Purification of Highly Conjugated Precipitins and Globulins Containing Reagins with Silk Hydrolysate

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PURIFICATION OF HIGHLY CONJUGATED PRECIPITINS AND GLOBULINS CONTAINING REAGINS WITH SILK HYDROLYSATE

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Four years ago one of us (BZR) reported that antigen could be identified in epithelial cells and histiocytes of allergic skin by immunofluorescent staining with conjugated human globulins containing reagins obtained from highly sensitive donors (1, 2). Purification by repeated alcohol precipitation (3) used in the original studies was not reproducible, however, with conjugated antibodies derived from less potent antisera.

During the past 4 years we have tried to improve the reproducibility, specificity and sensitivity of staining soluble antigen in tissues. Purification was attempted with the previously reported methods of adsorption (4–6) and with other published techniques (7–9). When effective, these methods purified only conjugates prepared with low proportions of fluorescein isothiocyanate (FITC) to yield low fluorescein/protein (F/P) ratios. The low fluorescence of the purified preparations was inadequate for unequivocal localization of soluble antigen in tissues.

Since adsorption with previously reported powders gave inadequate purification of highly conjugated antibodies, a search for new adsorbents with high affinity for dyes led us to ground human callouses, ground cattle horn, and various natural and synthetic textiles. The most satisfactory results were obtained by adsorption with a hydrolysate of silk. The powder was insoluble in water. Conjugates with high F/P ratios could be reproducibly purified to give only slight staining of epidermal cells and histiocytes in control sections of human skin. This degree of fluorescence was designated as 1+.

Since adsorption with previously reported powders gave inadequate purification of highly conjugated antibodies, a search for new adsorbents with high affinity for dyes led us to ground human callouses, ground cattle horn, and various natural and synthetic textiles. The most satisfactory results were obtained by adsorption with a hydrolysate of silk. The powder was insoluble in water. Conjugates with high F/P ratios could be reproducibly purified to give only slight staining of epidermal cells and histiocytes in control sections of human skin. This degree of fluorescence was designated as 1+.

The globulins from the three human and two rabbit sera were harvested, redissolved in buffered saline (0.15 M NaCl, buffered with 0.01 M phosphate to pH 7.6) and freed of sulfate ions by dialysis at 4°C against buffered saline.

For labeling with FITC the protein concentration of each of the globulins was adjusted to 2.5% (10) and conjugated by the procedure of Marshall et al. (11). Conjugates with dye/protein proportions ranging from 6 to 50 mg of dye/g of protein were prepared and adsorbed with silk hydrolysate before the final selection was made of 18 and 25 mg of FITC/g of protein as the two most suitable preparations.

Free fluorescein was removed from conjugated globulins by passage through a column charged with G-50 Sephadex medium (10). After estimating the fluorescein content and protein concentration by optical density at 490 and 280 μm, respectively, the eluate was lyophilized in small aliquots and stored at −20°C.

2. Preparation of silk hydrolysate and its use for adsorption. Approximately 117 g of untreated...
Purification of precipitins and globulins

Raw silk skeins were heated to boiling in 1.5 L of 20% sodium hydroxide. As the silk was boiled, the fibers swelled to a gelatinous consistency. Boiling with stirring was continued for approximately 1 hr, at which time almost all silk fibers had been reduced to a fine, punctiform material suspended throughout the sodium hydroxide solution. At this point the few remaining gelatinous strands of partially hydrolyzed silk were removed with a glass rod and the sodium hydroxide was quickly neutralized with concentrated hydrochloric acid to prevent further hydrolysis of the silk particles. After cooling in running water, the silk hydrolysate was harvested by filtration through a Büchner funnel on Whatman no. 1 filter paper and washed with distilled water until the washings lost all yellow color and were free of chloride ion.

