washed with water until neutral to litmus. Recrystallization from alcohol gave a 90% yield of 3,4-dibenzyloxybutyrophenone, m. p. 86–87°. It is reported to melt at 88°.[10]

α-Bromo-3,4-dibenzyloxybutyrophenone.—This bromoketone was prepared by the procedure of Bockmühl, Ehrhart and Stein,[9] who prepared and used the compound without isolating and characterizing it. To 335.5 g. (0.93 mole) of 3,4-dibenzyloxybutyrophenone dissolved in 1500 ml. of methylene chloride was added 110 g. (1.1 moles) of powdered calcium carbonate and then 149 g. (0.93 mole) of bromine in 400 ml. of methylene chloride. The excess calcium carbonate was dissolved with dilute hydrochloric acid, the methylene chloride layer separated, washed with water, and dried over sodium sulfate. After removing the solvent under reduced pressure the residue was recrystallized from alcohol. A 70% yield of cream-colored crystals, m. p. 100–101° was obtained.

Anal. Calcd. for $C_{24}H_{23}O_3Br$: C, 65.61; H, 5.28. Found: C, 65.48; H, 5.14.

α-Benzhydrylamino-3,4-dibenzyloxybutyrophenone Hydrochloride.—To 57.1 g. (0.13 mole) of α-bromo-3,4-dibenzyloxybutyrophenone in 175 ml. of absolute alcohol was added 47.7 g. (0.26 mole) of benzhydrylamine and the mixture refluxed three hours. The alcohol was completely removed under reduced pressure and 400 ml. of dry ether added. The precipitated benzhydrylamine hydrobromide was filtered and washed with dry ether. The ether solutions were combined and thoroughly shaken with 10% hydrochloric acid. An oil separated which slowly crystallized. It was filtered off, washed with water and with ether to remove traces of color. A 75% yield of cream-colored needles, m. p. 175–176° dec., was obtained.

Anal. Calcd. for $C_{37}H_{36}O_3NCl$: N, 2.42; Cl, 6.13. Found: N, 2.38; Cl, 6.09.

1-(3,4-Dihydroxyphenyl)-2-amino-1-butanol Hydrochloride.—To 28.9 g. (0.1 mole) of α-benzhydrylamino-3,4-dibenzyloxybutyrophenone hydrochloride, dissolved in 150 ml. of absolute alcohol, was added 0.5 g. of palladium sponge catalyst.[11] It was shaken at 55–70° under fifty pounds pressure until three equivalents of hydrogen had been used. After the alcohol was removed the residue was dissolved in water and the toluene and diphenylmethane removed by extracting with ether. The aqueous solution was boneblacked and further hydrogenated until the fourth equivalent of hydrogen had been used. The catalyst was removed and the solution taken to dryness under reduced pressure. The residue was boneblacked in absolute alcohol, an equal volume of acetone added and then dry ether until precipitation was complete. A 60% yield of colorless material was obtained. When completely dry it melted with decomposition at 199–200° (uncor.).

Anal. Calcd. for $C_{10}H_{16}O_3NCl$: N, 5.99; Cl, 15.17. Found: N, 6.17; Cl, 15.08.

Summary

1. The synthesis and characterization of 1-(3,4-dihydroxyphenyl)-2-amino-1-butanol has been described.

2. It has been shown that the intermediate benzhydrylaminoketone readily undergoes hydrogenolysis to the desired primary amine.

(10) I. G. Farbenind. A. G., British Patent 457,824.

(11) Willstätter and Waldschmidt-Leitz, *Ber.*, **54**, 123 (1921).

RENSSELAER, NEW YORK RECEIVED FEBRUARY 24, 1944

[CONTRIBUTION FROM ALLERGEN INVESTIGATIONS, BUREAU OF AGRICULTURAL AND INDUSTRIAL CHEMISTRY, AGRICULTURAL RESEARCH ADMINISTRATION, U. S. DEPARTMENT OF AGRICULTURE, AND THE ALLERGY CLINIC OF PROVIDENCE HOSPITAL]

The Chemistry of Allergens. IX. Isolation and Properties of an Active, Carbohydrate-Free Protein from Castor Beans[1]

BY JOSEPH R. SPIES, E. J. COULSON, DORRIS C. CHAMBERS, HARRY S. BERNTON AND HENRY STEVENS

The method of isolation, amino acid content and chemical and immunological properties of a non-toxic, allergenic, protein-polysaccharidic fraction, CB-1A, from castor beans were recorded in a previous publication.[1b]

The importance of the role of carbohydrate in the immunological specificity of pneumococcus organisms[2] and recognition of a possible analogous relationship in allergenic specificity[3] led to the isolation and chemical characterization of an essentially carbohydrate-free protein (CS-60C) from cottonseed.[4]

To determine similarly the significance of the polysaccharidic portion of the castor bean allergen, an attempt was made to isolate a carbohydrate-free protein from CB-1A, by electrophoresis, analogously to the previously described fractionation of CS-1A from cottonseed.[4,5] However, the final cathodic fraction, CB-60C, so obtained, contained 0.47% carbohydrate. Therefore, CB-1A was subjected to a varied and prolonged series of procedures involving chromatographic adsorption of the picrate, electrophoretic recovery of the protein from the picrate, electrophoresis of the protein, and solvent fractionation. The final active fraction, CB-65A, contained only a trace of carbohydrate as shown by chemical tests. The present paper describes the isolation of CB-60C and CB-65A and compares their chemical and immunological properties with those of CS-60C from cottonseed.

