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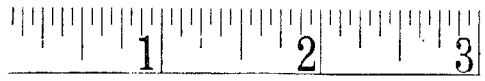

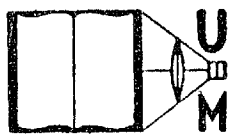

SOME CHEMICAL STUDIES OF THE NAVY BEAN (PHASEOLUS VULGARIS)

Arnold C. Ott

A THESIS

Submitted to the Graduate School of Michigan State College of Agriculture and Applied Science in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Chemistry

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I have appreciated the helpful assistance

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CONTENTS

I. Introduction.

It is quite probable that within the last two decades the scientific work done on soybeans has far overshadowed, in scope at least, that done on any other legume. Certainly there are many factors or characteristics inherent to the soybean which readily account for the attention they have received.

On the other hand, the common navy bean has been studied primarily from the standpoint of nutrition, and this to no great extent. Somewhat aside from the nutritional phase, is the rather extensive work of Ritthausen and Waterman, and others[1] on the proteins of the navy bean.

A partial impetus for this work came from a group representative of certain navy bean growers of Michigan. Their desire was to obtain a practical result in a relatively short time in order to alleviate in part the surplus cull beans as well as older storage beans. The fact is known that older navy beans (4-6 years) are much less permeable to water than newly grown beans. With this in mind it appeared plausible to study in part the chemical composition of the seed coats. To date, little work has been reported on the chemical compositon of the

navy bean seed coats. Schulze and Pfenninger[2] reported hemicelluloses to be a large constituent of the seed coats, the content of hemicelluloses reaching a maxium in the ripe bean. However, considerable work has been presented on the permeability of seed coats of the common bean to water and salt solutions.[3]

Among the most hydrophilic compounds present in plants are the closely related cell wall pectic substances. The building units of these pectic substances are for the most part uronic acids. A knowledge of the uronic acid content of the seed coats, or more properly, the polyuronide content of the seed coats, should lead toward an explanation of water sorption. Pentosans, one of the most important components of the numerous hemicelluloses, are believed to be closely related to polyuronides; furthermore, their determination has been grossly confused with the determination of polyuronides. Proteins are also well known to be functional in water sorption.[4]

The quantitative determination of crude lipids, unsaponifiable fraction, and sterols respectively was done on a mixture of strains of navy beans

grown in Michigan; a part of the same lot used for the seed coat study. Grimme[5] reported values for the crude lipid and unsaponifiable matter of the common navy bean. A survey of the literature, however, does not reveal any figures for the approximate amount of sterols or their characterization in the unsaponifiable fraction.

The common navy bean contains about two-thirds as much protein as does the common variety of soybeans. In this work it was desirable only to extract the total protein in the most efficient manner, and further ascertain its adaptability to plastic formation. Complete extraction has been effected with aqueous sodium sulfite solution and nearly complete precipitation of a globulin-type protein, white in color, results from the treatment with sulfur dioxide. This product so isolated is suitable for plastic formation.

It is rather widely known that sterols make up the major portion of the unsaponifiable fraction of various seed oils.

The seed oils of the various legumes contain different sterols. The unsaponifiable portion of alfalfa seed oil has been shown to contain the

isomeric spinasterols, with α- and β - spinasterol predominating in amount over the δ - spinasterol.[6] Medium and red clover seed oil - unsaponifiable (loc. cit.) contain relatively large amounts of stigmasterol. Stigmasterol occurs also as the major sterol, accompanied by sitosterols, in the unsaponifiable fraction of soybean oil.[7][8]

As previously mentioned no work has been found in the literature on the characterization of any of the sterols in the unsaponifiable fraction of the navy bean-oil proper. However, Likiernik[9] reported a substance over a half century ago, which he isolated from the oil of the seed coats of Phaseolus vulgaris. This substance he called paraphytosterol. The properties of this compound (m. p. 149-150°, $[\alpha]_D$ - 44.1°) are similar to those of γ - sitosterol and it is quite probable that these substances are the same.

In view of the facts presented, the crude sterols were isolated from the unsaponifiable fraction of the bean oil and some progress was made in separating individual compounds from the crude material.

Hydrocarbons are known to occur along with sterols in varying amounts in all seed oils. As a

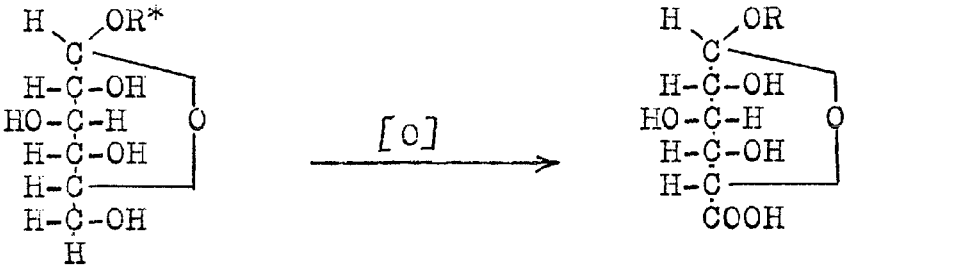
general rule, however, the amounts found are small in comparison to the amount of sterols. Indeed, their isolation from most seed oils for purposes of a thorough investigation hardly falls within the province of an ordinary technical examination. Evidence here exists for the presence of possibly two distinct hydrocarbons in navy bean oil.

II. Quantitative Study of Navy Bean Seed Coats.

A. Uronic Acids.

Uronic acids are widely distributed throughout the plant kingdom. They may arise as a result of the oxidation of ∝-D-glucose as represented in Equation I.

Equation I



∝-D-Glucose ∝-D-Glucuronic acid

D-Glucuronic and D-galacturonic acids[10] are the only uronic acids which have been isolated from higher plants. The latter occurs more abundantly than the former.

Mannuronic acid has been isolated from various algae by Nelson and Cretcher[11] and by Bird and Haas.[12]

An aldobionic acid, glucose-glucuronic acid has been shown by Heidelberger and Goebel[13] to be the fundamental building stone of the polysaccharide derived from Type III pneumococcus and to be an important constituent in the pneumococcus from

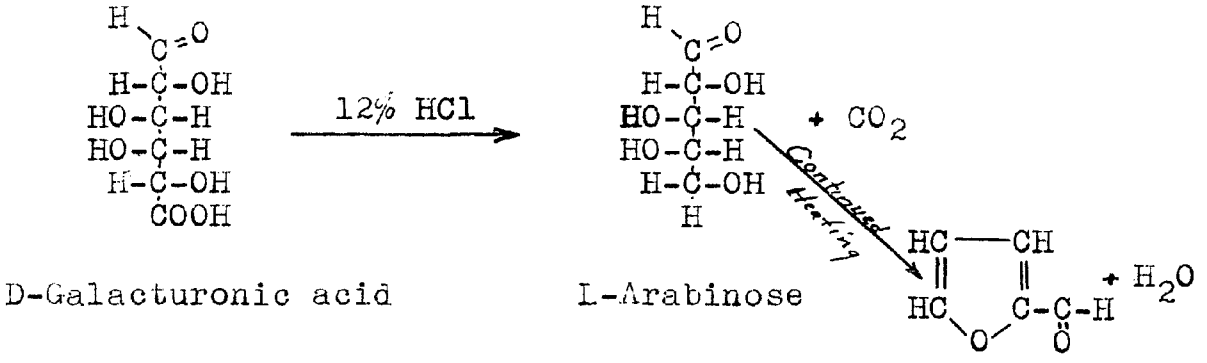
* R = sugar anhydride or some other residue

Friedlander's Bacillus.

The work of Norman, Bonner, Meyer, and Mark, Schneider, et al[14] lends evidence for the polygalacturonide chain structure which is believed to make up pectin.

Lefevre and Tollens[15] first demonstrated that uronic acids were decarboxylated by heating with 12% hydrochloric acid for several hours, yielding carbon dioxide along with a pentose, the latter yielding furfural. This is shown in Equation II.

Equation II



D-Galacturonic acid L-Arabinose Furfural

This idea is the basis for the improved quantitative method for the determination of uronic acids as proposed later by Dickson, Otterson, and Link.[16] Phillips, Goss, and Browne[17] offer a still further modification of the Dickson, Otterson,

and Link method.

A second method should be mentioned here; that developed by Tollens,[18] based on the color produced when glucuronic acid was heated with naphthoresorcinol in the presence of hydrochloric acid. However, the specificity of the naphthoresorcinol method was challenged a number of years ago by Mandel and Neuberg.[19] Many of the interferring substances, such as the lower dicarboxylic acids, occur in plants; therefore its use in the analysis of plant materials would be indeed subject to criticism.

Not to be overlooked is the fact that carbon dioxide is liberated from sources other than uronides. Whistler, Martin, and Harris[20] showed that aside from carbon dioxide coming from uronic acids, it came from cellulosic materials and starchy substances shown to be free of uronides. This fact, the difference in rate of carbon dioxide evolution between the rapidly reacting uronides and the slower reacting cellulose materials, gives access to a method for determining uronic anhydrides in the presence of large amounts of cellulosic materials.

Norman[21] in determining uronic acids in polysaccharides also points out that the rate of

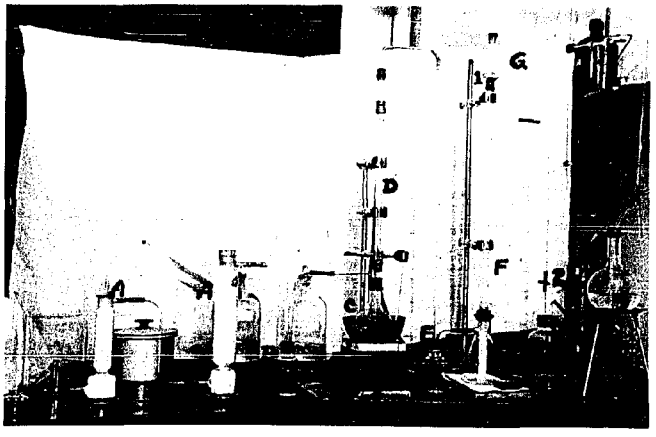
carbon dioxide liberation may indicate the presence or absence of uronic acids--the rate from polysaccharides being much slower, but yet continuous.

