups able to react with trinitrobenzenesulfonate. Although it proved very difficult to control the pH as portions of the anhydride were added to the reaction mixture, even the crude control achieved by adding sodium hydroxide dropwise allowed the reaction to proceed adequately.

*Step E.* We used a modified iodine monochloride method to iodinate the tyraminated derivatives either before addition of monosaccharides (mannose derivatives), just prior to esterification (galactose derivatives or underivatized Ficoll), or after addition of a stable DNP group. The reason for the failure of iodination of the mannosylated derivative was not examined, but it seems likely that the phenyl isothiocyanate intermediate reacted with the phenolic ring, thus blocking the possibility of further substitution with iodine (see below). The addition of iodine by other standard methods was not tried.

*Steps F and G.* For studies of the influence of antigen structure on immune complex metabolism, we chose to add monosaccharides for which specific receptors are known and for which suitable precursors were available. This meant that galactose and mannose were coupled by different methods. Although this difference might have led to irrelevant influences on the behavior of the complexes, suitable inhibition studies have demonstrated the dominant effect of the monosaccharide group (A. Rifai et al., unpublished experiments).

For the addition of galactose, we used the 2-imino-2-methoxyethyl 1-thioglycoside method (Lee et al., 1976). The activated thioglycoside was prepared from the commercially available cyanomethyl 1-thio-$\beta$-D-galactopyranoside and was used directly after synthesis without purification. We determined the efficiency of substitution as a function of the ratio of cyanomethyl 1-thio-$\beta$-D-galactopyranoside to AECM-F, allowing both the formation of the active intermediate and the coupling to go to virtual completion by using suitably long reaction times (Figure 2). The preparation chosen for study had 10.6 galactose groups per molecule of F70, an adequate number to assure rapid removal by the galactose-binding protein of the hepatocyte.

For the addition of mannose, we used the phenyl isothiocyanate method (McBroom et al., 1978; Smith et al., 1978). In this reaction, the phenyl isothiocyanate is formed from the reaction of thiophosgene with *p*-aminophenyl $\alpha$-D-mannopyranoside. The active intermediate was used without purification after the removal of unreacted thiophosgene. Initially, the degree of mannose coupling was estimated from the absorbance at 280 nm, assuming a molar extinction coefficient

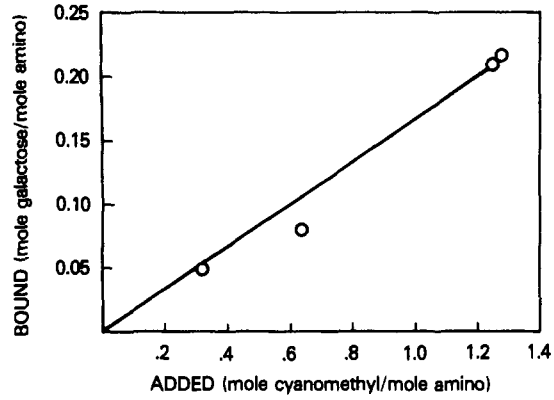


FIGURE 2: Reaction of cyanomethyl 1-thio-$\beta$-D-galactopyranoside with AECM-F70. Results are expressed as moles of the pyranoside added per mole of amino group and moles of galactose liberated from the product per mole of amino group (see Materials and Methods).

Table I: Substituents on Derivatized Ficolls[a]

| preparation | AECM | tyramine | sugar | DNP |
|---|---|---|---|---|
| DNP-GAECM-F70-tyr | 51 | 5 | 0 | 32 |
| DNP-GAECM-F70-tyr-Gal | 51 | 5 | 10.6 | 21 |
| DNP-GAECM-F70-tyr-Man | 51 | 5 | 21 | 31 |

[a] Moles per mole of F70.

of 1350 for the coupled aromatic ring. However, for two preparations for which we had a direct measurement of mannose liberated by acid hydrolysis, the estimate of coupling by absorbance was 1.83- and 2.03-fold higher than that by direct analysis. For the preparation used in these studies, only an absorbance measurement was made; too little material remained after biological studies for direct analysis. When corrected by a factor of 1.9 for the absorbance measurement, there were estimated to be 21 mannose groups per Ficoll. This preparation and several others had more substitutent groups—tyramine, mannose, and DNP—than free amino groups as determined by direct exhaustive dinitrophenylation (Table I). Although small errors in the methods for determining the substitution of each of these groups have contributed to the almost 13% apparent oversubstitution observed for the DNP-F70–Man, another contributing factor may have been the addition of mannose at other sites, for example, to the phenolic ring of the added tyramine. This has not been further investigated.