The washed silk hydrolysate appeared as a grey paste. When oven-dried at 56°C overnight, it became a light, spongy, friable grey mass, about 11% by weight of the original raw silk skeins. Before use, the dried material was crushed with mortar and pestle to a fine white powder. Microscopic examination revealed particles varying in size from about 20 μ to very small particles which passed through a 0.30 μ Millipore filter. No soluble protein was demonstrable by the Biuret method when the hydrolysate was stirred for 24 hr in 1 or 2% NaCl buffered with 0.01 M phosphates to pH 7.6. Solubility of the hydrolysate was obtained when the NaCl concentration was raised to 3%. The ability of the powder to bind dye was much greater than that of powdered liver, lung or cattle horn.

After various amounts of hydrolysate powder had been tried, the proportion chosen was 200 mg/ml of conjugate. Stirring was in the cold for 2 1/2 hr. The aim was to obtain a degree of nonspecific fluorescence rated as 1+ or less in control skin tissue containing no antigen and 3+ in skin challenged by antigen. Adsorption for longer than 2 1/2 hr or with higher proportions of the powder removed nearly all nonspecific and some specific staining. Such preparations were less desirable than those with slight nonspecific but intense specific staining. Centrifugation at 15,000 rpm for 10 min yielded 50% of the original volume. Following centrifugation, all except the finest hydrolysate particles were removed by filtration through a 0.30 μ Millipore filter.

Conjugates prepared with 18 mg FITC/g of protein, with their lower F/P values, could be more completely purified by adsorption with silk hydrolysate. The greater reduction of their nonspecific staining was, however, accompanied by a disproportionate reduction in specific fluorescence. While purified conjugates prepared with 25 mg FITC/g of protein gave slightly more nonspecific staining than those prepared with 18 mg/g of protein, they were more effective for staining antigen in tissues because the increased fluorescence due to the combined specific nonspecific staining compensated for the greater fluorescence of control sections. The preparations made with 25 mg FITC/g of protein were therefore selected for this study.

3. Fractionation of conjugates by gradient elution.

The F/P ratio of a conjugate is the expression of the over-all or average combination of FITC with protein. The ratio represents many gradations of fluorescent coupling within the conjugate, from light to very heavy (12). The conjugate can be subdivided into groups with narrower F/P ranges, each fraction composed of more nearly homogeneously labeled protein molecules, by adsorbing it on diethylaminoethyl (DEAE) or DEAE-Sephadex and eluting various fractions with gradient concentrations of NaCl (13) from 0.0 to 2.5 M NaCl (Table I).

The conjugated reaginic globulins and precipitins against egg-albumin were used for fractionation. As a result of adsorption with silk hydrolysate, three solutions from each of the two conjugates plus the recovered hydrolysate powder were available for study. The three conjugated solutions were: a) the unadsorbed conjugate (C-1), b) the purified fraction resulting from adsorption (C-2) and c) the material adsorbed on the silk hydrolysate after its elution from the powder (C-3).

The conjugated human globulins containing reagins and the rabbit precipitins in 2-ml volumes were each adsorbed with silk hydrolysate. They were then centrifuged at 40,000 rpm for 1 hr at 4°C. The supernatants (C-2) were removed as completely as possible with a fine capillary pipet. The conjugated rabbit precipitins yielded 80% of the original volume. The conjugated globulins

Raw silk skeins were kindly supplied by Mr. R. T. Knopf, president of the Belding-Heminway Co., Putnam, Connecticut.
TABLE I

Fractionation by gradient NaCl concentrations of conjugated anti-egg albumin precipitins and reagins

<table>
<thead>
<tr>
<th>NaCl for Elution</th>
<th>C-1 F/P</th>
<th>C-2 F/P</th>
<th>C-3 F/P</th>
<th>C-4 F/P</th>
<th>C-5 F/P</th>
<th>C-6 F/P</th>
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<tr>
<td></td>
<td>% P</td>
<td>F/P</td>
<td>% P</td>
<td>F/P</td>
<td>% P</td>
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<td>0.0</td>
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<tr>
<td>0.1</td>
<td>4.0</td>
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<td>7.0</td>
<td>2.8</td>
<td>7.7</td>
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</tr>
<tr>
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<td>4.3</td>
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<td>3.8</td>
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<td>6.1</td>
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<td>8.0</td>
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<td>4.4</td>
<td>2.3</td>
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* C-1 = total conjugate; C-2 = supernatant after adsorption; C-3 = conjugate eluted from silk hydrolysate.

