benzylidene glycerol were found to be 56.2 and 38.6°, respectively (Stimmel and King,[8] 62 and 32.5°). Thus, the melting points of the C_{10} to C_{18} saturated fatty acid beta monoglycerides are all higher than the corresponding benzylidene intermediates. In this, and previous, investigations the reverse relationship was evident for lower members (C_2 to C_8) of the series.

Summary

The new benzylidene glycerol intermediates, β-caproyl-α,α'-benzylidene glycerol and β-caprylyl-α,α'-benzylidene glycerol have been prepared, and subsequently reduced to the two new monoesters, β-monocaproin and β-monocaprylin, respectively.

Melting points of the new beta monoglycerides and their benzylidene intermediates increased with increasing length of the carbon chain of the fatty acids consistent with higher members of the aliphatic series.

PITTSBURGH, PA. RECEIVED MAY 24, 1943

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

The Chemistry of Allergens. VIII. Isolation and Properties of an Active Protein-polysaccharidic Fraction, CB-1A, from Castor Beans[1a,b]

BY JOSEPH R. SPIES AND E. J. COULSON

Isolation of the protein-polysaccharidic fraction, CS-1A[2a,b] which contained the principal allergens of cottonseed[1,3] and chemical characterization of the active constituents of CS-1A have been described in previous papers.[4] Similar studies on other substances are limited because suitably hypersensitive human subjects are not always available during the time required to develop isolation methods. Multiple allergenic sensitivity to cottonseed, nuts, and other oil-bearing seeds has long been recognized to form a distinct clinical grouping.[5] Therefore, it was assumed that the allergenic components of some of these other oil seeds might be *chemically* similar enough to CS-1A to permit their isolation by the procedure developed for obtaining CS-1A from cottonseed.

Castor beans, among several oil seeds subjected to the CS-1A procedure,[6] yielded 1.8% of a non-toxic allergenic protein-polysaccharidic fraction, CB-1A. The present paper describes the isolation, chemical and physiological properties and chemical composition of CB-1A.

Alilaire,[7] who first described sensitivity to the castor-oil plant, believed the allergen to be identical with the powerful toxalbumin, ricin. Ratner and Gruehl,[8] however, showed that castor beans contained an anaphylactogenic agent in addition to ricin. Barnard,[9] without giving experimental details, prepared non-toxic, allergenic extracts from castor beans. According to Barnard the allergen was water soluble, alcohol precipitable, heat stable and non-dialyzable. Grabar and Koutseff[10] separated a non-toxic allergenic fraction from castor beans which they called "ricin-allergene." Their preparation was water soluble, heat stable and dialyzable.

Experimental

Isolation of Allergenic Fraction, CB-1A.—Good quality shelled castor beans, *Ricinus communis*, U. S. Department of Agriculture Type 4, were kindly supplied for this investigation by Dr. Donald M. Crooks of the Bureau of

(1) (a) Presented before the Division of Biological Chemistry at the 105th meeting of the American Chemical Society held at Detroit, Michigan, April, 1943. Not copyrighted.

(1) (b) For Article VII of this series entitled "The Nature of the Unidentified Allergens of Cottonseed" see Spies, Chambers, Bernton and Stevens, *J. Allergy*, **14**, 7 (1942).

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

(3) Bernton, Spies and Stevens, *J. Allergy*, **13**, 289 (1942). This article and reference 1 contain conclusive evidence that CS-1A (and therefore subfractions obtained from CS-1A) was immunologically distinct from other allergens present in cottonseed.

(4) See Spies and Umberger, THIS JOURNAL, **64**, 1889 (1942), for the previous paper published in THIS JOURNAL.

(5) Coca, Walzer and Thommen, "Asthma and Hay Fever in Theory and Practice," Charles C. Thomas, Baltimore, Md., 1931, p. 394.

(6) Kapok seeds, black mustard seeds, flaxseed, croton beans, soybeans and pecan nuts also have been subjected to the CS-1A procedure. Yields of 0.9, 0.7, 0.07, 0.02, 0.02, and 0.00%, respectively, were obtained. The products contained nitrogen and carbohydrate in varying proportions and some possessed chemical properties similar to CS-1A. Results of these studies will be described when allergenic or antigenic properties have been determined.

