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The Chemistry of Allergens. II. Isolation and Properties of an Active Protein Component of Cottonseed[1]

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The human allergic response provides the only available criterion for recognizing allergens, the substances in the diet or environment that act as specific incitants of a distinct group of human disease reactions called allergies. For the most part, allergens are considered to be either complete or partial antigens and, by virtue of this classification, closely related to the native proteins. To isolate and characterize as chemical entities the allergens of cottonseed is the objective of this investigation.

The isolation of a potent allergenic fraction CS-1[1a] from cottonseed recently has been described.[2] The previously characterized proteins of cottonseed were also shown to possess no important degree of inherent allergenic activity.[3]

The chemical procedures used in isolating CS-1 included alcohol precipitations, boiling in aqueous solution, precipitation of impurities with basic lead acetate, and removal of lead with hydrogen sulfide. These procedures are more drastic than are usually employed in the isolation of a biologically active substance. However, each step leading to the separation of CS-1 resulted in demonstrable increase in specific activity.

The literature concerning the immunological specificity of native plant proteins and protein split products is extensive.[4] As recently as 1936 Landsteiner stated, "The most important studies on the specificity of plant proteins were made by Wells and Osborne." The active protein constituent of CS-1 corresponds essentially in chemical and physiological properties with the "so-called proteoses" separated from seeds by Wells and Osborne.[5] Their proteoses were apparently chemically distinct from the other reserve proteins of seeds, possessed strong anaphylactogenic activity and were stable to heating at 100°. Earlier, Schloss[6] had demonstrated that the proteoses from almonds and oatmeal were more active than the other proteins in producing local skin reactions on a boy hypersensitive to these foods.

Clinically important allergenic constituents of cottonseed have been concentrated in CS-1. This paper describes the isolation from CS-1 of a protein-like substance possessing an unusual combination of chemical and physiological properties.

CS-1 formed a picrate having allergenic activity equal to that of CS-1. Retention of activity by the yellow picrate (CS-3) suggested chromatographic adsorption for further purification, characterization, and evaluation of homogeneity. The active constituents of CS-3 were quantitatively adsorbed by activated aluminum oxide in a sharply defined yellow layer which was resolvable into two layers by development with the solvent.

Diagram I shows the results of a typical chromatographic analysis of the picrate.

The major allergenic fraction, CS-5, appeared to be chromatographically homogeneous or composed of chemical entities too closely related to allow fractionation by this technique. Fractions CS-5 and CS-6 exhibited no appreciable differences in clinical activity. Both gave specific cutaneous reactions on cottonseed-sensitive patients in threshold dilutions ranging from $1:10^5$ to $1:10^6$ and could not be differentiated by im-

(1) Not subject to copyright.

(1a) For convenience in reference a system of symbols has been adopted to designate fractions.

(2) Spies, Bernton and Stevens, *J. Allergy*, **10**, 113 (1939).

(3) Jones and Csonka, *J. Biol. Chem.*, **64**, 673 (1925), in their study of the proteins of cottonseed observed that a significant portion of the total nitrogen in sodium chloride extracts of cottonseed was lost during dialysis of the heat uncoagulable fraction. This fraction was precipitable by tungstic acid but was not isolated or further characterized. This non-coagulable, diffusible fraction is apparently contained in CS-1.

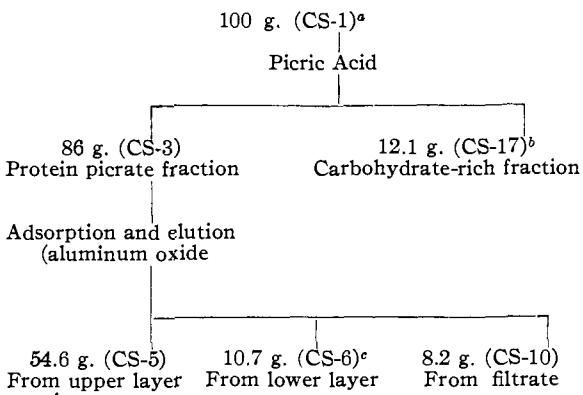
(4) For comprehensive reviews on these subjects see: H. Gideon Wells, "The Chemical Aspects of Immunity," The Chemical Catalog Co., N. Y., 1929; K. Landsteiner, "The Specificity of Serological Reactions," Charles C. Thomas, Baltimore, 1936; H. Zinsser, J. F. Enders and L. D. Fothergill, "Immunity—Principles and Applications in Medicine and Public Health," The Macmillan Co., New York, N. Y., 1939.

