[CONTRIBUTION FROM ALLERGEN INVESTIGATIONS, BUREAU OF AGRICULTURAL CHEMISTRY AND ENGINEERING, U. S. DEPARTMENT OF AGRICULTURE, AND THE ALLERGY CLINIC OF PROVIDENCE HOSPITAL, WASHINGTON, D. C.]

The Chemistry of Allergens. VI. Chemical Composition and Properties of an Active Carbohydrate-free Protein from Cottonseed*,[1]

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The importance of the role of carbohydrates in immunological specificity has increased since Heidelberger and Avery[2] first showed that polysaccharides determine the specificity of the pneumococcus organism. The possible analogous relationship of polysaccharides to allergenic specificity has made it seem worth while to record properties characterizing a carbohydrate-free allergenic protein fraction from cottonseed,[3]

Allergenic protein-polysaccharidic fractions containing less than 1% carbohydrate were described in a previous paper.[4] Some of these fractions which contained 0.9 to 3.0% carbohydrate were subjected to further electrophoresis to remove the carbohydrate. The material which migrated toward the cathode was used as starting product in three successive electrophoretic fractionations. The final cathodic fraction, CS-60C, was essentially free from carbohydrate as shown by chemical tests.[5]

Experimental

Apparatus.—Electrophoresis apparatus similar to that described and illustrated (Fig. 1) in the fourth article of this series[4] was employed, except that 125, 50 or 25 ml. cells were used, depending on the quantity of material to be fractionated. Side tubes were 10 mm. in outside diameter. Connection between cells was made with gum rubber tubing having 3 mm. wall thickness. *Danger of the current becoming grounded between the glass side-arm and the rubber connection was eliminated by coating the glass with petrolatum before sliding on the rubber tubing.*

Preparation of CS-60C.—Preliminary electrophoresis of previously described fractions[4] consisting of 1.0 g. of CS-51R, 1.5 g. CS-52R and 7.0 g. of CS-53R, which contained 0.9, 0.9, and 3.0 per cent. carbohydrate, respectively, was made in a 7-cell apparatus. The allergenic solution was placed in the center cell and an equal volume of distilled water was placed in each of the other cells. Electrophoresis was conducted with voltages ranging from 2500 to 5000 for six days. A general technique similar to that previously detailed[4] was used. The final *p*H in the cells ranged from 2.8 at the anode to 11.0 at the cathode. The substance which collected in cathodic cells having *p*H values higher than 6.5 was separated and isolated, as previously described, by alcohol precipitation. A combined total of 2.68 g. of solid was obtained in the first run. This solid was dissolved in 25 ml. of water and the solution was filtered from a slight amount of insoluble precipitate. The solution was placed in cell 2+ (25-ml. vol.) of a 6-cell apparatus and subjected to a second electrophoresis, using from 2500 to 5000 v. for five days. The *p*H in the cells ranged from 5.28 at the anode to 11.2 at the cathode. The substance which collected in cathodic cells having *p*H values from 7.69 to 11.0 was isolated by alcohol precipitation. The 563 mg. of solid thus obtained was dissolved in 25 ml. of distilled water and the solution (*p*H 9.4) was placed in cell 2+ of the 6-cell (25-ml. vol.) apparatus. After electrophoresis at 2500 v. for one day the solution in cell − was replaced with 25 ml. of distilled water. Electrophoresis was continued at 5000 v. for three days. The *p*H in the cells ranged from 4.49 at the anode to 10.6 at the cathode. Solutions in cells −, 1−, and 2− (*p*H, 7.52, 8.81 and 10.6, respectively) were combined and concentrated to 30 ml. in a vacuum desiccator over phosphorus

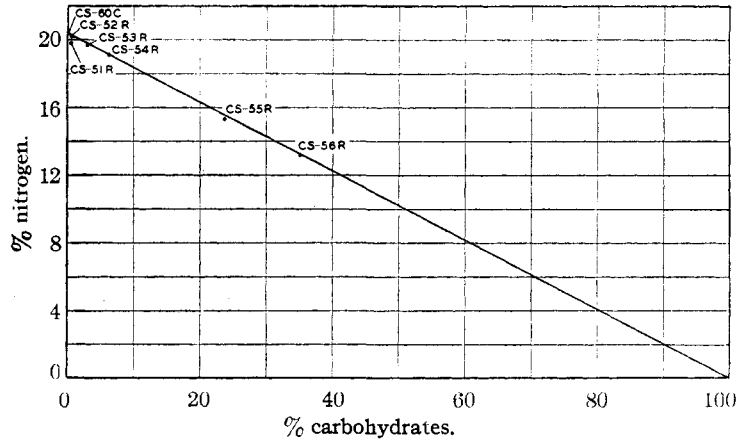

Fig. 1.—Curve showing the relationship between carbohydrate and nitrogen contents of allergenic fractions obtained by the electrophoretic fractionation of CS-1A.[4]

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(1) For Paper V of this series see Spies, THIS JOURNAL, **63**, 2994 (1941).

(2) Heidelberger and Avery, *J. Exptl. Med.*, **38**, 73 (1923).

