Diazotized *m*-Aminobenzyloxymethylcellulose as the Insoluble Matrix for an Immunoadsorbent Used in the Purification of Antigens and Antibodies*

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ABSTRACT: The use of diazotized *m*-aminobenzyloxymethylcellulose as the insoluble matrix for immunoadsorbents capable of purifying rabbit antibovine serum albumin and human C-reactive protein directly from serum has been explored. Based on the quantitative precipitin test and Lowry protein determinations, antibody with a specific activity (milligram of protein precipitable at the equivalence peak per milligram of total protein) of 0.98 has been isolated. In addition, C-reactive protein was recovered from appropriate immunoadsorbent columns, but in a less highly purified form. The yield of antibovine serum albumin per gram of adsorbent was 100 times that obtained for C-reactive protein.

Numerous reports on the immunospecific purification of antibodies by using various insoluble matrices have appeared in the literature since the publication by Campbell *et al.* (1951), in which diazotized *p*-aminobenzylcellulose was employed. Owing to the relatively high antibody yields per gram of immunoadsorbent reported by Gurvich and others (Gurvich, 1957, 1964; Kuzovleva and Gurvich, 1966), the quaternary ammonium halide, *m*-nitrobenzyloxymethylpyridinium chloride used by them as the initial reactant, was synthesized and the immunoadsorbent was prepared essentially by their methods.

The purification and isolation of antigens on immunoadsorbent columns sensitized with homologous antibodies have presented many difficulties. Although adsorption has been accomplished by numerous investigators (Manecke and Gillert, 1955; Williams and Stone, 1957; Drizlikh and Gurvich, 1964; Olovnikov, 1964; Stephen *et al.*, 1966; Kent and Slade, 1960) using various methods of coupling antibodies to insoluble adsorbents, few attempts at elution of the homologous antigens have succeeded. Weetall and Weliky (1965) reported the elution of human γ-globulin from a 1-g column of a diazotized aminoaryl derivative of CM-cellulose coupled with rabbit antihuman γ-globulin and recovered 0.4 mg out of 10 mg of the antigen. Avrameas and Thernynck (1966) recovered 1 mg of human serum albumin from each milliliter of human serum treated batchwise with an immunoadsorbent composed of rabbit antihuman serum albumin coupled to CM-cellulose. Elution of the adsorbed antigen was performed with a 0.2 M glycine–HCl buffer (pH 2.2). Drizlikh and Gurvich (1964) used diazotized *m*-aminobenzyloxymethylcellulose coupled to antigen to isolate the immunospecifically bound antigen–antibody complex. They concluded that this "sorbent–antibody" cannot be used to isolate pure antigens. In 1966, Gallop *et al.* prepared diazotized *m*-aminobenzyloxymethylcellulose by a modification of methods described by Gurvich *et al.* (1961). Antibodies to ovalbumin and human serum albumin were coupled to the diazotized *m*-aminobenzyloxymethylcellulose, but homologous antigen adsorbed from a known mixture of antigens was not eluted. Perhaps the best result was recently obtained by Chidlow *et al.* (1968), where about 10 mg of [^{131}I]human serum albumin was recovered from an antihuman serum albumin column containing 90 mg of antibody protein.

Materials and Methods

Preparation of Immunoadsorbent. Procedures utilized by Kursanov and Solodkov (1943) for the synthesis of *m*-nitrobenzyloxymethylpyridinium chloride and by Gurvich *et al.* (1959, 1961) for conversion of the pyridinium salt into *m*-nitrobenzyloxymethylcellulose were generally employed. In addition, we have utilized procedures reported by Moudgal and Porter (1963), Olovnikov (1964), and Gallop *et al.* (1966) to purify C-reactive protein directly from human serum. The *m*-nitrobenzyloxymethylcellulose was reduced according to Gurvich *et al.* (1959), prior to solution of 1 g in 34.6 ml of ammoniacal copper reagent. This was followed by precipitation with 40 ml of tepid water, which provided a fine gelatin-like precipitate representing only 10–20% of the total aminocellulose in solution. The precipitate was, therefore, centrifuged at 1500*g* and discarded. To the supernatant was added 4.0 ml of 2 N H_2SO_4 in order to obtain close to 90% of the aminocellulose. The resulting copper-treated aminocellulose was centrifuged at 1500*g* and the supernatant was tested for completeness of precipitation prior to discarding. The precipitated product was then neutralized by addition, with mixing, of 5.0-ml portions of 2 N H_2SO_4. The white aminocellulose was washed at least five times with distilled water prior to diazotization.

