Poly-DL-arginine Flavianate.—A solution of flavianic acid (5 g.) in water (5 ml.) was added to poly-DL-arginine sulfate (1 g.) in water (2 ml.) and the mixture left in the refrigerator for 48 hours. The supernatant liquid was decanted and the residue washed with cold water and alcohol and dried *in vacuo* over sulfuric acid. It was dissolved in hot dimethyl formamide (2 ml.), the solution filtered and poured into absolute ethanol (75 ml.). The yellow flocculent precipitate of poly-DL-arginine flavianate which separated out was centrifuged, washed several times with absolute ethanol and ether and dried *in vacuo* over sulfuric acid; yield quantitative, m.p. 214–220° (dec.).

Anal. Calcd. for poly-DL-arginine flavianate (*n* average = 80): C, 40.8; H, 3.8; N, 17.9; amino N, 0.04; S, 6.8. Found: C, 40.3; H, 4.1; N, 17.9; amino N, 0.5; S, 7.2.

Total Hydrolysis of Poly-DL-arginine Flavianate.—Poly-DL-arginine flavianate was hydrolyzed with 10% hydrochloric acid at 120° for 6 hours and the arginine content of the neutralized hydrolysate determined enzymatically and microbiologically.

Anal. Calcd. for 100 mg. poly-DL-arginine flavianate (*n* = 80): L-arginine, 18.5 mg. Found: L-arginine, 19.6 mg. (enzymatic method),[14] 18.0 mg. (microbiological method).[13]

Acknowledgment.—The authors are indebted to Miss S. Sicher for carrying out the microbiological assays.

REHOVOT, ISRAEL RECEIVED JANUARY 25, 1951

[CONTRIBUTION FROM ALLERGEN RESEARCH DIVISION, BUREAU OF AGRICULTURAL AND INDUSTRIAL CHEMISTRY, U. S. DEPARTMENT OF AGRICULTURE]

The Chemistry of Allergens. XI. Properties and Composition of Natural Proteoses Isolated from Oilseeds and Nuts by the CS-1A Procedure[1,2]

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

The procedure used to isolate the principal allergen of cottonseed, CS-1A, has been applied to the following oilseeds and nuts: Almonds, Brazil nuts, castor beans, coconuts, Barcelona and DuChilly filbert nuts, flaxseed, kapok seed, black mustard seed, peanuts, pecan nuts, soybeans, tung nuts and black walnuts and English walnuts. Typical natural proteoses possessing similar chemical, allergenic and antigenic properties were obtained from almond nuts, Brazil nuts, castor beans, cottonseed, Barcelona and DuChilly filbert nuts, flaxseed, kapok seed and mustard seed. Soybeans and peanuts yielded immunologically atypical fractions, and black walnuts yielded a fraction having immunological properties intermediate between those of the typical and atypical groups. The chemical composition, allergenic and antigenic properties of these substances are described. It is suggested that these substances be recognized as a class of native proteins and that they be designated by the term natural proteose proposed by Wells and Osborne, or the term natural proteone to show that, while these substances have chemical properties similar to those of non-antigenic derived proteoses or peptones they are highly antigenic preformed components of the seeds.

It has long been known that multiple sensitivity to cottonseed, nuts and other oilseeds is a frequently encountered clinical grouping.[3] Consideration of this clinical classification, after isolation of the principal allergen of cottonseed, CS-1[4] or CS-1A,[5] led to the assumption that allergens of other oilseeds and nuts might be similar enough to be isolated by a general procedure.[6,7] The validity of this speculation was shown by the isolation of the ricin-free allergenic fraction CB-1A from castor beans[7] by the same procedure originally developed for the isolation of CS-1A from botanically unrelated cottonseed. CB-1A was remarkably similar in composition and chemical and immunological properties to CS-1A, although differing in allergenic specificity. Kapok seed, which is botanically related to cottonseed, also yielded a similar allergen, KS-1A.[8] CS-1A and CB-1A, although not homogeneous, are important because they contain the most potent allergens of respective seeds, completely freed from other allergens and antigens present in the seeds. This has been conclusively shown by extensive passive transfer and cutaneous tests on sensitive human subjects[9,10] and by gross anaphylaxis and Schultz–Dale tests with guinea pigs.[11–14]

CS-1A and CB-1A provided relatively abundant and highly concentrated source materials for further studies designed to obtain homogeneous allergens or allergens sufficiently purified to permit determination of the chemical nature of the active components with reasonable certainty. The essentially carbohydrate-free natural proteoses[15] CS-60C[16] and CB-65A,[17] possessing full allergenic activity, were isolated from CS-1A and CB-1A and all evidence adduced showed that the allergenic and immunological specificities of CS-1A and CB-1A were inherent in the type of substance represented by CS-60C and CB-65A, respectively.

To determine the distribution of allergenic natural proteoses, several more oilseeds and nuts were subjected to the CS-1A procedure. The present paper describes results of this study with almond nuts, Brazil nuts, castor beans, cottonseed, coconuts, filbert nuts, flaxseed, kapok seed, mustard seed, pecan nuts, peanuts, soybeans, tung nuts, black walnuts and English walnuts.

(1) Not subject to copyright.

(2) Paper X, Spies, Coulson and Stevens, THIS JOURNAL, **66**, 1798 (1944).

(3) Bowman and Walzer, "Asthma and Hay Fever in Theory and Practice," Charles C. Thomas, Baltimore, Maryland, 1931, p. 394.

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

(5) Spies, Coulson, Bernton and Stevens, THIS JOURNAL, **62**, 1420 (1940).

(6) Spies, *ibid.*, **63**, 2994 (1941).

(7) Spies and Coulson, *ibid.*, **65**, 1720 (1943).

(8) Coulson, Spies and Stevens, *J. Immunol.*, **49**, 99 (1944). KS-1A contained a minor component that was serologically identical with CS-1A from cottonseed.