It will be noted that none of the eluted fractions of C-2 or C-3 had a fluorescein/protein (F/P) value as high as the over-all F/P ratio of either C-2 or C-3. This may have resulted from admixture of lightly conjugated with more highly conjugated material, thus lowering the F/P ratio of the latter. The arbitrary choice of a 15 ml volume (1 column volume) for elution could have resulted in incomplete removal of lightly conjugated material. The admixture of this with the eluate from the next, more concentrated salt gradient, would lower the F/P ratio of the eluted fraction.

The complicated technical manipulations needed to obtain C-3 fractions may have resulted in some protein denaturation. Its fractional elution differed materially from that of the other two fractions.

containing reagins yielded 70% of the original volume.

To obtain the material adsorbed and retained in the powder (C-3), the silk hydrolysate was washed by gentle suction through a small Büchner funnel with a large volume of 2% NaCl buffered to pH 7.6. A volume of 450 ml was needed before the washings were completely free of fluorescence when examined in the beam of an HBO-200 mercury vapor burner. This end point for determining the removal of all saline soluble dye and protein was more sensitive than were tests for protein.

The 2% saline washings, representing the reagin and precipitin C-3 fractions, were dialyzed to permit shell-freezing and lyophilization for reduction in volume. Each of the two lyophilized fractions was redissolved in a small volume of distilled water and the fluorescein and protein contents were estimated by optical density for the over-all F/P ratios.

The three fractions (C-1, C-2 and C-3) from the conjugated reagins and precipitins were adsorbed on DEAE-Sephadex columns equilibrated with 0.01 M phosphate (pH 7.6). Various NaCl concentrations in gradients from 0.0 to 2.5 M (Table I) were added in 15-ml columns to elute 15-ml fractions. The eluates were lyophilized to permit resolution in 1-ml volumes, and the total protein content, the fluorescein concentration and F/P ratios were estimated by optical density.

The two silk hydrolysate powders were still deeply yellow despite the large volumes of 2% NaCl used for washing and the absence of fluorescence in the final washings. They were strongly fluorescent under the microscope. The materials were lyophilized and extracted for 1 hr in a mechanical shaker with anhydrous acetone to determine if soluble, fluorescent dye could be dissociated by the treatment. No fluorescent material was obtainable by acetone extraction.

4. Skin used for staining. The marked affinity for acid dyes of epidermal cells, especially of the cornified layer, and of histiocytes made it more difficult to remove nonspecific staining properties from conjugates used on skin than on lung tissue of guinea pigs or on nasal mucosa of...
humans (14). Purified conjugates which gave little nonspecific staining of guinea pig lungs or of human nasal mucosa still gave a 1+ degree of nonspecific fluorescence of epithelial cells and histiocytes of skin.

Skin biopsies were obtained with a motor driven punch without local anesthesia as previously described (1) from six ragweed sensitive patients of whom, in addition, two were sensitive to egg albumin and one to cottonseed. Three of the six were tested by scratch method with ragweed pollen extract, the two sensitive to egg albumin with this antigen and the patient sensitive to cottonseed with the principal allergen of this material. In addition, a scratch test was made with 1:1000 histamine diphosphate on each patient as a control. The biopsies were obtained 20 min after the scratch tests.

Immediately after biopsy each tissue was frozen at -160°C in isopentane, cooled in liquid nitrogen to -170°C and dried in vacuo (15, 16). The dehydrated tissues were infiltrated with diethylene glycol distearate (17) and stored at -20°C. Sections, cut at 4 μm, were postfixed by floating for 1 min in 1% formaldehyde diluted by an equal volume of dioxane (3) and transferred to a drop of distilled water on a clean slide. Excess water was quickly removed with filter paper, and adherence to the slide was facilitated by pressure with a rubber-covered finger. The diethylene glycol distearate was dissolved in petroleum ether immediately before the tissues were covered with experimental reagents.

