briefly discussed. The relationships between frequency V_0, energy in ergs, and wave length in ångström units corresponding to bonds in the normal paraffin homologous series are shown graphically. For any one paraffin, the shift in frequency and wave length caused by change in density is appreciable. Measurements of ultraviolet absorption of normal paraffins in the vicinity of 1000 Å. would be of great interest.

MARCUS HOOK, PENNA. RECEIVED FEBRUARY 17, 1941

[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. IV. An Electrophoretic Fractionation of the Protein–Polysaccharide Fraction, CS-1A, from Cottonseed[1]

BY JOSEPH R. SPIES, HARRY S. BERNTON AND HENRY STEVENS

Isolation and properties of an allergenic protein–polysaccharide fraction, CS-1A, from cottonseed have been described.[2] This fraction is of interest because the components separated from it possessed an unusual combination of chemical stability and immunological potency and specificity. Moreover, active components have been obtained in quantity sufficient for investigation of their chemical composition.

Although the electrophoretic method of Tiselius and others is now widely employed in protein chemistry, the use of direct current at high voltage for fractionating unbuffered protein solutions in preparative work has not been exploited as much as may be warranted. The earliest extensive application of high voltage electrophoresis for fractionating biologically active substances was that of Williams and co-workers.[3] The theory of this type of electrophoresis has been discussed adequately by Williams and Truesdail.[3a] Du Vigneaud and Irving, *et al.*, have recently used this method in fractionating the posterior pituitary hormones.[4]

The present paper describes a large scale electrophoretic fractionation of CS-1A and properties of the separated components. The four cathodic fractions, CS-51R, CS-52R, CS-53R and CS-54R[5] were isolated by picric acid precipitation and were further purified by recovery and reprecipitations. These fractions were characterized by potent allergenic activity, high nitrogen content and by the absence of significant amounts of carbohydrate. The anodic fraction CS-56 contained 48% polysaccharidic carbohydrate, and was lower in nitrogen content and allergenic activity than the cathodic fractions. Fraction CS-55, recovered from the cell which originally contained unfractionated CS-1A, corresponded in composition to fraction CS-56. A denatured solid, CS-57, precipitated during electrophoresis in cells 1+ and 2+, Fig. 1. Of all the fractions separated, CS-57 was lowest in nitrogen content, exhibited the least allergenic activity and was not studied further.

Evidence of chemical differences in composition of the fractions was obtained by determination of total and protein nitrogen, carbohydrate, sulfur and cystine content. Table I contains a summary of these data on the fractions obtained from 400 g. of CS-1A, and on sub-fractions obtained from them by chemical procedures.

Heidelberger and Avery[6] working with pneumococci first showed that polysaccharides contribute importantly to the specificity of these organisms. Subsequent investigations have revealed that polysaccharides are no less significant than proteins in contributing to the immunological specificity of many bacteria.[7] The polysaccharides of pneumococci were resistant to treatment with acid at room temperature. However, warming with acid produced reducing sugars with accompanying loss in specific activity.[8] Later, Heidel-

(1) For Paper III of this series see Spies, Bernton and Stevens, THIS JOURNAL, **62**, 2793 (1940).

(2) (a) Spies, Bernton and Stevens, *J. Allergy*, **10**, 113 (1939); (b) Spies, Coulson, Bernton and Stevens, THIS JOURNAL, **62**, 1420 (1940).

(3) (a) Williams and Truesdail, *ibid.*, **53**, 4171 (1931); (b) Williams, Lyman, Goodyear, Truesdail and Holaday, *ibid.*, **55**, 2912 (1933); (c) Williams and Moser, *ibid.*, **56**, 169 (1934); (d) Williams, *J. Biol. Chem.*, **110**, 589 (1935).

(4) (a) Du Vigneaud, Irving, Dyer and Sealock, *ibid.*, **123**, 45 (1938); (b) Irving and du Vigneaud, *ibid.*, **123**, 485 (1938); (c) Irving, Dyer and du Vigneaud, THIS JOURNAL, **63**, 503 (1941).

(5) These four fractions are essentially equivalent to the protein fraction CS-13A previously described in Paper II of this series, 2b.

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

(7) Landsteiner, "The Specificity of Serological Reactions," Charles C. Thomas, Baltimore, Md., 1936, reviews the subject of carbohydrates with respect to serological specificity.

(8) Heidelberger and Avery, *J. Exptl. Med.*, **40**, 301 (1924). Heidelberger, Goebel and Avery, *ibid.*, **42**, 727 (1925).