Bowman and Mc Kinnis[22] in studying the pentose and uronic acid content of orange albedo worked out a method whereby pentoses and uronic acids may be determined simultaneously. This method, they point out, is supposed to do away with emperical constants.

(1) Experimental.

In this work the uronic anhydrides were determined by the method of Dickson et al.[16] The actual set-up patterned after the above authors is shown in Figure I.

Figure I



Apparatus Used for the Determination of Uronic Acids

The sample to be analyzed (about 1 gm.) is placed in the reaction flask C with 100 ml. of 12% hydrochloric acid. Connections are made and air is slowly drawn through the two soda-lime towers A and the water trap B containing sulfuric acid in order to yield dry carbon dioxide-free air. This operation is continued for 20 minutes, when the temperature will have reached about 100° C. using an oil bath on an electric hot plate. At this point any carbon dioxide in the system or that resulting from carbonates in the sample will have been removed. The carbon dioxide liberated from the polyuronides as a result of an increase in temperature passes through condenser D and through bottle E, which contains silver nitrate solution and is finally adsorbed in tube F. Tube F contains a known amount of standard barium hydroxide introduced through funnel G, this funnel being washed with carbon dioxide-free water. Heating is continued for 4-5 hours at 140° C. The suction is then shut off with pinch clamp 2, and the stopcock 1 on funnel G, which is fitted with a soda-lime tube, is opened to permit the solution in tower F to run into the flask. The excess of barium hydroxide is then titrated with standard hydrochloric acid using

phenolphthalien as an indicator.

To evaluate the accuracy of this apparatus samples of ∝ D-galacturonic acid monohydrate (Eastman) were used. The results are shown in Table I.

Table I

Authentic ∝-D-Galacturonic Acid Monohydrate (Eastman)

No.	Weight of Sample (gm)	Barium hydroxide neutralized by CO_2 (me.)	% Uronic Acid
1	0.1512	1.430	100.3
2	0.1216	1.148	100.1

In order to analyze the seed coats they were first loosened from the beans by placing the latter in distilled water for 15 minutes at 85° C. By use of rubber hand rollers the seed coats were removed from the beans. Rapid drying with warm air made final separations of the heavy bean from the seed coats relatively easy by means of an air blast. The seed coats were air dried, ground and passed through a 20-mesh sieve. It was desired to take the beans just as they come from the market. The lot contained mostly pea beans, together with some

robust and blue pod strains.

The ground seed coats were divided into four different lots. Lot I was left untreated; Lot II was treated with 1% acetic acid (after a commercial) process) for ten hours at 60° C. pH of 2.8; Lot III was treated with 0.8% sodium hydroxide for 10 hours at 60° C. pH of 11.5; while Lot IV was treated with 1% sodium bicarbonate solution for 10 hours at 60° C. and pH of 7.8. All these lots were washed seven times with distilled water and then air dried.

Moisture determinations on the above acquired seed coats were made by the oven-dry method in which the samples were dried to constant weight at 105-110° C. These figures are listed in Table II.

Table II

Moisture in Navy Bean Seed Coats

Treatment	None	1% CH_3COOH	1% $NaHCO_3$	0.8% NaOH
% Average Moisture	8.07	8.94	11.78	4.55

Table III gives the values for uronic acid content of the seed coats expressed as uronic acid

anhydride or polyuronide. Since one molecule of CO_2 is liberated from each polyuronide residue, according to Equation II, the percentage of CO_2 times 4 gives the percentage of polyuronide.

B. "Crude Pentosans."

By "crude pentosans" or "total pentosans" is meant the pentosans equivalent to the total furfural yielded from a given sample. Each residue weight of polyuronide yields one molecular weight of furfural (Equation II). However, Norris and Resch[23] found only about 42% of the furfural expected when uronic acids were determined in the presence of common plant materials. By reference to Kröber's tables[24] the pentosans equivalent to this corrected value of furfural given by the polyuronide, when deducted from the "total pentosans", gives the so-called "true pentosans".

(1) Experimental.

The same four lots of seed coats used in the polyuronide determination were also used for the determination of the "crude pentosan" content. The procedure for the determination of pentosans was essentially that published in the

Table III

Polyuronide Content of Navy Bean Seed Coats

No.	Weight of sample (gm.)	$Ba(OH)_2$ neutralized by CO_2 (me.)	% CO_2 (air dry basis)	% Uronide (air dry basis)	% Uronide (oven dry basis)
		Untreated			
1	1.5331	3.101	4.45	17.80	19.36
2	1.2492	2.510	4.42	17.68	19.23
3	1.3058	2.628	4.43	17.72	19.29
4	1.5707	3.254	4.56	18.24	19.85
5	1.5066	3.127	4.57	18.28	19.89
6	1.5090	2.999	4.37	17.48	19.02
	Treated with 1% $HC_2H_3O_2$ for 10 Hours @ 60°C. pH 2.8				
1	1.3289	2.646	4.38	17.52	19.24
2	1.4317	2.865	4.40	17.60	19.33
3	1.2159	2.435	4.41	17.64	19.37
4	1.4231	2.860	4.42	17.68	19.42
	Treated with 0.8% NaOH for 10 Hours @ 60°C. pH 11.5				
1	1.1245	2.150	4.21	16.84	16.93
2	2.1170	4.080	4.24	16.96	17.05
3	1.6308	3.175	4.28	17.12	17.20
4	1.0347	1.953	4.15	16.60	16.68
	Treated with 1% $NaHCO_3$ for 10 Hours @ 60°C. pH 7.8				
1	0.8964	1.749	4.29	17.16	19.49
2	1.9124	3.685	4.24	16.96	19.23
3	0.9124	1.749	4.22	16.88	19.14
4	1.2844	2.458	4.21	16.84	19.09

methods of Analysis of the A. O. A. C.[24]; steam distillation, however, was used for the recovery of furfural. This method involves the assumption that the pentosans consist of equal amounts of araban and xylan. Hockett et al.[25] have shown that even if D-lyxose and D-ribose were the furfural yielding substances, in place of arabinose and xylose, the error introduced would not be larger than that associated as a consequence of the difference between arabinose and xylose.

The procedure involves the treatment of the seed coat material with 100 ml. of 12% hydrochloric acid in 300 ml. distillation flask. The sample being of such a quantity as to yield not more than 0.300 gm. of phloroglucide. The flask was placed on a wire gauze connected to a water-condenser. The distillate obtained by steam distillation was passed through a small filter paper into a 500 ml. erlenmeyer flask sitting in ice. The acid boiled off was replaced from time to time by means of a separatory funnel in top of the distilling flask. Sealed into the stem of the separatory funnel was a tube fixed so as to spray the acid down the neck of the distilling flask.

Distillation was continued for several hours until a negative test for furfural with aniline hydrochloride paper is attained. To the distillate containing the furfural was added enough 1% phoroglucinol in 12% hydrochloric acid solution to have approximately a two-fold excess of phloroglucinol over the furfural expected. The phloroglucide precipitate was allowed to form over night before filtering through a weighed Gooch crucible. The precipitate was washed with 150 ml. of water and dried for 4 hours at 100-105° C., then cooled and weighed in a weighing bottle. The increase in weight was assumed to be due to furfural phloroglucide. To calculate the amount of pentosans the emperical tables of Kröber were used. The results are given in Table IV.

C. "True Pentosans".

As pointed out previously, the "true pentosans" were calculated from the "total pentosans" by correction for the furfural liberated from the polyuronides. For example, the percentage of polyuronide in the untreated seed coats is 19.39%; the furfural equivalent is 10.56%; the corrected

Table IV

Pentosan Content of Navy Bean Seed Coats

No.	Weight of sample (gm.)	Weight of phlorogluicide (gm.)	Pentosans (gm.)	% Crude Pentosans (air dry basis)	% Crude Pentosans (oven dry basis)
		Untreated			
1	0.7080	0.2058	0.1867	26.4	28.7
2	0.7766	0.2314	0.2092	26.9	29.3
3	0.7674	0.2268	0.2053	26.7	29.1
	Treated with 1% $HC_2H_3O_2$ for 10 Hours @ 60°C. pH 2.8				
1	0.9639	0.2872	0.2581	26.8	29.4
2	0.9440	0.2740	0.2465	26.2	28.8
3	1.0878	0.3303	0.2960	27.2	29.9
4	0.6476	0.1900	0.1729	26.7	29.3
	Treated with 0.8% NaOH for 10 Hours @ 60°C. pH 11.5				
1	0.7569	0.2080	0.1887	24.9	26.1
2	0.7213	0.1838	0.1676	23.3	24.4
3	0.6213	0.1697	0.1551	24.9	26.1
4	0.5357	0.1454	0.1355	25.3	26.5
	Treated with 1% $NaHCO_3$ for 10 Hours @ 60°C. pH 7.8				
1	0.8465	0.2417	0.2182	25.8	29.2
2	0.9446	0.2727	0.2454	25.9	29.4
3	0.5576	0.1597	0.1464	25.5	28.9
4	0.6422	0.1809	0.1651	25.8	29.2

value 4.44%, which in turn is equivalent, according to Kröber's tables, to 7.61% pentosans. This latter figure deducted from the "total pentosans" gives the "true pentosans". These results are given in Table V.

D. Protein.

The third factor of importance examined in water sorbability, namely, the protein,[4] was determined by a semi-micro Kjeldahl method for nitrogen on each treated portion of the navy bean seed coats. For comparison the factor 6.25 was used to convert nitrogen into protein. The results are shown in Table VI.