(7) Alilaire, *Ann. Inst. Pasteur*, **28**, 605 (1914).

(8) Ratner and Gruehl, *Am. J. Hygiene*, **10**, 236 (1929); these authors review the literature on castor bean allergy in *J. Allergy*, **2**, 1 (1930).

(9) Barnard, *J. Allergy*, **1**, 473 (1930).

(10) Grabar and Koutseff, *Compt. rend. soc. biol., Paris*, **117**, 700-703 (1934).

Plant Industry, Soils, and Agricultural Engineering. The seeds were crushed in a power grinder and de-fatted by through percolation with ether.

Kilogram lots of de-fatted castor beans were subjected to fundamentally the same procedure previously described for the isolation of CS-1A from cottonseed.[2b] The CS-1A procedure was developed from the following five observed properties of the allergen; it was water soluble, stable to boiling water, soluble in 25% but insoluble in 75% ethanol, and not precipitable by basic lead acetate.[2a] Because slight modifications were made in some details of the CS-1A procedure, the following additional description of the method is given starting at the point where the first departure was made. After the first precipitation of the concentrated aqueous extract of castor beans with ethanol (75%),[11] the suspension was cooled to 5–10° before separating the precipitate by centrifuging at 5°. The clear brownish ethanolic supernatant solution was discarded. The solid was dissolved and suspended in 1 liter of warm water by vigorous mechanical stirring. Ethanol was then added to 25% concentration. To this suspension was added 200 ml. (excess) 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. Ethanol was added to the solution to 75% concentration. The resulting suspension was cooled to 5° and the precipitate was isolated by centrifuging at 5°. When dried in a vacuum over phosphorus pentoxide a yield of 24.7 g. of a slightly brownish solid was obtained per kilogram of de-fatted seed. This solid was dissolved and suspended in 200 ml. of water and centrifuged in the batch bowl of the Sharples super-centrifuge at 45,000 r.p.m. for thirty minutes to remove colloidal lead salts. Excess lead in the clear solution was removed by adding 10% sodium carbonate solution to bring the *p*H to 9.6.[12] Most of the lead carbonate was removed by ordinary centrifuging. The slightly turbid solution was then clarified by centrifuging in the Sharples super-centrifuge at 45,000 r.p.m. for forty-five minutes. The *p*H was adjusted to 6.5 with dilute acetic acid and the solution was poured into five volumes of cold ethanol. The suspension was then cooled to 5°. The clear supernatant solution was decanted and the solid isolated by centrifuging at 5°. The solid was washed twice by stirring up with 150-ml. portions of cold 80% ethanol. The solid, CB-1A, was dried in a vacuum over phosphorus pentoxide. The white solid was ground to pass a 100-mesh sieve and equilibrated with air before analysis. The yield of CB-1A was 17.6 g. (dry) per kilogram of de-fatted seed.

Anal. (ash-water free basis) Found: N, 18.3, 18.4; N, ppt. by 5% trichloroacetic acid from 1% solution at 20°, 22.5; S, 2.29, 2.36; carbohydrate,[13] 3.12.

CB-1A was soluble in water but insoluble in organic solvents. Results of color tests on a 1% solution of CB-1A were: pinkish-purple biuret, purple ninhydrin, positive Millon, positive Molisch, no reduction of Benedict reagent before or after boiling for one hour in 3 *N* hydrochloric acid. Failure to obtain reduction of Benedict reagent after boiling a 1% solution of CB-1A for one hour with 3 *N* hydrochloric acid may be attributed to stability of the polysaccharidic linkages or to the low proportion of carbohydrate in CB-1A. Therefore, 273 mg. of CB-1A was dissolved in 2.0 ml. of 75% (weight %) sulfuric acid. The solution, after standing at room temperature for eighteen hours, was diluted to 15 ml. and refluxed for twenty-seven hours. The solution was then diluted to 50 ml. and the sulfuric acid was quantitatively removed by adding hot saturated barium hydroxide solution until the *p*H was 6.0. The barium sulfate was removed by centrifuging and the solution was filtered through a hardened paper. The clear solution was concentrated to 1.0 ml. and to this solution was added 1.0 ml. of Benedict reagent. This solution was boiled for three minutes and cooled. The heavy reddish-brown precipitate which formed showed that reduction had occurred. A concentrated solution of CB-1A did not reduce Benedict reagent before acid hydrolysis. Thus, 91 mg. of CB-1A was dissolved in 0.35 ml. of water and 0.35 ml. of Benedict reagent was added. The solution was boiled and left at 100° for three minutes. The solution assumed a brownish color but cuprous oxide did not precipitate.