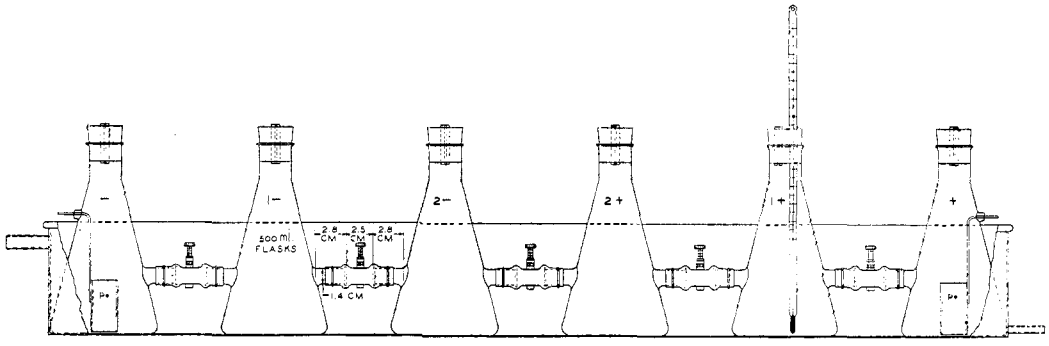

Fig. 1.—Electrophoresis apparatus.

berger, Kendall and Scherp[9] using precipitin tests with rabbit antisera demonstrated that the pneumococcus polysaccharides were labile to heat and to the chemical manipulations used in early isolation procedures. Apparently, progressive depolymerization of polysaccharide occurred which was readily detected with rabbit antisera but not with horse antisera. The role of polysaccharides in allergenic specificity has, therefore, been of interest.[10]

In previous fractionations of the polysaccharidic-protein CS-1A increased carbohydrate content has always been accompanied by decreased allergenic activity.[2a,b] Although the cathodic fraction CS-51R contained 20% nitrogen it gave a faintly positive Molisch test and contained 1% carbohydrate. The anodic fraction CS-56 which contained 10% nitrogen and 48% carbohydrate was demonstrably less active than CS-51R. The tentative assumption was made that the activity of CS-56 was due to the presence of the protein CS-51R in a mixture containing inactive polysaccharide. It was assumed, therefore, that if the activity of CS-51R were influenced by the indicated trace of polysaccharide, this activity would be destroyed by acid treatment, because in general peptide linkages are more stable than carbohydrate linkages. When fraction CS-51R was refluxed in 0.1 *N* acid for four hours the resulting fraction CS-51RH was still active. Fraction CS-51RH gave a barely distinguishable Molisch test. But it seemed unlikely that the small proportion of carbohydrate thus indicated could have survived the drastic acid treatment unchanged to contribute to the specificity of the allergenic reaction.[11] Peptide linkages were not ruptured during acid treatment as shown by the fact that there was no increase in amino nitrogen of the recovered fractions. By similar acid treatment the anodic fraction CS-56 was split into reducing pentoses and an active polysaccharidic-protein CS-56H which contained 37% non-reducing carbohydrate. Fraction CS-56 was further fractionated by picric acid precipitation. Fraction CS-56R, recovered from the anodic picrate, contained 13% nitrogen and 35% carbohydrate. Fraction CS-56S was obtained from the supernatant liquid after removal of the picrate. CS-56S, which contained only 2.6% nitrogen and 91% carbohydrate, was only one one-hundredth as active as CS-56R by cutaneous tests. No reducing sugar was obtained from CS-56R by refluxing for one hour with 3 *N* acid but boiling CS-56S for one minute produced carbohydrate which readily reduced Benedict reagent. When CS-56R was refluxed with 0.1 *N* acid for four hours an active protein CS-56RH was obtained. CS-56RH contained 14% nitrogen and 30% carbohydrate. It appeared, therefore, that part of the polysaccharide in CS-56 was chemically combined with protein and part was present as a contaminant.

The allergenic activity of fractions was compared by direct cutaneous testing and by passive transfer tests.[12] The cathodic fractions CS-51R, CS-52R, CS-53R and CS-54R were demonstrably more active than either the original CS-1A or the unfractionated anodic fraction CS-56, or the insoluble CS-57. Fraction CS-56R was as active as the cathodic fractions and was one hundred times more active than CS-56S. The acid treated fractions were not demonstrably less active than

(9) Heidelberger, Kendall and Scherp, *J. Expil. Med.*, **64**, 559 (1936).

(10) Early work dealing with 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, p. 734, *et seq.*; also Vaughan, "Practice of Allergy," C. V. Mosby Co., St. Louis, 1939, p. 607.

(11) Levine, *Proc. Soc. Exptl. Biol. Med.*, **27**, 830 (1930), discussed the sensitivity of the Molisch test and its lack of specificity for carbohydrate.