(5) Wells and Osborne, *J. Infectious Diseases*, **17**, 259 (1915), stated, "In their anaphylactic power these 'natural proteoses' differ sharply from proteoses obtained from animal proteins by digestion with enzymes or by chemical hydrolysis, such artificial products being almost if not entirely non-anaphylactogenic. Furthermore, those products of hydrolysis which result from heating vegetable proteins with acids, with water under pressure, or by peptic digestion, have, so far as we have tested them, no anaphylactogenic properties. From these facts it would seem that the vegetable 'proteoses' belong to a group of proteins which are chemically different from any heretofore recognized. They resemble highly soluble native proteins in their anaphylactogenic capacity and are probably quite as complex in their chemical constitution. Their designation as 'proteoses' is consequently improper." *Cf.* Fink, *J. Infectious Diseases*, **25**, 97 (1919).

(6) O. M. Schloss, *Am. J. Diseases Children*, **3**, 341 (1912).

DIAGRAM I

CHROMATOGRAPHIC ANALYSIS OF ALLERGENIC PROTEIN PICRATE FROM COTTONSEED



[a] Contained 5.1% water. [b] CS-17 was obtained from the filtrate, after removal of the picrate, by precipitation with 75% alcohol. Purification involving further precipitations with alcohol yielded a colorless substance containing 8.7% nitrogen. CS-17 does not reduce Benedict's reagent, but after acid hydrolysis has strong reducing properties, indicating its polysaccharidic nature. CS-17 possessed lower allergenic activity than CS-5, but evidence obtained in preliminary hydrolysis studies which are in progress indicates that this activity is inherent and not due to incomplete separation from the active portion of CS-5. [c] A chromatographic analysis of CS-6 with prolonged development failed to cause sufficient partitioning to indicate separation of chemically different components.

munological tests.[7] CS-10 obtained from the filtrate of the adsorption showed no allergenic activity and no antigenic properties.

CS-5 was demonstrated to be immunologically identical with antigen present in the unheated aqueous extract of cottonseed embryo. Intramuscular injection of 0.5 mg. of CS-5 in one arm produced wheals (15–40 mm. in diameter) in sites on the opposite arm passively sensitized with 0.05 ml. of serum from cottonseed-sensitive patients.[8]

A picric acid-free, nitrogenous compound (CS-13) was recovered from CS-5. CS-13 exhibited allergenic activity greater than CS-5 and was also immunologically identified with antigen present in the unheated aqueous extract of cottonseed embryo.

The method originally developed for the isolation of CS-1 has been modified and adapted to a larger scale process. The use of hydrogen sulfide in removing excess lead was eliminated to avoid possible introduction of sulfur into the product, CS-1A. CS-1A yielded an active picrate from which the picric acid-free compound CS-13A could be obtained analogously to CS-13. CS-13 and CS-13A gave the usual protein color and precipitation tests. The Molisch test was positive but the amount of carbohydrate present in these fractions was insufficient to reduce Benedict reagent even after acid hydrolysis. CS-13A contained 2.06% sulfur. Intramuscular injection of 0.01 mg. of CS-13A in one arm produced wheals (15–25 mm.) in sites on the opposite arm passively sensitized with 0.05 ml. of serum from cottonseed-sensitive patients.[9]

Available evidence indicates that the allergenic fractions CS-13 and CS-13A are protein in nature and native to the cottonseed.[10] The possibility is recognized, however, that these fractions may represent the allergenic group of some larger protein molecule, native to cottonseed. Further study would be required to reach a definite conclusion as to which hypothesis is correct. Designation of this type of substance as native protein is not provided for in existing protein classification systems. It is suggested, therefore, that the term natural proteose be adopted to describe them.

(7) The anaphylactic reaction of the guinea pig was used to demonstrate antigenicity. A detailed account describing the immunological reactions of allergenic constituents of cottonseed will be reported elsewhere.