5. Immunofluorescent staining. Four to six sections from each control and its related antigen-challenged tissue were mounted on the same slide in parallel rows. One set of slides was stained with purified, conjugated precipitins and the other set with the related, conjugated globulins containing reagins. Similarly, both reagin and rabbit antibody globulins in a 2% solution were used for inhibition of staining with rabbit and reaginic conjugates. Cross-inhibition (rabbit anti-egg and anti-ragweed antibodies to inhibit staining with globulins containing the related reagins and vice versa) was likewise done.

A generous amount of the principal allergen in cottonseed, CS-1A, was supplied through the kindness of E. J. Coulson, Ph.D., the Allergen Research Division, Bureau of Agricultural and Industrial Chemistry, United States Department of Agriculture, Washington, D.C.

The skin challenged with cottonseed was studied with reagins only. After staining for 20 min the sections were washed for 10 min in three changes of buffered saline, dehydrated and mounted with Fluormount.

The specificity of staining was demonstrated by the following: a) the staining of the control, histamine challenged skin which contained no antigen; b) the use of histamine-challenged allergic human skin for in vitro coating with related and unrelated antigen before staining; c) staining antigen-challenged tissues with antibody not related to the antigen used; d) inhibition of staining by the commonly used classical methods (4).

Inhibition of staining with unconjugated rabbit and human antibody globulins was at room temperature for 1 hr before staining for 20 min. Globulins containing reagins, heated for 4 hr at 56°C and rabbit precipitins from which antibody had been removed by addition of specific antigen, were used to demonstrate that no inhibition of staining occurred after such treatment. Unheated reaginic globulin against an unrelated allergen was added to the heated reaginic globulins to determine if inhibition properties could be restored. Failure to produce staining inhibition by antibodies not related to the antigen in the tissues was another control measure to demonstrate the specificity of fluorescence.

Work now in progress on the localization of antigen in actively sensitized monkeys indicates that control skin of normal human beings obtained after scratch test with antigen was not essential. Before monkeys are sensitized, skin obtained after scratch test with the antigen to be used fails to show the presence of antigen in the epidermis or dermis. After sensitization an immediate wheal type reaction can be produced by scratch test with the specific antigen; and the localization of the antigen is the same as in allergic human skin tested with antigen by the scratch method.

6. Optics and photography. A Leitz Ortholox microscope equipped with a dark-field condenser and an HBO-200 mercury vapor burner was used. The filters used for viewing sections were a BG 38 heat-absorbing filter, a fluted R diffusion filter (Leitz), a 2-mm UGI as a primary excitor filter and a Wratten 2A as a secondary barrier filter. For photography, the UGI filter was replaced by the Corning 5970 to screen out the arti-
fact haloes resulting from light reflection around the fine silk hydrolysate particles remaining in the conjugate. Kodak spectroscopic plates (103a-G), used in our previous studies (1, 2), were likewise used in this study.

RESULTS

Gradient fractionation with NaCl. Table I and Graphs 1a and 1b and 2a and 2b show the results of fractionation with NaCl concentrations from 0.0 to 2.5 M. It will be noted that the over-all F/P ratios of the C-2 fractions is only slightly lower than the parent C-1 fractions: 12.7% lower in the reaginic globulins and 17.5% lower in the precipitins. The reduction in the F/P ratios after adsorption with silk hydrolysate was even less in some of the other preparations that were studied. An occasional conjugate showed no change in the F/P ratio after adsorption, despite a striking reduction in nonspecific staining.

The reduction in nonspecific staining in the purified (C-2) fractions is not explainable by the assumption that highly conjugated material was removed from C-1 by the silk hydrolysate (Table I and Graphs 1a and 1b). The over-all F/P ratios of the removed materials (C-3 fractions) differed even less from the parent C-1 conjugates than did the C-2 fractions.