(12) The authors wish to acknowledge the assistance of Dorris C. Chambers in the clinical studies.

TABLE I

CHEMICAL PROPERTIES OF FRACTIONS OBTAINED IN AN ELECTROPHORETIC FRACTIONATION OF 400 G. OF CS-1A AND OF SUB-FRACTIONS OBTAINED BY CHEMICAL PROCEDURES[a]

Fraction[b]	Yield, g.	Total N content, %	Proportion of total N pptd. by 5% trichloroacetic acid,[c] %	Carbohydrate content,[d] %	Amino N content,[e] %	Total S content,[f] %	Cystine content,[g] % Found	Calcd.[h]
CS-1A		11.8	74.8	39.9	0.36			
CS-51R(−)	9.2	19.8	91.0	0.9	.53	2.29	7.60	8.58
CS-52R(1−)	8.8	20.2	89.7	0.9		2.24	8.45	8.40
CS-53R(2−)	7.1	19.7	84.6	3.0		2.04		
CS-54R(2+)	3.5	19.1	81.5	6.3		1.96		
CS-55(1+)	22.6	11.2	69.6	46.5				
CS-56(+)	84.5	10.0	69.8	48.0	.51	2.37	3.24	8.88
CS-57(1+, 2+)	79.7	7.94		64.6		0.98		
CS-56H		11.6	65.1	36.6	.52	2.04	4.21	7.64
CS-55R[i]		15.3	76.8	23.8				
CS-56S		2.61	0.0	91.0				
CS-56R		13.2	78.6	35.2				
CS-56RH		14.1	73.8	30.4				
CS-51RH		20.0	83.3	1.1	.59	2.49		

[a] The authors wish to acknowledge the technical assistance of Mr. E. J. Umberger, who made the microanalytical determinations reported in this table. [b] CS-1A was the starting material. Fractions for which yields are given represent the quantity of each fraction obtained from a total of 400 g. of CS-1A. Samples were dried in a vacuum over phosphorus pentoxide and ground to pass a 100-mesh sieve. The samples were then spread on a watch glass to become equilibrated with air. All weights and analyses are expressed on ash-water free basis. Ash was determined by ignition to constant weight in an electric furnace at 750°. Water was determined by heating the sample to constant weight in a vacuum Abderhalden drier at 110°. [c] The following procedure was used: 2 ml. of 10% TCA was added to 2 ml. of a 2% aqueous solution of the fraction in a 15-ml. graduated centrifuge tube. The mixture was heated on a water-bath at 70° for ten minutes. The resulting suspension was cooled at 20 ± 0.1° for twenty-four hours. The suspension was then centrifuged at 20 ± 1° in the capped tube until clear. Nitrogen was determined on the supernatant liquid by the Kjeldahl micromethod. *Cf.* Northrop and Kunitz, *J. Gen. Physiol.*, **16,** 267 (1932), also later papers. [d] Carbohydrate was determined by the method of M. Sørensen and Haugaard as modified by Heidelberger and Kendall, *J. Immunol.*, **30,** 267 (1936). Galactose, $[\alpha]^{20}D$ +79.7 (C = 3.00 g./100 ml.), obtained from the National Institute of Health through the courtesy of Dr. R. M. Hann was used as a standard for comparison. Determinations made with different concentrations of CS-56 indicated that the deduction of "blank" was unnecessary with the low concentrations of protein used. [e] Determined by the Van Slyke micromethod using the apparatus of F. C. Koch, *J. Biol. Chem.*, **84,** 601 (1929). [f] Sulfur was determined by the micromethod of Pregl. [g] Cystine determinations were made by Dr. W. C. Hess of Georgetown University. The protein was hydrolyzed with 20% hydrochloric acid both with and without titanium chloride and with constant boiling hydriodic acid. The methods of Sullivan and of Okuda gave concordant results. According to Dr. Hess no methionine is present. [h] Calculated on the basis of total sulfur content. [i] Fraction CS-55R was prepared from CS-55 similarly to CS-56R from CS-56.

the corresponding untreated fractions by cutaneous tests. Results shown in Tables II and III are typical of the methods used in comparison of cutaneous activity of all the fractions.[13]

TABLE II

COMPARISON OF CUTANEOUS ACTIVITY OF CS-1A (original), CS-51R (cathodic), CS-56 (anodic) AND CS-57 (insoluble) FRACTIONS

Patient[a]	Fraction[b] CS-1A	CS-51R	CS-56	CS-57	Time, min.	Dilution
R.D.	3+	4+	2+	1+	15	$1:10^6$
I.L.	0	2+	1+	0	15	$1:10^6$
M.W.	±	1+	0	0	30	$1:10^{5.7}$
Q.N.	±	4+	0	0	30	$1:10^{5.7}$
M.J.	0	3+	2+	0	15	$1:10^6$
G.W.	0	2+	±	±	30	$1:10^6$

[a] Cottonseed sensitive patients. [b] For comparative purposes tests were made simultaneously on the thigh with equal gravimetric dilutions of the fractions. See Paper I,[2a] Table I, for the method of cutaneous testing and interpretation of size of the wheal produced. Control tests on non-sensitive individuals were always negative.