(9) Bernton, Spies and Stevens, *J. Allergy*, **13**, 289 (1942).

(10) Spies, Chambers, Bernton and Stevens, *ibid.*, **14**, 7 (1942).

(11) Coulson, Spies and Stevens, *J. Immunol.*, **41**, 375 (1941).

(12) Coulson, Spies and Stevens, *ibid.*, **46**, 347 (1943).

(13) Coulson and Spies, *ibid.*, **46**, 367 (1943).

(14) Coulson, Spies and Stevens, *J. Allergy*, **21**, 34 (1950).

(15) Term proposed by Wells and Osborne, *J. Infectious Diseases*, **17**, 259 (1915).

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

(17) Spies, Coulson, Chambers, Bernton and Stevens, *ibid.*, **66**, 748 (1944).

TABLE I

SUBSTANCES EXAMINED FOR ALLERGENS, METHOD OF ISOLATION, YIELD AND ANALYSES

Substance	Source	Solvent used for defatting	Method of isolation	Yield of allergen,[a] %	Analyses, % Nitrogen	Analyses, % Carbohydrate[b]
Almond nuts—*Prunus amygdalus* Batsch.	Commercial	Pet. ether	1A	0.46	16.9	12.6
Brazil nuts—*Bertholletia excelsa* Humb. & Bonpl.	Commercial	Pet. ether	1A	0.81	17.6	6.9
Castor beans—*Ricinus communis* L.	U. S. Dept. Agr.	Ethyl ether	1A	1.76	18.4	3.1
Castor beans—*Ricinus communis* L.	Commercial (Brazilian Pomace)	Not extracted	1C	0.33	17.1	8.0
Cottonseed—*Gossypium spp.*	So. Reg. Res. Lab.	Depigmented and defatted	1A	1.38	12.1	36.4
Coconuts—*Cocos nucifera* L.	Commercial	Ethyl ether	1A	0.006	..	..
Filbert nuts—(Barcelona)—*Corylus avellana* L.	U. S. Dept. Agr.	Ethyl ether	1A	.30	17.1	10.5
Filbert nuts—(Barcelona)—*Corylus avellana* L.	Commercial	Pet. ether	H1B	.26	18.8	6.6
Filbert nuts—(DuChilly)—*Corylus avellana* L.	Commercial	Pet. ether	H1B	.33	19.2	6.0
Flaxseed—*Linum usitatissimum* L.	Commercial	Dehulled and defatted	1A	1.98	11.3	39.4
Kapok—*Ceiba pentandra* (L.) Gaertn.	Commercial	Benzene	1A	0.78	12.6	34.5
Mustard seed[c]—*Brassica nigra* (L.) Koch	Commercial	Ethyl ether	1A	0.58	11.1	40.7
Peanuts—*Arachis hypogaea* L.	Commercial	Ethyl ether	1A	.07	15.4	16.7
Peanuts—*Arachis hypogaea* L.	Commercial	Ethyl ether	1B	.18	15.2	10.6
Pecan nuts—*Carya illinoensis* (Wang.) Koch.	U. S. Pecan Field Lab. Albany, Georgia	Ethyl ether	1A	.00	..	..
Soybeans—*Glycine max* (L.) Merrill	U. S. Regional Soybean Lab., Urbana, Illinois	Ethyl ether	1B	.10	13.3	20.4
Tung nuts—*Aleurites fordii* Hemsl.	Commercial	Ethyl ether	1A	.008	5.7	..
Walnuts (Black) *Juglans nigra* (L.)	Commercial	Ethyl ether	1A	.40	17.9	11.2
Walnuts (English) *Juglans regia* (L.)	Commercial	Ethyl ether	1A	.00	..	..

[a] Calculated as per cent. of ash-free and water-free allergen in air-dried defatted meal. [b] Determined by the method of M. Sorensen and Haugaard as modified by Heidelberger and Kendall, *J. Immunol.*, **30** 267 (1936). Galactose $[\alpha]^{20}D$ +79.7 *c*, 3.0 g. 100 ml.) was used as standard. [c] This sample was contaminated with 3 to 4% *B. arvensis*.

Experimental

Source of Materials and Preliminary Treatment.—The seeds and nuts used in this study were obtained from commercial sources and government experiment stations, as shown in Table I. Identification and scientific names were obtained through the coöperation of S. F. Blake, H. L. Crane, F. J. Herman and Miss A. F. Musil of the Bureau of Plant Industry, Soils and Agricultural Engineering, U. S. Department of Agriculture.

Seeds and nuts were ground and then defatted by thorough percolation at room temperature with the solvents shown in Table I. Cottonseed that had been defatted and depigmented at the Southern Regional Research Laboratory by a special process[18] was a better source of CS-1A than was cottonseed that had been simply defatted with either benzene or ether, because less difficulty with stable suspensions was encountered in the isolation procedures. Commercial castor bean pomace has the advantage that preliminary grinding and defatting of toxic castor beans can be avoided, but the disadvantage that allergen-destructive treatment may have occurred before the pomace was received. CB-1A isolated from one lot of Brazilian pomace had the same antigenic activity as that obtained from untreated domestic castor beans,[2] but that obtained from a later lot of pomace had a lower antigenic potency. Flaxseed was difficult to handle because of the mucilaginous constituents in the hulls, and a relatively low yield of flaxseed-1A was obtained from the first lot studied. The flaxseed-1A described in this paper was obtained from seed dehulled and defatted by a flotation process to be described elsewhere. No difficulty was caused by mucilaginous components when dehulled seeds were used.

Isolation of Allergens by the CS-1A Procedure.—Fundamentally the same procedure was used to isolate the allergens from the seeds and nuts reported herein as was used to isolate CS-1A from cottonseed[4,5] and CB-1A from castor beans.[7] This procedure was based on the discoveries that the cottonseed allergen is soluble in water, soluble in 25% ethanol but insoluble in 75% ethanol, stable in boiling water and nonprecipitable by basic lead acetate. Any modifications in procedures used were made with consideration of the above-mentioned properties of the allergens. The previously published CS-1A procedure is not described here, but modifications and comments pertinent to its application are given below.