Table V

"True Pentosans"

(1) Type of Treatment	(2) Average "total pentosans" (dry basis)	(3) Furfural equivalent to uronide per 100 gm. (dry basis)	(4) % Pentosans equivalent to uronide-furfural	(5) % "true pentosans"
None	29.03	4.44	7.61	21.42
1% CH_3COOH	29.35	4.43	7.60	21.75
0.8% NaOH	25.78	3.88	6.66	19.12
1% $NaHCO_3$	29.20	4.41	7.56	21.64

Table VI

Percentage Protein in Seed Coats

	Treatment	Weight of sample (gm.)	% Nitrogen (oven dry basis)	% Protein (oven dry basis)
1.	None	1.5350	0.784	4.90
	"	1.3548	0.826	5.16
	"	1.3676	0.825	5.16
2.	CH_3COOH	1.6326	0.815	5.09
	"	1.2042	0.822	5.13
3.	NaOH	1.2389	0.489	3.06
	"	1.2911	0.475	3.05
	"	1.2575	0.472	2.95
4.	$NaHCO_3$	1.1515	0.739	4.62
	"	1.4519	0.716	4.78
	"	1.4098	0.758	4.74

Discussion

The total combined content of polyuronide and "true pentosans" of the untreated seed coats is in the neighborhood of 40%. Schulze and Pfenninger[2] found a maximum of 48.65% of hemicellulose in the ripened bean seed coats. The uronide content of the bean coats compares favorably with many of the higher yielding hemicellulose preparations from various plants discussed by Norman.[26]

A comparison of the moisture content of the four lots of seed coats gives an indication of the importance of the uronide and pentosans in the water retention of the dried coats. Treatment with 1% acetic acid gave seed coats of slightly increased moisture content, when air-dried, as compared with the air-dried, untreated coats. The sodium bicarbonate treated samples showed the highest moisture content, 11.78%, while the sodium hydroxide treated samples gave the lowest moisture content, namely 4.55%. The sodium hydroxide samples also showed greatest loss of uronide, "true pentosans", and protein. The sodium bicarbonate treatment gave a small definite loss of protein and no

appreciable loss of uronide or "true pentosan". If the water retention was correlated with the protein content, one would expect a decrease in the water retention of these bicarbonate treated samples; the opposite condition, however, was observed.

III. Quantitative Determination of Crude Lipids, Unsaponifiable Fraction, and Sterols on Ground Whole Beans

As pointed out before, Grimme[5] reported the value of the crude lipid content of the whole navy bean as 1.32%. This crude lipid had a saponification number of 189.2, iodine number of 135.7, fatty acids 87.51%, and unsaponifiable matter 5.85%.

Alternate extractions of the navy bean flour with ethyl alcohol and ethyl ether effected the means of obtaining the total lipids. This procedure originally proposed by Koch[27] and subsequently used in a modified form by Kumagawa and Suto,[28] Sando,[29] and Dill[30] is reported to extract a maximum amount of lipid material. It is well to keep in mind in using this method that sterols often occur in combination with sugars as characteristically insoluble glycosides or phytosterolins,[31][32][33][34] which as a consequence may be only partially extracted by the above procedure. A procedure for complete extraction of free and combined sterols should combine acid and alkaline hydrolysis of the whole plant tissue as preliminary steps to the extraction procedure. However, such a method would be even more involved; furthermore, the amount of glycoside

present in most plant tissues is very small compared to the total lipids. Also to be noted is the fact that the calcium salt of phosphatidic[35] acid is soluble in ether.

The separation of sterols from compounds of similar solubility involves as the first step, the separation of the glycerides which make up the bulk of any lipid extract. This separation is accomplished by saponification and extraction of the unsaponifiable fraction with suitable solvents. The success of this separation offers only technical difficulties in separating free sterols and those originally present as esters. Glycosides or phytosterolins are resistant to ordinary alkaline hydrolyses, thus these combined sterols would not be extractable to any degree.

After the unsaponifiable fraction has been freed of glycerides, there are still present substances, such as hydrocarbons, carotenoid pigments, aliphatic alcohols and cyclic alcohols. The separation of sterols from these compounds is classically accomplished by precipitation with digitonin.[36] The use of digitonin for the quantitative determination or separation of plant sterol mixtures is subject to criticism since sterol digitonides vary in solubility,

and, in some cases the digitonides may actually be more soluble than the sterol itself.[37] Also, there is known a naturally occurring sterol, viz., calosterol[38] from the juice of Calotropis Gigantea, which does not form a precipitate with digitonin. This means the hydroxyl group at C_3 must be trans to the methyl group at C_{10}. Furthermore, various lots of digitonin may have variable amounts of companion saponins present.[39] Then, too, digitonin preparations precipitate with certain alcohols other than sterols.[40] Consequently, any use of digitonin as a quantitative precipitating agent for plant sterols must be established on a strictly emperical basis.

Numerous methods exist for the determination of the main animal sterol (cholesterol).

In 1910 Windaus[41] suggested the precipitation of cholesterol in alcoholic solution with an alcoholic solution of digitonin with the subsequent gravimetric determination of the cholesterol digitonide, in which the chlesterol was precipitated with digitonin and the digitonide determined by a variation of the Lieberman-Burchard reaction. Bernoulli[43] determined cholesterol by measuring the red color developed when anhydrous zinc chloride

and acetyl chloride were added. Szent-Györgyi[44] used an oxidation method to determine micro amounts of cholesterol digitonide, while Sobel, Drekter and Natelson[45] determined cholesterol by precipitation with pyridine sulfur trioxide.

All these methods are subject to a great deal of criticism since each of them gives variable results.

Thornton* has examined the various color reactions for a suitable method of determination of soybean sterols. His work indicated that most of the methods now in use for determination of cholesterol are of little value in determination of crude soybean sterols. He made an extensive study of the Bernoulli reaction[43] and found that there was a characteristic transmission maxima at 440 mu. for various sterols, but that the extinction coefficient was different for the various individual sterols. The Bernoulli reaction[43] is further complicated by the fact that it gives color with carotenoid pigments and related substances and hence may be used only on pure sterol solutions.

The method utilized in this work is a modification of the Schoenheimer and Sperry[42] method. This modification was developed as part of a fellowship, held by L. C. King and subsequently by

* Private communication

the author, granted by the Upjohn Farm Relief Association.

A. Experimental.

(1) Determination of crude lipids--Three 25 gm. samples of 80-mesh navy bean flour were each transferred to 33 x 94 mm. paper thimbles. Each sample was covered with a cup-shaped filter paper, made by pressing a filter paper over the open end of a large test tube. The thimbles were then placed into continuous Soxhlet extractors. Each thimble was kept off the bottom of the chamber by means of a 2 cm. length x 8 mm. glass tubing. After 10 hours of continuous extraction with 95% ethanol, an 8-hour extraction with dry ethyl ether was made. Following this, a final 10-hour extraction was made with 95% ethanol. These combined extracts were collected into weighed 250 ml. beakers, and evaporated on a steam bath, over which blew a swift current of air from an electric fan. This air current prevented solvent-creeping, over-heating, and aided evaporation. When all the solvents appeared to have evaporated there was added to each beaker, with rotation 20 ml. of absolute ethanol, the contents warmed and mixed well. Experience has shown these last steps to be

helpful in removing the last traces of water. After drying the extracts, they were weighed to constant weight (usually 2-3 weighings). These values so obtained are the total or crude lipids and are shown in Table VIII.

(2) Determination of Unsaponifiable--To the crude lipid in the weighed beakers were added about 25 ml. of 95% ethanol and 3-4 ml. of 10 N sodium hydroxide. The beaker and contents were heated on the steam bath, with occasional rotation, for 1 hour. The cover glasses were removed and the sides of the beakers washed down with a stream of hot 10% ethanol-water solution. Heating was continued for an additional 30 minutes. The mixture was transferred to 250 ml. separatory funnel with the aid of hot water. The beakers were rinsed with about 50 ml. of "Skellysolve B" and this then added to the cooled mixture. The soap solution was extracted 5-6 times with "Skellysolve B" using a triangular funnel arrangement. The stubborn emulsions were broken by the addition of 10-12 ml. of 95% ethanol. The combined petroleum ether extracts were washed 3 times with 50 ml. portions of 10% ethanol-water solution. The extracts containing the

unsaponifiable were returned to dried 250 ml. beakers and evaporated to dryness. To each beaker were added 70-80 ml. of "Skellysolve B". The contents were warmed, then filtered into dry, tared 150 ml. beakers, and washed with warm "Skellysolve". This step removed some glycosides and other foreign material. The solvent was evaporated as before on a steam bath using a moving air current. The dried beakers and contents were weighed to constant weight. The weight so obtained gives the value for the unsaponifiable matter, the results of which are also listed in Table VIII.

(3) Determination of Sterols--To each of the beakers containing the unsaponifiable matter were added 50 ml. of 95% ethanol. Cover glasses were placed on the beakers and the latter were warmed on the steam bath with occasional swirling. The contents of the beakers were then quantitatively transfered to 200 ml. volumetric flasks.

For the determination of the sterols a 5 ml. aliquot was taken from each flask and pipetted into a 15 ml. centrifuge tube. To each tube were added 2.5 ml. of digitonin solution (1 gm. in 1000 ml. of water and the solution evaporated to 500 ml.).

The tubes were warmed gently on the steam bath to aid flocculation of the resulting digitonide. After 1-hour of warming the tubes and contents were allowed to stand over-night. The digitonides were concentrated under centrifugation for 15 minutes, after which time the supernatant liquid was almost completely separated from the settled digitonides. This separation was accomplished by means of a rubber bulb pipette. The settled digitonides were washed with 2 ml. of 10% ethanol-water solution and re-centrifuged. After again removing nearly all of the wash solution above the digitonide, the latter was dried at 80-90° C. for 1 hour. If in certain cases small amounts of digitonide adhered to the side of the tube, it could be washed down with a fine stream of hot glacial acetic acid or cold pyridine. Following this the tubes were finally dried in the vacuum oven for 1 hour at 55° and 25 inches of mercury. Two ml. of dry glacial acetic acid were then added and the loosely corked tubes heated on the steam bath until all of the digitonides had gone into solution. When the tubes had cooled to room temperature 2 ml. of C. P. acetic anhydride were added in such a way as to run down the sides of the tube. The tubes

were corked, set in the refrigerator (8° C.) for 15 minutes, 0.1 ml. of concentrated C. P. sulfuric acid carefully added to each tube, and the contents then thoroughly mixed. Immediately, the rack of tubes was placed in a 45° oven for exactly 1 hour after which time they were transferred to an 8° refrigerator. After 15 minutes the amount of light in the region of 650 mu, transmitted by the reddish-brown sterol solution was measured, using a Fisher "Electrophotometer". The actual values for the amount of sterols were read from a standard curve made up from known amounts of crude soybean sterols. These values are also given in Table VIII.