TABLE III

COMPARISON OF CUTANEOUS ACTIVITY OF ACID TREATED AND UNTREATED FRACTIONS

Patient[a]	Fraction[b] CS-51RH	CS-51R	CS-56H	CS-56
I.L.	4+	2+	4+	1+
R.D.	4+	4+	4+	3+
I.L.	3+	2+	2+	2+
M.J.	4+	4+	4+	4+
G.W.	4+	4+	2+	1+

[a,b] See Table II. All fractions were diluted $1:10^6$ and readings were taken after fifteen-minute interval.

The acid treated fractions CS-51RH and CS-56H retained reagin neutralizing power. A modi-

(13) Inherent limitations in the reliability of cutaneous tests for activity with cottonseed fractions probably involve errors of the order of ±25 to 50%.

fication of the Prausnitz–Küstner passive transfer technique[14] was used. Results in Table IV illustrate a typical passive transfer experiment. It is apparent that 10 micrograms of CS-51R and CS-51RH desensitized sites to a second subsequent tenfold quantity of the same fraction. Although CS-56H had less neutralizing power than CS-51RH a site desensitized to one of these was also desensitized to the other. However, using the more sensitive *in vitro* reagin neutralization method of Cooke, Stull and co-workers[15] it was demonstrated that at least one-half the reagin neutralizing power of the acid-treated fraction had been lost. The acid treated fraction CS-51RH retained antigenic properties as demonstrated by the production of anaphylaxis in guinea pigs.[16]

TABLE IV

COMPARISON OF REAGIN NEUTRALIZING POWER OF ACID TREATED COTTONSEED ALLERGENS, CS-56H, CS-51RH WITH UNTREATED CS-51R

Recipient[a]	Injection no.[b]	Allergenic solution injected in left arm[d] Quantity, micrograms[c]	CS-51RH, mm.	CS-51R[e], mm.	CS-56H, mm.
	1	10	15		
D.C.	2	1000	0		
	3	1000			0
	1	10	12		
I.J.	2	100	0		
	3	100			0
	1	10	12		
J.H.	2	100	0		
	3	100			0
	1	0.1		0	
L.S.	2	10		10	
	3	100		0	
	1	10		22	
V.G.	2	100		0	
	1	100			17
C.G.	2	1000			0
	3	100		0	
	1	100			14
C.P.	2	1000			0
	3	100	0		
	1	10			8
E.J.	2	100			16
	3	100	0		
	1	10			5
H.J.	2	100			±
	3	100	0		

[a] Cottonseed non-reactors were used as recipients. Sites were sensitized on the upper right arm with 0.05 ml. of serum from a cottonseed sensitive subject I.J. [b] The numbers refer to the order number of successive intramuscular injections of allergenic solution. At least seventy-two hours elapsed between the original sensitization and the first injection and also between later successive injections. [c] Quantity of allergen contained in 1 ml. of sterile physiological salt solution. [d] The numbers refer to the average diameter of the wheal, in mm., produced in the right arm fifteen to sixty minutes after intramuscular injection of allergen in the left arm. [e] Previous similar experiments using I.J. serum demonstrated that 1 microgram of CS-51R produced no reaction but 10 micrograms produced wheals and desensitized the sites to subsequent tenfold quantities of CS-51R. Similar results have been obtained with serum from other cottonseed sensitive patients.

(14) Coca, Walzer and Thommen,[10] pp. 356–362; *cf.* Lippard and Schmidt, *Am. J. Diseases Children*, **54**, 288 (1937).