(8) Thereafter, all sensitized sites failed to respond to a second similar injection of CS-5. However, a subsequent injection of 0.5 ml. of an unfractionated water extract of cottonseed (1:11) produced moderate wheals of less than one-half the size induced by the initial injection of CS-5. These results showed that CS-5 contained the principal allergenic constituent of cottonseed. Also demonstrated was the fact that the unfractionated extract contained more than one allergen capable of inducing positive reactions in sites passively sensitized with serum from cottonseed-sensitive subjects. Clinical observations have previously prompted the hypothesis that foods or pollens contain multiple allergens. For the first time the validity of this hypothesis has been experimentally demonstrated by actual isolation of one of the allergenic components. Clinical significance of these observations will be presented elsewhere. *Cf.* Lippard and Schmidt, *Am. J. Diseases Children*, **54**, 288 (1937).

(9) This was the threshold dilution, as 0.001 mg. of CS-13A produced slight reaction only in some cases.

(10) In chemical properties CS-13 corresponds to the proteoses except, however, that it does not give a precipitate with nitric acid and is more diffusible. The best evidence available, however, for regarding CS-13 as a complete or native protein is based on immunological data. While the subject is somewhat controversial, weight of opinion supports the view that complete proteins are required for antigenicity. P. Hartley, "A System of Bacteriology," vol. 6, His Majesty's Stationery Office, London, 1931, page 231, states, "The results which have been obtained in carefully conducted experiments in which due regard has been paid to chemical changes produced by hydrolysis and to the properties of the materials used led to the conclusion that antigenic function, so characteristic a property of native proteins, is lost when the large protein molecule is broken down into smaller fragments. At what stage in the breakdown of the proteins the property is lost is unknown but all available evidence supports the view that this occurs very early and before substances known as proteoses have been formed." Zinsser and Bayne-Jones, "Textbook of Bacteriology," 8th edition, 1939, p. 166, state, "The antigenic substance of proteins is easily destroyed by cleavage. Even the higher cleavage products, polypeptides and proteoses, no longer show antigenic properties."

Experimental[11]

Isolation of Active Fraction (CS-1A).—The fat was removed from choice quality, ground cottonseed embryo by extraction with benzene at temperatures not exceeding 25°.[12]

Three kilograms of benzene-extracted cottonseed was mechanically stirred for at least two hours with 18 liters of distilled water. The cake was separated by centrifuging and discarded. The extract was filtered through cheesecloth and heated to 100° in 12-liter flasks in an autoclave. After cooling, the coagulum was filtered off on cheesecloth. The filtrate was concentrated under reduced pressure (water pump) so that the volume of the combined extracts from 6 kg. of cottonseed was reduced to 5 liters. During this concentration the extract was allowed to flow into a 12-liter, three-necked distilling flask heated at 92–95° on a water-bath at a rate slow enough to prevent excessive foaming. It was also necessary to occasionally remove the concentrate from the distilling flask to facilitate evaporation.

Ethanol[13] was added slowly to the concentrated extract (5 l.) with stirring to 75%. The sticky brown coagulum which formed was collected and the brown solution was decanted and discarded. The coagulum was dissolved in 5 liters of distilled water by mechanical stirring and warming with a glass steam coil. When solution and uniform suspension was complete, ethanol was added to 25%. To this suspension was added 2.5 liters of 10% basic lead acetate (of the type used for sugar analysis) dissolved in 25% ethanol solution. The precipitated lead salts were separated by centrifuging and discarded. Colloidal lead salts suspended in the solution were further removed by passing through a Sharples super-centrifuge at 50,000 r. p. m. To the slightly turbid solution ethanol was again added with stirring to 70% concentration. The resulting suspension was allowed to settle at −7° overnight. The clear supernatant liquid was decanted and discarded. The solid was freed from most of the ethanol by suction on a Büchner funnel. When dried in a vacuum desiccator over phosphorus pentoxide and ground, 100 to 130 g. of a brown powder was obtained. The next step can be carried out directly, however, without drying the solid. The brown solid obtained from 6 kg. of cottonseed was dissolved and suspended in 1200 ml. of distilled water by vigorous mechanical stirring for two hours. The suspension was centrifuged in 250-ml. lots in the batch bowl of the Sharples super-centrifuge for twenty minutes at 45,000 r. p. m. A viscous, brownish, slightly turbid solution was obtained. To the centrifuged solution 60 ml. of 10% sodium carbonate solution was added. The suspended lead carbonate was removed by centrifuging in the batch bowl of the Sharples super-centrifuge at 45,000 r. p. m. for thirty minutes. The slightly turbid solution was then filtered with pressure through a no. 7 Seitz clarifying pad. The average *p*H of the solutions thus obtained was 9.5.[14] The *p*H was adjusted to 6.1–6.3 with 50% acetic acid solution. This solution was poured slowly with stirring into five volumes of cold ethanol. The suspension was adjusted to *p*H 6.1–6.3 with 20% sodium hydroxide and cooled overnight at −7°. The supernatant solution, which should be clear, was siphoned off and the solid was obtained by centrifuging. The solid (CS-1A) was dried in a vacuum over phosphorus pentoxide and powdered. The average yield from twelve 6-kg. lots of cottonseed was 68.4 g. A dried sample of CS-1A equilibrated with air contained 10.7% total nitrogen and 3.6% water. Clinical comparison of CS-1 and CS-1A showed that they possessed equal activity. CS-1A did not reduce Benedict reagent but gave strong reduction after acid hydrolysis. Preparations of CS-1 contained 14.7 to 15.8% nitrogen and undoubtedly the lower nitrogen content of CS-1A was due to a greater proportion of polysaccharide.