Experimental

Apparatus.—The high voltage electrophoresis apparatus described and illustrated in papers IV[5] (Fig. 1) and VI[4] of this series was used except that 250, 125, 50 or 25 ml. cells

(1) (a) Not copyrighted. (b) For Article VIII of this series see Spies and Coulson, THIS JOURNAL, **65**, 1720 (1943).

(2) First demonstrated by 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 Vaughn, "Practice of Allergy," C. V. Mosby Co., St. Louis, Mo., 1939, p. 607.

(4) Spies and Umberger, THIS JOURNAL, **64**, 1889 (1942).

(5) Spies, Bernton and Stevens, *ibid.*, **63**, 2163 (1941).

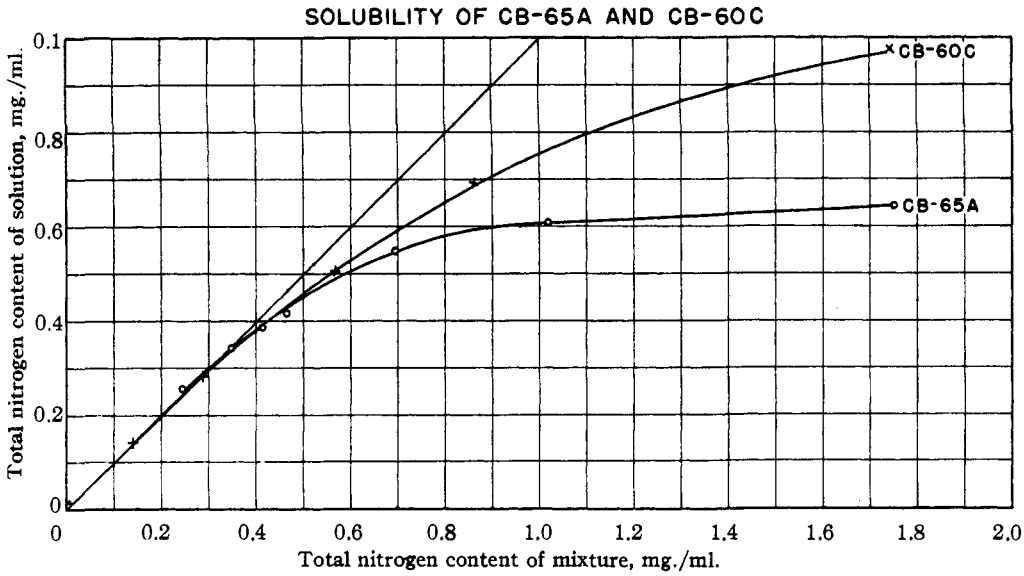

Fig. 1.—Solubility curves of CB-60C and CB-65A: weighed quantities of the protein fractions were placed in glass stoppered tubes. Samples of CB-60C were dissolved in 1.5 ml. of 0.05 *M* acid potassium phthalate buffered at *p*H 5.0. One ml. of absolute ethanol was added to each solution. Samples of CB-65A were dissolved in 0.75 ml. of the same buffered solution and 0.75 ml. of absolute ethanol was added to each. The tubes were fastened in a larger stoppered tube and the suspensions were equilibrated by slowly rotating the tubes in a water-bath at 5 ± 0.1° for at least twenty hours. The excess solid collected on the walls of the tubes during equilibration, leaving a clear supernatant solution, which was decanted and analyzed for total nitrogen content.

were employed, depending on the quantity of material fractionated. Cylindrical (10 × 25 mm.) platinum gauze electrodes were used in terminal cells.

Preparation of CB-60C.—CB-60C was obtained from CB-1A[6] by an electrophoretic fractionation similar to that used to obtain CS-60C [4] from the cottonseed allergen, CS-1A. That portion of CB-1A which migrated toward the cathode was used as starting material in five successive fractionations. The principle of the procedure has been discussed before[4,5] and, therefore, will not be given in detail here. However, pertinent data are assembled in Table I. In the initial fractionation a solution of 15.3 g. of CB-1A dissolved in 200 ml. of water was used. In each fractionation the starting solution was placed in cell 2− of a 6-cell apparatus and an equal volume of distilled water was placed in each of the other cells. The volumes of the cells used were 250, 125, 50, 50 and 25 ml. for fractionations numbered one to five, respectively. Direct current at voltages ranging from 2500 to 7500 was employed. Temperature within the cells was maintained at 15 to 25°. Anodic and cathodic fractions were precipitated by pouring the respective solutions into five volumes of ethanol followed by adjustment of the *p*H to 6.0–6.5 with dilute acetic acid or sodium hydroxide. Fractions, separated by centrifuging, were dried in a vacuum over phosphorus pentoxide. The dried fractions were ground to pass a 100-mesh sieve and were equilibrated with air before analysis.