Discussion

It is recognized that picric acid precipitations and recovery of the cathodic fractions CS-51R, CS-52R, CS-53R, and CS-54R, accomplished some fractionation in addition to that effected by electrophoresis. However, the fraction CS-56R, recovered from the picrate obtained from the anodic fraction, CS-56, contained only 13% total nitrogen as compared with 19 to 20% for the cathodic fractions. Furthermore, the active fraction CS-56RH obtained by acid treatment of CS-56R, contained 14% nitrogen compared with 20% nitrogen for the acid treated cathodic fraction CS-51RH. Electrophoresis alone accomplished a qualitatively similar fractionation. When a solution containing 25 g. of CS-1A, in cell 1+, was subjected to electrophoresis the first fraction, CS-39b, which migrated to cell 1− contained 17.7% nitrogen and 8.4% carbohydrate. In a similar experiment, but with CS-1A in cell 1− at the start, the first fraction, CS-49, obtained from cell 1+, contained 7.8% nitrogen and 51% carbohydrate. CS-49 was demonstrably less active than CS-39b. This fact led to the adoption of the procedure described for the electrophoretic fractionation. Passive transfer experiments showed that CS-49 and CS-39b cross neutralized sensitized sites, thereby demonstrating the presence of the same active group in each.

The activity of fractions CS-51R, CS-52R and CS-51RH appeared to be due to the protein component alone although these fractions contained 1% carbohydrate. This same or a slightly modified protein component appeared to be combined with varying proportions of polysaccharidic carbohydrate as represented by fractions CS-53R,

(15) Cooke, Barnard, Hebald and Stull, *J. Exptl. Med.*, **62**, 733 (1935); Stull, Cooke, Sherman, Hebald and Hampton, *J. Allergy*, **11**, 439 (1940).

(16) The authors are indebted to Dr. E. J. Coulson of this Laboratory for immunological tests. Studies in progress to determine quantitative antigenic relationships of cottonseed allergenic fractions will appear elsewhere.

CS-54R, CS-55R, CS-56H, CS-56R and CS-56RH. Available evidence supports the view that the polysaccharidic portion of these fractions is in chemical combination with protein rather than present as a contaminant. Contaminating polysaccharide would have been removed (for example CS-56S) in the picric acid precipitations and recoveries involved in preparing CS-53R, CS-54R, CS-55R and CS-56R. Also, anodic fractions CS-56H, CS-56R and CS-56RH contained 37, 35 and 30% carbohydrate, respectively. If the polysaccharide were not chemically combined the different procedures used in isolating these fractions would undoubtedly have produced a greater difference in carbohydrate content.

The electrophoretic technique employed effects separation of these substances because the net charge of the protein-polysaccharide components would be different from that of the protein alone. If the polysaccharide contained acidic groups or was combined with basic groups of the protein then the greater the proportion of combined carbohydrate the lower would be the *p*H of the isoelectric point of that compound. Thus fractions CS-51R, CS-52R, CS-53R, CS-54R, CS-55R and CS-56R containing 1, 1, 3, 6, 24 and 35% polysaccharide, respectively, were isolated from cells in which the *p*H was 9.4, 6.4, 5.7, 5.0, 4.1 and 3.0, respectively. This theory also accounts for the results obtained in the picric acid fractionation described in Paper III.[1] This picric acid fractionation method was based on the solubility depressing effect on the protein picrate exerted by excess picric acid in the solvent by the common ion effect of the law of mass action. Thus, if chemically combined polysaccharide decreased the basicity of the protein complex, the picric acid combining power would also be decreased. Excess picric acid in the solvent would exert a smaller solubility depressing effect on the picrate of those fractions containing the most combined carbohydrate. In accordance with this theory it is of interest to point out that the protein component of the most soluble picrate fraction CS-5-1RE contained 16.8% nitrogen as compared to 18.9% for the corresponding component of the less soluble fraction CS-5RE.[17] The results shown in Fig. 2, Paper III could also be accounted for by this theory.

(17) The carbohydrate content of these fractions is not available. However, results of carbohydrate determinations on the fractions given in Table 1 show that carbohydrate content is inversely proportional to the nitrogen content.

Available evidence indicates that CS-1A is a mixture of compounds containing the same active protein combined with varying proportions of polysaccharidic carbohydrate. Whether such a mixture exists in cottonseed or is produced by a progressive depolymerization caused by isolation procedures, as has been shown to occur with the pneumococcus polysaccharides,[9] cannot be decided by available evidence.