(3) The possible non-protein nature of allergens is discussed by Coca, Walzer and Thommen, "Asthma and Hay Fever in Theory and Practice," Charles C. Thomas, Baltimore, 1931, pp. 734 *et seq.*; also Vaughan, "Practice of Allergy," C. V. Mosby Co., St. Louis, Mo., 1939, p. 607.

(4) Spies, Bernton and Stevens, THIS JOURNAL, **63**, 2163 (1941).

(5) A Molisch test made on a 1% solution of CS-60C was negative. A control test using an equal volume of a 0.002% solution of galactose was distinctly positive by comparison. Using the orcinol method [Heidelberger and Kendall, *J. Immunol.*, **30**, 267 (1936)] 0.2% carbohydrate was indicated in CS-60C. This value is so near the lower limit of the method that it is indecisive and may be due to "blank."

pentoxide. The clear solution was then filtered through a porous platinum filter to avoid contamination with filter paper hairs. The colorless solution was then frozen in a 250-ml. conical centrifuge cup. After freezing the ice was dissolved by addition of 120 ml. of absolute ethanol and the suspension was stirred gently. This procedure eliminated the *p*H adjustment required to effect precipitation of the protein when its solution was poured into alcohol. The suspension was then centrifuged and the solid was washed with two 25-ml. portions of cold 80% ethanol. The white solid, designated as CS-60C, was dried in a vacuum over phosphorus pentoxide. A yield of 204 mg. was obtained. CS-60C was ground to a powder and equilibrated with air before analysis. CS-60C was completely soluble in water and gave protein color tests like those described in previous publications for the precursor fractions.

Discussion

The chemical composition and some properties of CS-60C are shown in Table I. Carbon and hydrogen content were of the usual order of magnitude encountered in proteins. The nitrogen content of CS-60C was higher than that found in most proteins, owing to the relatively large proportion of arginine present in cottonseed allergenic fractions.[1] Fraction CS-60C had a levo optical rotation of 140.

Substantiating evidence for the absence of carbohydrate in fraction CS-60C is contained in Fig. 1 where carbohydrate contents of the previously described[4] electrophoretic fractions CS-51R, CS-52R, CS-53R, CS-54R, CS-55R, and CS-56R are plotted as abscissas against their nitrogen contents as ordinates. A straight line starting on the abscissa at 100% carbohydrate passed through these points and intersected the ordinate representing zero per cent. carbohydrate at 20.4 which is the percentage of nitrogen actually found in CS-60C.

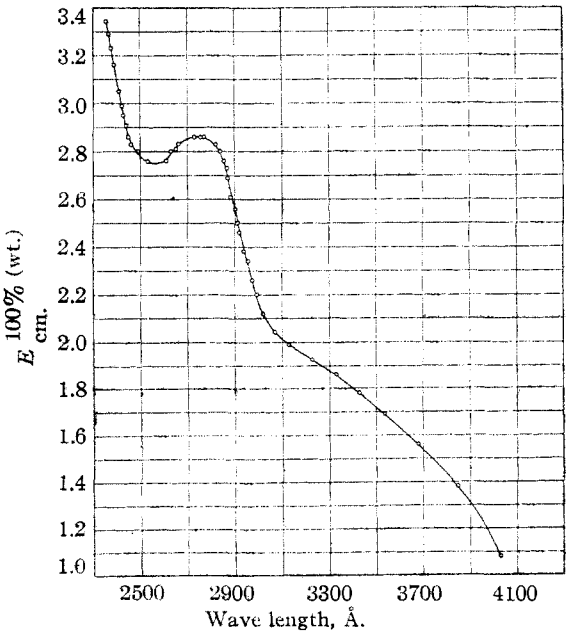

Fig. 2.—Ultraviolet absorption curve of CS-60C in water (concentration 0.413%).

TABLE I

CHEMICAL COMPOSITION AND PROPERTIES OF CS-60C[a]

Nitrogen	20.4
Nitrogen pptd. by 5% trichloroacetic acid [20 ± 0.1°][b]	86.6
Carbon	48.2
Hydrogen	6.58
Sulfur	2.35
$[\alpha]^{20}D$ 1% water solution	−140[c]
$[\alpha]^{20}D$ 2% water solution	−135

[a] All analyses are expressed on an ash and water-free percentage basis. CS-60C contained 0.58% ash and 7.94% water. Analyses were made by the micro methods of Pregl. [b] *Cf.* Paper IV, of this series, Table I.[4] [c] Optical rotation was kindly determined by Dr. E. Yanovsky of the Bureau of Agricultural Chemistry and Engineering.