The appearance of the used powder suggested a clue for the marked reduction in nonspecific staining properties of adsorbed conjugates. After washing the silk hydrolysate with large volumes of 2% NaCl to remove all traces of soluble fluorescent material and protein, the powder was still deeply yellow and highly fluorescent. Since all soluble, fluorescent material and more than 95% of the protein contained in the parent conjugates had been recovered in the C-2 and C-3 fractions, two possible explanations suggest themselves for the fluorescence of the used silk hydrolysate and reduction in nonspecific staining. Either a very small amount of highly conjugated protein was firmly adsorbed on the hydrolysate, or noncovalently bound FITC had been removed from the conjugate and was bound to the many free amine groups of the hydrolysate. So firmly bound to the hydrolysate was the fluorescent material that it could not be removed either by saline washings or by vigorous extraction of the dried hydrolysate with a large volume of anhydrous acetone.

The antibody titer after adsorption with silk hydrolysate. The total antibody N/ml of a 1% globulin solution of rabbit anti-egg albumin was determined at the equivalent point before and after conjugation and after adsorption with silk hydrolysate at 4°C for periods up to 24 hr. Before conjugation the antibody N content was 1.45 mg/ml of 1% globulin; after conjugation, 1.15 mg/ml; after adsorption for 24 hr, 1.0 mg/ml. Adsorption of this and of the other two precipitin conjugates for longer periods showed no appreciable reduction in precipitin titers during the first 4 hr of stirring at 4°C. Thereafter, the precipitin titer dropped sharply.

Staining of allergic human skin

Histamine control and antigen-challenged. The control tissues from the histamine-challenged allergic skin areas showed a 1+ degree of nonspecific fluorescence of epithelial cells and histiocytes (Fig. 1). The tissues challenged with ragweed extract, with egg albumin and with cottonseed protein showed a 3+ fluorescence of the various epithelial elements, of histiocytes and pericapillary cells (pericytes). Histiocytes in the superficial part of the dermis stained more intensely than those in the deep dermis. Less intense staining was present in fibrocytes, ground substance and endothelial cells (Fig. 2). The multiplicity of the structures stained gave the tissue an appearance of general fluorescence, compared to the histamine control sections. Intensity of fluorescence was nearly as great after staining with conjugated globulins containing reagins as after staining with conjugated related precipitins. Localization of staining in antigen-challenged tissues was the same with reagin and precipitin conjugates (Figs. 2 and 3).

Specificity of immunofluorescence was demonstrated by the methods mentioned previously. While complete inhibition of staining was not attained, approximately a 75% degree of inhibition was noted with either unconjugated reaginic globulins or with rabbit precipitins (Figs. 4 and 5). Precoating with the unconjugated globulins was followed by staining with either precipitin or reagin conjugates. No inhibition of staining was observed when the tissues were precoated with unrelated, unconjugated antibody, with heated reaginic globulins or with precipitins from which antibodies had been removed with specific antigen (Figs. 6 and 7). Addition of unheated, unrelated reaginic globulins
Graph 1a - F/P Ratios of Eluted Fractions

Graph 1b - Percentage of Protein Eluted in Fractions

Graph 2a - F/P Ratios of Eluted Fractions

Graph 2b - Percentage of Protein Eluted in Fractions

Graphs 1 and 2. The results of fractional elution, shown in Table I, are graphically reproduced in 1a and 2a. Note the similarity of the F/P ratios obtained with any given salt concentration from the unfractionated material (C-1), the purified material (C-2) and the material eluted from the hydrolysate (C-3).

Graphs 1b and 2b represent the percentage of protein contained in each of the eluted fractions.
to the heated unconjugated human antibody globulins did not result in inhibition of staining.