The allergenic fractions separated in this investigation possess remarkable stability. These compounds, therefore, might be suitable for correlating chemical degradation and progressive loss of antigenicity.[18]

Experimental

Apparatus.—The electrophoresis apparatus shown in Fig. 1 possesses advantages found in the cell described by Williams[3d] and is more simply constructed. This apparatus consisted of six cells made from 500-ml. Erlenmeyer flasks with 15-mm. side-tubes sealed to the flasks 50 mm. from the bottom. Connection between cells was made with short pieces of heavy-wall *gum rubber* tubing held in place with copper wire. The rubber connection withstood 5000 v. and replacement was not necessary until after four weeks of continuous use. The usual laboratory type of rubber tubing will not withstand effects of the high voltages employed. Each of the two terminal cells contained a 25 × 45 mm. cylindrical platinum foil electrode with sealed-in platinum lead wires (18 B & S gage). The cells were closed with cork stoppers provided with 1-mm. bore capillary tubes. By circulating cold water around the cells in a copper trough the temperature within the electrophoresis system was maintained between 20 and 30°. Mixing of cell contents when the current was interrupted was prevented by closing the rubber connecting tubes with pinchcocks. Diffusion of ampholytes during electrophoresis, in this apparatus, is minimized by the high potential electric barrier across the tubes which connect cells containing solutions at different *p*H.

High voltage direct current was obtained with a three-phase, full-wave rectifier employing six mercury rectifying tubes.[19]

Electrophoresis of CS-1A.—A solution of 25.0 g. of CS-1A in 400 ml. of distilled water (*p*H of solution 7.2) was placed in cell 1+, Fig. 1. The same volume of distilled water was placed in each of the other cells. Toluene was employed as a preservative in each cell. Direct current at 2000 v. was applied to the system for four hours, after which the voltage was increased to 2500 for forty-four hours. The current, initially 0.3 ma., gradually rose to a maximum of about 35 to 45 ma. in twenty-four hours and then declined to 16 ma. at the end of forty-eight hours. The temperature was maintained between 20–30°. After this preliminary forty-eight hour period the current was interrupted and the solution was removed from cell + and

(18) Landsteiner,[7] p. 18, discusses this question.

(19) The electrical apparatus, with needed safety appliances, was made by a commercial firm according to the specifications of Dr. M. J. Horn of this Bureau.

reserved for examination. The contents of cells − and 1− were removed and discarded. The *p*H of the solution in each cell was: +, 2.5; 1+, 3.9; 2+, 6.4; 2−, 10.1; 1−, 11.3; −, 11.7.[20]

After placing 400 ml. of distilled water in cells +, 1−, and −, direct current at 4000 v. was applied to the system for twenty-four hours. The current, initially 10 ma., rose to 20 ma. in three hours and then gradually dropped to 5 ma. After twenty-four hours the content of cell + was removed and combined with the solution previously obtained from cell +. The solution was withdrawn from cell − and discarded. The *p*H of each solution at the end of this period was: +, 3.3; 1+, 3.9; 2+, 5.1; 2−, 6.6; 1−, 9.8; −, 11.2.

The final phase of the electrophoresis was carried out after 400 ml. of distilled water had been added to cells + and −. Direct current at 5000 v. was now applied to the system for ninety-six hours. The current reached a maximum of 13 ma. in one hour, then fell to 5.0 ma. in twenty-four hours and remained constant during the rest of the run. After ninety-six hours the content of cell + was combined with the solutions previously obtained from that cell. The solutions from the other cells were stored separately. The *p*H of each solution was: +, 3.0; 1+, 4.1; 2+, 5.0; 2−, 5.7; 1−, 6.4; −, 9.4. Solutions obtained from cells − and 1− in the preliminary 2500 and 4000 volt runs contained inorganic cations with a negligible quantity of active protein, and were therefore discarded.

Isolation and Purification of the Cathodic Fractions [CS-51R(−); CS-52R(1−); CS-53R(2−); and CS-54R(2+)].—The active protein fractions from cells −, 1−, 2− and 2+ were isolated and further purified by the following uniform procedure.

The combined solution from each cell obtained from four 25-g. portions or 100 g. of CS-1A was filtered with pressure through a No. 7 Seitz clarifying pad and cooled to 7°. To this solution was added 400 ml. (excess) of a saturated aqueous solution of picric acid. The resulting suspension was cooled overnight at 5° and centrifuged at 5°. The separated picrate was stirred with 250 ml. of cold water, recovered by centrifuging, and dried in a vacuum over phosphorus pentoxide. The average yields of picrate obtained from individual cells from four 100-g. lots of CS-1A were as follows: −, 7.0 ± 0.3 g.; 1−, 9.6 ± 0.7 g.; 2−, 7.4 ± 0.4 g.; 2+, 4.5 ± 1.3 g.