Weighed quantities of defatted meals were stirred mechanically for three hours or longer with distilled water in the proportions of 6 to 10 liters of water to 1 kilogram of meal for the initial extraction. Some extracts were concentrated by evaporation in a current of air instead of by vacuum distillation. Chloroform was used as preservative during such evaporations.

Precipitation with excess basic lead acetate is a critically important step in obtaining allergens immunologically free from other allergens and antigens in the seeds and nuts. It is essential that excess lead be used for the precipitation of these other allergens and that the lead salts be completely separated. To determine the quantity of basic lead acetate required for complete precipitation, 20 ml. of the appropriate solution was placed in a 40-ml. centrifuge tube, and 3 ml. of basic lead acetate solution was added with stirring. The suspension was centrifuged, and another portion of basic lead acetate was added to the clarified supernatant solution. The point where no precipitate formed was determined by repeated tests and the calculated amount of basic lead acetate was then added to the main solution. In the previously described procedure, 10% basic lead acetate (the type used for sugar analysis) in 25% ethanol solution was used. The precipitation may also be carried out in water solution by using 10% basic lead acetate in water.

The bulk of precipitated lead salts was removed by centrifuging. If colloidal lead salts remained in solution after this preliminary centrifuging, it was found convenient, in

(18) Boatner and Hall, *Oil and Soap*, **23**, 123 (1946).

most cases, to remove them by Seitz filtration (Size 14 filter). However, some extracts were too viscous for Seitz filtration and could only be clarified by supercentrifuging.

Excess lead was removed from clarified solutions by adding 10% sodium carbonate until the *p*H of the solution was 9.4 to 9.8. Precipitated lead carbonate was removed by centrifuging, and any colloidal lead carbonate was removed by Seitz filtration or by centrifugation in the batch bowl of the Sharples supercentrifuge, care being used to avoid undue heating. Clear solutions were tested for complete removal of lead by further addition of a few drops of sodium carbonate solution. The *p*H of solutions was adjusted to about 6.2 with 50% acetic acid solution as soon as possible after removal of lead, brom cresol purple being used as indicator. Preparations obtained in this way were designated by the suffix -1A.

"1B" Procedure.—In some cases, a milder isolation procedure was used by avoiding heating and adjusting the solution to *p*H 9.6. In these cases, suspended protein in the original water extract was flocculated by bringing the *p*H to about 4.7 with 50% acetic acid solution. The precipitate was removed by centrifuging and discarded. The *p*H of the solution was then adjusted to about 5.6 with sodium hydroxide solution either before or after concentration by evaporation at room temperature. Excess lead in these cases was removed by hydrogen sulfide precipitation. The rest of the procedure was the same as the 1A procedure. These preparations were designated by the suffix -1B.

Heated 1B Preparations.—Samples of Barcelona and DuChilly filbert nut-1B were dissolved in water (about 1% solution) and heated in a boiling water-bath for 1 hour. The solutions were cooled, and the small amount of coagulum was centrifuged off and discarded. The solutions were filtered and made 0.1 *N* in sodium acetate buffered at *p*H 5.6. The allergens were precipitated with three volumes of ethanol and isolated as usual. Recoveries of 81 and 85% were made from Barcelona filbert-1B and DuChilly filbert-1B, respectively. The heat treatment had no evident effect on immunologic potency or specificity. These preparations were designated by the suffix -H1B.

"1C" Procedure.—In this procedure heat was used as in the 1A procedure but hydrogen sulfide, instead of sodium carbonate, was used to precipitate excess lead. Considerable difficulty was sometimes experienced in removing the colloidal lead sulfide in this method. There was no indication that the use of sodium carbonate caused any decrease in potency of the allergens. These preparations were designated by the suffix -1C.

All allergen fractions were finally dried in a vacuum desiccator over phosphorus pentoxide or calcium chloride; the latter was preferred because slight discoloration occurred on the surface of some samples dried with phosphorus pentoxide. Samples were ground to pass 60, 80 or 100 mesh sieves and equilibrated with air before analysis. All of the fractions were practically white, and all, except mustard-1A, were soluble in water. Mustard-1A contained some denatured material and was only partially soluble.

Determination of Amino Acids.—Amino acids were determined microbiologically, except that tryptophan was determined by general procedure N as described by Spies and Chambers.[19] Microörganisms were obtained from the American Type Culture Collection, 2029 M St., Washington, D. C.

Hydrolysis.—One hundred milligrams of sample was dissolved in 2 ml. of 3 *N* hydrochloric acid (prepared from constant boiling acid) in a 17 × 90 mm. Pyrex tube. After complete solution at room temperature, the tubes were placed upright in a Pyrex desiccator to which had been added enough water to fill the desiccator with water vapor. The desiccator was evacuated to 40 mm. pressure and heated in an autoclave for 8 hours at 15 pounds pressure (120–125°). The hydrolysate was cooled and filtered quantitatively through a hardened paper, water being used for washing and making the final volume 20 ml. Suitable dilutions of this solution were used for analyses. Amino acids in the hydrolysates were considered to be in the L-form.

Amino Acid Standards.—Best quality commercial, specially prepared or recrystallized amino acids were used for standards. Each amino acid so used had the theoretical nitrogen content, as determined by the Kjeldahl micromethod. L-forms of arginine, recrystallized cystine, glutamic acid, histidine, proline and tyrosine were used. L-Methionine was prepared as previously described.[20,21] Samples of L-serine, L-threonine and L-valine, resolved by enzymatic hydrolysis of respective chloroacetyl derivative,[22] were obtained through the kindness of Dr. J. P. Greenstein. DL-Alanine and glycine were recrystallized. Except for aspartic acid only the L-forms in standard DL-mixtures were utilized by the microörganisms. Under the conditions of the test both forms of aspartic acid were utilized.