Diazotization and Antigen Coupling. Previously reported diazotization procedures (Gurvich *et al.*, 1961; Moudgal and Porter, 1963) were somewhat modified. Thus, 1 g of aminocellulose was added to a solution of 3.0 *G* of $NaNO_2$ in 200 ml of 5% HCl at 4°. The suspension was agitated for 30 min prior to filtration on Teflon filter paper (Bel-Art Products, Pequannock, N. J.). The filter cake was washed twice with cold distilled water and twice with 0.2 M sodium borate (pH

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TABLE I: Effects of Neutralization and/or Dialysis against Divalent Cations on Precipitate Formation in Diazotized *m*-Aminobenzyloxymethylcellulose–Bovine Serum Albumin Eluates.[a]

Treatment of Eluates	Sample on Column		Eluate Protein			Sp Act. mg of Ab/ mg of Protein	Combining Ratio mg of Ab Protein/ mg of Aq Protein
	Protein (mg)	Ab at Equiv (mg)	Supernatant[b] (mg)	Precipitate (mg)	Ab at Equiv		
			Experiment 1				
Not Neutralized							
A. H_2O dialyzed	350	26.0	13.2	1.26	15.2	1.15	8.2
B. $MgCl_2$ (10^{-4} M) dialyzed	350	26.0	12.7	0.69	11.8	0.93	6.5
C. $MgCl_2$ (10^{-6} M) dialyzed	350	26.0	12.2	0.90	11.2	0.92	6.6
Neutralized[c]							
A. $MgCl_2$ (10^{-4} M) dialyzed	262	19.5	9.4	0.75	7.8	0.83	7.1
B. $CuCl_2$ (10^{-6} M) dialyzed	262	19.5	1.3				
C. $ZnCl_2$ (10^{-4} M) dialyzed	262	19.5	8.1				
			Experiment 2[d]				
Not Neutralized							
A. H_2O dialyzed	463	39.1	17.0	1.39	16.6	0.98	7.2
B. $MgCl_2$ (10^{-4} M) dialyzed	463	39.1	17.2	1.81	14.6	0.85	7.1
Neutralized							
A. H_2O dialyzed	483	40.6	17.2	2.32	15.1	0.88	8.1
B. $MgCl_2$ (10^{-4} M) dialyzed	483	39.1	16.1	1.91	14.3	0.89	7.3

[a] The term "Effluent" refers to the fraction washed through immunoadsorbent columns at pH 7.2. "Eluate" refers to the fraction washed through at pH 3.2. [b] After centrifugation at 10,000*g*. [c] With 0.1 N NaOH prior to dialysis. [d] See Figure 1 for protein in tubes nearest the equivalence point.

8.6) prior to adding to 100 ml of a 1% solution of bovine serum albumin in the same buffer. After stirring 1 hr at room temperature, the reaction mixture was placed at 4° and agitated for an additional 16–20 hr. The resulting immunoadsorbent was washed by resuspension three times in distilled water and three times in saline on a fritted glass funnel. The filter cake was finally resuspended in 100 ml of 0.1 M sodium barbital buffer (pH 9.2), saturated with β-naphthol, and incubated at room temperature for 1 hr and overnight at 4°. All steps through this final treatment with β-naphthol were performed in the dark, due to the light sensitivity of diazo groups. The resultant couple of diazotized *m*-aminobenzyloxymethylcellulose and bovine serum albumin was washed thoroughly with saline until the optical density at 220 mμ was less than 0.050.[1]

Purification of Immunoglobulins. Immunoglobulin fractions in goat antihuman C-reactive protein[2] serum were purified on DEAE-cellulose columns similar to those used by Levy and Sober (1960). We found, however, that hemoglobin present in hemolyzed serum was not separated from the γ-globulin fraction when the pH 6.3 buffer of these workers was employed. On the basis of a report by Gondko *et al.* (1964) in which myoglobin was separated from hemoglobin on DEAE-Sephadex using 0.05 M Tris-HCl (pH 8.6), 200 ml of serum was passed over DEAE-cellulose columns equilibrated with the same buffer. The dimensions of this column were 4.9 × 20 cm. The resulting γ-globulin fraction was free of contaminating proteins as determined by cellulose acetate electrophoresis.