TABLE I

DATA CONCERNING THE ISOLATION OF CB-60C BY ELECTROPHORETIC FRACTIONATION OF 15.3 G. OF CB-1A

Fractionation No.	*p*H Range[a] Anodic[b]	Cathodic[c]	Yield, g. Anodic	Cathodic	Carbohydrate content,[e] % Anodic	Cathodic
1	2.80–5.40	6.02–11.3	5.92	8.13	4.15	1.61
2	4.39–5.64	6.31– 9.57	3.11	3.27	1.94	0.97
3	4.98–5.68	6.51–10.1	0.86	1.89	1.09	.73
4	4.68–6.70	7.39–10.5	0.80	0.78	0.80	.55
5	4.90–7.43	8.12–10.0	0.26	0.34[d]	0.51	.47

[a] The *p*H of solutions was determined with a Beckman *p*H meter. [b] The anodic *p*H range is for solutions taken from cells +, 1+ and sometimes 2+. In each case the anodic fraction was isolated from the combined contents of these cells. [c] The cathodic *p*H range is for solutions taken from cells −, 1−, 2− and sometimes 2+. In each case the cathodic fraction was isolated from the combined contents of these cells. [d] This fraction was designated CB-60C. [e] Percentages are on an ash and water free basis.

Preparation of CB-65A.—CB-65A was obtained from CB-1A by a prolonged series of procedures involving chromatographic adsorption of the picrate, electrophoretic recovery of protein from its picrate, electrophoresis of the protein and solvent fractionation.

Picrate Formation.—A 10-g. composite sample, consisting of 3.6 g. of CB-1A and 6.4 g. of anodic fractions of CB-1A (obtained in preparing CB-60C), was dissolved in 200 ml. of water. To the solution was added 300 ml. of a saturated water solution (25°) of picric acid. The resulting suspension was stored overnight at 5° and the picrate was separated by centrifuging at 5°. The yellow picrate was washed once by stirring it up with 75 ml. of cold water followed by centrifuging. The solid (CB-3) was dried in a vacuum over phosphorus pentoxide. The yield of CB-3 was 12.43 g.

Chromatographic Adsorption.—CB-3 (12.43 g.) was dissolved in 1500 ml. of 50% ethanol (volume concentration) by warming and stirring. The slightly turbid solution, obtained by filtering, was passed slowly through a 44 × 135 mm. column of activated aluminum oxide (Brockmann).[7] The column was washed with 100 ml. of 50% ethanol. The picrate was adsorbed in a well-defined 30 mm. layer at the top of the column. Two smaller 2-mm. layers formed

(6) Isolation and properties of CB-1A from castor beans have been described.[1b] CB-1A contained 18.4% nitrogen, 2.33% sulfur and 3.12% polysaccharidic carbohydrate.

(7) Obtained from Merck and Co., Rahway, N. J.

under the main one,[8,9] but these were not separated. The aluminum oxide–picrate adsorption compound was removed and dried over phosphorus pentoxide (yield 67 g.).

Elution of the Picrate.—Sixty-seven grams of the aluminum oxide–picrate adsorption compound was suspended in 150 ml. of water (*p*H of the suspension was 5.0) and 0.5 *N* sodium hydroxide was added dropwise with mechanical stirring until the *p*H was 9.0.[10] The suspension was centrifuged and the solution was removed and filtered through a hardened paper. The residue was similarly re-extracted five times using 100 ml. portions of water. The final *p*H of each extract was 9.5, 9.5, 9.5, 9.7, and 10.0, respectively. The extracts were combined (650 ml.) and 300 ml. of saturated picric acid solution (excess) was added. The resulting suspension was stored overnight at 5°. The picrate was separated by centrifuging and washed once with 50 ml. of cold water. After drying in a vacuum desiccator over phosphorus pentoxide, 6.99 g. of yellow picrate, CB-5, was obtained.[11]

Recovery of Protein by Electrophoresis.—It was previously observed, with a cottonseed allergenic picrate, that the protein could be recovered by electrophoresis.[12] The protein picrate was soluble in 50% dioxane but the freed protein was insoluble in this solvent. Under the influence of a direct current picric acid migrated toward the anode and the protein moved to the cathodic cell where it was partially precipitated. A similar recovery of protein was carried out on CB-5 as follows:[13]