CB-1A induced positive cutaneous reactions on castor bean sensitive patients in dilutions of $1:10^6$. Positive passive transfer reactions were obtained with 1×10^{-10} g. of CB-1A.

Methods Used in Determining Amino Acids in CB-1A.— The methods used for determining the amino acid content of CB-1A were similar to those employed for the cottonseed allergenic fractions as previously described.[14] The hydrolyzate from a single 3-g. sample was used to determine humin, ammonia, histidine, arginine, lysine, glutamic acid, and the unidentified fractions of nitrogen in the dicarboxylic and monoamino acid groups. Results are given in Table II. References to methods and pertinent analytical data are contained in footnotes to Table II.

On the basis of the Adamkiewicz–Hopkins test it was previously reported that cottonseed allergenic protein fractions contained no tryptophan.[14] However, on addition of copper sulfate to the glyoxylic acid reagent, as described by Winkler,[15] a positive qualitative test for tryptophan resulted with CS-51R.[16] But this test was clearly negative on CB-1A. Fraction CS-51R contained 0.8% tryptophan using the quantitative method, based on Winkler's observation, described by Shaw and McFarlane.[17] However, Shaw and McFarlane reported only from 0.9 to 1.3% tryptophan in various casein samples while others have reported much higher values.[18,19,20] The method of

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

(12) A commercially available apparatus with a glass electrode and a mechanical stirrer was used to determine *p*H. The *p*H of alcoholic solutions or suspensions was determined colorimetrically on a spot plate.

(13) Carbohydrate was determined by the orcinol 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.) was used as a standard for comparison.

(14) Spies, THIS JOURNAL, **63**, 2994 (1941).

(15) Winkler, *Z. Physiol. Chem.*, **228**, 50 (1934).

(16) Spies, Bernton and Stevens, THIS JOURNAL, **63**, 2163 (1941); CS-51R was a subfraction of CS-1A obtained by electrophoresis.

(17) Shaw and McFarlane, *Can. J. Research*, **16B**, 361 (1938).

(18) Jones, Gersdorff and Moeller, *J. Biol. Chem.*, **62**, 183 (1924), reported 2.2%. These authors used zein to which known quantities of tryptophan were added as a standard.

(19) Sullivan, Milone and Everett, *ibid.*, **125**, 471 (1938), reported 2.4%.

(20) *Cf.* Mitchell and Hamilton, "The Biochemistry of the Amino Acids," The Chemical Catalog Co., New York, N. Y., 1929, pp. 159–161.

Shaw and McFarlane also gave low values for casein in this Laboratory. A sample of casein supplied by Dr. D. B. Jones was found to contain only 1% (ash-water free basis) tryptophan by their procedure whereas the May and Rose[21,22] method gave a value of 1.8% (ash-water free basis) on the same sample. Details of the tryptophan determination by the May and Rose method follow.

The color was developed in an incubator at 35–37°. Under these conditions maximum color developed in ninety-eight hours in the CS-51R solutions and in one hundred and ninety and two hundred and thirty-six hours, respectively, in the casein and tryptophan standards.[23] Colorimetric comparison was made with the Evelyn Photoelectric Colorimeter using filter 580. Results, calculated on comparisons made at maximum color development, were corrected for the slight color developed in the CS-51R and casein "blank solutions." Fraction CS-51R contained 1.46% tryptophan using tryptophan as standard and 1.86% using casein (2.2% tryptophan) as standard. However, this casein standard compared to the tryptophan standard in this run contained 1.8% tryptophan. Further determinations were not made because comparison of methods for determining tryptophan was not an objective of this work.

The May and Rose test for tryptophan on CB-1A was clearly negative after one hundred and sixty-eight hours.