Control allergic skin histamine-challenged coated with related and unrelated antigen. On each slide the top row of sections were coated for 20 min with a 1:2000 solution of the antigen related to the antibody and the bottom row with a 1:2000 solution of an unrelated antigen. The tissues from patients sensitive to egg albumin and to ragweed antigen were stained with their related conjugated reaginic globulins and conjugated precipitins, while those from the cottonseed patient were stained with conjugated anti-cottonseed reaginic globulins only.
Figure 5. Inhibition of staining in the skin of an egg albumin sensitive patient challenged with egg albumin. The tissue was coated with unconjugated rabbit anti-egg albumin precipitins and stained with conjugated rabbit anti-egg albumin precipitins. Note the similarity of inhibition resulting from reagins (Fig. 1) and from precipitins (Fig. 5) (X 300).

Figure 6. Failure of inhibition by reaginic globulins heated to 56°C for 4 hr is demonstrated in the antigen-challenged skin of an egg albumin sensitive patient by staining with conjugated specific reagins after coating the section for 1 hr with the heated unconjugated globulins containing reagins (X 300).

Figure 7. Failure of inhibition in a section from the same tissues as in Figure 6 by unconjugated rabbit anti-egg albumin precipitins from which antibodies had been removed with specific antigen. Note that, as in Figure 6, no inhibition occurred (X 300).

Figure 8. Histamine-challenged skin from an egg albumin sensitive patient. The sections were coated with 1:2000 solution of egg albumin and stained with anti-egg conjugated reaginic globulins. Note that the intensity of staining is less than that seen in skin challenged in vivo (Fig. 2). Specific staining is present in epithelial cells, in histiocytes (h) and in pericytes (p). It is not demonstrable in capillary endothelial cells or fibrocytes (X 300).

Like the uncoated, histamine-challenged sections from allergic skin, those coated in vitro with unrelated antigens showed only one plus fluorescence of epithelial cells and histiocytes. The sections from such tissues coated with the antigen to which the patient was sensitive when stained with either conjugated related reaginic globulins or precipitins showed 2+ staining of cells and histiocytes. Unlike skin challenged in vivo with antigen, in vitro coated sections showed
no staining of fibrocytes, endothelial cells or ground substance (Fig. 8). And, as previously reported (2, 14), intensity of fluorescence was less in tissues challenged with antigen in vitro than in those challenged with antigen in vivo.

Inhibition of staining with either reagins or precipitins related to the antigen used for coating was effective whether conjugated globulins containing reagins or precipitins were used for staining. Failure to inhibit staining occurred with antibody not related to the antigen used, with heated reaginic globulins or with rabbit antibody globulins from which precipitins had been removed by antigen.

**DISCUSSION**

Two properties account for the high dye-binding characteristics of silk hydrolysate. The reduction of the material to such minute particles that the smallest could not be removed by filtration through a 0.30 μ Millipore filter greatly increased the available adsorbing surface area over any other adsorbent; and hydrolysis of the silk exposed many more reactive amine groups to combine with the FITC than are available in native proteins used for adsorption.

Conjugates with F/P values as high as 12.5 × 10⁻³, when purified, had variable F/P ratios. In some conjugates little or no reduction occurred in the F/P ratios. More frequently, the F/P ratios were reduced from the original 12.5 value to 9 × 10⁻³.

Despite the high F/P ratios of such purified conjugates, they could be used at protein concentrations of 10 mg/ml for staining soluble antigen in tissues. Such purified conjugates yielded a 1 + nonspecific staining in control allergic tissues and a 3 + specific staining in tissues challenged in vivo with antigen.

Purified conjugates with high F/P ratios are more satisfactory than those with low F/P ratios for the localization of soluble antigen in tissues. In a recent thorough study, Goldstein et al. (10) advised the use of 6 to 8 mg FITC/g of protein for conjugation. They purified these conjugates by gradient elution through DEAE-cellulose columns (13). The F/P values of the staining solutions ranged between 2 and 3.5 × 10⁻³ and staining was done with less than 2 mg protein/ml, a fifth of the protein concentration used in our studies. When attempts were made to use conjugates prepared by these methods for staining the tissues in the present study, the specific fluorescence was too low for satisfactory localization of antigen.