A solution of 6.80 g. of CB-5 in 200 ml. of 50% aqueous dioxane was placed in cell 2− (250 ml. volume) of a 6-cell apparatus. An equal volume of 50% dioxane was placed in each of the other cells. Direct current at 4000 to 5000 volts was applied at the electrodes for twenty-seven hours. The current, initially at 5.1 ma., increased to a maximum of 18.5 ma. The temperature in the cells was kept at 14 to 21°. After the electrophoresis, the orange solution in anodic cell + was removed and replaced with 200 ml. of fresh solvent. Electrophoresis was continued at 4000 volts for twenty-six hours. The orange solution in cell + was again replaced with fresh solvent and electrophoresis continued for twenty-two hours. Solution in cells + and 1+ was then replaced with fresh solvent. Electrophoresis was continued at 5000 volts. At twenty-six, twenty-two and twenty-six hour intervals solvent in cells +, 1+ and 2+ was replaced with fresh solvent. After a final seventy-two hour period at 6000 volts, during which the current was 4.0 to 4.1 ma., the electrophoresis was stopped. Solutions in cells +, 1+, 2+ and 2− were discarded. Solutions in cells − and 1− were decanted and poured into 600 ml. of cold ethanol. Precipitation occurred when the *p*H was adjusted to 6.0–6.5 with dilute sodium hydroxide. The suspension was cooled to 10° and the solid was separated by centrifuging. This solid was combined with the gummy substance which had precipitated in cell − and in the rubber tube connecting cells − and 1−. The combined solids were stirred up in 200 ml. of water. The *p*H of the suspension was adjusted to 6.4 with dilute sodium hydroxide. The suspension was then centrifuged and a small amount of insoluble matter was discarded. The clear brownish-yellow solution was poured into five volumes of cold ethanol and precipitation occurred when the *p*H was adjusted to 6.2 with a few drops of 10% acetic acid. The suspension was cooled to 5° and the solid was separated by centrifuging at 5°. The solid was washed once with 150 ml. of cold 80% ethanol and dried in a vacuum over phosphorus pentoxide. The yield was 4.82 g. of brownish solid which contained 18.7% nitrogen and 0.41% carbohydrate.

Chromatographic adsorption and elution of the 5.77 g. of picrate obtained from this recovered solid was then repeated as described before. A yield of 3.97 g. of eluted picrate was obtained. This picrate was dissolved in 200 ml. of 50% dioxane and the protein was recovered electrophoretically by essentially the same procedure as just described. This electrophoresis required two hundred and sixteen hours. The voltage employed was 4000 and the temperature in the cells was maintained at 7 to 12°. After the final run, solutions in cells +, 1+, 2+ and 2− were discarded. The solid, isolated as previously described from the solution in cells − and 1−, was combined with the gummy precipitate in cell −. The combined solids were then dissolved in 200 ml. of water. A small amount of insoluble matter, separated by centrifuging, was discarded. Solid was isolated from the solution by ethanol precipitation and centrifugation. A yield of 3.01 g. of brownish colored substance containing 18.9% nitrogen and 0.31% carbohydrate was obtained.

Electrophoresis of the Protein.—A solution containing 2.91 g. of solid, recovered from the picrate and containing 0.31% carbohydrate, in 50 ml. of water (*p*H of the solution was 5.65) was placed in cell 2− (50 ml. volume) of a 6-cell apparatus. Fifty ml. of water was placed in each of the other cells and direct current at 3500 volts was applied to the system for twenty-three hours. The temperature was kept at 15 to 19°. A small amount of dark-colored precipitate separated in all of the cells. After the electrophoresis the *p*H of solutions in the cells was: +, 4.94; 1+, 5.30; 2+, 5.44; 2−, 5.93; 1−, 6.33; −, 7.00. The solutions in cells + and − were replaced with 50 ml. of water and electrophoresis was continued at 6000 volts for forty-eight hours at a temperature of 16 to 17°. The *p*H of solution in the cells was then: +, 4.63; 1+, 5.17; 2+, 5.39; 2−, 5.73; 1−, 6.25; −, 7.19.

The solution removed from cell −, after the first run, and the solution from cells −, 1−, 2− and 2+ of the second run were combined (*p*H 5.92). The combined solution, after boiling for fifteen minutes with 1 g. of activated charcoal, was cooled and centrifuged. Then it was filtered through a hardened paper and solid was isolated from the filtrate by ethanol precipitation. A yield of 1.45 g. of solid containing some colloidal carbon was obtained.

Solvent Fractionation and Reprecipitations.—The solid obtained by electrophoresis (1.45 g.) was dissolved in 50 ml. of water and boiled with 300 mg. of carbon. The carbon, removed by centrifuging, was washed with 10 ml. of water. The wash solution was combined with the main solution which was filtered through a Seitz sterilizing pad.[14] Solid was separated from the clear slightly yellow solution by precipitation with five volumes of ethanol. A yield of 1.27 g. was obtained. This solid was dissolved in 50 ml. of water and decolorized with carbon. This combined solution and washings (75 ml.) was passed through a Seitz sterilizing pad and 25 ml. of ethanol was added to the filtrate. This solution was kept at −6° overnight. The white suspension which formed on standing was decanted from a small amount of gummy solid that had collected on the bottom of the container. This residual solid, which contained most of the color, was discarded. The decanted suspension was poured into 350 ml. of cold ethanol. The

(8) A similar layering was observed in a previous study of CS-5, the analogous picrate of the cottonseed allergen, Spies, Coulson, Bernton and Stevens, THIS JOURNAL, **62**, 1420 (1940). No difference was demonstrable in clinical activity of the three layers from CS-5. Therefore, in this study the layers were not separated.