*Step H.* The final synthetic step, esterification with 2,4-dinitrophenol, proved troublesome until the need for anhydrous conditions and pure (amine-free) dimethylformamide was appreciated. With extensively purified DMF and the rigid exclusion of water during subsequent chromatography, it was possible to achieve a reasonably high degree of esterification of the free carboxyl groups (Table I). Since esterification was carried out at room temperature for 2 h, some of the newly formed ester bonds may have broken down prior to gel filtration in the cold. The fractions eluted from the column were tested for carbohydrate, for the alkali-liberated absorbance at 360 nm, to indicate ester-linked DNP groups, and for radioactivity when radioiodinated polymer was used. Superimposable peaks by these three methods were always found in the exclusion volume of the LH-20 column. Pooled material from this peak was pale yellow before alkalinization. A large, trailing, deep yellow peak of unreacted 2,4-dinitrophenol was not significantly enhanced by alkalinization and contributed negligibly to the alkali-liberated absorbance used to estimate the degree of esterification of the carbohydrate-containing peak.

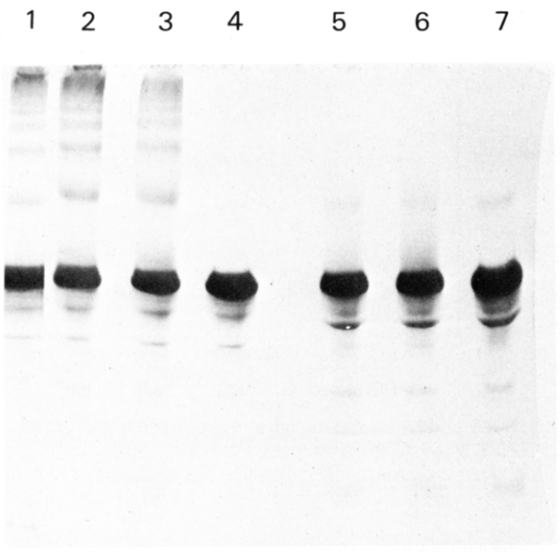


FIGURE 3: Gradient polyacrylamide gel (2–16%) electrophoresis in the presence of sodium dodecyl sulfate of mouse IgG treated with various reagents. Mouse anti-DNP (50 μg/10 μL) treated with (1) 2.9 μg of DNP-GAECM-F70–Man, (2) 3.8 μg of DNP-GAECM-F70, (3) 4.5 μg of DNP-GAECM-F70–Gal, and (4) 2 μL of DMF. Nonimmune mouse γ-globulin (37 μg/10 μL) treated with (5) 3.8 μg of DNP-GAECM-F70, (6) 4.5 μg of DNP-GAECM-F70–Gal, and (7) 2 μL of DMF. IgG was reacted with reagents for 5 min at 4 °C before quenching with 1 μL of ethanolamine. Nonimmune mouse γ-globulin was not cross-linked by DNP-GAECM-F–Man (results not shown).