Dialysis of CB-1A.—A solution containing 100 mg. of CB-1A in 10 ml. of water was placed in a cellophane tube[24] (previously tested for leaks) and dialyzed against 30-ml. portions of distilled water using toluene as a preservative. Development of osmotic pressure inside the membrane during dialysis proved that there were no leaks. The outer solution was removed and replaced with fresh water at intervals. Results in Table I, show that 58.4% of the total nitrogen in CB-1A dialyzed in three hundred and sixty-two hours.

TABLE I

DIALYSIS OF CB-1A

Consecutive interval number	Time of dialysis, hr.	Dialyzate, ml.	Nitrogen content of dialysate, mg./ml.	Proportion of total nitrogen, %
1	24	27.3	0.058	8.7
2	24	28.6	.045	7.0
3	27	27.3	.045	6.7
4	93	28.0	.093	14.2
5	96	28.2	.080	12.3
6	98	29.0	.060	9.5

The dialyzate solutions were combined and concentrated to 25-ml. in a vacuum desiccator over phosphorus pentoxide. To the dialyzate was added five volumes of cold ethanol and precipitation occurred after adjusting the pH to 5.7–6.0 with dilute acetic acid. The solid, recovered by centrifuging, was washed once with 20 ml. of cold 80% ethanol and dried in a vacuum over phosphorus pentoxide. A yield of 41 mg. of white solid, containing 18.9% total nitrogen, was obtained.

(21) May and Rose, *J. Biol. Chem.*, **54**, 213 (1922).

(22) Holm and Greenbank, THIS JOURNAL, **45**, 1788 (1923).

(23) Eastman Kodak Co. crystalline *l*-tryptophan containing 13.68% nitrogen (theory 13.72%) was used as standard.

(24) The cellophane tubing used was 0.00028 mm. thick according to the manufacturer.

One-tenth mγ of CB-1A dialyzate produced positive passive transfer reactions when tested with serum from a castor bean sensitive subject (A. M.) on two recipients similarly to tests shown in Table IV.

The dialyzate was anaphylactogenic as shown by tests on guinea pigs.

TABLE II

Comparison of the Amino Acid Content of Allergenic Fractions from Castor Beans (CB-1A) and Cottonseed (CS-51R) (results are expressed as per cent. of the total nitrogen)

Nitrogen in the form of	CB-1A[a]	CS-51R[a,14,16]
Humin	0.1	0.1
Ammonia	13.6	15.0
Cystine[b]	5.0	4.5
Histidine[c]	1.0	0.1
Arginine[d]	26.6	32.8
Lysine[e]	3.2	3.8
Glutamic acid[f]	8.6	14.2
Tyrosine[g]	1.1	1.7
Tryptophan[h]	0.0	1.1 to 1.4
Mono amino fraction[i]	19.3	8.0
Dicarboxylic acid fraction[j]	8.6	3.5
Total	87.1	84.8

[a] Fractions CB-1A and CS-51R contained 18.4 and 19.8% nitrogen; 2.33 and 2.29% sulfur; and 3.12 and 0.9% carbohydrate, respectively. All analyses were on an ash-water free basis. It is recognized that the values obtained by isolation procedures are minimal. [b] Determined by the Sullivan method. Cystine sulfur accounted for 90 and 89% of the total sulfur in CB-1A and CS-51R, respectively. [c] Determined by the Kapeller–Adler method, *Biochem. Z.*, **264**, 131 (1933), after separation by the silver salt method of Kossel and Kutscher as modified by Vickery and Block, *J. Biol. Chem.*, **93**, 109 (1931). [d] This value was obtained by the flavianic acid method of Vickery, *ibid.*, **132**, 325 (1940). *Anal.* Calcd. for $C_{16}H_{20}O_{10}N_6S$: N, 17.2; S, 6.56. Found: N, 17.2, 17.2; S, 6.56, 6.67. The arginine nitrogen of CB-1A represented 22.7% of the total nitrogen when determined as the monoflavianate after silver salt purification. *Anal.* Found: N, 17.4; S, 6.47. [e] Lysine was separated by the silver salt method and isolated as the picrate which decomposed between 253 and 258°. *Anal.* Calcd. for $C_{12}H_{17}O_9N_5$: N, 18.7. Found: N, 18.8, 18.8. [f] The dicarboxylic acids were separated from the monoamino acids by precipitation of their barium salts with alcohol. Jones and Moeller, *ibid.*, **79**, 429 (1928). Glutamic acid was then determined as the hydrochloride which decomposed sharply at 201–202°. *Anal.* Calcd. for $C_5H_{10}O_4N$ Cl: N, 7.63. Found: N, 7.42, 7.58. [g] Determined by the Folin–Marenzi method using 100-mg. samples., *ibid.*, **83**, 89 (1929). [h] May and Rose method.[21] [i] This unidentified nitrogen was considered the monoamino acid fraction because the barium salt was soluble in alcohol. This value has been corrected for all of the tyrosine, although some tyrosine also occurs in the dicarboxylic acid fraction. [j] This unidentified nitrogen occurred in the filtrate after precipitation of glutamic acid hydrochloride.

