

Comparison of the protein-binding capacities of cyanogen bromide-activated polysaccharides

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Techniques for chemically coupling allergens to insoluble polysaccharides have permitted the development of the radioallergosorbent test (RAST) to measure reaginic antibodies. To date these allergens have been those contained in crude commercial allergy extracts. Because these allergens are not well characterized, quantitative measurements of the amounts coupled to the polymers have not been possible. In this study several different polysaccharide polymers were examined for their ability to bind bovine serum albumin (BSA) and ragweed antigen E (AgE) by use of different activation procedures with cyanogen bromide. The capacity of the polymers to bind these antigens was assessed by examining the allergen-particle complex for uptake of radioactivity (BSA) or by measurement of allergen by radioimmunoassay (AgE). Sepharose polymers were the most efficient in binding antigen but tended to trap uncoupled antigen in the gel interstices. Microcrystalline cellulose bound more of both antigens than did Sephadex polymers. The AgE bound to cellulose did not differ immunologically from that of pure AgE IV-C. This suggests that there was no loss of antigenic determinants during the coupling procedure.

Since the introduction of techniques for chemically coupling proteins to polysaccharides by means of cyanogen bromide,^{1, 2} the coupled polymers have been employed in affinity chromatography,³ in solid-phase radioimmunoassay,^{4, 5} and for in vitro measurement of reaginic antibodies by the radioallergosorbent test (RAST).⁶ The quantities of protein coupled to polymers in these studies have been estimated by amino acid analysis and by the ability of the antibody polymer complexes to bind antigen.^{2, 4} However, comparatively little information is available about the quantities of material coupled to various polysaccharide particles or about the preferable polymer for use in the RAST procedure. In this study both of these questions were examined by coupling proteins to a variety of polymers.

MATERIALS AND METHODS

Reagents

Avicel microcrystalline cellulose was purchased from Brinkmann Instruments, Inc., Westbury, N. Y., and the Sephadex and Sepharose were purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. The actual weights of washed Sepharose polymers were estimated by centrifuging measured aliquots ($1,120 \times g$ for 15 minutes) and heating the packed gel to

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dryness in a vacuum desiccator at 88° C. over P_2O_5 . Sephadex G-25UF was a gift from Pharmacia Fine Chemicals through the courtesy of Mr. Erlan Lindgren. Cyanogen bromide and N,N-dimethylformamide (DMF) were purchased from Fisher Scientific Company, Chicago, Ill. Crystalline bovine serum albumin (BSA) was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio (Lot No. 4300) and from Mann Research Laboratories, New York, N. Y. (Lot No. V3924).

AgE protein

Purified AgE IV-C⁷ (3.8 mg. per milliliter) for use in the radioimmunoassay described below was kindly supplied by Dr. F. C. McIntire. Analysis of AgE IV-C by disc electrophoresis revealed 2 rapidly migrating major bands and a series of faint bands. When studied by immunoelectrophoresis with potent rabbit antisera to crude ragweed pollen, a single band was seen with one serum and 2 bands, one major and one minor, were seen with another serum.

A partially purified AgE preparation was obtained from short ragweed pollen (Lot No. G-91, Greer and Greer, Princeton, W. Va.) by a modification of the procedure of King and associates.^{7, 8} After defatting with diethyl ether, 200 Gm. of pollen was extracted with 1,200 ml. of distilled water for 5 hours at 25° C. The pH was adjusted from 6.1 to 7.1 with ammonium hydroxide and the extract concentrated to 83 ml. by ultrafiltration with the use of a Diaflow PM-10 membrane (Amicon Corporation, Lexington, Mass.). After centrifugation to remove insoluble material, 80 ml. was applied to a 4 × 95 cm. Sephadex G-25 column equilibrated with pH 7.5, 0.025M Tris-HCl buffer. AgE activity was detected by immunodiffusion analysis with antiserum to AgE, and fractions rich in AgE were pooled and concentrated. Aliquots were applied to a 5 × 92 cm. Sephadex G-100 column equilibrated with 0.05M Tris-HCl, 0.1M NaCl (pH 7.3). AgE emerged with an elution volume of 1,110 ml., whereas chymotrypsinogen (molecular weight 22,500) eluted at 1,175 ml. and BSA (molecular weight 69,000) eluted at 790 ml. The concentration of AgE in the final preparations measured by radioimmunoassay as described below was 2.3 mg. per milliliter, while the total protein concentration measured by biuret assay with human serum albumin as a standard was 4.6 mg. per milliliter.