The recovery and purification of the active protein from the picrate obtained from cell − is described below. This procedure is typical of that used on the picrates from the other cells. To 28.0 g. of protein picrate dissolved in 2.1 liters of 0.05 *N* sodium hydroxide, was added, with vigorous stirring, 2.1 liters of commercial absolute ethanol.[21] The *p*H of the clear yellow solution was adjusted to 6.3 with acetic acid, 4.2 liters more ethanol was added and the suspension was kept at 5° for two days. The clear supernatant liquid was decanted and discarded. The gummy residue was centrifuged at 5° and then dissolved in 700 ml. of water. The solution was boiled with activated carbon,[22] cooled and centrifuged. The solution was filtered through a Seitz sterilizing pad and poured into 3 liters of cold ethanol. Precipitation occurred when the *p*H was adjusted to 6.3. The suspension was cooled at 5° overnight and the solid was recovered by centrifuging at 5°. After drying in a vacuum over phosphorus pentoxide, 17.0 g. of a brownish colored solid was obtained.

This solid was dissolved in 1700 ml. of water and to the solution was added 400 ml. of a saturated aqueous solution of picric acid. After standing overnight at room temperature the picrate was separated by centrifuging. The picrate was washed with 125 ml. of water, centrifuged and dried in a vacuum over phosphorus pentoxide; yield 19.5 g.

The protein was recovered as previously described. A yield of 12.9 g. of a tan-colored solid was obtained. This solid was dissolved in 650 ml. of water and the *p*H of the solution was adjusted to 5.9. The solution, decolorized by boiling with carbon, was cooled, centrifuged and filtered through a Seitz sterilizing pad. The solution was poured into 2.6 liters of cold ethanol and when cooled to 8° the *p*H was adjusted to 6.3. The suspension was cooled overnight and the solid recovered by centrifuging. The solid was washed first with 200 ml. of cold 80% ethanol, and then with 200 ml. of cold ethanol. The solid, CS-51R, was dried in a vacuum over phosphorus pentoxide. A 1% aqueous solution of CS-51R gave color tests like those previously described for CS-13A.[2b]

Isolation of CS-56 from Cell +.—The combined solution from cell + (4.8 l.) obtained from 100 g. of CS-1A was filtered through a No. 7 Seitz clarifying pad and poured into 8 liters of cold ethanol. Precipitation occurred without further *p*H adjustment. After standing for several days at 5° the supernatant liquid was decanted and discarded. The solid (CS-56) was recovered by centrifuging and dried in a vacuum over phosphorus pentoxide. The average yield of CS-56 from four 100-g. lots of CS-1A was 21.4 ± 1.1 g.

CS-55 from Cell 1+.—The combined solution (1.6 l.) from cell 1+ from 100 g. of CS-1A was filtered through a no. 7 Seitz pad and poured into 4 liters of cold ethanol. The *p*H was adjusted to 6.3 with dilute acetic acid and the solution was cooled at 5° for several days. The solid (CS-55) was isolated similarly to CS-56. The average yield of CS-55 from four 100-g. lots of CS-1A was 5.7 ± 0.4 g.

CS-57 from Cells 1+ and 2+.—The insoluble matter which precipitated in cells 1+ and 2+ during electrophoresis was stirred with 95% ethanol. The sticky brown mass was centrifuged and dried over phosphorus pentoxide. This solid (CS-57) was obtained as an amorphous light brown water insoluble powder. The average yield from four 100-g. lots of CS-1A was 20.0 ± 0.4 g.

CS-51RH Obtained by Acid Treatment of CS-51R.—A solution of 500 mg. of CS-51R in 10 ml. of 0.100 *N* sulfuric acid was refluxed for four hours. The sulfuric acid was quantitatively neutralized with barium hydroxide and the barium sulfate removed by centrifuging. The solution was boiled with carbon, cooled, centrifuged and filtered through a Seitz sterilizing pad. The clear colorless solution (40 ml.) was poured into 160 ml. of cold ethanol. Adjustment

(20) The *p*H of aqueous solutions was determined with a Beckman *p*H meter. The *p*H of alcoholic suspensions, later referred to, was determined colorimetrically on a spot plate.

(21) Throughout this work commercial absolute ethanol was used; concentrations are expressed as volume per cent.

(22) In all cases of carbon decolorization the carbon was washed with a small quantity of water and the extract was combined with the main solution.

of the *p*H to 6.3 caused precipitation. After cooling overnight at −7° the solid was centrifuged off, washed with 10 ml. of cold 80% ethanol, and dried in a vacuum over phosphorus pentoxide. A yield of 226 mg. of white solid (CS-51RH) was obtained. A 1% solution of CS-51RH gave the following color tests: pinkish biuret, deep purple ninhydrin, strongly positive Millon test, very faint Molisch test and no reduction of Benedict reagent.