Method of Analysis.—Essentially the method and medium of Henderson and Snell[23] were used. The *p*H of the sample was adjusted to that of the medium before mixing. The volume of the medium was 1 ml., and that of sample was also 1 ml. Organisms were allowed to grow for 66 to 72 hours in the dark at 30°. The samples were then diluted to 50 ml., and the lactic acid was titrated with 0.05 *N* sodium hydroxide, brom thymol blue being used as indicator. At least two determinations were made at each of five concentration levels. Conventional standard curves were obtained. Results were calculated from at least three concentration levels that fell in the useful part of the standard curve. The determinations of alanine and cystine were not as satisfactory as the others by the method used although good results were obtained.

Evaluation of Reliability of Results.—Microbiological determination of amino acids has not yet become standardized. Therefore, evaluation of the accuracy of the results was made by comparison with values obtained by other workers using a different method. This was done by determination of the amino acids in crystalline B-lactoglobulin[24] and comparing results with those of Stein and Moore[25] who used partition chromatography on starch columns (Table III). The eighteen amino acids determined accounted for 96.4% of the total nitrogen, exclusive of amide or ammonia nitrogen. When the value of amide nitrogen used by Stein and Moore was added the total recovery of nitrogen was 103.3% as compared to 99.6% obtained by Stein and Moore. Individual percentage differences between values obtained in this work and those of Stein and Moore ranged from 0 to 35.4% while the average deviation was ±10.9%.

The amino acid content of casein[26] was also determined, and exclusive of amide nitrogen 96.5% of the total nitrogen was accounted for in the eighteen amino acids determined. In general, good agreement of values with those recently published by Gordon, *et al.*,[27] and Henderson and Snell[23] was obtained. Further evidence of the reliability of the microbiological values for arginine and tyrosine was furnished by comparison with the values obtained chemically on three representative allergen preparations. Arginine was isolated as the analytically-pure monoflavianate (shown by nitrogen and sulfur analysis) by the method of Vickery.[28] Tyrosine was determined colorimetrically by the method of Lugg.[29] The values obtained by microbiological and chemical methods are in good agreement as shown in Table IV.

In view of the foregoing considerations, the values obtained microbiologically for the amino acids are regarded as entirely satisfactory for demonstrating the composition of the allergen preparations.

Other Analytical Methods.—Nitrogen was determined by a Kjeldahl micromethod using mercuric sulfate as catalyst. Arginine monoflavianates were reduced with hydriodic acid before Kjeldahl digestion. Sulfur was determined by the micromethod of Pregl. Water was determined by heating the sample for 3 hours in an Abderhalden vacuum drier at 110° followed by weighing in a closed system. Ash was determined by ignition in an electric furnace for 1.5 hours, during which time the temperature rose to 750°. All analytical results are expressed on an ash-free and water-free basis.

Cutaneous Tests.—A scratch that just penetrated the epidermis was made on the forearm or thigh of the sensitive

(19) Spies and Chambers, *Anal. Chem.*, **21**, 1249 (1949).

(20) Spies, *J. Biol. Chem.*, **182**, 439 (1950).

(21) Spies and Chambers, *ibid.*, **183**, 709 (1950).

(22) Price, Gilbert and Greenstein, *ibid.*, **179**, 1169 (1949).

(23) Henderson and Snell, *ibid.*, **172**, 15 (1948).

(24) The sample used was described in reference 19.

(25) Stein and Moore, *J. Biol. Chem.*, **178**, 79 (1949).

(26) Prepared as described in reference 19.

(27) Gordon, Semmett, Cable and Morris, THIS JOURNAL, **71**, 3293 (1949).

(28) Vickery, *J. Biol. Chem.*, **132**, 325 (1940).

(29) Lugg, *Biochem. J.*, **31**, 1422 (1937); *ibid.*, **32**, 775 (1938).

TABLE II

AMINO ACID CONTENT OF OILSEED ALLERGENS

Results expressed as per cent. of total nitrogen of the allergen in the indicated amino acid

Substance	Alanine[a]	Aspartic acid[a]	Arginine[b]	Cystine[c]	Glycine[a]	Glutamic acid[d]	Histidine[a]	Isoleucine[a]	Leucine[a]	Lysine[a]	Methionine[a]	Phenylalanine[a]	Proline[a]	Serine[a]	Threonine[a]	Tryptophan	Tyrosine[a]	Valine[a]	Total
Almond-1A	4.9	4.0	18.4	3.7	12.5	19.2	4.2	1.0	1.4	7.5	1.1	0.7	1.4	2.6	2.4	0.3	0.8	1.2	87.3
Brazil nut-1A	5.6	3.7	35.4	2.7	3.6	16.6	4.2	0.4	2.7	2.3	5.2	.3	3.4	2.9	0.0	0.0	0.7	0.5	90.2
Castor bean-1A (Brazilian)	6.6	3.8	29.9	2.1	4.8	19.6	1.3	2.1	2.4	4.9	0.5	.2	0.9	9.1	.8	.0	1.1	2.8	92.9
Castor bean-1A (domestic)[e]	4.7	2.4	27.1	1.5	5.9	21.1	1.6	2.7	2.5	4.9	.4	.2	0.9	9.2	.0	.0	1.2	3.3	89.6
Cottonseed-1A	7.1	5.2	27.5	3.1	3.0	20.5	3.4	0.5	1.0	6.5	.6	1.0	2.6	1.7	1.5	.6	1.2	0.8	87.8
Filbert nut-1A (Barcelona)	8.4	4.1	38.6	3.6	3.5	23.1	0.9	.6	2.6	1.5	2.4	0.2	1.3	1.7	1.3	.1	1.1	.9	95.9
Filbert nut-H1B (Barcelona)	6.5	4.4	36.9	3.1	3.3	20.6	1.3	.6	2.0	3.0	1.6	.4	1.3	1.8	2.0	.2	1.3	.8	91.1
Filbert nut-H1B (DuChilly)	5.9	4.0	39.4	3.0	3.6	20.3	1.4	.5	1.8	2.8	1.5	.4	1.5	1.7	1.8	.2	1.4	.7	91.9
Flaxseed-1A	8.5	5.6	20.2	1.7	9.9	19.8	2.1	2.0	2.8	6.6	0.8	1.1	1.2	3.1	2.7	1.4	0.6	2.0	92.1
Kapok-1A			22.1[f]	3.3[g]												0.3	1.4[h]		27.1
Mustard-1A (Black)	8.7	3.6	17.7	2.9	9.1	14.4	5.3	2.1	3.0	7.5	.9	1.2	5.0	3.2	4.5	.7	0.8	2.4	93.0
Peanut-1B	6.8	5.0	10.8	0.5	29.4	7.9	5.2	1.4	1.7	12.3	.6	0.9	2.0	5.8	3.8	.4	1.8	2.0	98.3
Soybean-1A	11.7	8.7	17.6	0.1	6.2	11.3	3.9	1.9	1.8	14.5	.7	1.0	1.8	4.3	5.3	.6	1.3	2.4	95.1
Walnut-1A (Black)	6.4	3.4	36.8	2.1	7.2	19.6	3.0	1.0	1.2	3.9	.5	0.8	2.0	2.6	1.5	Trace	0.7	0.8	93.5