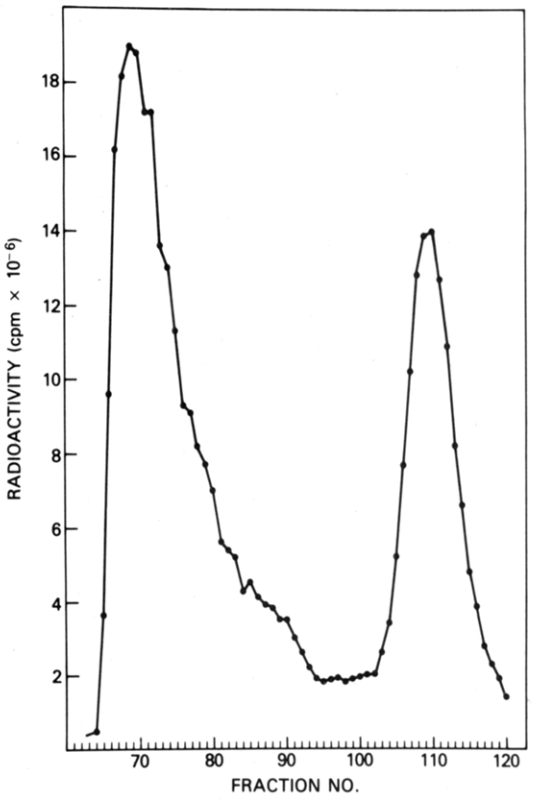


FIGURE 4: Gel chromatographic profile of mouse anti-DNP antibody, 2.5 mg/mL ($1.65 \times 10^5$ cpm/μg), treated with 350 μg of DNP-GAECM-F400 for 5 min; tandem columns (1.6 × 100 cm) of AcA22 and AcA34, equilibrated with BBS (pH 8.0), were used. Fractions (3 mL) were collected at a constant elution rate of 7 mL/h.

When stored in carefully capped plastic vials at −80 °C, the DNP-esterified Ficolls did not lose their ability to cross-link anti-DNP antibodies over several months.

*Step I.* Stable polymers could be made similar to those described (Inman, 1975) by coupling 2,4-dinitrobenzene-sulfonate to AECM-Ficolls with or without 1-tyramine or monosaccharides. These can be stored at room temperature after lyophilization and be radioiodinated later for convenience.

*Cross-Linking of Anti-DNP Antibodies.* Because the DNP ester groups can react nonspecifically with free amino groups, it was necessary to find conditions under which the DNP-Ficoll esters would react negligibly with amino acids outside the antigen-binding region to ensure that the stable IgG oligomers formed were indeed linked by polymer through the binding sites. This is necessary in order that the relationships of Fab and Fc regions of different immunoglobulin molecules in the same oligomer resemble the relationships when antibodies react noncovalently with antigen.

As had been found with bis(DNP) pimelic ester (Plotz et al., 1979), at high concentrations of antibody (greater than 7 mg/mL), some cross-linking of nonspecific mouse γ-globulin occurred even if the γ-globulin solution had been freed of DNP-reactive antibodies by passage over the TNP-Sepharose. At 5 mg/mL or below, however, no significant cross-linking was evident by $NaDodSO_4$–polyacrylamide gel electrophoresis.

In preliminary experiments, it was found that cross-linking occurred over a wide range of Ficoll/antibody ratios. For convenience in later experiments, a volume of antibody solution at 1–5 mg/mL was mixed with 0.1 or 0.2 volume of antigen solution (stored at −80 °C at 2–5 mg/mL in DMF). With F400 derivatives, a molar ratio of 0.05–0.10 (weight ratio, 0.13–0.25) gave excellent cross-linking. The yield was highest at pH 8.0. The reaction took place in a bath of melting ice, and the reaction time was limited to 5 min since nonspecific cross-linking began to occur thereafter. The reaction was quenched by allowing an excess of ethanolamine to react with any remaining DNP ester groups.

Under these conditions, each of the DNP-Ficolls cross-linked anti-DNP antibodies to yield a variety of oligomers, and there was no detectable cross-linking of nonspecific mouse γ-globulin (Figure 3). A small amount of IgG dimer was present in both anti-DNP and γ-globulin solutions before the reaction along with a variety of small molecular weight species which did not contribute to cross-linking (unpublished observations). It is likely that the bands appearing immediately above the monomer immunoglobulin represent two immunoglobulin molecules linked to a single or at most two Ficoll molecules. Because the presence of a large amount of carbohydrate in a molecule introduces considerable uncertainty into molecular weight determination by polyacrylamide gel electrophoresis, it is impossible to identify the species precisely even with markers of known weight (Leach et al., 1980). Furthermore, the Ficolls in these preparations were heterogeneous in molecular weight, contributing additional uncertainty. Studies with Ficolls of restricted size are under way to characterize the species more exactly.