Discussion

Comparison of the amino acid content of the

castor bean and cottonseed allergenic fractions, Table II, reveals considerable similarity in composition. Both CB-1A and CS-51R are relatively rich in arginine (27 and 33% of the total nitrogen, respectively), moderately rich in lysine and relatively poor in histidine. This relative proportioning of basic amino acids is a recognized characteristic of proteins of oil seeds.[25] Cystine in

TABLE III

TOXICITY OF UNFRACTIONATED WATER EXTRACT OF CASTOR BEANS[a] TO GUINEA PIGS[b]

Animal	Total nitrogen in dose,[e] γ/kilogram body weight	Results
4666 ip[c]	1970	Died overnight
4634 ip	1450	Died overnight
4619 ip	750	Died overnight
4655 sc[d]	139	Died in 21 hours
4620 sc	115	Died in 21 hours
4654 sc	76	Died in 42 hours
4651 sc	66	Died in 42 hours
4667 sc	40	Died in 42 hours
4676 sc	33	Died in 42 hours
4723 sc	16	Died in 91 hours
4628 sc	13	Survived,
4701 sc	8	developed
4685 sc	8	necrotic area

[a] Five grams of de-fatted castor beans was extracted overnight with 95 ml. of distilled water saturated with toluene. The residue was centrifuged and the supernatant solution was filtered. The filtrate contained 0.828 mg./ml. total nitrogen. Eighty-one per cent. of the nitrogen was precipitated by phosphotungstic acid and 74% was precipitated by 5% trichloroacetic acid. [b] Female guinea pigs weighing from 195 g. to 317 g. were used. [c] Injected intra-peritoneally. [d] Injected subcutaneously. [e] The dosages were diluted to 1 ml. with 0.85% sodium chloride solution.

TABLE IV

ANTIGENIC CAPACITY AND ABSENCE OF TOXICITY OF CB-1A USING GUINEA PIGS[a]

Animal	Total CB-1A nitrogen in sensitizing dose, γ/kilogram body weight	Toxic effect[c]	Total CB-1A nitrogen in shocking dose,[d] γ/kilogram body weight	Antigenic effect[e]
4593 ip[b]	56	None	316	F. A. (4 min.)
4595 ip	58	None	3.3	F. A. (3 min.)
4602 ip	54	None	1.6	None[f]
4510 ip	546	None	82	F. A. (3 min.)
4534 ip	539	None	363	F. A. (3 min.)
4543 ip	5390	None	36	F. A. (5 min.)
4560 ip	5760	None	10	F. A. (3 min.)
4579	..	...	407	None

[a] Guinea pigs weighing from 290 to 310 g. at the time of sensitization, were used. [b] Injected intraperitoneally. [c] During twenty-one days of observation. [d] Injected into the median basilic vein. [e] F. A. = fatal anaphylaxis. [f] This animal was probably not sensitive.