The standard solutions were made from a stock solution consisting of 225 mg. of recrystallized crude soybean sterols dissolved in 95% ethanol made up to 500 ml. at 25° C. From this stock solution proper aliquots were pipetted into seven 200 ml. volumetric flasks so that when made up to the mark with 95% ethanol the flasks contained 10 mg., 20 mg., 25 mg., 30 mg., 35 mg., 40 mg., and 50 mg. of crude soybean sterols respectively. From each one of these flasks containing the standard solutions were taken a 5 ml. aliquot and treated exactly as

outlined above in the sterol determination. The resulting standard curve with the percent transmission plotted against the mgs. of crude soybean sterols using a 650 mu. filter in the Fisher "Electrophotometer" is shown in Figure II. The readings, from which the curve was plotted, are given in Table VII.

Table VII

Transmission Values for Standard Curve

Solution Number	Wt. of Crude Soybean Sterol per 200 ml. of sample	% Transmission at 1 Hour and 45°C. Filter 650 mu.						
		Set I	Set II	Set III	Set IV	Set V	Set VI	Average
1	10 mg.	83.2	85.5	85.0	85.4	84.3	84.5	84.7
2	20 "	70.1	72.6	72.6	----	71.6	73.6	72.1
3	25 "	----	65.6	65.6	65.2	65.4	63.6	65.1
4	30 "	----	59.3	59.3	58.9	60.5	61.0	59.8
5	35 "	----	55.3	55.3	----	55.0	54.6	55.1
6	40 "	----	50.0	50.0	47.8	48.3	48.7	49.0
7	50 "	38.2	38.3	38.4	39.8	41.2	41.1	39.5

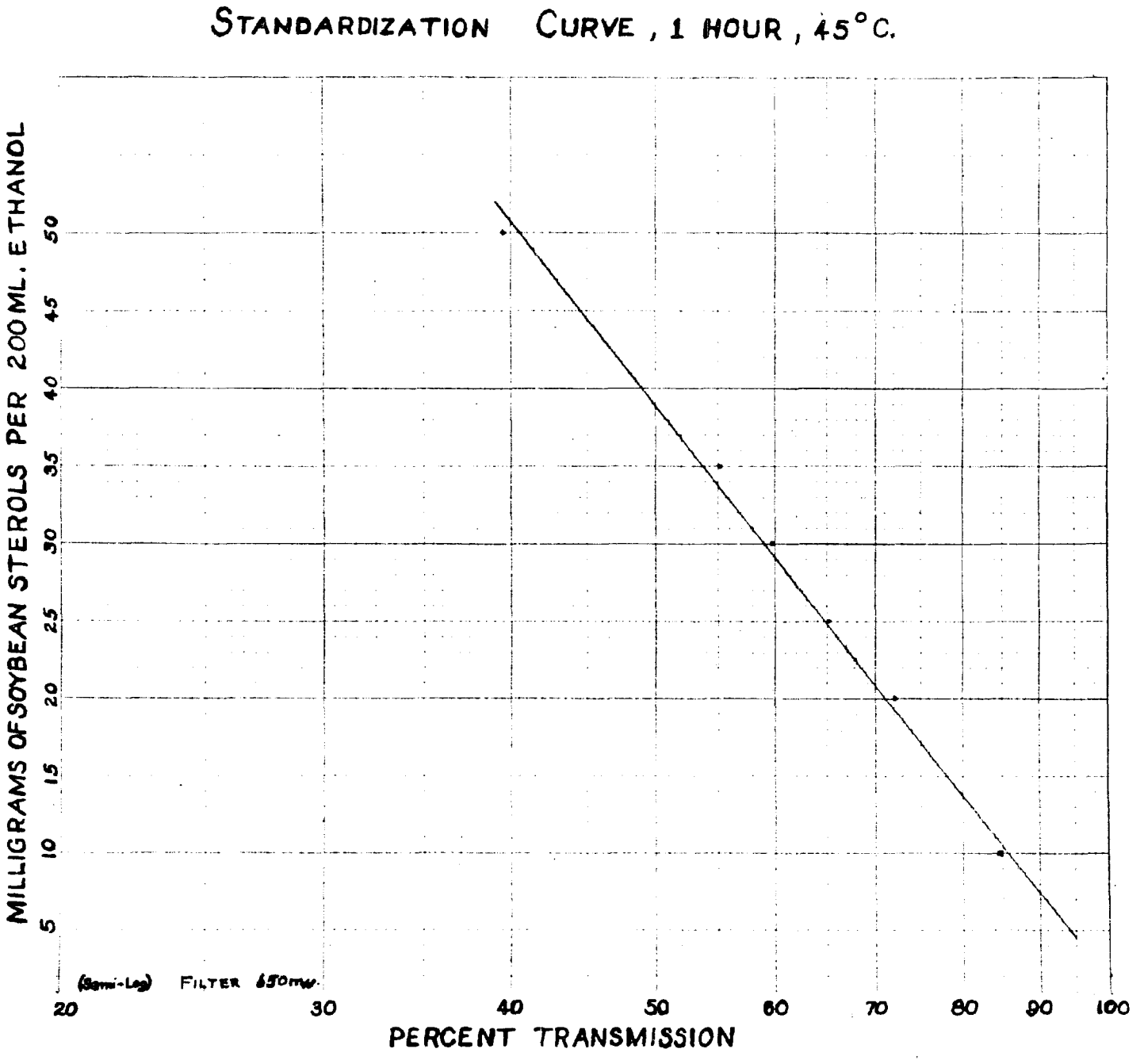

STANDARDIZATION CURVE, 1 HOUR, 45° C.
MILLIGRAMS OF SOYBEAN STEROLS PER 200 ML. ETHANOL
5
10
15
20
25
30
35
40
45
50
(Semi-Log) FILTER 650mμ
20
30
40
50
60
70
80
90
100
PERCENT TRANSMISSION

FIGURE II

Table VIII

Crude Lipid, Unsaponifiable, and Sterols on Navy Bean Flour

(25.0 gm. samples) (% moisture on oven dry basis = 8.29)

Sample No.	Crude Lipid (gm.)	% Crude Lipid on basis of seed (air dry basis	% Crude Lipid on basis of seed (dry basis)	Unsaponifiable fraction (mg.)	% Unsaponifiable on basis of seed (air dry basis)	% Unsaponifiable on basis of crude lipid	% Transmission at 1 hour, 45°C. filter 650. mu.	Crude sterols per 200 cc. (mg.)	% Sterols on basis of unsaponifiable
1	0.6642	2.66	2.90	38.4	0.15	5.78	67.0	23.2	60.4
2	0.6572	2.63	2.87	38.7	0.15	5.83	67.7	22.7	58.7
3	0.6676	2.67	2.91	38.6	0.15	5.89	68.0	22.4	58.1

Discussion

The values obtained for the crude lipid agree reasonably well considering the fact that the crude extract is handled and weighed in relatively large containers.

The results obtained for the unsaponifiable show better agreement than is normally attained on samples containing very large amounts of fatty acids and a small amount of unsaponifiable matter. A reasonable agreement among such a set of values lends evidence for the fact that 5 or 6 1-minute extractions of the soaps with "Skellysolve" is sufficient to remove the unsaponifiable matter. The use of a small container for the final weighing of the unsaponifiable is also desirable.

The values for the crude sterols compare reasonably well with the figure obtained from the large-lot extraction, subsequently to be reported. The justification for the use of the standard, made from crude soybean sterols, on the determination of the crude sterols in navy beans lies in the fact that the sterol fractions of these two legumes are quite similar.

The characteristics of the light transmission of the reddish-brown colored solution used in the

sterol determination shows no characteristic absorption maxima. Consequently, no particular part of the curve is ideally adopted for a color determination. However, a portion was selected which was conveniently covered by a standard filter (red filter o50 mu.) supplied with the Fisher "Electrophotometer", and which gave a reasonable dispersion of transmission values as a function of concentration of the sterol.

Without doubt, much is to be desired in this method. However, if extreme care is taken to avoid moisture-contamination of the dried digitonide and adherence to "exact" duplication of steps in both standard and unknown samples, reasonable comparative results can be obtained by an individual worker.

IV. Extraction of Total Protein from Ground Whole Navy Beans

A. Experimental.

Several solutions were tried for the purpose of extracting the total protein from the ground, air dry beans. The total protein of the bean flour calculated from the total nitrogen was about 26%. In all cases 10 gm. of the 80-90 mesh flour together with 150 ml. of extracting solution were used in centrifuge bottles (250 ml.). These mixtures were allowed to stand for 4 hours with frequent stirring. They were then centrifuged at 2000 r. p. m. for 10 minutes, and 50 ml. portions of the clear supernatant liquid were pipetted into Kjeldahl flasks and the protein content determined. Results in Table IX are given as protein (N x 6.25).

Table IX

Effectiveness of Extracting Solutions on Navy Bean Protein

No.	Extracting Solution	Average % Protein Air dry Basis
1	Distilled water	10.7
2	0.4% NaOH	22.7
3	10.0% NaCl	22.5
4	0.2% NaOH	22.3
5	0.5% Na_2SO_3	24.3

The use of sodium sulfite solution for extracting the protein from soybeans is mentioned by Horvath.[46]

Aside from the results of Table IX favoring 0.5% sodium sulfite solution for extraction, there is also obtained after precipitation with sulfur dioxide, a more highly desirable product with regards to plasticity and color.