Antiserum to AgE

Two rabbits were immunized with AgE IV-C in complete Freund's adjuvant by intradermal injection of antigen, 0.2 mg. at time zero and 1.0 mg. at 10 weeks and again at 14 weeks. The animals were bled 2 weeks after the last injection, and serum from one of the rabbits (R2) was used for detection of AgE in column fractions by immunodiffusion and in the AgE polymers by radioimmunoassay. This antiserum reacted with crude extracts of whole ragweed to form single bands in Ouchterlony immunodiffusion analysis. These bands gave a reaction of identity with AgE IV-C.

Measurement of AgE by Radioimmunoassay

AgE IV-C was radioiodinated by use of the chloramine-T method,⁹ and 93 to 98 per cent of the radioactivity in the final preparations was precipitated by phosphotungstic acid. The radioimmunoassay was performed in essentially the same manner as that described previously for IgE.¹⁰ The resulting inhibition curves were logit transformed and the concentrations of AgE were determined from this regression line. Finally, the slopes of the regression lines with AgE and related materials were compared in order to determine if antigenic differences existed. The application of radioimmunoassay for detection of AgE activity and for antigenic comparison of AgE activity in various materials will be described elsewhere.¹¹

Radioiodination of BSA

BSA was radioiodinated by the chloramine-T procedure. BSA (50 mg.) in a volume of 0.1 ml. was added to a polyvial containing 2.9 mc. of $Na^{131}I$ (Cambridge Nuclear, Inc., Cambridge, Mass.) and reacted with chloramine-T and sodium metabisulfite.⁹ The labeled protein was separated from free ^{131}I by gel filtration on a column of Sephadex G-100. Ninety-two

TABLE I. Coupling of BSA-¹³¹I to activated Sephadex G-25SF

Polymer	Quantity of BSA- ¹³¹ I reacted (mg.)	Wash		BSA- ¹³¹ I coupled	
		No. 1 (% of total counts)	No. 2 (% of total counts)		
				%	μg./mg.
Sephadex G-25SF, activated	1.3	20.7	1.0	1.7	0.11
	6.6	19.5	0.7	0.6	0.20
	13.2	20.1	0.7	0.4	0.26
	26.1	20.4	0.9	0.3	0.39
Sephadex G-25SF, unactivated	6.6	21.6	1.2	0.1	0.03

The procedure of Wide⁵ for activation and coupling was followed. Although the polymer was washed 6 times, only the radioactivity in the first 2 washes is shown, as no counts above background were detected in the last 4 washes.

per cent of the radioactivity was incorporated into the protein, and the radioactivity was quantitatively precipitated by phosphotungstic acid. The resulting BSA-¹³¹I had an activity of 0.05 μc per microgram. In some experiments radiolabeled protein was diluted with unlabeled BSA in order to have sufficient material for coupling.

RESULTS

In initial experiments we activated Sephadex G-25SF by use of the procedure described by Wide.⁵ In a well-ventilated chemical hood 5.0 Gm. of cyanogen bromide was dissolved in 250 ml. of distilled water. Dry Sephadex G-25SF (5 Gm.) was added to 200 ml. of this solution and mixed by continuous magnetic stirring. The pH was raised to 11.0 and maintained between 10.5 and 11.0 for 2 minutes by addition of 1N NaOH. The total base consumption was 25 mEq. After 2 minutes the mixture was filtered through a fritted glass funnel and washed successively with 500 ml. of ice water, 200 ml. of 50 per cent acetone, and finally 100 ml. aliquots of 70 per cent, 90 per cent, and 100 per cent acetone. After air-drying for 30 minutes under suction, the activated Sephadex was stored at -20° C. The final yield was 5.4 Gm. Individual 200 mg. aliquots of this activated Sephadex were mixed with 1.3, 6.6, 13.2, and 26.1 mg. of BSA-¹³¹I. The reaction volumes were adjusted to 1.0 ml. by the addition of pH 8.4 0.1M NaHCO₃, and the mixtures were magnetically stirred for 24 hours at 25° C. The coupled polymer was separated by centrifugation (1,120 × g for 15 minutes) and washed successively with two 10 ml. aliquots of 0.5M NaHCO₃ (pH 8.4). A third wash consisted of 5 ml. of 0.1M acetate buffer (pH 4.0) with rotation of the polymer at room temperature for one hour. The fourth wash was 5 ml. of 0.1M acetate buffer (pH 4.0) overnight at 4° C. with rotation. After 2 final brief washes with 10 ml. of phosphate-buffered saline (pH 7.4), the quantities of BSA-¹³¹I associated with the polymer were measured in a gamma scintillation counter. As shown in Table I, increasing the amount of BSA in the coupling reaction resulted in a corresponding increase in the quantities of protein coupled. That the activation increased the capacity of the polymer to bind protein is shown by the six- to sevenfold increase in binding with activated G-25SF compared to the inactivated polymer. However, only very small amounts of the total BSA-¹³¹I in the system were coupled to the Sephadex.