Coupling of Goat Antihuman C-Reactive Protein to Diazotized m-Aminobenzyloxymethylcellulose. DEAE-cellulose-purified goat antihuman C-reactive protein (500 mg) was dissolved in 40 ml of 0.005 M Tris buffer (pH 7.2) and centrifuged at 10,000*g* for 10 min to remove any insoluble material. The clear supernatant was diluted to 475 ml with the same buffer prior to addition of 28 ml of bis-diazotized benzidine at a concentration of 0.06 mg/ml. After stirring 30 min, 2 g of diazotized *m*-aminobenzyloxymethylcellulose, previously washed with buffer, was added. Stirring was continued for 1 hr at room temperature and overnight at 4°. Distilled water and saline were used in the washing process until $OD_{220\,m\mu}$ was less than 0.10. Resuspension in 200 ml of β-naphthol-saturated sodium barbitol buffer (0.1 M, pH 9.2) was carried out as described above under "antigen coupling."

Immunoadsorbent Columns. The β-naphthol-treated immunoadsorbents were washed with saline until $OD_{220\,m\mu}$ of filtrate was less than 0.05. After thorough mixing with a slurry of "plain" cellulose (Carl Schleicher & Schuell, Keene, N. H.) equal to 20 times the original diazotized *m*-aminobenzyloxymethylcellulose weight, the column was poured and

[1] All optical density readings reported were obtained using a 1.0-cm light path.

[2] We wish to express our thanks to Dr. Harrison F. Wood, School of Medicine, Yale University, for the purified C-reactive protein used in immunizing our goats.

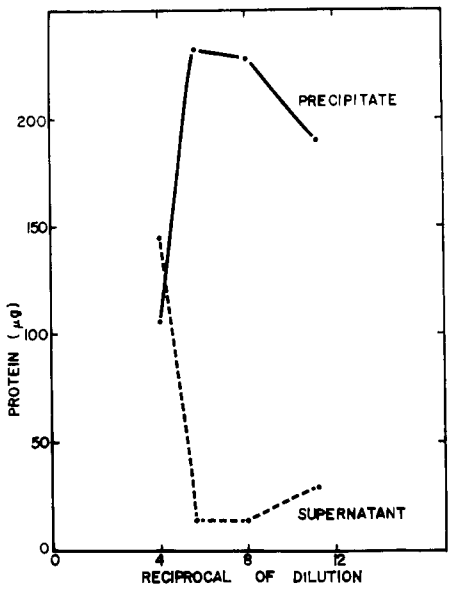

FIGURE 1: The recovery of bovine serum albumin–antibovine serum albumin protein at or near the equivalence point in the quantitative precipitin assay. To 0.2-ml aliquots of serial dilutions of 0.1% bovine serum albumin, 0.2-ml portions of purified antibovine serum albumin were added. After appearance of floccules, all tubes were stored at 4° for at least 5 days prior to centrifuging at 4000*g* for 15 min.

washed with either 1% or 1 M NaCl (pH 3.2) and 0.0075 M saline phosphate (SP) buffer[3] (pH 7.2) until $OD_{220\,m\mu}$ was less than 0.05 for both buffers. To the column, finally equilibrated with the SP buffer, an appropriate volume (see above) of serum was added. Nonadsorbed serum proteins were washed through the column with the SP buffer, followed by elution of adsorbed proteins with either 1% or 1 M NaCl (pH 3.2).[4] The eluted antigen or antibody solution was deionized by Diaflo membrane ultrafiltration (Amicon Corp., Lexington, Mass.) or by dialysis, and subsequently lyophilized.