[a] *Leuconostoc mesenteroides* P-60 was used for these determinations. [b] *Streptococcus faecalis* R. 8043 and *Leuconostoc mesenteroides* P-60 were used for these determinations. [c] *Lactobacillus arabinosus* 17-5 and *Leuconostoc mesenteroides* P-60 were used for these determinations. [d] *Lactobacillus arabinosus* 17-5 and *Streptococcus faecalis* R. 8043 were used for these determinations. [e] A fraction obtained by precipitation of CB-1A with picric acid and subsequent recovery of the picric acid-free allergen was used. [f] Determined by the method of Vickery.[28] [g] Determined by method of Sullivan, *Proc. Am. Soc. Biol. Chem.*, 21, 14 (1927). [h] Determined by the method of Lugg.[29]

subject. The thigh is more sensitive than the forearm; consequently somewhat higher dilutions will give positive reactions on the thigh than on the forearm. A few milligrams of solid substance, moistened with a few drops of 0.1 *N* sodium hydroxide, or water solutions of the allergens were placed on the scratch and worked in with a clean wooden toothpick. The intensity of the reaction was determined by the size of the wheal produced in 15 to 30 minutes. A key to interpretation of size of wheals is given by Table I, reference 4. Control tests were negative.

Passive Transfer Tests.—Recipients were sensitized on the upper arm in each of several (usually four) sites with 0.05 ml. of serum from a subject having the appropriate sensitivity. After 24 to 72 hours, 0.025 ml. of the appropriate dilutions of allergens in sterilized physiological salt solution, were injected into the sites. The reaction intensity was determined by the size of the wheal produced in 15 to 30 minutes. Control tests on normal skin of recipients showed no non-specific reactions (*cf.* Table V in reference 7).

Anaphylaxis and Schultz-Dale Tests.—Details of immunological tests with guinea pigs are given in papers by Coulson, *et al.* (see references, 11, 12 13 and 14 for examples of these methods).

Results and Discussion

Yields, and nitrogen and carbohydrate contents, of the allergens from fifteen different oilseeds or nuts are shown in Table I. The yields, based on defatted, air-dried meals, ranged from negligible quantities from pecan nuts, English walnuts, coconuts and tung nuts to a maximum of 1.98% from dehulled flaxseed. Yields are minimum, because the isolation procedure was designed to completely separate the major allergen from other allergens and antigens present in the meals rather than to effect quantitative recovery. As discussed previously,[7] failure to isolate an allergen similar to CS-1A from an oilseed by the CS-1A procedure does not necessarily mean that a somewhat similar allergen is not present. Such failure could result if the allergen were precipitated by basic lead acetate. In such cases, other means would have to be devised to isolate an allergen of this type. However, difficulty probably would be encountered in obtaining the allergen immunologically distinct from other allergens present because undoubtedly it is the property of non-precipitability with basic lead acetate that makes possible such effective separation of CS-1A type allergens. The difficulty of separating allergens by other means is illustrated in Paper VII[10] of this series which describes attempts to isolate a second allergen from cottonseed immunologically distinct from CS-1A.

The carbohydrate contents of the allergens ranged from 3.1% in castor bean-1A to 40.7% in mustard-1A. This carbohydrate is undoubtedly polysaccharidic because, except for Brazil nut-1A, no appreciable reduction occurred when equal volumes of 2% allergen solutions and Benedict reagent were heated, whereas 0.25 mg. of galactose added to 0.5 ml. of 2% flaxseed-1A solution gave definite reduction. The polysaccharide of CS-1A did not contribute to its allergenic specificity, but it did enhance the sensitizing capacity of CS-1A, presumably by combination with specific protein to form a larger molecule and hence a better sensitizer.[30]

The protein nature of the allergens is shown by their amino acid composition. Results of amino acid analysis of thirteen allergen preparations are shown in Table II. Eighty-seven to 98% of the total nitrogen of the allergens was accounted for in eighteen amino acids except for kapok-1A where only 27% of the nitrogen was determined in four amino acids because insufficient sample was available for complete analysis. The nitrogen unaccounted for is probably amide nitrogen present as ammonia in hydrolysates. Generalizations and possible relationships of amino acid composition to allergenic and antigenic properties are discussed below.