In an effort to increase the quantities of bound BSA-¹³¹I, we used the

TABLE II. Coupling of BSA-¹³¹I to polymers

Polymer	Cyanogen bromide for activation (Gm.)	Coupling supernatant (% of total counts)	Wash			BSA coupled	
			No. 1 (% of total counts)	No. 2 (% of total counts)	No. 3 (% of total counts)		
						%	μg/mg.
Sephadex G25SF	1.0	93.0	2.7	0.4	0.3	1.4	2.8
	3.0	89.0	5.4	0.6	0.2	1.4	2.8
Microcrystalline cellulose	1.0	77.4	4.3	1.2	0.5	18.0	36.0
Sepharose 4B	1.0	12.8	3.3	1.8	2.8	99.9	199.8

See text for description of procedure.

procedure described by Rejnek and associates¹² for activation of polymers. Quantities (500 mg.) of swollen Sephadex G-25SF, dry microcrystalline cellulose, and Sepharose 4B were washed with distilled water. Each polymer was suspended in pH 8.4 0.1M NaHCO₃ to produce a total volume of 20 ml. and cooled in a 4° C. ice-water bath in a beaker containing a magnetic stirring bar and a pH electrode. The pH of the mixture was raised to 11.0 by addition of 4M NaOH. Cyanogen bromide dissolved in 1.0 ml. of N,N-dimethylformamide (DMF) was added dropwise to the mixture, while the pH was maintained at 11.0 and the temperature at 4° C. After 30 minutes the polymer was transferred to a sintered glass funnel and washed with several aliquots of cold pH 8.0, 0.2M H₃BO₃-0.04M NaOH-0.16M NaCl (borate saline). The material was resuspended in 10 to 20 ml. of borate saline, and the pH was raised to 8.4 with 4M NaOH. With continuous magnetic stirring 100 mg. of BSA-¹³¹I in 10 ml. of borate saline was added dropwise. Reaction volumes in all cases were 30 ml. The reaction mixtures were stirred overnight at 4° C. The polymer was then washed successively with cold 10 ml. volumes of borate saline, 0.1N HCl, borate saline again, and then resuspended in 20 ml. of borate saline. Coupling yields were determined by measurement of radioactivity. The results are shown in Table II and indicate that the more porous Sepharose 4B gel bound much more protein than did the other polymers. However, nonspecific trapping of radioactivity seemed to be greater with Sepharose 4B, as shown by the number of counts in the third wash. The quantities of protein coupled per milligram of Sephadex G-25SF were greater than in the first experiment (Table I). Increasing the amount of cyanogen bromide by threefold in the activation step did not result in any increase in coupling efficiency. Microcrystalline cellulose bound approximately thirteenfold more BSA than did G-25SF. For all polymers except Sepharose 4B the recovery of radioactivity approximated 100 per cent.

Additional experiments were carried out to compare coupling of BSA-¹³¹I to several polymers. Activation (1.0 Gm. of CNBr) and coupling procedures were performed as described in the preceding paragraph with the exception that 2 additional washing steps were employed after the coupling step. The fourth wash was an overnight wash with 10 ml. of 0.1M acetate buffer (pH 4.0). The fifth wash was a brief wash in 10 ml. of borate saline. Sephadex G-25SF, G-100SF, Sepharose 4B, and Sepharose 2B were activated and coupled to BSA-¹³¹I. The results are shown in Table III. The G-25 and G-100 polymers had previously