In using the sodium sulfite solution for the protein extraction, 66 gm. of 80-mesh bean flour were added to one liter of 0.5% sodium sulfite solution and this mixture was stirred with an electric stirrer for 2½ hours. The particles were allowed to settle out. The decanted solution was passed through the Sharpless super-centrifuge in which the revolving bowl was lined with a celluloid sheet to remove any particles not in solution. In order to precipitate the protein 100 ml. aliquots were placed in 250 ml. centrifuge bottles, dilute sodium hydroxide added until a slight amber color appeared and then 50% sulfuric acid until precipitation just started. To another aliquot there was added sodium hydroxide as above and sulfur dioxide passed in until precipitation appeared complete. Another

aliquot was treated with sodium hydroxide and precipitation made with 50% acetic acid; the same result was accomplished with another aliquot with phosphoric acid. All these solutions were centrifuged for fifteen minutes and Kjeldahl determinations run on the mother liquors. The mother liquor from the aliquot treated with sodium hydroxide and sulfur dioxide was clearest in appearance and contained the least protein. A Kjeldahl determination on a 50 ml. aliquot resulted in a figure for the protein left in solution of 0.4 gm. per 100 ml. of supernatant liquid. The sulfite mother liquor previously contained 1.6 gm. of protein per 100 ml., or in other words, 75% of the protein was precipitated.

In order to get a more definite picture of the corresponding pH of the solution when maximum precipitation was effected by the SO_2 treatment, a continuous stream of SO_2 was passed into a solution of protein extracted with sulfite and made alkaline with 5 molar sodium hydroxide at pH of 11.5. Aliquots were removed, then allowed to stand and the relative precipitation noted. The results are shown in Table X.

Table X

pH Corresponding to Maxium Precipitation

Time	pH	Result
0 min.	11.5	No precipitate
18 min.	11.3	x
23 min.	10.2	x
25 min.	6.3	x x
28 min.	6.0	x x x x x x (clearest separation)
30 min.	4.9	x x x x x x (mother liquor cloudy)
33 min.	4.10	x x x x x x (very fine ppt.)
35 min.	2.00	x x x x x x (very fine ppt.)

The best textured precipitate was obtained between a pH of 5 and 6 (about 0.3-0.5% with respect to SO_2). It was later found better to precipitate from a solution made slightly less basic with NaOH, namely pH 10-11.

The protein obtained is a heavy, white, glossy semi-solid which had very good plastic properties. When rubbed between the thumb and fore-finger the protein became very smooth, transparent and dried to a fine film.

A few attempts for plastic formation were made with this protein. For this purpose a special brass mold was used through which live-steam could be passed (90-95° C.).

Number 1.

Base ---------- protein
Glutinizer ------- lactic acid
Indurating agent - 20% formaldehyde
Pressure --------- 2,000 lb. / sq. in.
Time ---------- 15 minutes
Temperature ------ 90° C.

The protein, of about 40% moisture, and lactic acid were kneaded in a mortar until the mixture became tacky or rubbery, a little (1-1 formalin) was then added and the mixture placed in a mold. It was immediately pressed and held for 15 minutes.

The product after heating in a 50° oven for 1 week was amber in color, hard and brittle.

Number 2.

Base ---------- protein
Glutinizer ------- lactic acid
Indurating agent - 30% formaldehyde
Pressure --------- 4,000 lb. / sq. in.
Time ---------- 15 minutes
Temperature ------ 90° C.

The protein was worked up as in #1. The product had a somewhat grainy appearance, was rubbery, and brown in color.

Number 3.

Base ---------- protein
Glutinizer ------- ortho phosphoric acid (75%)
Indurating agent - 30% formaldehyde
Pressure --------- 4,000 lb. / sq. in.
Time ---------- 15 minutes
Temperature ------ 95° C.

The product had a high elasticity, i.e., bent and snapped back quickly into original position. A higher pressure gave a clearer product.

Number 4.

Base ---------- protein
Glutinizer ------- 10% NaOH
Indurating agent - 40% formaldehyde
Pressure --------- 2,000 lb. / sq. in.
Temperature ------ 90-95° C.
Time ---------- 15 minutes.

The protein was kneaded in a mortar with NaOH until it became amber colored and rubber-like. It was then immersed in formalin, kneaded, and pressed in the mold. The product was clear, amber colored,

hard and brittle.

An inferior type of thermosetting plastic was made from the ground beans without extracting any of the protein. To 15 gm. of 80-mesh navy bean flour in a 500 ml. 3-necked flask was added 0.6 mole of redistilled phenol, and 0.5 mole of trioxmethylene. The mixture was stirred for three hours at 80° C. with an electric stirrer, during which time the mixture became darker and more turbid. It was transferred to a large watch glass and placed in an oven at 50° C. for two weeks. There resulted an amber colored, glassy mass containing dispersed particles. The latter were probably due to insoluble carbohydrate material and not protein since globulin-type proteins are soluble in hot phenol.

A much better product was obtained when 15 gm. of 80-mesh bean flour was mixed with a minimum of water, ptyalin added, and the mixture then agitated in a Waring blender. This mixture was allowed to stand for three hours. It then was made slightly acid with 3% acetic acid and 0.3 mole of trioxmethylene was added. After removal from the blender the preparation was heated to 80° C., 2 gm. of phenylisocyanate added, and the maroon colored product poured on a watch glass.

After five days at 50°C. there resulted a hard, glossy, thermosetting plastic with quite a homogeneous texture, relatively insoluble in water.

Discussion

Navy bean proteins consist chiefly of the globulin type[47] proteins which are soluble in dilute neutral salt solutions. According to Osborne[48] about 3% of the total navy bean proteins are alkali-insoluble and nearly salt-insoluble, which may account for the amount unextracted by the 0.5% sodium sulfite solution.

Since proteins are easily hydrolyzed by strong alkaline solutions, thereby affecting unfavorably the plasticity of the products obtained therefrom, it is well to employ weak alkaline solutions having reducing properties, such as a 0.5% solution of sodium sulfite.

Sulfurous acid (SO_2) has a triple advantage of producing a precipitate of good plastic quality, of bleaching the product, and of preventing its oxidation by air.

V. Extraction of Oil and Acquisiton of Unsaponifiable Fraction from Ground Navy Beans

As far as can be ascertained there is no other account given for the extraction of the total lipids from navy beans aside from that of Grimme.[5] As pointed out on page 4, Likiernik[9] appeared to be the only worker to have isolated any sterols from any part of the navy bean. He investigated only the unsaponifiable fraction of the oil obtained from the seed coats.

A. Experimental.

(1) Extraction of oil--Forty kilograms of navy beans were finely ground in a hammer mill and extracted six times with cold ethyl ether. The extraction was accomplished by the use of a 20-gallon galvanized garbage can having a brass faucet located about 2 inches from the bottom. The flour was held in a cloth sack which was fastened to rings inside and near the top of the can. The sack rested on two test tube racks.

The extract was filtered and the last traces of solvent distilled off in the presence of an atmosphere of carbon dioxide. The yield was 0.83 kilograms of yellow oil, which represented 1.9% of the original bean.

(2) Saponification of the oil--To the entire lot of oil, contained in a 12 liter round bottom flask, were added 4 liters of ethyl alcohol and 1 liter of 50% KOH (in H_2O). This mixture was stirred on the steam bath for two hours. Following saponification 4 liters of water were added and the unsaponifiable was exhaustively extracted from the soaps with "Skellysolve B". The extracts were concentrated to about 50%, washed free of alkali, and the remainder of the solvent distilled off. The yield was 48.7 gm. of a yellow colored semi-solid, which represented 5.9% unsaponifiable on basis of crude lipid.

(3) Separation studies on crude sterols--The entire amount of unsaponifiable matter was taken up in 500 ml. of petroleum ether and steam was passed into the solution until near saturation occurred. The solution was allowed to stand over-night. A mass of beautiful white crystals separated; yield of first crop 22.5 gm.; m. p. 138-144°; $[\alpha]_D^{27}$ -44° (35.4 mg., 2.1 ml. chloroform, l = 1 dm., α_D^{27} -0.742°, average reading). The Liebermann-Burchard reaction was distinctly positive. The mother liquor was evaporated to 300 ml. and allowed to stand for 24 hours. A

second crop of fine crystals was obtained; yield 7.2 gm.; m. p. 128-131°; $[\alpha]_D^{26}$ -38° (38.6 mg., 2.1 ml. chloroform, l = 1 dm., α_D^{26} -0.698 average reading). Again the mother liquor was concentrated to 100 ml. and set aside over-night. A third fraction of soft, waxy-like material was obtained; yield about 2 gm. The total amount of crude sterols acquired amounted to about 29 to 30 gms. or 59 to 62% sterols on basis of unsaponifiable.

(a) Chromatographic adsorption of crude sterol derivatives.

An attempt was made to separate the navy bean sterols by chromatographic adsorption of the colored esters[49] of the crude sterols. The colored esters were formed by esterifying the crude sterols with the colored azobenzene-p-carboxyl chloride. The azoyl chloride was prepared from the azobenzenemoncarboxylic acid and thionyl chloride in the presence of anhydrous of sodium carbonate.

Preparation of azobenzenemoncarboxylic acid-- This was accomplished by the method of Alway.[50] Para-nitrobenzoic acid (1 mole) was dissolved in ethanol and mixed with about 6 mole of glacial acetic acid. To this solution with agitation were added

2 moles of zinc dust in small portions. The temperature of the mixture was kept between 5-10° C. The cold yellow solution of p-hydroxylamino-benzoic acid was poured into an aqueous 10% ferric chloride solution and the mixture warmed to 45° and held at this temperature for 1 hour. The solid material was filtered and washed well with water. This solid material was then extracted with warm ethanol. The p-nitrosobenzoic acid dissolved while a gray insoluble substance remained. From the dark green filtrate the p-nitrosobenzoic acid separated upon evaporation of the solvent. This crude product was dissolved in a minimum of warm glacial acid and to this were added 25 gm. of redistilled aniline. The mixture is stirred while heating for about three hours, after which time a brown substance separated. This was filtered off, washed with water, dissolved in alcohol, Norite added, and again filtered. Upon evaporation the crude brown acid separated. Following three recrystallizations bright red crystals were obtained; yield 10 gm., m. p. 240-242° C.