(9) The filtrate from the adsorption was evaporated to dryness and 845 mg. of yellow powder (CB-10) was obtained. Clinical tests on castor bean sensitive subjects, using solid obtained from previous similar adsorptions, showed that CB-10 was completely inactive. The solid (CS-10) similarly obtained from cottonseed also showed no clinical activity, and was not antigenic.

(10) A commercially available *p*H meter employing a glass electrode with mechanical stirrer was used.

(11) CB-5 from another similar adsorption contained 18.9% total nitrogen and 31.6% picric acid. See Spies, Bernton and Stevens, THIS JOURNAL, **62**, 2793 (1940), for the method used for determining picric acid.

(12) Spies, *ibid.*, **63**, 1166 (1941).

(13) The rapid method of recovering the allergenic protein from its picrate by ethanol precipitation at the isoelectric point, reference 8, was not used, in this case, because protein recovered from CB-5 by this method contained 0.93% carbohydrate whereas that recovered by electrophoresis contained 0.41%.

(14) Seitz pads were washed before use by filtering through them 100 ml. of boiling water and 130 ml. of cold water to remove traces of soluble carbohydrate.

resulting suspension was cooled to 4° and centrifuged. The solid was washed once with cold 80% ethanol and dried as usual. A yield of 601 mg. of almost colorless material was obtained. This solid was dissolved in 35 ml. of water and stirred at room temperature with 200 mg. of carbon. The carbon was removed and the solid was isolated as usual. A yield of 503 mg. of solid containing 19.0% nitrogen and 0.22% carbohydrate was obtained.

Four hundred and twenty-two mg. of this solid was dissolved in 25 ml. of water. The practically clear, colorless solution was filtered through a washed hardened filter paper into a 250 ml. conical centrifuge cup. To this solution was added 8.3 ml. of ethanol. The solution, which remained clear at room temperature, was cooled at −7° for three hours. The resulting suspension was centrifuged at −7°. The supernatant solution was discarded. The solid was washed once with 25 ml. of cold 80% ethanol and dried in a vacuum over phosphorus pentoxide. A yield of 314 mg. of white solid (CB-65A) was obtained. This solid was ground to pass through a 100-mesh sieve and equilibrated with air before analysis.

CB-65A was completely soluble in water. Results of color tests on a 1% solution of CB-65A were: pinkish biuret, deep purple ninhydrin, positive Millon, and Benedict test for reducing sugars, negative.

Determination of Carbohydrate.—Carbohydrate was determined on protein fractions by the orcinol method of M. Sørensen and Haugaard[15] as modified by Heidelberger and Kendall.[16] Galactose, $[\alpha]^{20}D$ +79.7; *C*, 3.00 g./100 ml., was used as a standard and color comparison was made with a Duboscq colorimeter. Test showed that no color (blank) developed on heating protein fractions in the acid solution without orcinol.

Carbohydrate in CB-65A was determined by the orcinol method, using a photoelectric colorimeter for measuring the color developed. Twenty-eight mg. of CB-65A was placed in a 125-ml. Erlenmeyer flask with 1.0 ml. of water, 2.0 ml. of a 2% orcinol solution in 25% sulfuric acid, and 15.0 ml. of 60% sulfuric acid. The solution was heated in a water-bath, in the dark, at 80° for twenty-five minutes. After cooling at −7° the solution was warmed to room temperature and the intensity of the color was read on an Evelyn photoelectric colorimeter using filter No. 520. The reagent (blank) contained 1.0 ml. of water, 2.0 ml. of the orcinol reagent and 15.0 ml. of 60% sulfuric acid. Negligible color developed when a blank was run on a solution containing 27.8 mg. of protein,[17] 1.0 ml. of water, 2.0 ml. of 25% sulfuric acid and 15.0 ml. of 60% sulfuric acid. The carbohydrate content of CB-65A, calculated from color readings in comparison with the curve obtained with solutions containing 0.01, 0.05, and 0.10 mg. of galactose was 0.11%.

A Molisch test on a 1% solution of CB-65A, while difficult to read, was considered doubtfully positive.