In an attempt to preserve antibody activity after elution from diazotized *m*-aminobenzyloxymethylcellulose–bovine serum albumin columns, it was thought that immediate neutralization and dialysis against divalent cations might be of value. Consequently, 10^{-4} and 10^{-6} M $MgCl_2$, 10^{-6} M $CuCl_2$, and 10^{-4} M $ZnCl_2$ were employed in dialysate fluids.

Protein Determinations. The Lowry (Lowry *et al.*, 1951) modification of the Wu (1922) procedure using the Folin–Ciocalteu (1927) phenol reagent was followed with Labtrol (Dade Reagents, Inc., Miami, Fla.) as a standard protein solution.

Quantitative Precipitin Test. Procedures outlined by Campbell *et al.* (1964) were generally followed. A constant volume of antibody solution or serum inactivated at 56° for 30 min was rapidly added to serial dilutions of 0.1% bovine serum albumin. Antibody protein was calculated from the protein in the bovine serum albumin–antibovine serum albumin precipitate at the equivalence point, as determined by the Folin-insoluble protein method. The known amount of bovine serum albumin protein at the equivalence point was subtracted from the total amount of protein in the precipitate to give microgram of antibody protein.

Double Diffusion in Agar. Microscope slides precoated with 0.5–1.0 ml of 0.1% agar in water were employed. A 1% solution of Noble agar containing 0.1% sodium azide and 7.5% glycine in distilled water was applied to the slides just before use. In order to standardize the depth of agar in the diffusion layer, 1.5 ml of liquified gel was routinely applied. Filter paper disks 0.25 in. in diameter were saturated with appropriate antigen or antibody solution and placed on the solidified agar surface according to a given template. Pictures were taken at various times after incubation in a moist chamber at 37°.

Analytical Ultracentrifuge Determination. Sedimentation velocity experiments were carried out in the Spinco Model E analytical ultracentrifuge using a double-sector cell with a 1.2-cm light path. The rotor speed was 52,000 rpm and the distance the boundary moved at 8-min intervals between 4 and 52 min after the rotor had reached full speed was determined using a photoscanner at 280 mμ. The rotor temperature was maintained at 20° throughout the run.

Cellulose Acetate Electrophoresis. Antigen preparations were checked for purity with Sepraphore III cellulose polyacetate strips and an appropriate electrophoresis chamber (Gelman Instrument Co., Ann Arbor, Mich.). The high-resolution buffer, supplied by Gelman, was dissolved in distilled water to an ionic strength of 0.027, pH 8.6. A current of 2.0 mA/strip was applied for 45 min to 1 hr. Protein bands were developed with a 0.2% solution of ponceau S in 5% trichloroacetic acid. Excess dye was removed by washing in 5% acetic acid.

Results

Purification of Rabbit Antibovine Serum Albumin. In order to test the efficiency of our immunoadsorbent in the purification of antibodies, several columns were made in which bovine serum albumin was coupled to diazotized *m*-aminobenzyloxymethylcellulose as described above. In all of these, a portion of the eluted solids was insoluble in 1% NaCl after desalting and lyophilization. Whether or not these represent only euglobulins was not investigated; however, the protein present in these precipitates was always 5–10% of that in the 10,000*g* supernatant. As shown in Table I, an attempt was made to eliminate this loss of eluted material. The effect of dialysis of the eluted antibovine serum albumin solutions against 10^{-4} and 10^{-6} M $MgCl_2$, 10^{-6} M $CuCl_2$, and 10^{-4} M $ZnCl_2$ as compared with distilled water is shown. The results indicate that these cations have little or no beneficial effect on the formation of eluate solids during dialysis and lyophilization. On the other hand, dialysis against distilled water resulted in specific activities very close to 1.0 for the lyophilized antibovine serum albumin. The exceedingly high specific activity in the top row of Table I cannot be explained at this time. It is most likely due to an indeterminate error, since good precision in duplicate or similar experiments has been observed throughout this work.