Allergenic activity was determined by cutaneous and passive transfer testing where suitably sensi-

(30) Coulson, Spies and Stevens, *J. Immunol.*, **62**, 171 (1949).

tive subjects were available. The maximum dilution of allergens that gave positive cutaneous reactions varied from $1:10^4$ to $1:10^6$ as shown in Table V. The threshold quantities of allergen required to cause positive passive transfer reactions varied from 0.1 to 100 millimicrograms (Table V). These results cannot be used as a basis for an absolute comparison of potency of the different allergens because of variations in the cutaneous sensitivity of allergic subjects and variations in reagin contents of the serums of sensitive subjects. The results, however, do show the extremely small quantities of these allergens required to induce allergic manifestations.

That CS-1A and CB-1A contained the principal allergens of cottonseed and castor beans, respectively, immunologically distinct from other allergens present in the seeds has been the main reason for the extensive chemical and immunological studies previously recorded. It was of interest, therefore, to determine whether the newly isolated allergens were similarly distinct from unidentified allergens present in respective seeds and nuts. Immunologic distinctness of CS-1A in tests on human subjects has been demonstrated in two ways: (1) by *in vivo* or *in vitro* neutralization of reagins specific for CS-1A in serum from cottonseed sensitive subjects and subsequent demonstration of residual reagins specific for unidentified components of the seed[5,9,10]; and (2) some cottonseed sensitive subjects gave positive cutaneous reactions to whole cottonseed but no reaction to solid CS-1A, Type I, while others reacted to whole seeds and to CS-1A diluted to $1:10^6$, Type II.[4] The reagin neutralization method is time consuming, and, until such a study can be made, clinical evidence for the distinctness of allergens in the seeds and nuts examined is based on results of cutaneous tests. The results (Table VI) show the immunological distinctness of the 1A allergens and that at least two allergens are present in castor beans, cottonseed, filbert nuts, flaxseed and black walnuts. Similar demonstration with the other allergens is dependent on availability of suitably sensitive allergic subjects.

A method for demonstrating the serological distinctness of antigens and also whether antigenic specificity was altered during the isolation process is provided by the Schultz–Dale technique using guinea pigs. This method has the advantage that sensitization can be developed artificially and, therefore, is not dependent on chance encounter with subjects possessing appropriate sensitivities. By this method it was conclusively demonstrated that almond nut-1A, Brazil nut-1A, castor bean-1A, cottonseed-1A, Barcelona filbert nut-1A and -H1B, DuChilly filbert nut-H1B, flaxseed-1A, kapok-1A[8] and mustard seed-1A were highly antigenic, being serologically distinct from other antigens in the seeds from which they were isolated, and that the specificities were not altered during isolation. Barcelona filbert nut-1B and DuChilly filbert nut-1B had the same antigenic specificities and therefore were not serologically distinguishable. From these studies it was concluded that all of the substances mentioned had antigenic properties typical of the natural proteoses. However, peanut-1A and soybean-1A were not typical of the other natural proteoses isolated by the 1A procedures. Soybean-1A exhibited very little, if any, antigenic capacity and therefore was not studied extensively.

Peanut-1A was antigenic but exhibited a specificity differing from that of any component of either aqueous or saline extract of peanut. This suggests a structural alteration of the protein during the isolation procedure. Peanut-1B, isolated by a milder procedure, was antigenic and was serologically identifiable with some component in unfractionated peanut extract. Peanut-1B was serologically distinct from peanut-1A. The antigenic capacity of peanut-1B was destroyed by heat. Hence it is unlikely that peanut-1B was the precursor of peanut-1A.

Black walnut-1A appeared to have antigenic characteristics intermediate between those of the typical natural proteoses and those of atypical peanut-1A and soybean-1A. Details of the immunological studies will appear elsewhere.

Demonstration of class characteristics of the natural proteoses on the basis of isolation by a general procedure and similar potent allergenic and antigenic properties made it pertinent to scrutinize the amino acid composition for distinguishing class characteristics. With but two exceptions each of the allergens contained all of the eighteen amino acids determined; castor bean-1A contained no tryptophan, and Brazil nut-1A contained no tryptophan or threonine. The outstanding general composition characteristics are the relatively high arginine and glutamic acid contents. Arginine nitrogen ranged from 17.7% in mustard-1A to 40.1% in DuChilly filbert nut-H1B. This latter value is higher than found in most proteins except the protamines, which contain up to 90% arginine. Glutamic acid nitrogen ranged from 14.4% for mustard-1A to 23.1% for filbert nut-1A. DuChilly filbert nut-H1B contained a combined total arginine and glutamic acid nitrogen content of 60.5%, the highest for any allergen. In general, the allergens were poor in those amino acids containing an aromatic nucleus. The highest proportions of nitrogen in these amino acids were 1.4% tyrosine in filbert nut-1A and kapok-1Al 1.5% tryptophan in flaxseed-1A and 1.2% phenylalanine in mustard-1A. Obermeyer and Pick,[31] suggested that the presence of amino acids containing an aromatic nucleus is necessary for antigenicity but later work has raised serious doubt regarding the validity of this hypothesis.[32,33] None of the allergens were free from amino acids containing the aromatic nucleus.

Outstanding individual composition characteristics of the allergens were the 9.1% serine nitrogen content of castor bean-1A, which is almost three times that found in any other allergen, and the 5.2% methionine nitrogen content of Brazil nut-1A, which is unusually high and more than twice that in any of the other allergens.

(31) Obermeyer and Pick, *Wein klin. Woch.*, **19**, 327 (1906); *cf.* Wells, "The Chemical Aspects of Immunity," The Chemical Catalog Co. (Reinhold Publ. Corp.), New York, N. Y., 1929, pp. 28, 93.

(32) Landsteiner, "The Specificity of Serological Reactions," Harvard University Press, Cambridge, Mass., 1945, pp. 59, 60.

(33) Boyd, "Fundamentals of Immunology," Interscience Publishers, Inc., New York, N. Y., 1943, p. 86.