(25) Mitchell and Hamilton, *loc. cit.*, pp. 182–188

TABLE V

THRESHOLD QUANTITY OF CB-1A REQUIRED TO PRODUCE POSITIVE PASSIVE TRANSFER REACTIONS WITH SERUM FROM A CASTOR-BEAN SENSITIVE PATIENT[a]

CB-1A injected,[b] millimicrograms,[c] mγ[c]	Recipient[d] (wheal size in mm.) P. S.	F. F.	M. P.	E. H.
10	16 × 17	13 × 12	22 × 16	16 × 16
1.0	13 × 13	11 × 12	12 × 10	12 × 11
0.1	8 × 8	10 × 10	9 × 8	10 × 11
0.01	±[e]	±[e]	±[e]	±[e]

[a] This serum (A. M.) gave positive passive transfer reactions with CB-1A when diluted $1:10^2$ but not when diluted $1:10^3$. *Cf.* Coca and Grove, *J. Immunol.*, **10**, 445 (1925). [b] Quantity of CB-1A (contained in 0.025-ml. sterile physiological salt solution) injected into each sensitized site. [c] *Chem. Eng. News*, **18**, 491 (1940). [d] Recipients were uniformly sensitized on the upper arms with 0.05 ml. of serum. The tests of each series were conducted simultaneously. The size of the wheal produced in fifteen to thirty minutes is given in mm. CB-1A gave no non-specific reactions in normal skin. Positive reactions were also obtained using serum from another castor bean sensitive patient (L. C.) with 1×10^{-9} g. of CB-1A. [e] Quantities of CB-1A from 0.01 mγ to as low as 0.00001 mγ injected into sensitized sites gave reactions distinguishable from the reaction given by a control injection of 0.025 ml. of physiological salt solution into a similar sensitized site. However, there was no apparent gradation in the size of these ± reactions caused by tenfold decreases in quantity injected. Therefore, we have chosen the quantity of allergen ten times larger than that which produced the first ± reaction as the threshold amount required to produce a positive passive transfer reaction.

CB-1A and CS-51R (7.8 and 7.6%, respectively, on a weight basis) accounted for 90% of their total sulfur content. CB-1A contained no tryptophan in contrast with CS-51R which contained 1.5 to 1.9% of this amino acid. CB-1A is more potent antigenically than CS-51R.[26] Tryptophan, therefore, is not necessary for antigenicity of this type of naturally occurring protein.

Physical properties of CB-1A indicated low molecular weight as compared to other natural antigens. CB-1A, like CS-1A, dialyzed through cellophane membranes. This confirmed the observation of Grabar and Koutseff regarding dialyzability of their "ricinallergene."

Owing to the presence of ricin the unfractionated water extract of castor beans is highly toxic. Results in Table III show that death, in guinea pigs, was produced in ninety-one hours by injection of 16 γ of total nitrogen from an unfractionated water extract of castor beans per kilogram of body weight. However, as shown in Table IV, no primary toxic effect resulted in

(26) Unpublished data.

twenty-one days from injection of 5760 γ of CB-1A nitrogen per kilogram of body weight or 360 times the quantity of nitrogen from the unfractionated water extract which caused death.

The guinea pigs injected with CB-1A became sensitive and fatal anaphylaxis resulted from a subsequent injection of CB-1A or unfractionated castor bean extract. The minimum shocking and sensitizing dose of CB-1A nitrogen was approximately 0.33 γ and 8.4 γ per guinea pig, respectively.[26] That CB-1A was immunologically distinct from other antigens present in castor beans was demonstrable by the Schultz–Dale technique.[27] CB-1A and the cottonseed allergen, CS-1A, did not cross react in appropriately sensitized guinea pigs, thus indicating no common specificity.

CB-1A, when diluted 1:10^{9}, produced positive cutaneous reactions on two hypersensitive subjects. Results in Table V show that 1×10^{-10} g. of CB-1A caused positive passive transfer reactions using a serum of moderate potency.

The remarkable similarity in chemical composition, physical and physiological properties of the allergenic proteins from cottonseed and castor beans is apparent. *The significance of this similarity is enhanced because the castor bean allergen was isolated by a procedure developed independently for isolating the cottonseed allergen, CS-1A.* Allergists have long regarded cottonseed and castor beans as sources of allergens of the highest potency and they have recognized that multiple sensitiveness to oil seeds and nuts forms a distinct clinical grouping.[28] Recognition of this clinical grouping prompted the prediction[14] that allergens *chemically* similar to the cottonseed allergen, CS-1A, might occur in other members of the oil-seed group. Isolation of CB-1A by the CS-1A-procedure has fulfilled this prediction.