In an effort to prove the concept of total precipitation of our purified antibovine serum albumin with bovine serum albumin using the quantitative precipitin test, protein determinations were carried out on several supernatants in tubes nearest calculated equivalence points. As shown in Figure 1 where pro-

[3] This buffer is made with sodium phosphate and 0.85% NaCl.

[4] Various other eluting buffers were tried with less success.

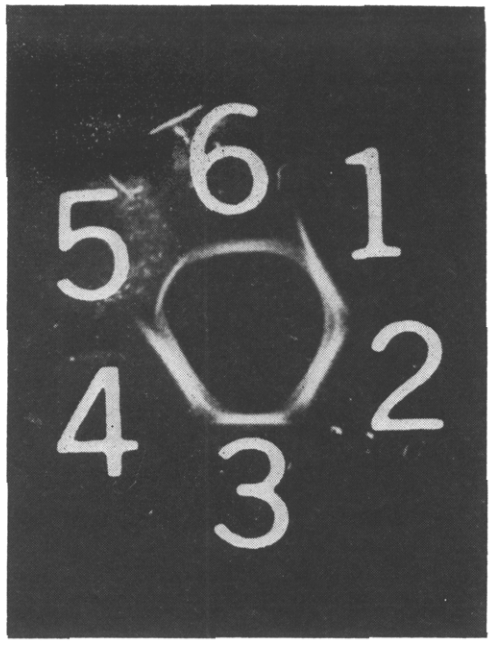

FIGURE 2: Gel diffusion of C-reactive protein fractions. (1) Serum effluent, (2) C-reactive protein-positive serum 759, (3) C-reactive protein-positive serum 758, (4) repeat of C-reactive protein-positive serum 759, (5) antigen eluate from 480 mg of diazotized *m*-aminobenzyloxymethylcellulose–anti-C-reactive protein column (no. 1, Table II), and (6) Difco C-reactive protein. Goat anti-C-reactive protein was placed on center disk.

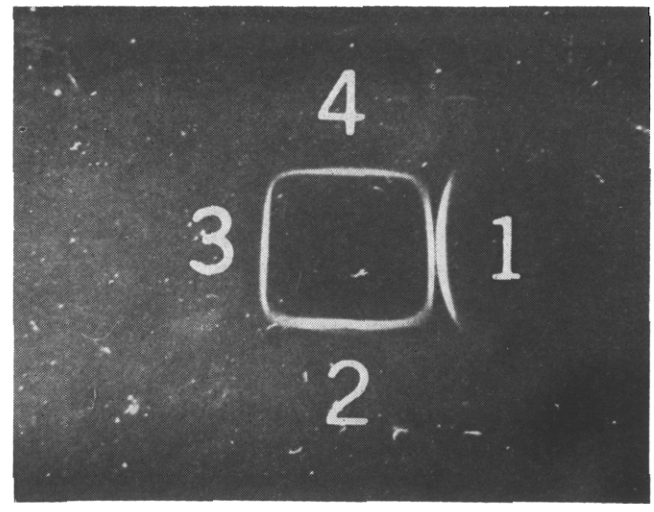

FIGURE 3: Gel diffusion of C-reactive protein fractions. (1) Pooled human C-reactive protein-positive serum, (2) purified C-reactive protein used for immunization of the goat from which our anti-C-reactive protein was obtained, (3) antigen eluate from a 4-g diazotized *m*-aminobenzyloxymethylcellulose–anti-C-reactive protein column (no. 7, Table II), and (4) Difco C-reactive protein. Goat anti-C-reactive protein was on center disk.

teins were determined on the supernatants and precipitates of tubes in a series of dilutions in a quantitative precipitin test, the supernatant proteins showed an inverse relationship to precipitated protein complexes. In many experiments supernatant protein was negligible at or near the equivalence point. These results indicate that our purified antibovine serum albumin is immunospecifically pure.

Analytical ultracentrifuge data established an *s* value equal to 6.2 ± 0.2 S at the 95% confidence level. Within the limits of the photoscanner and adsorbance at 280 mμ, no impurities could be detected.