Preparation of Allergenic Protein Picrate (CS-3).—To the solution of 50 g. of CS-1 in 800 ml. of water there was added with stirring 1 liter of a saturated aqueous solution of picric acid (25°). The picrate suspension was cooled overnight at 5° and the yellow precipitate was separated by centrifuging. The picrate precipitate was washed by stirring with 500 ml. of water and recentrifuged. The resulting solid (CS-3) was dried in a vacuum over phosphorus pentoxide; yield, 42.5 g.

Chromatographic Adsorption of Allergenic Picrate.—Approximately 20-g. lots of finely ground CS-3 were dissolved in 5 liters of 50% ethanol by warming (50–60°) and stirring. The clear filtered solution was allowed to flow slowly through a 44 × 188 mm. column of activated aluminum oxide[15] (Brockmann).

A sharply defined yellow layer formed at the top of the column. Development with 500 to 1500 ml. of 50% ethanol resolved the original layer with formation of two layers.

Elution of Picrate.—Ninety-one grams of the aluminum oxide–picrate adsorption compound from the upper layer (from 20 g. of CS-3) was stirred for ten minutes with 500 ml. of 0.05 *N* sodium hydroxide. The solid was separated by centrifuging and eluted with three more 400-ml. portions of 0.05 *N* alkali. To the combined alkaline extracts there was added with stirring an equal volume of a saturated aqueous solution of picric acid (excess). The precipitated picrate was separated by centrifuging and washed by stirring with 500 ml. of water. The picrate (CS-5) was dried in a vacuum over phosphorus pentoxide. The average yield of eluted picrate from this layer was 64%. CS-5 contained 18% total nitrogen, was insoluble in water and organic solvents, moderately soluble in 50% aqueous ethanol, and very soluble in 50% dioxane.

Recovery of Active Protein from the Picrate.—To a solution containing 15 g. of eluted picrate dissolved in 1150 ml. of 0.05 *N* sodium hydroxide an equal volume of ethanol was added with vigorous mechanical stirring.

(11) Microanalyses were carried out by Thomas H. Harris and Ernest J. Umberger. Dorris C. Chambers assisted in the clinical studies.

(12) A modification of the extractor described by Drake and Spies, *Ind. Eng. Chem., Anal. Ed.*, **5**, 284 (1933), was used which permitted extraction of 25-pound (11-kg.) lots with solvent cooled to room temperature.

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

(14) The *p*H of aqueous solutions was usually determined with a Beckman *p*H meter. The *p*H of alcoholic suspensions, later referred to, was determined colorimetrically on a spot plate using brom cresol purple indicator.

(15) Obtained from Merck & Company, Rahway, N. J. Celite (Johns Manville Co.) adsorbs CS-5 weakly. Commercial silica gel does not adsorb it at all from 50% ethanol solution.