TABLE III. Coupling of BSA-¹³¹I to polymers

Polymer	Coupling supernatants (% of total counts)	Wash					BSA coupled	
		No. 1 (% of total counts)	No. 2 (% of total counts)	No. 3 (% of total counts)	No. 4 (% of total counts)	No. 5 (% of total counts)		
							%	μg/mg.
Sephadex G-25SF	94.4	1.7	0.4	0	0	0.2	1.0	2.0
Sephadex G-100SF	73.1	5.0	0.6	0.2	0.3	0.2	24.9	49.8
Sepharose 4B	8.0	2.0	1.2	0.9	0.3	0.5	99.1	198.2
Sepharose 2B	9.4	2.8	1.5	1.7	0.7	0.7	83.5	167.0

Each polymer (500 mg.) was activated by reaction with 1.0 Gm. of CNBr in 1.0 ml. of DMF for 30 minutes at 4° C. The activated polymers were reacted with 100 mg. of BSA-¹³¹I overnight at 4° C. The polymers were washed 5 times as described in the text. Reaction volumes during the activation step were 21 to 22 ml. and during the coupling step were 30 ml., with the exception of Sepharose 2B; reaction volumes here were 10 ml. greater in both steps.

been swollen at room temperature for a minimum of 36 hours. The results indicated that the Sepharose gels bound more protein than did the Sephadex gels. However, the Sepharose gels were also more difficult to wash free of unbound protein. Unactivated Sepharose 2B and 4B carried through the same procedures as a control contained approximately 5 per cent and 2 per cent of total BSA-¹³¹I, respectively, at the completion of the washing procedure. The quantity of BSA-¹³¹I bound to G-25SF was approximately the same as in the experiment shown in Table II.

In order to test the Sephadex G-25UF, of which we had only a small quantity, we scaled down the coupling procedure. Quantities (50 mg.) of polymer were swollen, washed as previously described, and taken up in sufficient 0.1M NaHCO₃ (pH 8.3) to produce a total volume of 2 ml. The polymer was activated at 4° C. for 30 minutes at pH 11.0 with the use of 100 mg. of CNBr in 0.1 ml. of DMF. The activated polymer was washed with cold borate saline and resuspended in 2 ml. of borate saline. BSA-¹³¹I (10 mg.) was reacted with the activated polymer by tumbling overnight at 4° C. Reaction volumes in all cases were approximately 3 ml. The polymer was then washed 5 times with 2 ml. volumes of the same solutions and for the same lengths of time as described in the previous paragraph. Experiments were also conducted to determine the effect of increasing the coupling stage to 3 days, and the results are shown in Table IV. Considerably less BSA-¹³¹I was coupled to Sepharose 4B in this experiment than in those done previously. This was also evident with the other gels as well. Only a slight increase in the quantity of protein coupled was noted when the incubation period was prolonged to 3 days. Comparison of results with microcrystalline cellulose and G-25UF revealed greater binding by the cellulose polymer.

In another series of experiments partially purified ragweed AgE was coupled to polymers, and the quantity of AgE in the washes and on the final polymer were measured by radioimmunoassay. As shown in Table V experiments were performed with quantities of AgE from 1 to 5 mg. coupled to 50 mg. quantities of polymer. The activation and coupling conditions were identical to those

TABLE IV. Scaled-down procedure for coupling of BSA-¹³¹I to polymers

Polymer	Coupling supernatant (% of total counts)	Wash					BSA coupled	
		No. 1 (% of total counts)	No. 2 (% of total counts)	No. 3 (% of total counts)	No. 4 (% of total counts)	No. 5 (% of total counts)		
							%	μg/mg.
Overnight								
Sephadex G-25UF	96.6	5.4	0	0	0.6	0	1.5	3.0
Sephadex 4B	43.6	5.0	1.9	0.6	1.2	0	40.6	81.2
3 Days								
Sephadex G-25UF	88.4	3.3	0.1	1.3	1.5	0.3	2.1	4.2
Sephadex G-50F	92.2	1.8	0	0	2.8	0.2	0.1	0.2
Microcrystalline cellulose	81.8	2.4	0	0	1.2	0	8.6	17.2

Each polymer (50 mg.) in a volume of 2 ml., pH 8.4, 0.1M NaHCO₃ was activated by reaction with 100 mg. of CNBr in 0.1 ml. of DMF for 30 minutes at 4° C. The activated polymer was reacted with 10 mg. of BSA-¹³¹I at 4° C. and washed 5 times.