TABLE III

COMPARISON OF AMINO ACID CONTENT OF CRYSTALLINE β-LACTOGLOBULIN WITH THAT OBTAINED BY STEIN AND MOORE

Expressed as % total nitrogen in the form of the amino acid[a]

Amino acid	Found, this Laboratory[b]	Found, Stein and Moore[26]	Difference
Alanine	6.9	7.15	− 4.3
Aspartic acid	7.6	7.80	− 2.6
Arginine	6.3	6.00	+ 4.8
Cystine + cysteine	1.9	2.54	−31.6
Glutamic acid	12.4	11.64	+ 6.5
Glycine	2.0	1.66	+15.0
Histidine	2.8	2.83	0.0
Isoleucine	6.2	4.01	+35.4
Leucine	10.7	10.61	+ 0.9
Lysine	14.4	15.45	− 7.6
Methionine	2.2	1.94	+13.6
Phenylalanine	2.0	2.06	− 5.0
Proline	4.5	4.01	+11.1
Serine	4.4[c]	3.39	+22.7
Threonine	3.6[d]	3.71	− 2.8
Tryptophan	2.3	1.71	+26.1
Tyrosine	1.9	1.81	+ 5.3
Valine	4.3	4.31	0.0
Amide-NH_3	...	6.93[e]	....

[a] Values for methionine and cystine + cysteine and tryptophan were determined by Brand, *et al.*, THIS JOURNAL, 67, 1524 (1945). [b] Nitrogen content of crystalline β-lactoglobulin 15.4%. Results on ash-free and water-free basis. [c] Corrected by dividing value obtained by 0.90; *cf.* Rees, *Biochem. J.*, 40, 632 (1946). [d] Corrected by dividing value obtained by 0.95; *cf.* Rees, ref. *c*. [e] Determined by Warner and Cannan, *J. Biol. Chem.*, 142, 725 (1942).

TABLE IV

COMPARISON OF VALUES FOR ARGININE AND TYROSINE IN ALLERGENS AS DETERMINED MICROBIOLOGICALLY AND CHEMICALLY

Nitrogen in Amino Acid Expressed as per cent. of Total Nitrogen

Allergen	Arginine Micro-biol.	Arginine Chem.[a]	Arginine Differ-ence	Tyrosine Micro-biol.	Tyrosine Chem.[b]	Tyrosine Differ-ence
Black walnut-1A	36.8	37.4 ± 0.5	1.6	0.7	0.7	0.0
Filbert-1A	38.6	36.6 ± 0.4	5.3	1.1	1.1	0.0
Mustard-1A	17.7	15.7	12.0	0.8	0.9	11.8

[a] Determined by the method of Vickery.[28] [b] Determined by the method of Lugg.[29]

The atypical immunological properties of soybean-1A and peanut-1A may result from fundamental differences in structure and composition of these preparations. Evidence for this hypothesis is that peanut-1A has significantly lower arginine nitrogen and that both preparations have lower glutamic acid nitrogen than do the typical natural proteoses. Also the lysine nitrogen contents of peanut-1A and soybean-1A are significantly higher than those of the other allergens. It is of possible significance that peanut-1B contained 29.5% glycine nitrogen, which is three to ten times that of any other allergen studied.

Schloss[34] first clearly associated proteoses from almond nuts and oats with their allergenic properties, as shown by their capacity to cause positive cutaneous reactions in dilute solution on a sensitive subject. The proteoses isolated by Schloss were water soluble, heat stable and dialyzable. Later Wells and Osborne[15] isolated and studied the anaphylactogenic reactions of proteoses from various seeds and nuts. Their preparations were soluble in water, stable to heating in solution at 100°, strongly anaphylactogenic, and immunologically distinct from other reserve proteins of the seeds. The chemical properties of these proteoses were similar to those of derived proteoses, but, to distinguish these anaphylactogenic proteoses from non-anaphylactogenic derived proteoses, Wells and Osborne proposed the term "natural proteose." A further distinguishing characteristic of the natural proteoses is that their specificities are the same as those contained in unfractionated water extracts of respective seeds and that no new specificities result from isolation procedures. Although sporadic reference to allergenic activity of proteoses has since been made, the further detailed study deserved by this class of proteins was not made until their independent rediscovery in this Laboratory. On the basis of the work of Schloss and of Wells and Osborne and the evidence presented in these studies, it is suggested that this type of substance be recognized as a class of native proteins and that

(34) Schloss, *Am. J. Diseases of Children*, 3, 341 (1912).

TABLE V

RESULTS OF CUTANEOUS AND PASSIVE TRANSFER TESTS WITH OILSEED ALLERGENS

Allergen	Highest dilution giving positive reaction cutaneous	Threshold quantity giving positive passive transfer reaction, millimicrogram
Almond nut-1A	..[a]	...[a]
Brazil nut-1A	..[a]	...[a]
Castor bean-1A	$1:10^6$	0.1
Cottonseed-1A	$1:10^6$	1.0[b]
Filbert nut-1A (Barcelona)	$1:10^5$	...[c]
Flaxseed	$1:10^4$	10
Kapok-1A	$1:10^6$	<100[d]
Mustard-1A (Black)	$1:10^4$	10
Peanut-1B	$1:10^4$	...[a]
Soybean-1A	..[a]	100
Walnut-1A (Black)	$1:10^6$	...[a]

[a] Sensitive subjects not available for test. [b] Determined on CS-60C, a carbohydrate-free subfraction of CS-1A.[16] [c] Serum from subject cutaneously sensitive to B-Filbert nut-1A did not passively sensitize sites on four recipients. [d] Threshold value was not determined.