Failure to isolate an allergenic protein similar to CS-1A or CB-1A from an oil seed (*e. g.*, pecan nuts[6]) by the CS-1A procedure does not necessarily mean that a similar substance is not present. Such failure could occur in case the allergen, although quite similar to CS-1A, were precipitable by basic lead acetate. In such cases other means have to be devised to isolate an allergen of the CS-1A type. Difficulty, however, probably would be encountered in obtaining the allergen immunologically distinct from other allergens in the same seed without use of basic lead acetate. Undoubtedly the property of non-precipitation with basic lead acetate made possible the isolation of CS-1A immunologically distinct from other allergens of cottonseed.[1,3] The difficulty of effecting such complete separation of allergens, by other means, is illustrated in Paper VII,[1] which describes an attempt to isolate from cottonseed a second allergen immunologically distinct from CS-1A.[29]

Likeness of the cottonseed allergen, CS-1A, to the "natural proteoses" of Wells and Osborne[30] has been discussed in previous papers.[31] Fraction CB-1A from castor beans is another example of this type of naturally occurring antigen.[32]

The authors are indebted to Dr. Harry S. Bernton and to Dorris C. Chambers for clinical tests. Microanalytical determinations were also made by Dorris C. Chambers.

Summary

A non-toxic, allergenic, protein-polysaccharidic fraction, CB-1A, has been isolated from castor beans by the procedure developed for isolating the allergenic fraction, CS-1A, from cottonseed. CB-1A represented 1.8% of the de-fatted castor bean meal. Chemical properties and amino acid content of CB-1A were remarkably similar to those of the previously characterized cottonseed allergen. Both CB-1A and CS-1A were characterized by relatively high proportions of arginine and cystine but CB-1A in contrast with the cottonseed allergen contained no tryptophan. Both proteins also contained histidine, lysine, tyrosine and glutamic acid in addition to unidentified nitrogenous compounds in the monoamino and dicarboxylic acid groups.

That the potent toxic albumin, ricin, had been removed from CB-1A was shown by tests on guinea pigs. No primary toxic effect resulted in twenty-one days from injection of 5760 γ of CB-1A nitrogen per kilogram of body weight. Injection of 16 γ of nitrogen from an unfractionated water extract of castor beans caused death in ninety-one hours. CB-1A was a powerful antigen; the minimum shocking and sensitizing dose of CB-1A nitrogen was approximately 0.33 and 8.4 γ per guinea pig, respectively. That CB-1A

(27) Detailed results of immunological tests will be published elsewhere.

(28) Coca, Walzer and Thommen, *loc. cit.*, p. 393–394, 406.

(29) *Cf.* Coulson, Spies and Stevens, "A Study of the Homogeneity of Cottonseed Globulin Preparations by Anaphylactogenic Reactions," to be published.

(30) Wells and Osborne, *J. Infectious Diseases*, **17**, 259 (1915).

(31) See footnotes 4, 5, and 10, ref. 2b and ref. 14 for a discussion of this subject.

(32) *Cf.* Coulson, Spies and Stevens, *J. Immunol.*, **46**, 347–389 (1943).

was immunologically distinct from other antigens present in castor beans was shown by the Schultz–Dale uterine strip method.

Positive passive transfer reactions were produced with 1×10^{-10} g. of CB-1A. Positive cutaneous tests, on castor bean hypersensitive subjects, were obtained with CB-1A diluted 1:10^6.

WASHINGTON, D. C. RECEIVED APRIL 23, 1943

[CONTRIBUTION FROM THE COLLEGE OF PHARMACY, UNIVERSITY OF MICHIGAN]

Antispasmodics. V

BY F. F. BLICKE AND NATHANIEL GRIER[1,2]