Preparation of the acid chloride of azobenzenemonocarboxylic acid--To 2.7 gm. of the dry

colored acid were added 7 gm. of anhydrous sodium carbonate and 33.8 ml. of thionyl chloride. This mixture was refluxed for 1½ hours and the product taken up in low boiling petroleum ether. The sodium carbonate was removed by filtration and the filtrate was then evaporated to dryness on the steam bath. Recrystallization of the residue from petroleum ether at 0° gave bright red crystals which melted at 93-94°.

Preparation of sterol esters of azobenzenemonocarboxylic--Seven-tenths of a gram of crude navy bean sterols (dried in a vacuum at 100° for two hours) and 0.6 gm. of azobenzenemonocarboxyl chloride were dissolved in 20 ml. of dry pyridine contained in a 50 ml. glass-stoppered flask. The solution was heated on the steam bath for 1½ hours. Water was added and the crude steryl azobenzenemonocarboxylate esters, filtered, and washed. The dried product was recrystallized from absolute ethanol. The violet colored crystals melted at 133-135° C.

Chromatography of the esters--The adsorption tube used was 2 cm. in diameter and 80 cm. in length with a 10 cm. capillary attached to one end. A small

pack of cotton, followed by 3 cm. of sand, and this in turn followed by small portions of activated anhydrous aluminum oxide (80-mesh). Each portion was packed quite firmly by tapping on the side of the tube while slight suction was applied. The column was treated with 1-1 petroleum ether and benzene until there was left about 1 cm. of solvent over the alumina. Two-tenths of a gram. of the above described steryl esters were dissolved in 15 ml. of C. P. benzene and the resultant, deep red solution poured into the tube and the suction turned off. A few milliliters of benzene were added to wash down the last traces of the mixture. A liter separatory funnel was then stoppered tightly into the tube, and pure high-boiling petroleum ether was allowed to drop onto the column at the same rate as the solvent was dropping from the capillary (10-20 drops per minute). The petroleum ether was added only after the colored compounds had disappeared from the top of the column. The adsorbent was never allowed to become dry during the experiment.

Following the addition of about 50 mls. of petroleum ether a violet colored zone formed,

4 cm. in length about 12 cm. from the top of the column. Upon washing the column with 1½ liters of petroleum ether, the colored zone had moved down about 20 cms. more without any development of a distinct chromatogram. The zone was about 3 cm. in length with a distribution of violet color toward the top of the zone diffusing into a reddish-brown near the bottom. A further attempt to develop a chromatogram was made by washing the column with 500 ml. of 1-1 petroleum ether and benzene. This effected nothing except to carry the colored zone down beyond the middle of the column. A still further attempt was made by washing the column with 300 ml. of 1-3-3 absolute ethanol, petroleum ether, and benzene. The violet and the reddish-brown colored parts still persisted in staying together.

Discussion

Ladenburg et al[49] have shown that mixtures of the azobenzenemonocarboxylic esters of the following sterols were separated on adsorption columns; cholesterol from stigmasterol, stigmasterol from ergosterol, and cholesterol from ergosterol. Cholesterol, stigmasterol, and ergosterol, as a mixture, were each separated from one another. However, the ester of β -sitosterol could not be separated to any degree of efficiency from cholesterol, stigmasterol or ergosterol. It is now conceded that the separability of sterols on alumina columns depends primarily upon differences between the number of double bonds in the molecule, and not upon the position of the double bonds or the structure of the side chain.

From the results of the present work one would conclude that the individual sterols in the navy bean sterol mixture are of the same general type.

(b) Electrophoretic separation of crude navy bean sterols--According to Moyer[51] particles of <u>suspensions</u> prepared by grinding crystals of purified cholesterol with ice at -10°

show a remarkably uniform electrophoretic mobility. In fact, the mobility-pH curve of cholesterol (in $\frac{M}{150}$ acetate buffers) suspension prepared as above is in shape quite similar to that of a protein with an isoelectric point at pH of 3.2.

Trial preparations of buffer mixtures--In this work it was desired to have a buffer system which would maintain the crude sterols in solution at 0.5° C. and also have at this temperature a specific conductivity of about 10^{-3} mhos.

Buffer 1.

Ethanol (95%)	70 mls.
Pyridine (redistilled)	30 mls.
Acetic acid (glacial)	30 mls.
Sodium acetate	0.8 gm.
Specific Conductivity	3.08×10^{-3} @ 0°
p^H	4.7

In buffer #1 a 0.2% solution of crude navy bean sterols crystallized within 24 hours @ 0° C.

Buffer 2.

Ethanol (95%) ---------- 60 mls.
Pyridine (redistilled) --- 30 mls.
Acetic acid (glacial) ---- 10 mls.
Chloroform ---------- 0.5 ml.
Specific conductivity ---- 0.22×10^{-3} @ 0° C.
P^{H} ---------- 4.4

In buffer #2 a 0.2% solution of crude navy bean sterols gave no crystallization within 24 hours @ 8° C.

Buffer 3.

Ethanol (95%) ---------- 60 mls.
Pyridine (redistilled) --- 30 mls.
Acetic acid (glacial) ---- 10 mls.
Chloroform ---------- 0.5 ml.
Sodium acetate ---------- 0.1 gm.
Ethanolamine ---------- 16 ml.
Specific conductivity ---- 0.79×10^{-3} @ 0° C.
P^{H} ---------- 9.3

In buffer #3 a 0.2% solution of crude navy bean sterols gave no crystallization within 24 hours @ 0° C.

Buffer 4.

Ethanol (95%) ---------- 60 mls.

Pyridine (redistilled) --- 30 mls.

Acetic acid (glacial) ---- 10 mls.

Chloroform ---------- 0.5 ml.

Sodium acetate ---------- 0.1 gm.

NH_3 bubbled through for 3 minutes

P^H ---------- 8.7

In buffer #4 a 0.2% solution of crude navy bean sterols gave no crystallization within 24 hours @ 0° C.

These experiments showed all buffers except #1 to be satisfactory, however #4 was chosen for its slightly higher specific conductivity.

An electrophoretic analysis of the mixture was attempted using the technique described by Tiselius.[52] The navy bean crude sterols from fraction 1 and 2, twice recrystallized, were dissolved in sufficient buffer solution #4 to make a 0.2% solution. This buffer has a pH of 8.7 and an ionic strength of about 0.1. The sample was dialyzed against frequent changes of the same buffer until the specific conductivity of the sample and buffer showed that an equilibrium had been reached. Electrophoresis

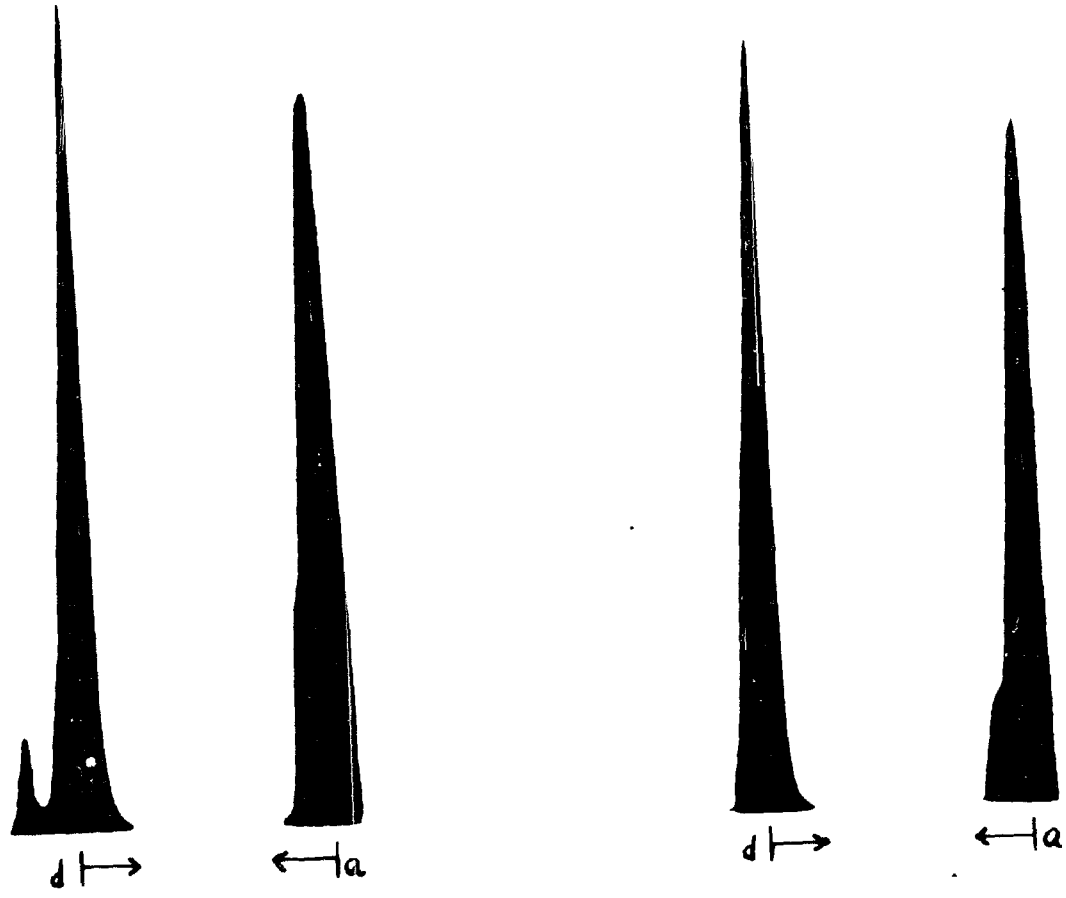
was then carried out for 6,000 seconds at a potential gradient of 4.2 volts per cm. in one case, Figure IIIa, and for 12,000 seconds at a potential gradient of 4.5 volts per cm. in another case, Figure IIIb. Photographic records were made using the Longsworth-Schlieren scanning technique.(53)

Figure IIIa shows the electrophoretic patterns obtained after electrophoresis for 6,000 seconds. The descending pattern reveals a small peak migrating as an anion. The concentration of this material, as determined from the relative areas of the two peaks, is low. The large peak shows no tendency to migrate and remains electrophoretically homogeneous. The ascending pattern would seem to indicate the presence of a small amount of material migrating toward the cathode. However, the separation from the large amount of electrophoretically inert material is incomplete.