Comparison of Antigenic Potency of CB-1A and CB-65A, Using Guinea Pigs.—Comparison of CB-1A and CB-65A with regard to the median sensitizing dose (SD-50) and the median lethal dose (LD-50) in previously sensitized guinea pigs, was made from dosage-response curves according to the method of computation of Bliss.[18] The Bliss method, originally developed for the evaluation of toxicological studies, has been adapted to the quantitative comparison of antigenic potency of cottonseed allergenic fractions.[19]

The SD 50's of CB-1A and CB-65A were 63.6 ± 13γ and 746 ± 240γ (on ash and water free basis), respectively. From the complete dosage response curve the LD 50 of CB-1A was found to be 2.38 ± 0.36γ. Owing to the limited quantity of CB-65A available the mortality ratio was determined at only the critical level. Each of 10 guinea pigs (previously sensitized with 1.0 mg. of CB-1A) was injected intravenously with 2.7γ of CB-65A. The mortality ratio was six fatal to four non-fatal anaphylactic reactions. This mortality ratio was identical with that previously observed when similarly sensitized animals were injected with 2.7γ of CB-1A. The shocking capacity of CB-65A is, therefore, very close to that of CB-1A.

(15) M. Sørensen and Haugaard, *Comp. rend. trav. lab. Carlsberg*, **19**, no. 12 (1933).

(16) Heidelberger and Kendall, *J. Immunol.*, **30**, 267 (1936).

(17) This sample was an intermediate fraction obtained from CB-1A.

(18) Bliss, *Ann. Appl. Biol.*, **22**, 134 (1935); **22**, 307 (1935).

(19) Coulson and Spies, *J. Immunol.*, **46**, 367 (1943).

Discussion

Results of elementary analyses and some properties of CB-65A and CB-60C are compared, in Table I, with those of the analogous protein fraction CS-60C, from cottonseed. The nitrogen content of CS-60C was higher than that of CB-60C or CB-65A, probably because of the higher proportion of arginine in the cottonseed allergen.[1b] Carbon and hydrogen contents of the three fractions were of the order of magnitude usually found in proteins. Like CS-60C, both CB-60C and CB-65A were levorotatory. There was, however, no marked difference between the rotatory power of CB-60C and CB-65A. This indicated that changes which might have occurred in CB-65A as a result of the more drastic fractionation had not taken place in that part of the molecule which determines optical rotation. The solubility curves of CB-60C and CB-65A, Fig. 1, indicated that neither was chemically homogeneous. CB-65A, however, was more nearly homogeneous, by this criterion, than was CB-60C.

Eighty-seven per cent. of the nitrogen of CS-60C was precipitable by trichloroacetic acid (TCA) in contrast with only 27% for CB-60 and none for CB-65A. No radical change in constitution or composition of CB-60C appeared to have occurred as a result of fractionation because 22.5% of the nitrogen of precursor fraction CB-1A was precipitable by TCA. However, some change appeared to have occurred in CB-65A either by molecular rearrangement as a result of the drastic fractionation or by the

TABLE II

COMPARISON OF CHEMICAL COMPOSITION AND PROPERTIES OF CB-65A AND CB-60C WITH CS-60C[a]

Determination[b]	Fraction CB-65A	CB-60C	CS-60C
Nitrogen	19.0	19.1	20.4
Nitrogen pptd. by 5% trichloroacetic acid at 20 ± 0.1°[c]	0.0	26.7	86.6
Carbon	47.6	50.1	48.2
Hydrogen	6.95	6.36	6.58
Sulfur[d]	2.57	2.53	2.35
$[\alpha]^{20}D$ 1% water solution	−49.5	−45.0[e]	−140

[a] The carbohydrate-free allergenic fraction, CS-60C, obtained from cottonseed has been previously described (ref. 4). [b] All analyses are expressed on an ash- and water-free percentage basis. CB-65A and CB-60C contained 1.0 and 0.13% ash and 7.30 and 7.09% water, respectively. Analyses were made by micro methods. [c] See Paper IV of this series, Table I, for the method used. [d] In precursor fractions cystine sulfur accounted for 90% of the total sulfur. [e] Optical rotation of CB-60C was determined with a 2% water solution.

TABLE III

COMPARISON OF THE THRESHOLD QUANTITY OF CB-65A AND CB-1A REQUIRED TO PRODUCE POSITIVE PASSIVE TRANSFER REACTIONS USING SERUM FROM A CASTOR BEAN SENSITIVE SUBJECT[a]

Quantity of fraction injected.[b] Millimicrogram, mγ[c]	Recipients[d] (wheal diameter in mm.) F. F. CB-1A	F. F. CB-65A	M. P. CB-1A	M. P. CB-65A	E. H. CB-1A	E. H. CB-65A
10	12 × 13	13 × 13	16 × 22	16 × 21	16 × 16	16 × 17
1	11 × 12	12 × 12	10 × 12	12 × 18	11 × 12	13 × 15
0.1	10 × 10	10 × 11	9 × 8	9 × 13	10 × 11	11 × 12
0.01	±[e]	±	±	0	±	±