CS-60C was further characterized by determination of the ultraviolet absorption curve,[6] Fig. 2. An absorption maximum occurred at 2750 Å. which coincides with that found for tyrosine.[7] The fractions from which CS-60C was obtained contained approximately 5% tyrosine.[1] No absorption maximum corresponding to that of phenylalanine was found.[7] The ultraviolet absorption of CS-60C corresponds in general to that which would be predicted from its composition.[1,8]

The solubility curve of CS-60C, Fig. 3, indicated that it was a solid solution.[9]

Whether this protein fraction was a mixture of allergenic components, closely related structurally, or consisted of a single active constituent associated with inactive contaminant cannot be decided from present evidence. However, in view of the variety and drastic nature of the processes used to isolate CS-60C, it seemed unlikely that a small proportion of active substance could have persistently remained associated with a preponderance of inactive material. It seemed more probable that CS-60C represented a mixture of proteins whose structural variations were too slight to permit effective chemical fractionation

(6) The authors are indebted to Dr. P. A. Cole of the National Institute of Health for determination of the ultraviolet absorption curve.

(7) Smith, *Proc. Roy. Soc.* (London), **B104**, 198 1929).

(8) *Cf.* Schmidt, "Chemistry of Amino Acids and Proteins," Charles C. Thomas, Baltimore, Md., 1938, pp. 552 *et seq.*

(9) For comparison the solubility curve of a precursor fraction CS-51R, is included in Fig. 3.

or perhaps too slight to impart immunological identity even if they could be separated.

Fraction CS-60C was antigenic as demonstrated by the property of producing anaphylactic sensitivity and shock in guinea pigs.[10]

The threshold quantity of CS-60C required to incite passive transfer reactions is shown in Table II. These results show that 1×10^{-9} g. of CS-60C was capable of producing positive reactions using a serum of moderate potency.[11]

TABLE II

THRESHOLD QUANTITY OF CS-60C REQUIRED TO PRODUCE POSITIVE PASSIVE TRANSFER REACTIONS WITH SERUM FROM A COTTONSEED SENSITIVE PATIENT[a]

CS-60C Injected,[b] micrograms	Recipients[c] T.W.	N.W.	H.B.
1	19 × 17		
0.1	15 × 15	15 × 13	12 × 15
.01	12 × 14	11 × 11	9 × 11
.001	6 × 7	10 × 10	0
.0001	0	±	0
.00001	..	0	..

[a] This serum (E.S.) gave positive passive transfer reactions to cottonseed allergen when diluted 1:10 and in one case 1:10^2. *Cf.* Coca and Grove, *J. Immunol.*, **10**, 445 (1925); also Levine and Coca, *ibid.*, 11, 411, 435 and 449 (1926). [b] Quantity of CS-60C (contained in 0.025 ml. of sterile physiological salt solution) injected into each sensitive site. [c] Recipients were uniformly sensitized on the upper arms with 0.05 ml. of serum in each of five sites. The tests of each series were conducted simultaneously. The numbers refer to the diameter (in mm.) of the wheals which formed within fifteen to thirty minutes. CS-60C produced no non-specific reactions in normal skin. Qualitatively similar results were obtained using serum from another cottonseed sensitive patient (G.W.).

(10) The authors are indebted to Dr. E. J. Coulson for immunological tests: *cf.* Coulson, Spies and Stevens, *J. Immunol.*, **41**, 375 (1941).

(11) The authors wish to acknowledge their indebtedness to Dr. Harry S. Bernton for clinical facilities and to Dorris C. Chambers for assistance in conducting the tests. The clinical evidence showing that CS-1A and fractions derived from it are immunologically distinct from other allergens present in cottonseed has been described by Bernton, Spies and Stevens, *J. Allergy*, **13**, 289 (1942).

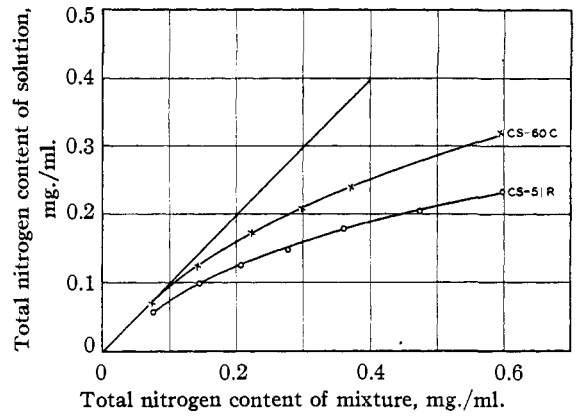

Fig. 3.—Solubility curves of CS-51R and CS-60C: weighed quantities of the protein fractions were placed in glass-stoppered tubes and then dissolved in 1.5 ml. of 0.05 *M* acid potassium phthalate solution buffered at *p*H 5.0. One ml. of absolute ethanol was added to the solution. The suspension was equilibrated by slowly rotating the tubes at 5 ± 0.1° for at least eighteen hours. The excess solid collected on the walls of the tubes during equilibration, leaving a clear supernatant solution which was analyzed for total nitrogen.

Summary

1. A carbohydrate-free allergenic protein, CS-60C, has been isolated from previously described protein-polysaccharidic fractions from cottonseed. Its chemical composition, optical rotation and ultraviolet absorption curve were determined. Solubility data indicated that CS-60C was not homogeneous but probably represented a mixture of active proteins whose structural variations were too slight to permit effective chemical separation.

2. Fraction CS-60C was antigenic as shown by tests on guinea pigs. Positive passive transfer reactions were produced with 1×10^{-9} g. of CS-60C.

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