



## The Chemistry of Allergens

### XVII. Disc Electrophoresis and Gel Diffusion of the Carbohydrate-Free Allergenic Protein, CB-65A, from Castor Beans<sup>1,2</sup>

R. S. MORRIS, JOSEPH R. SPIES, AND E. J. COULSON

*Allergens Laboratory, Eastern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture, Washington, D.C.*

The carbohydrate-free protein mixture, CB-65A, which contains the principal allergen(s) of castor beans, was resolved into four bands by disc electrophoresis on a 7.5% polyacrylamide gel. Gel diffusion analysis revealed that these four bands all contained antigens with identical antigenic specificities.

The castor bean allergenic protein fraction, CB-1A, is a complex mixture of proteins and proteins combined with varying amounts of polysaccharidic carbohydrate (1, 2). Chemically distinct components of CB-1A contain a major common or identical antigenic specificity (3). This could be due to both (a) active protein combined with varying proportions of polysaccharidic carbohydrate and (b) proteins with structural differences too slight to impart distinct specificities. There is no evidence to distinguish unequivocally between these possibilities (3).

CB-65A, the carbohydrate-free allergenic protein mixture isolated from CB-1A, retains both the allergenic and the antigenic specificities of CB-1A (2, 4). Layton *et al.* (5) separated CB-65A into five bands by paper-strip electrophoresis. All five bands elicited skin reactions in castor bean-sensitive individuals. No evidence was presented as to whether each band contained a distinct specificity.

<sup>1</sup> Paper XVI of this series: SPIES, J. R., AND COULSON, E. J., *J. Biol. Chem.* **239**, 1818 (1964).

<sup>2</sup> Presented in part at the 48th annual meeting of the Federation of American Societies for Experimental Biology, Chicago, Illinois, April 12-17, 1964.

Spies and Coulson (3) found that the electrophoretogram of CB-65A had four bands while the immunoelectrophoretogram and gel double-diffusion pattern each had three bands or groups of compounds. Available evidence did not permit conclusions regarding the specificity relationships of the components of CB-65A.

Superior resolution of protein mixtures can often be achieved by electrophoresis on polyacrylamide gels. This paper describes disc electrophoresis of CB-65A and determination of the specificity relationships of the resolved components by gel double-diffusion analysis.

#### PROCEDURE

*CB-65A* was prepared from CB-1A by a prolonged procedure consisting of the formation of the picrate of CB-1A, the chromatographic adsorption of the picrate, the electrophoretic recovery of the protein from the picrate, high voltage electrophoresis of the protein, and solvent fractionation (2).

*CB-1A rabbit antiserum.* Rabbits were immunized to CB-1A by a series of inoculations of CB-1A in Freund's complete adjuvant as described for the preparation of CB-13E rabbit antiserum (6).

*Disc electrophoresis.* The method for disc electrophoresis developed by Ornstein and Davis (7) was used. Stock solutions were obtained from

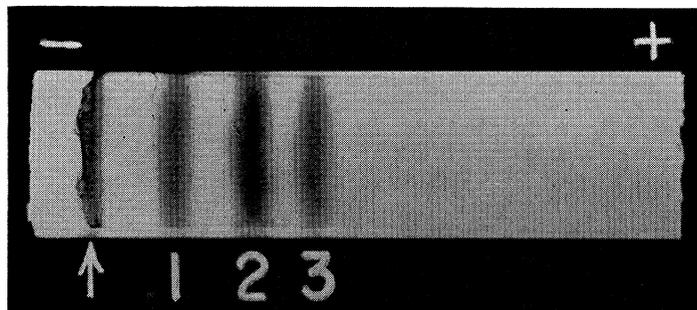


FIG. 1. Disc electrophoresis of CB-65A. Running gel, 7.5% polyacrylamide; electrode buffer, Tris-glycine, pH 8.3,  $\Gamma/2$  0.01.

Canal Industrial Inc., Bethesda, Maryland.<sup>3</sup> The gel column (5 mm i.d.  $\times$  65 mm) contained 0.75 ml of 7.5% polyacrylamide running gel, 0.16 ml of 2.5% polyacrylamide spacer gel, and 0.16 ml of 2.5% polyacrylamide gel containing 76  $\mu$ g of CB-65A. Both electrode vessels contained glycine (0.29%)-2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) (0.06%), pH 8.3. Electrophoresis was conducted at room temperature in a four position apparatus with platinum electrodes for 1 hour at 135 V and a constant current of 2.5 ma per column.

The gel cylinder was removed from the column and stained in 1% Amido Schwarz in 7% acetic acid. The gel was washed in 80-ml portions of 7.5% acetic acid for successive periods of 1, 16, 6, 2, and 16 hours to remove excess dye. The time intervals were chosen solely for convenience. The dye-protein complex is immobile in acidic solution. Fixed protein does not diffuse from the gel during destaining. It is unlikely that the relatively mild washing with acetic acid had any denaturing or inactivating effect on the antigenic components, as the castor bean allergen is extremely stable. For example, no loss of precipitating capacity of CB-1A occurred on heating at 110° at pH 5.9 for 1 hour (8). Heating CB-1A at 100° for 32 hours at pH 4.0 did not destroy all of its precipitating capacity or reagin neutralizing capacity (6). Furthermore, CB-1A retains precipitating capacity after gel filtration in 1 *N* acetic acid.

Alternatively, the gel cylinders can be destained electrophoretically. The procedure is not always optional. Johnson *et al.* (9) found that certain histone fractions had no bands when destained electrophoretically but had several bands when destained by solvent extraction; they attributed this to the removal of smaller constituents during electrophoretic destaining.