[a] This serum (A. M.) gave positive passive transfer reactions to test with castor bean allergen when diluted 1:10^3 but not when diluted 1:10^3; *cf.* Coca and Grove, *J. Immunol.*, **10**, 445 (1925). [b] Quantity of fraction (contained in 0.025 ml. of sterile physiologic salt solution) injected into each sensitized site. [c] *Chemical and Engineering News*, 18, 491 (1940). [d] Subjects who reacted negatively to cutaneous test with powdered de-fatted castor bean meal and also negatively to intracutaneous test with 0.1 γ of CB-1A were used as recipients. Recipients were uniformly sensitized with 0.05 ml. of serum in each of four sites on the upper arms. All tests of a series were conducted simultaneously. The size of the wheals which formed in fifteen to thirty minutes are in mm. A negative control test was made on the unsensitized skin of the recipient at the time of the passive transfer tests. [e] See Paper VIII, footnote *e* of Table V for comments on the ± reaction.

removal of constituents precipitable by TCA. Whichever possibility occurred CB-65A suffered no demonstrable loss in capacity to produce positive passive transfer reactions as compared with precursor fraction CB-1A. Results of this clinical comparison, Table III, show that 1 × 10^{-10} g. of both CB-1A and CB-65A produced positive passive transfer reactions of equal size. However, alteration in the antigenic potency of CB-65A was demonstrable, using guinea pigs as test animals. The median lethal dose of CB-65A was the same as that of CB-1A but the median sensitizing dose of CB-65A was more than eleven times as great as that of CB-1A.

An interpretation analogous to the conclusions drawn from more exhaustive studies of cottonseed allergenic fractions may explain these observations. From chemical evidence previously presented,[5] it was suggested that the cottonseed allergenic fraction, CS-1A, was a mixture containing principally a specifically active protein and compounds of this same protein chemically combined with varying proportions of polysaccharidic carbohydrate. Later the active, essentially carbohydrate-free protein, CS-60C, was isolated.[4] Antigenic studies on excised uterine muscle from sensitized guinea pigs substantiated the view that carbohydrate did not determine antigenic specificity.[20] Carbohydrate, however, had a marked influence on antigenic potency[21] of fractions separated from CS-1A which, as in the cases of CB-1A and CB-65A, was manifested only in the sensitizing capacity.[19] Thus the median lethal doses of CS-51R[5] and CS-56R,[5] which contained 0.9 and 35.2% carbohydrate, respectively, was 5.61 ± 0.39 γ of nitrogen and 6.75 ± 0.77 γ of nitrogen, respectively. The median sensitizing dose of CS-51R was 91.7 ± 15.7 γ of nitrogen or more than eight times as high as that of CS-56R which was 11.0 ± 2.0 γ of nitrogen. Fraction CB-65A retained the same antigenic specificity as characterized CB-1A. Therefore the decreased sensitizing capacity of CB-65A may be owing to the removal of protein-polysaccharidic compounds from CB-1A having relatively high molecular weight and being, therefore, more effective in sensitizing power.

An alternate explanation of the decreased sensitizing power of CB-65A is that sensitization and shock may be produced by different functional groupings in the antigenic molecule. One grouping might therefore be altered without causing change in the other.

It is noteworthy that analogous manifestations of antigenicity in humans (capacity to incite positive passive transfer reactions) and in animals (shocking power) were unaffected by the fractionation required to obtain CB-65A. No comparison of the sensitizing capacity of this fraction for human subjects and animals was attempted because of the hazard of establishing in human subjects a permanent hypersensitivity to castor bean allergen.

Final decision as to whether protein or carbohydrate is responsible for the allergenic specificity of such highly purified substances as CS-60C and CB-65A cannot be made on the basis of chemical tests for carbohydrate. Even if tests for carbohydrate were unequivocally negative the possibility would still remain that sufficient carbohydrate was present to produce clinical reactions. As shown in Table III, solutions of CB-65A containing 4.0 × 10^{-9} g./ml. were clearly detectable by passive transfer technique. This is beyond the sensitivity of chemical tests on solutions of pure sugars. If the activity of CB-65A were owing to the 0.1% carbohydrate indicated by the orcinol method, then the concentration of active substance in CB-65A detectable by passive transfer would be 4.0 × 10^{-12} g./ml. At present there is no basis for accepting either 4.0 × 10^{-9} or 4.0 × 10^{-12} g./ml. as the approximate sensitivity of a castor bean allergenic preparation by the passive transfer technique. More decisive than chemical tests is the fact that CB-1A, which contained at least thirty times as much carbohydrate as did CB-65A, was not

(20) Coulson, Spies and Stevens, *J. Immunol.*, **46**, 347 (1943).

(21) Antigenic activity of a compound consists of two measurable manifestations, capacity to sensitize and capacity to shock.

demonstrably more effective in producing passive transfer reactions, or in shocking sensitized guinea pigs.