The *p*H of the clear solution was adjusted to 6.3 with 50% acetic acid. A second 1150 ml. volume of ethanol was then added and the suspension was allowed to stand at 5° overnight. The separated solid was centrifuged off and extracted with 400 ml. of water. The insoluble portion was separated and discarded. The solution was brought to *p*H 6.3 and, after stirring with decolorizing carbon, was filtered through a Seitz sterilizing pad. The clear solution was poured into 3 volumes of cold ethanol. The *p*H was adjusted to the coagulation point and the solution was allowed to stand at 5°. The coagulation point in 75% ethanol lies between *p*H 6.1 and 6.3 and is very sharp. The solid was separated and redissolved in 500 ml. of water. The solution was boiled with activated carbon, filtered, and reprecipitated with ethanol as before. The solid, CS-13, was centrifuged off and dried in a vacuum over phosphorus pentoxide; yield, 2.9 g. *Anal.* Found: N, 17.9.

CS-13 was soluble in water but insoluble in organic solvents. Results of color tests were: pinkish biuret, deep blue ninhydrin, positive Millon, negative Hopkins–Cole, faintly positive Molisch, no reduction of Benedict reagent after acid hydrolysis. Results of precipitation tests were: precipitates with phosphotungstic acid, picric acid and 2.5% trichloroacetic acid after boiling and cooling. No precipitates with basic lead acetate nor mercuric acetate. All tests were conducted on a 1% aqueous solution of CS-13. CS-13 diluted $1:10^6$ gave strong cutaneous reactions on cottonseed-sensitive patients.

CS-13A.—In an electrophoretic system the picric acid precipitable fraction of CS-1 and CS-1A migrated toward the negative electrode. In an electrophoretic fractionation of CS-1A (which will be described separately) the active substance in the cell containing the negative electrode was precipitated as the picrate. From this fraction the picric acid-free protein was isolated as follows: 2.9 grams of picrate was dissolved in 230 ml. of 0.05 *N* sodium hydroxide and 230 ml. of ethanol was added. The *p*H of the clear solution was adjusted to 6.3 with 10% acetic acid. To the suspension was added 460 ml. of cold ethanol and the *p*H again carefully adjusted to the coagulation point (6.1–6.3). The suspension was cooled overnight at −5°. The precipitated solid was centrifuged off. The gummy solid was dissolved in 75 ml. of warm water and the solution was boiled with carbon, cooled, centrifuged, and filtered through a Seitz sterilizing pad. The filtrate was poured into 300 ml. of cold ethanol. If coagulation does not occur, adjustment of *p*H to 6.3 is necessary. The suspension was cooled at −5° overnight. A yield of 1.47 g. was obtained by centrifuging and drying the solid over phosphorus pentoxide.

Further purification consisted of again reprecipitating with picric acid and recovering the picric acid-free solid (1.0 g.) as described before. This solid was dissolved in 50 ml. of water and the solution was boiled with carbon, cooled, and centrifuged. The solution was filtered through a fritted glass filter and poured into 190 ml. of cold ethanol. Coagulation occurred without *p*H adjustment. The suspension was cooled to −5°, centrifuged and the solid dried in a vacuum over phosphorus pentoxide. A yield of 0.79 g. of white solid (CS-13A) was obtained. A 1% solution of CS-13A gave the same reactions as those obtained with CS-13. A dried sample of CS-13A equilibrated with air was used for analysis. *Anal.* Found: C, 43.4; H, 6.78; ash, 0.51; N, 18.1 (micro Kjeldahl); S, 2.04, 2.09 (micro); H_2O, 9.4; P, neg. CS-13A diluted $1:10^6$ gave strong cutaneous reaction on cottonseed-sensitive patients.

Summary

1. The active component of the protein picrate obtained from a concentrate of allergenic constituents from cottonseed embryo was adsorbed quantitatively on aluminum oxide.

2. The adsorbed picrate fractions were eluted and recovered without loss of allergenic activity.

3. A protein possessing an unusual combination of chemical and physiological properties was recovered from the active picrate. The term natural proteose is suggested to designate proteins of this class.

4. Unfractionated aqueous extracts of cottonseed contain a substance or substances other than those present in CS-5 capable of inducing positive reactions in sites passively sensitized with serum from cottonseed-sensitive patients.

WASHINGTON, D. C. RECEIVED JANUARY 5, 1940