The patterns obtained after electrophoresis of the same sample for 12,000 seconds are shown in Figure IIIb. The small peak migrating toward the anode is no longer apparent. This is probably due to the concentration gradient, and therefore, the refractive index gradient becomes so small that

the peak cannot be detected. The ascending pattern again shows the presence of a small amount of migrating material. However, the mobility of this material is so low that it still has not separated from the main components.

FIGURE III



a. b.

ELECTROPHORETIC PATTERNS

Discussion

Figure IIIa reveals a small peak, probably a non-steroid impurity because of the low concentration, migrating as an anion-negatively charged. The rest of the pattern shows no migrating material. The ascending side reveals some material (very low mobility) migrating as a cation-positively charged. Most of the material is inert.

Figure IIIb, descending pattern, does not reveal any small peak (explained above) and ascending pattern shows the same material as the other ascending pattern. Large peaks in each pattern represent electrophoretically inert material. No actual separation was effected.

It is to be noted that in this work, unlike that of Moyer's, the sterols were in solution as chemical individuals, or possibly associated, and not as hydrated colloids.*

* Acknowledged credit is here given to Dr. C. R. Hardt for the electrophoretic work and the developing of the patterns.

B. Irradiation of Crude Navy Bean Sterols.

Occurrence of vitamin-D activity, as a consequence of irradiation of a given seed oil or the crude sterols therefrom, is generally attributed to the presence of small amounts of ergosterol. However, this does not exclude the possibility of other activatable provitamin-D's, yet unisolated, being present in the seed oils.

For this experiment a total of 0.117 gm. of material, made up of equal parts from all the fractions of the unsaponifiable portion, was irradiated with ultra-violet light in ethyl ether for 5 minutes. The irradiated solution was then made up to 100 ml. with ethyl ether. Suitable aliquots were taken from this stock solution for assay by the standard biological method. The results of this assay indicated an activity of 700 U. S. P. units per gram of crude navy bean unsaponifiable material.*

* The author wishes to thank Dr. C. A. Hoppert for the Vitamin-D assay.

(c) Isolation of Sterols from the Unsaponifiable Fraction of Navy Bean Oil by Fractional Crystallization.

Crystallizations of sterols or their derivatives have resulted in the isolation of numerous sterols from plants.

Only paraphytosterol[9] has been reported, as far as the writer can determine, as a sterol constituent of a portion of the navy bean.

(1) Experimental.

One and one-half grams of crude sterols were taken from each of the first two fractions (pages 47 and 48). To this combination of 3 gm. of crude sterols, in a 125 ml. erlenmeyer flask, were added 60 ml. of acetic anhydride. The mixture was heated for 1½ hours under reflux. The volume of the solution was then decreased to the extent of saturation. After cooling for 1 hour at 12° C. the crude acetates were filtered.

Bromination of the crude steryl acetates--Three and one-tenth grams of crude acetates were dissolved in 30 ml. of ethyl ether, and to this solution were added 38 ml. of bromine-acetic acid solution (5 gm.

bromine in 100 ml. of glacial acetic acid) following the procedure of Windaus and Hauth.[54] After allowing the solution to stand in the refrigerator, the insoluble tetrabromides were filtered off and washed with cold ethyl ether. Yield 1.4 gm., m. p. 190-194°. This would correspond to about 25% sterol (on the basis of stigmasterol) in the crude mixture. Six recrystallizations of the tetrabromide from chloroform-methanol mixture gave a melting point of 194-196° C.

Stigmasteryl acetate--To a solution of 1.2 gm. of the tetrabromide acetate in 12 ml. of glacial acid were added 1.2 gm. of zinc dust. This mixture was refluxed for 1½ hours, filtered hot, diluted with water and extracted with ethyl ether. The ether layer was washed with a dilute sodium sulfite solution and then with water, and the ether finally removed by evaporation on the steam bath. Yield of the crude acetate was 580 mg. It was recrystallized 4 times from ethanol and twice from 2-1 methanol-chloroform mixture; m. p. 139-140° C.; $[\alpha]_D^{25}$ -54.0° (37.4 mg., 2.1 ml. of chloroform, 1 dm., α_D^{25} -0.962°, average reading).

Anal.* 3.074 mg. gave 2.949 mg. HOH; 9.150 mg. CO_2

C, 81.18% ; H, 10.73%

Calc'd. for $C_{31}H_{50}O_2$, C, 81.88% ; H, 11.09%

Stigmasterol--Four-hundred milligrams of the above acetate were hydrolyzed by refluxing for 1 hour with sufficient 10% alcoholic potassium hydroxide. Water was added to the mixture and the latter extracted with ethyl ether. The ether solution was washed with a dilute sodium carbonate solution, then with water, and evaporated to near dryness on a steam bath. The residue was recrystallized from 95% ethanol 3 times; m. p. 168-169° C.; $[\alpha]_D^{27}$ = -47.3° (28.6 mg., 2.1 ml. of chloroform, l = 1 dm., α_D^{27} -0.644°)

Anal. 3.121 mg. gave 3.153 mg. H_2O; 9.552 CO_2

C, 83.47% ; H, 11.70%

Calc'd $C_{29}H_{48}O$, C, 84.40% ; H,11.30%

Stigmasterol benzoate--About 300 mg. of the free sterol were dissolved in 2 ml. of dry pyridine, and to this was added 0.6 ml. of benzoyl chloride. The mixture was heated on the steam bath for 2½ hours in a 25 ml. glass-stoppered flask and the mixture

* Microanalyses are by Dr. T. S. Ma, University of Chicago

then allowed to stand over-night. The contents were poured into an ice cold 5% sulfuric acid solution. The ethyl ether extract of the mixture was washed with dilute sodium carbonate solution, and finally with 10% ethanol solution. The contents were evaporated to dryness on the steam bath, and then recrystallized 3 times from ethanol and once from a methanol-chloroform mixture; m. p. 160.5-161.5°;

$[\alpha]_D^{24}$ -24.5° (152.3 mg., 2.1 ml. of chloroform, l = 1 dm., α_D^{24} -1.779°, average reading).

Anal. 2.959 mg. gave 2.602 mg. H_2O; 9.108 CO_2

C, 83.95% ; H, 9.83%

Calc'd $C_{36}H_{52}O_2$ C, 83.65% ; H, 10.15%

Nearly all of the remainder of fractions 1 and 2 plus fractions 3 and 4 were combined and converted into the acetates by dissolving in 250 ml. of acetic anhydride. The mixture was refluxed for 2 hours and allowed to stand over-night. Floating on the top of the solution was a wax-like cake, yellow in color, and in the solution proper crystals were noted. The flask and contents were warmed slightly (about 40° C.) in order to effect solution of the crystals. The mixture was filtered warm,

thus collecting the wax-like cake on the filter. The residue (about 3 gm.) was set aside.

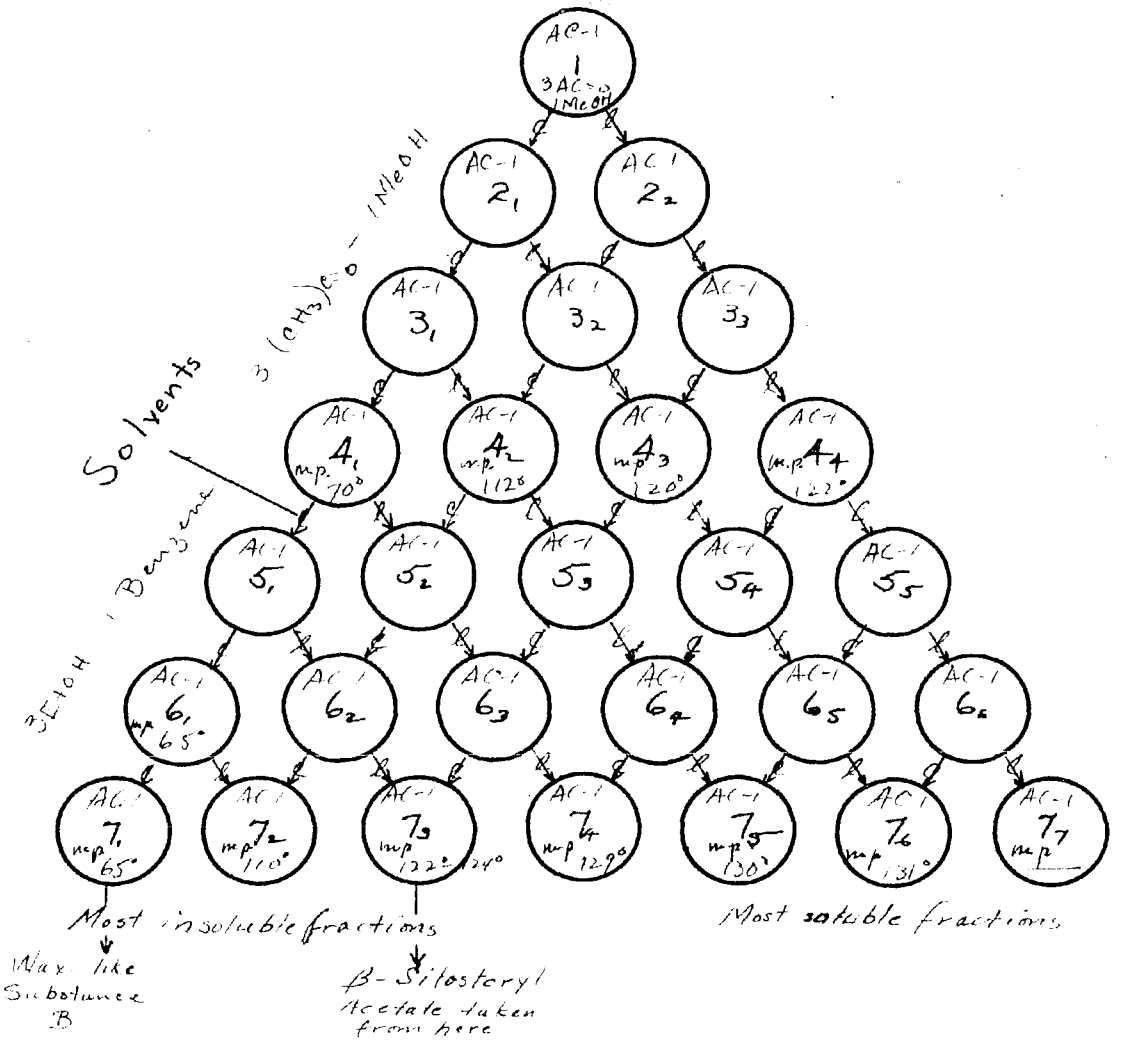
The volume of the filtrate was reduced until complete crystallization had nearly occurred. The contents were then cooled for 1 hour in the refrigerator, after which time the mixture was filtered and washed with cold glacial acetic acid. The mixture of supposed acetates (about 10 gm.) was brominated, in the way previously described for stigmasterol isolation in order to effect the removal of the stigmasterol present. The stigmasteryl acetate tetrabromide was filtered off and the derivatives in the filtrate were debrominated in the usual manner with zinc dust.