When a reaction mixture of anti-DNP antibodies and DNP-GAECM-F400–tyr was separated by gel filtration on tandem columns of AcA22 and AcA34, a complicated pattern emerged (Figure 4). (IgG and Ficoll can be separated from oligomers satisfactorily with AcA34 alone.) This would be expected from a consideration of the possible molecular species which might be formed when a bivalent antibody reacts with a multivalent antigen. When individual fractions from this column were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 5), it was apparent that although a number of peaks were present there was a dominant species, and this could be purified by repeating the gel filtration. On repeated gel filtration, there was no change in size of the

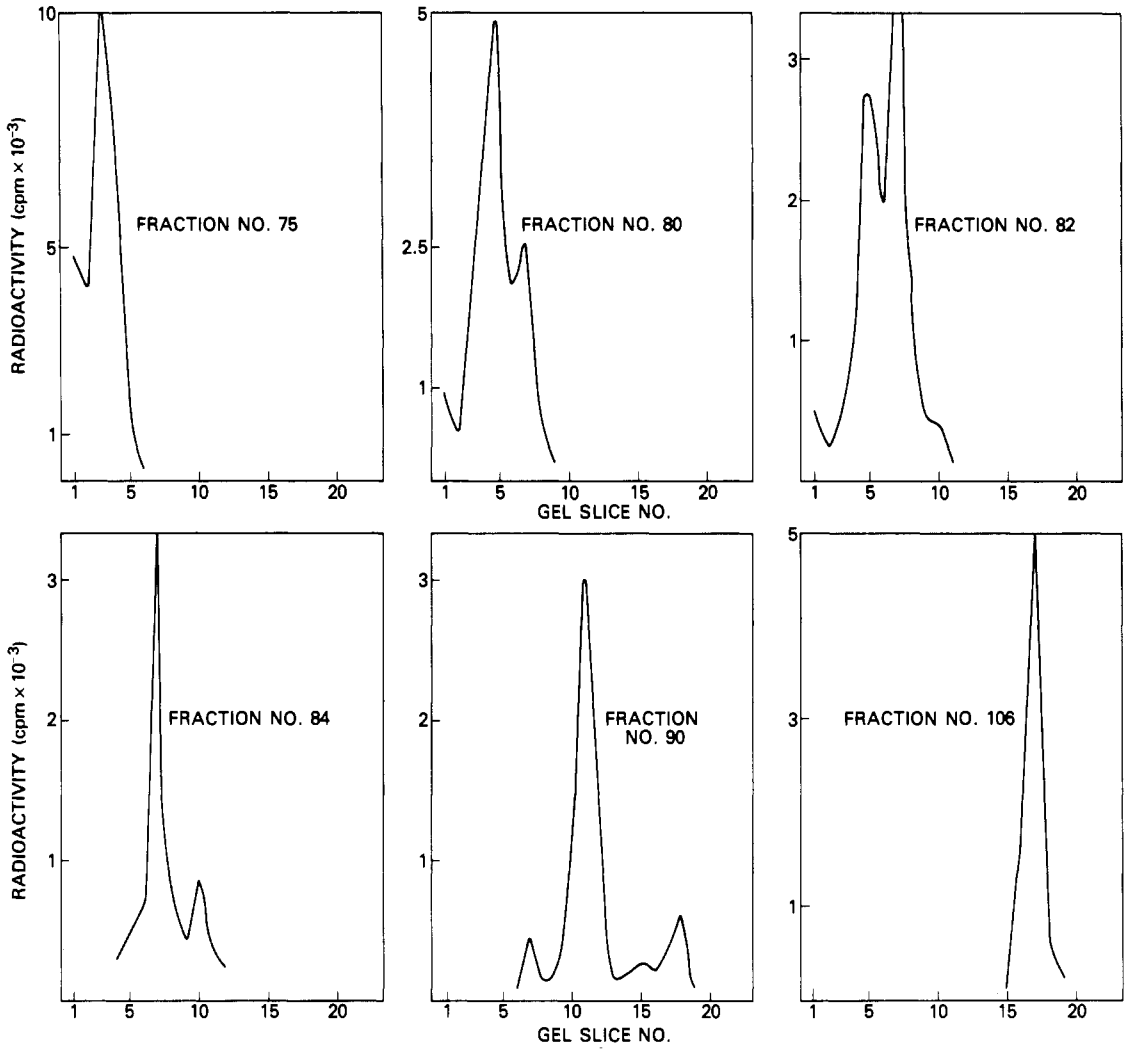


FIGURE 5: Gradient polyacrylamide gel electrophoresis of mouse anti-DNP antibody cross-linked with DNP-GAECM-F400. Samples obtained from gel chromatography fractions (see Figure 4) were analyzed on a 2–16% polyacrylamide slab gel in the presence of sodium dodecyl sulfate. The number on each panel corresponds to the selected fraction. Gels were sliced and counted as described under Materials and Methods.

Table II: Yield of Anti-DNP Oligomers with DNP-F70 and DNP-F400

| preparation | antigen | | | antibody | | molar ratio | | oligomer yield (%) |
|---|---|---|---|---|---|---|---|---|
| | mg | Ficoll[a] | DNP[a] | mg | IgG[a] | Ficoll/IgG | DNP/Fab | |
| $DNP_{32}$-GAECM-F70 | 0.7 | 10 | 320 | 2.0 | 12.5 | 0.80 | 12.8 | 42 |
| $DNP_{21}$-GAECM-F70-Gal | 0.9 | 13 | 273 | 2.3 | 14.3 | 0.91 | 9.5 | 37 |
| $DNP_{31}$-GAECM-F70-Man | 0.6 | 8.6 | 267 | 2.0 | 12.5 | 0.69 | 10.7 | 53 |
| $DNP_{235}$-GAECM-F400 | 0.35 | 0.87 | 205 | 2.5 | 15.6 | 0.056 | 6.6 | 63 |
| $DNP_{215}$-GAECM-F400-Gal | 0.25 | 0.62 | 134 | 2.2 | 13.8 | 0.045 | 4.8 | 35 |
| $DNP_{172}$-GAECM-F400-Man | 0.36 | 0.87 | 150 | 2.2 | 13.8 | 0.063 | 5.4 | 33 |

[a] Millimoles X $10^6$.

oligomers. With repeated concentration by negative pressure, there was no aggregation.