<sup>3</sup> The use of a trade name, distributor, or manufacturer is for identification only and implies no endorsement of the product or its manufacturer.

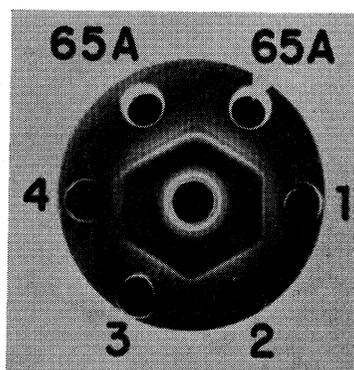


FIG. 2. Gel double diffusion of components of CB-65A. CB-1A rabbit antiserum is in the central well.

To perform gel diffusion analysis of stained discs, it is essential to remove excess acetic acid from the disc. Excess acetic acid causes nonspecific precipitation of antiserum in the Ouchterlony plate. Furthermore, the dye-antigen complex does not dissociate in acid solution. Each of the stained discs was cut from the gel cylinder. To leach out excess acetic acid, the discs were washed for 17 hours in 20 ml of distilled water containing toluene as a preservative. The water was decanted, and a fresh 20-ml portion of distilled water was added to each disc. The discs were washed for an additional 4 hours. The pH of the final wash solution was 5.2. The dye-protein complex does not diffuse out of the discs under acidic conditions; this was shown by the absence of dye from the wash water and the absence of fading of the discs.

*Gel double diffusion.*<sup>4</sup> The discs were analyzed by the Ouchterlony technique (10). The bottom of a 60-mm diameter Petri dish was coated with

<sup>4</sup> This procedure for gel diffusion analysis of stained discs has also been successfully applied to bovine serum albumin and may be useful with other antigens as well.

silicone. Then 8 ml of molten 0.5% Oxoid, Ionagar No. 2 in pH 8.6 barbitone acetate buffer (11), was added to the plate. Dehydrated Oxoid barbitone acetate buffer (Consolidated Laboratories, Inc., Chicago Heights, Illinois) was reconstituted by adding 8.8 gm of granules to 1 liter of distilled water; merthiolate (0.02%) was added as a preservative.

The Ouchterlony pattern was formed with a Feinberg agar cutter (Consolidated Laboratories, Inc.).<sup>3</sup> CB-1A rabbit antiserum was added to the central well and allowed to diffuse into the agar for 21 hours. The stained discs were then placed into four of the outer wells, and these wells were filled with barbitone acetate buffer. CB-65A (0.0065 mg N per milliliter) was added to two adjacent wells. Diffusion proceeded at  $24^{\circ} \pm 1^{\circ}$ . The patterns were observed daily and were photographed on the fifth day.

#### RESULTS

CB-65A was resolved into four bands by electrophoresis on 7.5% polyacrylamide gel (Fig. 1). Three intense bands and one faint band were located 5-6, 9-11, 13-14.5 and 17-18 mm, respectively, from the cathode end of the gel. The bromophenol blue "tracking dye" migrated 31.5 mm.

Figure 2 shows the gel diffusion pattern of the four bands obtained from CB-65A and of the original CB-65A. The single line of precipitate from each disc joined in a reaction of identity; the ends of this line joined with the major inner line of precipitate of the original CB-65A. Band 1 had a second line of precipitate which joined with the outer line of CB-65A. Additional lines of precipitate might have been obtained from the other bands if sufficient CB-65A had been available.

#### DISCUSSION

The results of the present study demonstrate unequivocally that electrophoretically

separable components of the carbohydrate-free allergenic protein mixture, CB-65A, have an identical or closely similar antigenic specificity. This is attributed to proteins with structural differences too slight to impart immunological distinctness. In view of these results and results described previously (3), demonstration of inherent antigenic or allergenic properties in chemically distinct components of CB-1A, either protein or polysaccharidic protein, cannot be taken as evidence of distinct antigenic or allergenic specificities.

#### REFERENCES

1. SPIES, J. R., AND COULSON, E. J., *J. Am. Chem. Soc.* **65**, 1720 (1943).
2. SPIES, J. R., COULSON, E. J., CHAMBERS, D. C., BERNTON, H. S., AND STEVENS, H., *J. Am. Chem. Soc.* **66**, 748 (1944).
3. SPIES, J. R., AND COULSON, E. J., *J. Biol. Chem.* **239**, 1818 (1964).
4. COULSON, E. J., SPIES, J. R., STEVENS, H., AND SHIMP, J. H., *J. Allergy* **21**, 34 (1950).
5. LAYTON, L. L., GREENE, F. C., DEEDS, F., AND GREENE, T. W., *Am. J. Hyg.* **75**, 282 (1962).
6. SPIES, J. R., COULSON, E. J., BERNTON, H. S., STEVENS, H., AND STRAUSS, A. A., *Ann. Allergy* **18**, 393 (1960).
7. ORNSTEIN, L., AND DAVIS, B. J., "Disc Electrophoresis." Preprinted by Distillation Products Industries, Rochester, N. Y. (1962).
8. SPIES, J. R., COULSON, E. J., BERNTON, H. S., WELLS, P. A., AND STEVENS, H., *J. Agr. Food Chem.* **10**, 140 (1962).
9. JOHNSON, L. D., DRIEDGER, A., AND MARKO, A. M., *Can. J. Biochem.* **42**, 795 (1964).
10. OUCHTERLONY, O., "Diffusion-in-Gel Methods for Immunological Analysis. Part II," *Progr. Allergy* **6**, 30 (1962).
11. OWENS, J. A., *Analyst* **81**, 26 (1964).