Since γ -sitosterol,[55] the most insoluble of the sitosterol complex, is also precipitatable as a bromide, constant observation was made in this work for γ -sitosterol, but no evidence was found for its presence. However, its presence in small quantities is not excluded.

The debrominated acetates were taken up in sufficient 1-1 acetone-chloroform mixture to effect what was considered to be a half-saturated solution.

The contents were allowed to stand over-night. The solid acetates, designated as AC-1, were removed by filtration. The filtrate was subsequently designated as AC-2. The AC-1 fraction was subjected to triangular fractionation using a 3-1 acetone-methanol mixture and later a 3-1 ethanol-benzene mixture. The separation of β -sitosteryl acetate is shown in Figure IV. The separation of a wax-like substance was also effected in this fractionation.

Figure IV



Separation of β-Sitosteryl Acetate
and a Wax-like Substance

β-Sitosteryl acetate--Following the final recrystallization from ethanol the compound melted at 123-124°; $[\alpha]_D^{29}$ -38.5° (40.5 mg., 2.1 ml. of chloroform, l = 1 dm., α_D^{28} -0.744°, average reading).

Anal. 2.970 mg. gave 2.912 H_2O ; 8.886 mg. CO_2

C, 81.59%, H, 10.96%

Calc'd. $C_{31}H_{52}O_2$ C, 81.52%; H, 11.47%

β-Sitosterol--The above acetate was saponified with 10% alcoholic potassium hydroxide and further worked up as usual. Three recrystallizations from methanol-chloroform mixture yielded the free sterol; m. p. 136-138° C.; $[\alpha]_D^{25}$ -37.8° (19.2 mg., 2.1 ml. of chloroform, l = 1 dm., α_D^{25} -0.346°, average reading).

Anal. 2.929 mg. gave 2.957 mg. H_2O ; 8.989 mg. CO_2

C, 83.41%; H, 11.25%

Calc'd. $C_{29}H_{50}O$ C, 83.99% H, 12.15%

β-Sitosteryl benzoate--Fifty milligrams of free sterol were reacted with 0.2 ml. of benzoyl chloride in 1.5 ml. of dry pyridine. The mixture was heated on the steam bath for 1½ hours and allowed to stand over-night. The contents were poured into 10 ml. of cold 5% sulfuric acid. The mixture was extracted with ethyl ether. The fourth recrystallized

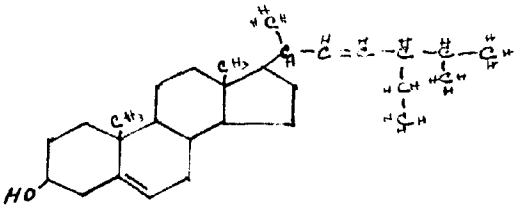
product from ethanol melted at 145-146° C.; $[\alpha]_D^{25}$ -13.5° (19.4 mg., 2.1 ml. of chloroform, l = 1 dm., α_D^{25} -0.125°, average reading).

The crystalline acetates of the AC-2 fraction were obtained by evaporating the solvent on the steam bath. The residue was taken up in ethanol and acetates hydrolyzed by addition of a 10% solution of potassium hydroxide. The solution was worked up as usual. The crude sterols so obtained were dried and then reacted with sufficient 3,5-dinitobenzoyl chloride to yield the esters. Triangular fractionation was used again in an attempt to isolate one of the three known "α-sitosterols" by taking advantage of the fact, as did Wallis and Fernholz[56] that the corresponding 3,5-dinitrobenzoates differ greatly in their solubility. The α_1-sitosteryl-m-dinitrobenzoate is by far the most insoluble while the α_3-sitosterol derivative is the most soluble. As a result of this attempt, the most insoluble 3,5-dinitrobenzoate obtained had a m. p. of 222-223° C.; $[\alpha]_D^{28}$ -17.3° (23.6 mg., 2.1 ml. of chloroform, l = 1 dm., α_D^{28} -0.195°, average reading). The free sterol melted at 155-157° C.; $[\alpha]_D^{25}$ -55.8° (15.6 mg., 2.1 ml. of chloroform, l = 1 dm., α_D^{26} -0.415°, average reading).

From the free sterol the acetate was made which melted at 134-135° C. The Liebermann-Burchard reaction was positive. Since all the derivatives of the "α-sitosterols" have a positive value for the optical rotation with a relatively large magnitude, one is inclined to conclude from the above observations that no "α-sitosterols" are present in the navy bean oil. The high, negative value of rotation for the sterol is of interest since it appears to be the highest of the phytosterol group. A mycosterol, ergosterol, has a rotation value of -132.0°.

Discussion

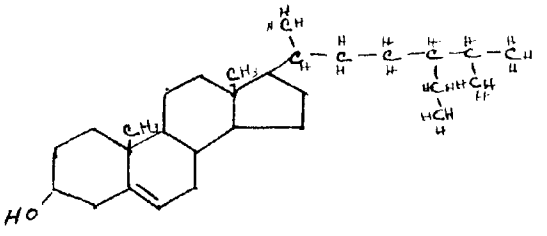
The navy bean oil herein studied, making up about 2.5% of the bean, consists of 5.-6.0% unsaponifiable. The unsaponifiable consists of about 55-60% crude sterols, of which about 23-25% consists of stigmasterol.



Stigmasterol

This percentage compares closely with that obtained from soybeans by Kraybill et al.[57] They found about one-half of the unsaponifiable to be crude sterols and of this portion stigmasterol was present to about 20-25%.

It is estimated that β-sitosterol occurs to the extent of 5-7% of the navy bean crude sterols.



β-Sitosterol

"Sitosterol", the common plant sterol, has been resolved into three isomeric sitosterols ($C_{29}H_{50}O$).[55] The order of their insolubility in organic solvents is γ, β, and α-sitosterol. β-Sitosterol has been isolated in the pure state from cotton seed oil.[58] Cinchol, the sterol isolated

from carrots and cinchona bark is now considered to be identical with β -sitosterol.(59)

C. Isolation of Probable hydrocarbons.

(1) Experimental.

The wax-like residue obtained from on top of the acetylated mixture of crude sterols mentioned on page 65 was recrystallized from acetone and benzene eight times raising the melting point from 65-68° to 72-73° C. The white, waxy-like material, designated as A, was insoluble in cold concentrated sulfuric acid and did not decolorize bromine-chloroform solution. No further characterization was made.

A substance more soluble in acetic anhydride was separated in the fractionation of -sitosterol, page 68. This was called substance B. The results of the qualitative tests were the same as those designated for A above. After thirteen recrystallizations from an ethanol-benzene mixture and from p-dioxane the substance melted at 65-66.5° C. No further study was made.

Summary

(1) From Tables III and V it is seen that the polyuronide and the "true pentosans" content of the navy bean seed coats are about 19% and 21%, respectively, of their dry weight.

(2) The protein is about 5% of the dry weight of the seed coats.

(3) Treatment of the seed coats with 0.8% sodium hydroxide lowers the content of both polyuronide and "true pentosans", while 1% acetic acid and 1% sodium bicarbonate have little effect.

(4) Protein of the seed coats is slightly lowered by sodium bicarbonate treatment and noticeably by treatment with sodium hydroxide.

(5) There is an indication that polyuronides and "true pentosans" are involved in water retention in the dried seed coats.

(6) From Table VIII it is seen that the crude lipid and unsaponifiable are about 2.65%, and 0.15%, respectively, of the air dry seeds.

(7) A modified method based on the Liebermann-Burchard reaction has been presented for the determination of crude sterols.

(8) The crude sterols of the navy bean oil unsaponifiable are about 59%.

(9) The proteins of the ground whole navy beans have been effectively extracted with 0.5% sodium sulfite solution and precipitated satisfactorily with sulfur dioxide.

(10) The protein is adaptable to plastic formation.

(11) Three separation studies on crude sterols separated from navy bean oil have been attempted, namely, chromatographic, electrophoretic, and triangular fractionation. Only the latter was successful.

(12) A vitamin-D activity of 700 U. S. P. units per gram of crude navy bean oil unsaponifiable matter was observed.

(13) Stigmasterol and β -sitosterol have been isolated and characterized.

(14) An unidentified sterol with a high negative rotation has been isolated.

(15) Two different probable saturated hydrocarbons have been isolated from the navy bean oil.

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