TABLE VI

SUMMARIZED DATA SHOWING CUTANEOUS RESPONSE OF TYPE I AND TYPE II ALLERGIC SUBJECTS TO ALLERGENS (1A) AND CORRESPONDING UNFRACTIONATED SUBSTANCE (US)

Substance	Cutaneous response: Type I subjects[a] 1A	Type I subjects[a] US	Type II subjects[b] 1A[c]	Type II subjects[b] US
Castor bean	±	2+	$1:10^6$	4+
Coconut	0	3+	...	...
Cottonseed	0	4+	$1:10^6$	4+
Filbert	0	4+	$1:10^5$	4+
Flaxseed	0	4+	$1:10^4$	4+
B. Walnut	0	4+	$1:10^6$	4+

[a] Type I subjects give positive cutaneous reactions to whole substance but no reaction to 1A fraction put on scratch in solid form. [b] Type II subjects gave positive cutaneous reactions to unfractionated substance and at least a 2+ reaction to $1:10^4$ dilutions of 1A fraction. [c] Maximum dilution of 1A allergen which gave at least a 2+ reaction.

either the term "natural proteose" proposed by Wells and Osborne, or the term "natural proteone" be adopted for their designation. The latter term is suggested as an alternate because it is recognized that the allergens have chemical properties similar to those of both proteoses and peptones (*cf.* footnote 10 in reference 5). It is suggested that consideration be given adoption of possibly a more suitable class name for the natural proteoses in any future revision of the protein classification system.

It is recognized that each of the allergens described herein, although immunologically distinct from other allergens and antigens present in the seed, is chemically a complex mixture.[35,17] However, the isolation of carbohydrate-free allergenic proteins from both cottonseed (CS-60C)[16] and castor beans (CB-65A)[17] by prolonged, drastic and varied fractionation has left little room for doubt that the specifically active constituent is protein in nature. Undoubtedly, similar carbohydrate-free allergens could be isolated from the other 1A fractions by application of similar techniques.

The information gained on composition and important chemical and immunological properties that characterize the typical natural proteoses, as represented by the allergenic fractions almond nut-1A, Brazil nut-1A, castor bean-1A, cottonseed-1A, Barcelona filbert nut-1A and -H1B, DuChilly filbert nut-H1B, flaxseed-1A, kapok-1A and mustard-1A, may be summarized as follows. They are soluble in water and in dilute ethanol (up to 25% concentration) at room temperature. They are insoluble in 75% ethanol and organic solvents. The allergens are stable in boiling water and resist drastic chemical treatment; an electrophoretic subfraction CS-51R, from CS-1A, retained allergenic[35] and antigenic[36] properties, although of decreased potency, after being refluxed for 4 hours in 0.1 *N* hydrochloric acid. These substances are not precipitated by basic lead acetate, which is important in the purification procedure. They are partially dialyzable. The allergens are composed of known amino acids and in general are characterized by relatively high proportions of arginine and glutamic acid. They possess potent allergenic activity; positive cutaneous reactions were obtained with dilutions up to $1:10^6$ and positive passive transfer reactions were produced by 0.1 to 100 millimicrograms. They are potent antigens producing sensitization and fatal shock in guinea pigs. These substances are preformed in the seeds and are immunologically distinct from other allergens and antigens in the seeds.

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

(36) Coulson and Spies, *J. Immunol.*, **46**, 377 (1943).

WASHINGTON 25, D. C. RECEIVED JANUARY 11, 1951

[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH U. S. PUBLIC HEALTH SERVICE, FEDERAL SECURITY AGENCY]

The Acid-catalyzed Conversion of Codeinone to 8-Hydroxydihydrocodeinone

BY STEPHEN P. FINDLAY AND LYNDON F. SMALL

It has been discovered that the α,β-unsaturated ketone, codeinone, dissolved in dilute mineral acids, slowly combines with water. This reaction involves hydration of the ethylenic bond, the product being 8-hydroxydihydrocodeinone. The reaction rate is approximately proportional to the hydrogen ion concentration. Unlike codeinone, the α,β-unsaturated ketone, the bainone, does not react with water in the presence of dilute acids.

During a study of the chromic acid oxidation of codeine chromate,[1] which gives codeinone (II), it was observed that the product was contaminated by considerable quantities of phenolic impurities, but attempts to characterize them failed. Because of the disposition of codeinone to rearrange in hot dilute[2] and hot concentrated[2] hydrochloric acid it was thought that these impurities arose from the action of the acidic reaction medium and that one might establish their identity by ascertaining the action of cold, dilute acids on the isolated ketone. First of all, it was observed that on long standing codeinone, dissolved in normal hydrochloric acid, lost about half its optical rotatory power, but that no morphothebaine, which alone of the two acid-produced rearrangement products of codeinone[2] is optically active, could be recovered from the solution. Instead a new base was discovered which had nearly the same melting point (200°) and specific rotation in alcohol ($[\alpha]^{20}D$ −135°) as morphothebaine but which gave a distinctive color test with concentrated nitric acid[3] and was found by analysis to differ from codeinone (m.p. 184°) by the elements of water

$$C_{18}H_{19}NO_3(\text{codeinone}) + H_2O \xrightarrow{H^+} C_{18}H_{21}NO_4$$

In subsequent experiments it was found that the best yields of the new base were obtained by dissolving codeinone in a large excess of 1.5 *N* hydrochloric acid and keeping three weeks. The new base was also obtained when hydrochloric acid was replaced by dilute sulfuric acid. The rate of hydration of codeinone was approximately proportional to the hydrogen ion concentration.

The new base furnished a crystalline hydrochloride, which, with respect to melting point and water solubility, was similar to codeinone hydrochloride. It reacted readily with hydroxylamine hydrochloride and acidic 2,4-dinitrophenylhydrazine to give the corresponding ketone derivatives. The retention of the keto group indicated that the transformation had involved no rearrangement of the carbon skeleton. It liberated one and a half atoms of ac-

(1) S. P. Findlay and L. F. Small, THIS JOURNAL, **72**, 3247 (1950); *cf.* F. Ach and L. Knorr, *Ber.*, **36**, 3067 (1903).

(2) L. Knorr, *ibid.*, **36**, 3074 (1903).

(3) *Cf.* W. Klee, *Arch. Pharm.*, **252**, 211 (1914).