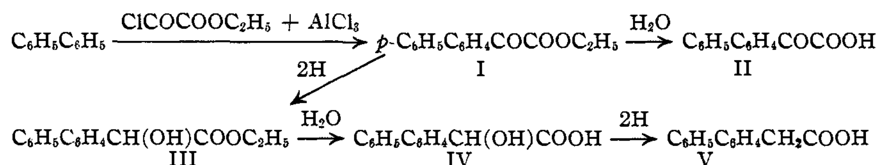
The two principal naturally-occurring antispasmodics are atropine and papaverine; the former is a basic ester, the latter a cyclic amine. In the search for synthetic antispasmodics the structures of these two compounds have served as patterns for the preparation of a wide variety of basic esters and amines.[3]

At the present time three antispasmodics of the ester type are on the market: Syntropan (the primary phosphate of β,β-dimethyl-γ-diethylaminopropyl tropate), Trasentin (the hydrochloride of β-diethylaminoethyl diphenylacetate)[4] and Demerol (Dolantin, the hydrochloride of ethyl 1-methyl-4-phenylpiperidine-4-carboxylate).[5]

Since Trasentin is not only an effective antispasmodic but is relatively easy to synthesize, the preparation and pharmacological study of esters, similar in structure to this substance, offers an inviting field.

In this paper (Table III) are described hydrochlorides of basic-alkyl esters of the general type p-$C_6H_5C_6H_4CH(R)COOR'$: R = hydrogen, phenyl cyclohexyl, methyl, ethyl or propyl; R′ = β-diethylaminoethyl, β-piperidinoethyl, β-dibutylaminoethyl, γ-diethylaminopropyl or γ-piperidinopropyl.

p-Xenylacetic acid was obtained by the series of reactions outlined below



We were able to increase the reported yield of ethyl p-xenylglyoxylate (I) by a modification of Rousset's[6] procedure. Hydrolysis of the ester yielded p-xenylglyoxylic acid (II), while catalytic reduction converted it into ethyl p-xenylhydroxyacetate (III). The latter was hydrolyzed to p-xenylhydroxyacetic acid (IV), and this acid was reduced with red phosphorus and iodine to p-xenylacetic acid (V).

The substituted p-xenylacetic acids, $C_6H_5C_6H_4$-$CH(R)COOH$, were obtained according to method A[7] or B.[8]

The esters were prepared by two procedures: (C) the required acid, dissolved in isopropyl alcohol, was heated with a molecular equivalent amount of the basic alkyl chloride[9]; (D) the necessary acid chloride was allowed to react with two molecular equivalents of the basic alcohol in benzene solution whereupon a part of the basic alcohol precipitated as the hydrochloride, and the desired ester base remained in solution.

Our products were examined pharmacologically by Dr. C. W. Geiter and Dr. A. N. Lands of Frederick Stearns and Company. A detailed ac-

(1) This paper represents part of a dissertation submitted to the Horace H. Rackham School of Graduate Studies by Nathaniel Grier in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University of Michigan.

(2) Frederick Stearns and Company Fellow.

(3) Cheney and Bywater (THIS JOURNAL, **64**, 970 (1942)) have described a number of morpholinoalkyl esters which were tested for antispasmodic activity by Rowe (*J. Am. Pharm. Assoc.*, **31**, 57 (1942)). A number of references to the literature of this subject are to be found in the article by Cheney and Bywater. Recently Burtner and Cusic (THIS JOURNAL, **65**, 262 (1943)) published the syntheses of a number of basic esters of arylacetic acids which have been examined pharmacologically by Lehmann and Knoefel (*J. Pharmacol.*, **74**, 217, 274 (1942).

(4) Trasentin-H is the hydrochloride of β-diethylaminoethyl cyclohexylphenylacetate.

(5) Syntropan and Trasentin, like atropine, are esters in which the basic nitrogen is in the alcoholic radical of the ester; in Demerol, however, the basic nitrogen is found in the acyl group.

(6) Rousset, *Bull. soc. chim.*, [3] **17**, 809 (1897).

(7) Grignard (*Ann. chim. phys.*, [7] **27**, 548 (1902)) was the first to study the action of aliphatic Grignard reagents on ethyl phenylglyoxylate.

(8) McKenzie and Ritchie (*Ber.*, **70**, 33 (1937)) showed that ethylphenylhydroxyacetic acid can be obtained in good yield from ethylmagnesium bromide and phenylglyoxylic acid.

(9) General method of Horenstein and Pählicke, *ibid.*, **71**, 1654 (1938).