Purification of C-Reactive Protein. Based on the work of Olovnikov (1964), the DEAE-cellulose purified anti-C-reactive protein was polymerized in the presence of bis-diazotized benzidine just prior to coupling to diazotized *m*-aminobenzyloxymethylcellulose. The resulting diazotized *m*-aminobenzyloxymethylcellulose–anti-C-reactive protein columns were employed in the isolation of immunospecifically pure C-reactive protein from human C-reactive protein-positive serum. Figure 2 illustrates results obtained when 2 M NaSCN was used to elute the C-reactive protein. Although antigen was eluted by this chaotropic ion (Dandliker *et al.*, 1967), giving a band of gel diffusion which coalesced with C-reactive protein purchased from Difco (Detroit, Mich.), columns treated with this ion could not be reused (Table II). In addition, this ion seemed to bind quite strongly to the C-reactive protein isolated, and indeed contributed somewhat to protein color developed by the phenol reagent. Also, in Table II are shown data which indicate that columns eluted with 1 M NaCl (pH 3.2) were obtained with 1.5-mg/0.1 ml concentrations of deionized and lyophilized antigen eluate. Coalescence is present with both Difco C-reactive protein and the C-reactive protein preparation employed for immunization of the goat from which our anti-C-reactive protein immunoglobulin was isolated. It is of interest that all human sera, whether normal or C-reactive protein positive, tested by gel diffusion against the goat antihuman C-reactive protein, gave a precipitin band. In Figure 3, the C-reactive protein-positive serum gave at least two precipitin bands, only one of which coalesced with the Difco antigen. After isolation of C-reactive protein in our eluates, however, the second band does not appear. Of additional interest in Table II is the small amount of protein in our eluted C-reactive protein in relation to the dry weight. Since the per cent protein in our dried eluates decreased from 16.7% in the 1.5-g columns to 7.0% in the 4-g column, this may be a function of the amount of diazotized *m*-aminobenzyloxymethylcellulose involved. This point needs to be further investigated.

The data in Table III indicate that some nonspecific adsorption of C-reactive protein occurs when diazotized *m*-aminobenzyloxymethylcellulose is coupled with normal goat γ-globulin. Concentrations of eluate twice those employed to demonstrate specific binding of C-reactive protein (Table II) were required to show weak bands in the Ouchterlony assay. Table III also shows that normal human serum inhibits the nonspecific adsorption of C-reactive protein from patients' sera. The columns were run in the order listed in the table.

Cellulose acetate electrophoresis of eluted C-reactive protein indicated the presence of weak protein bands with mobilities similar to those present in the C-reactive protein injected into goats for antibody production.

Discussion

In order to establish an immunoadsorbent technique which could serve as a tool in the purification of both antigens and antibodies, various methods appearing in the literature were

TABLE II: Diazotized *m*-Aminobenzyloxymethylcellulose–Goat Anti-C-Reactive Protein Columns. Elution of C-Reactive Protein

Column	Column Size (mg of Diazotized *m*-Aminobenzyloxy-methylcellulose)	C-Reactive Protein-Positive Serum (ml)	Eluting Buffer (M), pH	Eluate Dry Wt (mg)	Protein in Eluate[a] (mg)	Ouchterlony Done on Eluate[b]
1	480	3.0	NaSCN (2), 6.0	96.1		+
2	480 (reuse)	3.0	NaSCN (2), 6.0	12.2		−
3	300	3.0	NaSCN (2), 6.0	21.8	1.6	+
4	300 (reuse)	3.0	NaSCN (2), 6.0	22.0	0.8	−
5	1500	7.9	NaCl (1), 3.2	7.78	1.3	++
6	1500 (reuse)	Serum effluent from column 5	NaCl (1), 3.2	4.50	0.6	+
7	4000	6.0	NaCl (1), 3.2	10.0	0.7	++
8	4000 (reuse)	6.0 (NHS)[c]	NaCl (1), 3.2	5.20		−
9	4000 (reuse)	Serum effluent from column 7	NaCl (1), 3.2	5.50		++

[a] 10,000 *g* supernatant. [b] Done on 1.5-mg/0.1 ml concentrations by dry weight. [c] NHS = normal human serum.