The obvious fact that biological tests on castor bean allergenic preparations are more sensitive than chemical tests for a given constituent provides justification for having employed the involved procedures used to isolate CB-65A. That CB-65A was obtained by such prolonged, varied, and drastic fractionation must be regarded as substantiating evidence that its activity is inherent and not owing to the presence of a difficultly removable contaminant. Osborne, Mendel and Harris[22] used similar reasoning in deciding that the powerful toxic action of ricin, from castor beans, was inherent in the albumin fraction exhibiting maximum toxicity and not owing to a difficultly removable contaminant.

Summary

An essentially carbohydrate-free allergenic protein, CB-65A, has been isolated from fraction CB-1A from castor beans by a prolonged series of procedures involving chromatographic adsorption of the picrate, electrophoretic recovery of the protein from its picrate, electrophoresis of the protein, and solvent fractionation. Elementary analyses and properties of CB-65A are compared with those of an analogous fraction CS-60C from cottonseed.

Positive passive transfer reactions were produced by 1×10^{-10} g. of CB-65A. Fraction CB-65A was antigenic as shown by tests using guinea pigs. CB-65A was as potent as precursor fraction CB-1A in producing passive transfer reactions and in shocking guinea pigs, but its sensitizing capacity was approximately one-eleventh that of CB-1A. Available evidence indicates that the principal allergenic and anaphylactogenic specificities of both CB-1A from castor beans and CS-1A from cottonseed are inherent in the essentially carbohydrate-free protein fractions CB-65A and CS-60C obtained from them.

(22) Osborne, Mendel and Harris, *Am. J. Physiol.*, **14**, 259 (1905).

WASHINGTON, D. C. RECEIVED FEBRUARY 17, 1944

[CONTRIBUTION FROM THE INSTITUTE OF PAPER CHEMISTRY]

The Action of Ultraviolet Light upon Cellulose. I. Irradiation Effects. II. Post-Irradiation Effects[1]

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Irradiation Effects[2a,b]

Although a number of studies of the effect of ultraviolet light on cellulose have been reported, the results are in many ways contradictory and incomplete, especially with respect to the role of oxygen and the mechanism of the reaction. The objectives of this investigation were to clear up existing discrepancies and to gain information as to the reaction mechanism.

Apparatus, Materials and Methods

Irradiation Apparatus.—The apparatus used in this investigation (Fig. 1) consisted of a gas purification train, an exposure chamber with a magnetic stirrer and capable of being evacuated, a mercury vapor lamp, and a gas analysis train with provision for the quantitative estimation of water, carbon dioxide, formaldehyde, hydrogen, carbon monoxide and organic combustibles.

The gas used as the atmosphere surrounding the cellulose sample during the experiment was passed first through a calibrated flowmeter of the orifice type. The gas line leading from the flowmeter had a side connection leading to a bubbler, in which the water level was maintained about two inches above the gas outlet. This side tube served as a safety valve, never permitting the gas pressure in the exposure chamber to rise above two inches of water. From the flowmeter, the gas passed through a calcium chloride tower to remove water vapor, a copper gauze furnace to remove oxygen, an Ascarite–Anhydrone bottle to remove carbon dioxide and the last traces of water vapor and, finally, through a three-way stopcock into the exposure chamber. The copper gauze furnace was of the type described by Savage and Ordal[4]; it consisted of 40-mesh copper wire gauze wound on a Calrod heating element (General Electric Company), the assembly being encased in a tight-fitting Pyrex tube. When oxygen was used as the gaseous atmosphere, the furnace was turned off; otherwise, the gas received the same purification treatment.

The outer shell of the exposure chamber consisted of an open-top brass cylinder 2.25 inches high by 10.25 inches in diameter (inside dimensions). Inside of this was a concentric, rotatable, open-top brass cylinder 1.5 × 10 inches (outside dimensions) supported on ball bearings. Two pieces of soft iron were fastened to the bottom of the inner cylinder, so that it might be turned by a motor-driven electromagnet rotating in a horizontal plane below the outer shell. Direct current was fed to the coils of the magnet by means of slip rings, about 50 watts being required for satisfactory operation of the agitator. The cellulose was placed in the inner cylinder. When the latter rotated, the material was mixed by stationary plows attached to the upper rim of the outer shell. The plow holder was removable, allowing the inner cylinder to be taken out. A mercury-in-glass thermometer, shielded from direct radiation, was attached to the plow holder. To keep the temperature down during irradiation, a fan blew air over the exposure chamber.

The cover consisted of four pieces (each 5.625 inches square) of Vycor high-silica ultraviolet-transmitting glass (Corning Glass Works), sealed in place with beeswax-

(1) Original manuscript received December 14, 1942.

(2) (a) A portion of a thesis submitted in partial fulfillment of the requirements of The Institute of Paper Chemistry for the degree of Doctor of Philosophy from Lawrence College, Appleton, Wis. This work was carried out under the direction of Emil Heuser. Presented before the Division of Cellulose Chemistry at the 106th Meeting of the American Chemical Society, Pittsburgh, Pa., Sept., 1943.

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(4) Savage and Ordal, *Science*, **91**, 222 (1940).