described for BSA in the preceding paragraph, except that buffers near neutrality were used. Washes No. 1, No. 2, and No. 3 were borate saline; wash No. 4 was an overnight wash with 0.01M K₂HPO₄-KH₂PO₄-0.15M NaCl, pH 7.4 (phosphate-buffered saline); wash No. 5 was 0.1M phosphate buffer, pH 7.5. Only small quantities of AgE were coupled to polymers, but again cellulose bound more protein, as shown in 2 final experiments in which 50 mg. of AgE was reacted with 500 mg. quantities of polymer. The poor percentage recovery of AgE in the supernatant and washes was probably due to the elapsed time between the coupling experiments and the radioimmunoassay for AgE; samples were stored at 4° C. for several weeks and probably lost some AgE activity during this time. One preparation of cellulose-AgE was analyzed by radioimmunoassay, and the slope of the inhibition curve was compared to that of purified AgE IV-C. No significant difference in slopes was found, which suggested that antigenic determinants associated with AgE were not destroyed during the coupling procedure.

DISCUSSION

The cyanogen bromide method for coupling of proteins to polysaccharides developed by Axen and associates¹ has proved useful for preparing reagents for affinity chromatography, for coupling of antibodies to polymers for solid-phase radioimmunoassay, and for the radioallergosorbent test for IgE antibodies. In the RAST the antigens coupled to polysaccharides have been those contained in commercial allergy extracts. Because these antigens are not well characterized, quantitative measurements of the amounts coupled to the polymers have not been possible. In this study we compared the 2 coupling methods and several polysaccharides in terms of the binding of BSA and ragweed AgE. In initial experiments we activated Sephadex G-25SF with the use of the procedure

TABLE V. Coupling of ragweed AgE to polymers

Polymer	AgE reacted (mg.)	Coupling supernatant (% of total AgE activity)	Wash		AgE activity associated with polymer	
			No. 1 (% of total AgE activity)	No. 2 (% of total AgE activity)		
					%	μg/mg.
Microcrystalline cellulose (50 mg.)	1.1	67.0	2.3	0.1	0.2	0.04
Sephadex G-25UF (50 mg.)	5.0	87.0	3.2	0.2	0.05	0.05
Sephadex G-100SF (50 mg.)	3.0	59.0	3.3	0.1	0.03	0.02
Microcrystalline cellulose (500 mg.)	50.0	68.0	3.8	0.3	0.3	0.30
Microcrystalline cellulose (500 mg.)	50.0	74.0	2.9	0.8	0.15	0.15

The procedure employed was as in Table IV (for 50 mg. polymer) and in Table III (for 500 mg. polymer), except that the wash procedure was altered. Only buffers near pH 7.0 to 8.0, such as borate and phosphate, were employed. Less than 0.1 per cent of the starting material was recovered in the final 3 washes.

described by Wide⁵ for activation and coupling. The yields, in terms of BSA bound, were small. Consequently, we modified the conditions for activation of the polysaccharides by dissolving cyanogen bromide in DMF as described by Rejnek and associates,¹² and we used polymers such as Sepharose, known to have a high capacity for coupled proteins. As expected, the quantity of protein coupled to Sepharose gels was large as compared to that coupled to Sephadex or cellulose. Increasing the quantity of cyanogen bromide in the activation step had no effect on the amount of protein coupled in one experiment. Sephadex G-100 and G-25SF bound considerably less protein than did Sepharose 4B by factors of 4 and 100, respectively. Comparison of the results in Table III suggests that although the Sepharose polymers have the greatest capacity to bind antigen, it is difficult to wash them free of residual antigen. Thus, for use in a test such as the RAST, the propensity of these Sepharose polymers to trap antigen could be a serious limiting factor in that IgE might be trapped in the interstices of the gels. In the results shown in Table IV we compared a variety of polysaccharides to bind BSA, including ultrafine G-25 (particle size of 1 to 10 μ).⁵ Despite claims to the contrary,⁵ we could not demonstrate the superiority of this material, at least in its ability to bind BSA, and in this experiment our results suggested that microcrystalline cellulose had a greater binding capacity.

In experiments with ragweed AgE the quantity of material coupled was measured by radioimmunoassay. In general the amounts coupled were considerably less than in experiments with BSA. Again, cellulose appeared to have a greater capacity to bind AgE than did Sephadex G-25SF or G-100SF. Comparison of the immunologic reactivities of the AgE-cellulose polymer and AgE IV-C by radioimmunoassay did not reveal a difference in the slopes of the inhibition curves, which suggests that there is no loss of antigenic determinants during the coupling procedure.

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