TABLE III: Diazotized *m*-Aminobenzyloxymethylcellulose–Normal Goat γ-Globulin Immunoadsorbent Columns. Nonspecific Adsorption.[d]

Column	Column Size (mg of Diazotized *m*-Aminobenzyl-oxymethylcellulose)	C-Reactive Protein-Positive Serum (ml)	Eluate Dry Wt (mg)	Protein in Eluate[a] (mg)	Ouchterlony Done on Eluate[b]
1	1800	3.0	7.2		±
2	1800 (reuse)	3.0	3.5		+
3	1800 (reuse)	3.0[c] 3.0	2.7		−
4	1800 (reuse)	3.0	3.5		+
5	1200	3.0	11.4	0.45	−

[a] 10,000*g* supernatant. [b] Done on 3.5-mg/0.1 ml concentrations by dry weight. [c] NHS followed without elution by C-reactive protein-positive serum. [d] The eluting buffer was 1 M NaCl (pH 3.2).

employed. Procedures outlined by Gurvich (Gurvich, 1957, 1964; Kuzovleva and Gurvich, 1966) and utilized by Moudgal and Porter (1963) and Gallop *et al.* (1966) were employed in an effort to obtain the high antibody yields reported by these investigators. Characterization of the resulting antibody preparations was carried beyond the point previously reported, in order to be certain that nonspecific absorption of serum components present in the hyperimmune sera from which antibody was isolated had not occurred. The complete absence of protein in supernatant obtained at the equivalence point in some of our quantitative precipitin tests supports the view that our antibody preparations are 90–98% pure and also confirms the stoichiometric relationship traditionally thought to be required for Ag–Ab precipitation. The combining ratios listed in Table I also confirm the fact that the antigen has multiple combining sites. Whether or not the variations in this ratio from one preparation to another are significant was not investigated. However, since so many assays are required to arrive at each of these figures, it seems reasonable to say that the molar ratio of antibody to Ag is about 3.1.

The protective effects of neutralization and divalent cations on eluted antibodies have not been established. In expt 1 of Table I there was some indication that $MgCl_2$ reduced the loss of protein during dialysis, but these results were reversed in expt 2. In both experiments, the highest specific activities were obtained in the absence of neutralization and divalent cations.

The recovery of C-reactive protein directly from C-reactive protein-positive serum by use of anti-C-reactive protein immunoadsorbents demonstrates the use of the immunoadsorbent technique in the isolation of an agent associated with inflammatory processes. The obvious use of immunoadsorption for the detection and purification of other serum components specifically related to diseases of unknown etiology is being explored. In addition, the utilization of immunoadsorbents in the one-step purification of a specific protein present in mixtures as complex as serum has many advantages over

classical procedures involving $(NH_4)_2SO_4$ fractionations, column chromatography, and gel filtration. Thus milligram quantities of a highly purified protein antigen can be obtained in essentially one purification step from an immunoadsorbent sensitized with the homologous antibody.

In our purification of C-reactive protein it is important to realize that the antigen isolated from human pleural exudates and employed in the immunization of our goat gave three or four lightly stained bands by cellulose acetate electrophoresis. These protein bands coincided very well with the lightly stained bands obtained using our diazotized *m*-aminobenzyloxymethylcellulose–anti-C-reactive protein-purified antigen. The fact that antibodies made to our initial C-reactive protein antigen gave a precipitin band with all normal sera tested in in the Ouchterlony assay indicates the presence of a normal serum component in addition to the C-reactive protein. A C-reactive protein preparation recently obtained from the same laboratory,[2] however, does not contain this contaminant.

It is not surprising that the C-reactive protein preparations utilized in our work give multiple bands when subjected to cellulose acetate electrophoresis, since the work of Hokama and others (Riley *et al.*, 1965; Hokama *et al.*, 1967) indicates that C-reactive protein isolated by precipitation techniques as well as by DEAE-cellulose chromatography is an aggregated complex with a mobility of 7S γ-globulin. During electrophoresis, however, dissociation can occur giving components with mobilities similar to pre- and postalbumins. The association of C-reactive protein with mucopolysaccharide components, reported by these same investigators, also explains the low amounts of protein in relation to dry weight obtained for our immunospecifically purified preparations.

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