While the best results in this study on 2.5% β conjugated globulins were obtained by stirring for 212 hr with 200 mg of hydrolysate/ml of conjugate, the procedure advisable for any new conjugate with an F/P ratio between 8 and 12 × 10⁻³ is to adsorb small aliquots in an ice bath for 212 hr with 150 and 200 mg hydrolysate. Staining of control and antigen-challenged tissues with the purified samples will permit the selection of the optimum proportion of powder to be used on the total preparation. Either 1-ml amounts may then be adsorbed with the selected proportion of powder immediately before staining or the total conjugate may be adsorbed and divided into small aliquots of about 0.2 ml for lyophilization and storage at −20°C. Thus far, purified, lyophilized conjugates have been stored at −20°C for over 3 months without change in staining properties. Further experience is necessary to determine the desirability of longer storage for purified, lyophilized conjugates.

The insolubility of the silk hydrolysate in 2% NaCl permitted study of the effect of adsorption on reducing nonspecific staining of conjugates. The reduction in nonspecific staining probably was due primarily to the removal of loosely bound dye from the conjugated proteins. Since this dye cannot be removed from the conjugate by prolonged dialysis or by adsorption on Sephadex, it seems probable that it represents a portion of FITC which was not covalently conjugated. The dye in such loosely bound conjugates is attracted by the positively charged radicals in tissue proteins more strongly than by the covalent protein-dye molecules of the conjugate and results in nonspecific staining. The free amine groups of the silk hydrolysate offer strong competition to the covalent, FITC-saturated groups of the serum globulins for the noneovaletly bound dye. Their removal by the hydrolysate reduces the nonspecific staining properties of the conjugate.

The specific staining of antibody-bound antigen in epithelial cells of allergic human skin is confirmed in this study. The presence of antibody in epithelial cells is not the result of its overflow and adsorption into the cells during the antigen-antibody reaction, since antibody is demonstrable in epithelial cells of allergic human skin after in
**in vitro** application of antigen to tissue which had not been previously challenged. In another study (14) localization of antibody-bound antigen was demonstrated in the surface epithelial and gland cells of the allergic human nasal mucosa. A question still to be answered is whether antibody is present in other epithelial cells covering other surfaces which separate tissues from the external environment.

The similarity in staining intensity and in inhibition effectiveness of human reagins and rabbit precipitins raises an important question which is not answered in this work. The conjugated rabbit anti-egg precipitins contained 1 mg antibody nitrogen/ml of 1% globulin solution. The human anti-egg globulins containing reagins presumably contained no more than 0.1 µg of antibody nitrogen/ml of serum (18-20). Nevertheless, the staining intensity of conjugated precipitins and the inhibition capacity of unconjugated reaginic globulins and precipitins were similar. This was true in both anti-ragweed and anti-egg reagins and precipitins. It appears that the relative titers of reagins and precipitins cannot be assessed by the intensity of their fluorescent staining.

It is noteworthy that the staining results, indicating the presence of antibody in epithelial cells and in other tissue elements, were obtained with both reaginic and precipitin antibodies in an inhalant case (ragweed), in an ingestant case (egg albumin) and in a case whose symptoms were due to either ingestion or inhalation of the allergen (cottonseed).

**SUMMARY**

Highly conjugated human globulins containing reaginic antibodies against egg albumin, ragweed pollen and cottonseed and conjugated rabbit precipitins against egg albumin and ragweed pollen were purified by adsorption with silk hydrolysate. The purified antibodies were still highly conjugated and gave satisfactory specific staining of antigen in allergic human skin. The reduction in nonspecific staining was probably due to adsorption of noncovalently bound dye by the silk hydrolysate.

The presence of antibody-bound antigen in epithelial cells of challenged, allergic skin was confirmed. The epithelial cells of histamine-challenged, allergic skin were capable of binding specific antigen when this was supplied **in vitro**.

The human reaginic globulins and the rabbit precipitins gave similarly effective staining and inhibition results and the localization of antigen demonstrable with either antibody was the same.

**REFERENCES**