The yield of oligomers varied among the different DNP-Ficolls. As Table II shows, the yield of oligomers with the three preparations described and with three similar preparations of F400 ranged from 33 to 64%, with no obvious correlation between yield and Ficoll/IgG or DNP/IgG ratios.

## Discussion

The family of reagents described here allows the preparations of covalently cross-linked oligomers composed of antibodies and a large ($M_r$ 70000 or 400000) water-soluble polymer. Under suitable conditions of antibody and polymer concentration, time, and temperature, the reaction is specific for anti-DNP antibodies since the antibodies react initially noncovalently with the DNP esters on the polymer to concentrate the active ester in the antigen-binding site.

The Ficoll polymer offers the possibility of a wide variety of substitution onto either aminoethyl, carboxymethyl, or glutarylated derivatives (Inman, 1975). Because it has proved possible to add more than one kind of substituent, it has been possible to react anti-DNP antibodies with monosaccharide-substituted, radiolabeled DNP-Ficoll. The anti-DNP antibodies react as well and specifically with the substituted as with the unsubstituted DNP-Ficolls. Thus, purified anti-DNP antibodies can be specifically covalently cross-linked to a variety of antigens. The model immune complexes formed in this way are being used to study the influence of antigen structure on the biological behavior of the complex (A. Rifai et al., unpublished experiments).

Because there are many DNP esters on each polymer molecule and because they are randomly distributed on it, a

variety of molecular species could form, and this is what was observed. It seems likely, however, that by limiting the density of haptenic groups on the polymer and by altering the antibody/polymer ratio in the reaction mixture, particular species could be favored. This property might be useful in exploring hew Fc region density in antibody oligomers affects the properties of the complex.