CS-56H Obtained by Acid Treatment of CS-56.—A solution of 44.3 g. of CS-56 in 600 ml. of 0.100 *N* sulfuric acid was refluxed for four hours. The protein component was isolated as described for CS-51RH. A yield of 22.4 g. was obtained. After two more reprecipitations involving carbon decolorization, a final yield of 17.0 g. of white solid, CS-56H, was obtained. Results of color tests on a 1% solution of CS-56H were: pinkish biuret, deep purple ninhydrin, positive Millon, positive Molisch, no reduction of Benedict reagent.

By evaporation of the alcoholic solution remaining after the first precipitation of CS-56H a yield of 10.7 g. of a brown hygroscopic powder containing 2.9% nitrogen was obtained. This is 26.8% of the original CS-56. A 1% solution of this carbohydrate mixture gave strongly positive Bial and aniline hydrochloride tests indicating its pentose nature. It contained 61% reducing sugar calculated as glucose. It yielded an unstable phenylosazone which melted constantly at 154–156° after several recrystallizations from 30% ethanol.

Fraction CS-56R.—To a solution of 4.5 g. of CS-56 in 250 ml. of water was added 100 ml. of a saturated solution of picric acid (excess). After standing overnight the suspension was centrifuged for one hour in the batch bowl of the Sharples supercentrifuge at 45,000 r. p. m. Ordinary centrifuging would not separate the stable picrate suspension. The gummy picrate was dissolved in 175 ml. of 0.05 *N* sodium hydroxide and the protein recovered by the method described for the cathodic picrates. A yield of 1.13 g. of white solid CS-56R was obtained. A 1% solution of CS-56R gave no reduction of Benedict reagent even after boiling for one hour with 3 *N* hydrochloric acid.

Fraction CS-56S.—The supernatant solution obtained by centrifuging the picrate of CS-56 (above) was poured into 1400 ml. of cold ethanol and cooled at 3° overnight. The solid was separated by centrifuging and washed with 20 ml. of cold ethanol. A yield of 1.27 g. of white solid (CS-56S) was obtained after drying in a vacuum over phosphorus pentoxide. A 1% solution of CS-56S did not reduce Benedict reagent, but after boiling one minute with 3 *N* acid the neutralized solution gave strong reduction.

CS-56RH.—A solution containing 500 mg. of CS-56R in 10 ml. of 0.1 *N* sulfuric acid was refluxed for four hours. The solid was isolated as described for CS-51RH. A yield of 167 mg. of CS-56RH was obtained. A 1% solution of CS-56RH gave no reduction of Benedict reagent.

Summary

1. In an electrophoretic fractionation of the cottonseed allergenic fraction CS-1A, a protein migrated toward the cathode and a polysaccharidic-protein migrated to the anode.

2. The evidence presented indicates that CS-1A is a mixture containing a specifically active protein and active compounds of this protein chemically combined with varying amounts of polysaccharidic carbohydrate.

3. The cutaneous activity of the cathodic and anodic fractions was not decreased by refluxing with 0.1 *N* acid for four hours.

4. The reagin neutralizing capacity of the fractions was decreased but not destroyed by the acid treatment.

5. A simple apparatus is described for large-scale, high voltage electrophoretic fractionation of water soluble ampholytes which is suitable for preparative work.

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[CONTRIBUTION FROM THE DEPARTMENT OF RESEARCH IN PURE CHEMISTRY, MELLON INSTITUTE OF INDUSTRIAL RESEARCH]

Cinchona Alkaloids in Pneumonia. IX. Quaternary Salts

BY MARY A. CLAPP, ALICE G. RENFREW AND LEONARD H. CRETCHER

Alkyl halides may be added readily to the quinuclidine nitrogen of cinchona alkaloids with the formation of quaternary salts. Reports in the literature offer rather conflicting evidence concerning the antipneumococcic action of such cinchona derivatives. The most encouraging results were the findings of Felton and Dougherty[1] that the small antipneumococcic power of dihydroquinine was enhanced in certain aromatic quaternary salt derivatives prepared by Jacobs and Heidelberger,[2,3] in particular, in dihydroquinine *p*-chloroacetylaminophenol and dihydroquinine 4-chloroacetylaminopyrocatechol. Morgenroth and Schnitzer[4] confirmed the observations of the intensified antipneumococcic action of quaternary salts of dihydroquinine with chloroacetanilide and *p*-chloroacetylaminophenol. However, similar quaternary derivatives of optochin

(1) Felton and Dougherty, *J. Expll. Med.*, **35**, 761 (1922).

(2) Jacobs and Heidelberger, THIS JOURNAL, **41**, 2090 (1919).

(3) Jacobs, "The Harvey Lectures," 1923–24.

(4) Morgenroth and Schnitzer, *Z. Hyg. Infectionskrank.*, **103**, 441 (1924).