A feature of the DNP ester based affinity-labeling reagents that has proved useful is the presence of open antigen-binding sites on the antibodies in the complex. Although it has been argued that interaction of antigen with the Fab region of the antibody can cause conformational changes in Fc responsible for properties of the complex, the preponderance of evidence suggests that such changes do not occur and that the properties of a complex are determined rather by the antigen-induced aggregations of Fc regions on different molecules (Metzger, 1978). With the DNP ester group of reagents, the DNP group is the hapten, but the covalent bond forms because the group activates the ester bond, allowing a covalent bond to form between the activated carboxyl and a nearby amino group on the antibody; the DNP group is liberated as 2,4-dinitrophenol. Residual ester bonds on the polymer are cleaved by reaction with water or with ethanolamine added to stop the reaction. The free hapten is easily separated from the oligomer by gel filtration chromatography. The model complex then has free antigen-binding sites, but it does not aggregate because there are no longer haptenic groups in the polymer. The model complexes are therefore free to bind to hapten-coated erythrocytes so that their properties can be studied on cell surfaces as well as in solution (Segal et al., 1979; Jones et al., 1979). Furthermore, much larger model complexes can be formed by repeated addition of DNP-Ficolls to stable oligomers already formed (A. Rifai, unpublished experiments).

Finally, although we have used glycosylated Ficolls in order to study the interaction of immune complexes with monosaccharide-specific receptors, the amino and carboxyl intermediates offer the possibility to add other substituent groups in order to explore in a broader way the biological properties of immune complexes.

## Acknowledgments

We are grateful to John Inman, Y. C. Lee, David Zopf, and Bo Nilsson for much helpful advice and discussion and to Charlette Cureton, Evelyn Burrell, and Felicia Barankovich for excellent secretarial assistance.

## References

Christian, C. L. (1960) *J. Exp. Med. 84*, 112–121.
Helmkamp, R. W., Goodland, R. L., Bale, W. F., Spar, I. L., & Mutschler, L. E. (1960) *Cancer Res. 20*, 1495–1500.
Inman, J. K. (1974) *Methods Enzymol. 34*, 30–58.
Inman, J. K. (1975) *J. Immunol. 114*, 704–709.
Ishizaka, T., Ishizaka, K., Salmon, S., & Fudenberg, H. (1967) *J. Immunol. 99*, 82–88.
Jones, J. F., Plotz, P. H., & Segal, D. M. (1979) *Mol. Immunol. 16*, 889–897.
Keim, P., Vigna, R. A., Morrow, J. S., Marshall, R. C., & Gurd, F. R. N. (1973) *J. Biol. Chem. 248*, 7811–7818.
Knutson, D. W., Kijlstra, A., & van Es, L. A. (1977) *J. Exp. Med. 145*, 1368–1381.
Krantz, M. J., & Lee, Y. C. (1976) *Anal. Biochem. 71*, 318–321.
Laurent, T. C., & Granath, K. A. (1967) *Biochim. Biophys. Acta 136*, 191–198.
Leach, B. S., Collawn, J. S., & Fish, W. W. (1980) *Biochemistry 19*, 5734–5741.
Lee, Y. C., Stowell, C. P., & Krantz, M. J. (1976) *Biochemistry 15*, 3956–3963.
McBroom, C. R., Samanen, C. H., & Goldstein, I. J. (1978) *Methods Enzymol. 50*, 212–219.
Metzger, H. (1978) *Contemp. Top. Mol. Immunol. 7*, 119–152.
Plotz, P. H., Kimberly, R. P., Guyer, R. L., & Segal, D. M. (1979) *Mol. Immunol. 16*, 721–729.
Segal, D. M., & Hurwitz, E. (1976) *Biochemistry 15*, 5253–5258.
Segal, D. M., Taurog, J. D., & Metzger, H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2993–2997.
Segal, D. M., Guyer, R. L., & Plotz, P. H. (1979) *Biochemistry 18*, 1830–1835.
Smith, D. F., Zopf, D. A., & Ginsburg, V. (1978) *Methods Enzymol. 50*, 169–171.
Tung, A. S., Shyre-Te, J., Sato, S., & Nisonoff, A. (1976) *J. Immunol. 116*, 676–681.
Wright, J. K., Tschopp, J., & Jaton, J. C. (1980) *Biochem. J. 187*, 767–774.