The diacetate of coumestrol was about equal in activity to coumestrol, taking into account the weight of the two acetate groups.

Cheng and coworkers (*11*) under their bioassay conditions found daidzein to be the most potent of the four isoflavones, while biochanin A and genistein had about equal potencies. This apparent variation in results between that of Cheng's laboratory and that included in this study may be due to differences in bioassay procedure. One major difference between their procedure and ours is that in their procedure the excised mouse uteri were fixed in Bouin's fluid, then blotted before weighing.

The authors found formononetin to have definite activity, although less than that of other isoflavones. This is contrary to the findings of at least three other laboratories (*5*, *8*, *18*) which reported formononetin to be inactive by either oral administration or subcutaneous injection at levels of 10 to 50 mg. per mouse. Cheng *et al.* (*11*) reported that the feeding of formononetin to mice at a level of 2.5 mg. per day for 4 days produced a slight increase in uterine weight.

Our results, Table I, show that coumestrol is from 30 to 100 times more potent than the isoflavones. Estrone, the natural animal estrogen, and the synthetic diethylstilbestrol are respectively about 200 and 3000 times more potent than coumestrol under the conditions of our bioassay.

Despite the lower potencies of the forage estrogens, when compared to diethylstilbestrol, they are present in certain forages in sufficient quantities as to be influential in animal nutrition and physiology. Thus, Oldfield *et al.* (*16*) have shown that alfalfa meal, selected for high coumestrol content, can cause increased weight gains in wether lambs. Furthermore, the even less potent isoflavones, notably genistein, have been shown to be present in Australian subterranean clover (*7*) in sufficient quantities to cause reproductive problems in sheep.

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COTTONSEED PROTEINS

Isolation and Chromatographic Characterization of Low Molecular Weight Cottonseed Proteins

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Cottonseed protein is the term assigned to the composite mixture of individual proteins found in the defatted seed. Its value for food or feed is dependent upon the chemical content and physical characteristics of the individual proteins.

Jones and Csonka (*6*), using classical fractionation procedures, isolated six fractions from cottonseed. The α- and β-globulins, glutelin, and pentose protein composed the major fractions. Protamine-type protein was absent.

Spies and coworkers (*12*, *13*) have found the relatively minor water-soluble fraction to consist of a series of proteins varying widely in pentose content.

Since lysine is a major determinant of the nutritive value of cottonseed protein (*9*), the isolation of a lysine-rich fraction in any comprehensive study would be of particular interest. Both the pentose protein of Jones and the water-soluble fraction of Spies were seemingly high in the basic amino acids.

With this carbohydrate content and salt solubility of cottonseed protein in mind, a preliminary fractionation attempt was made using a NaCl–aqueous ethanol solvent system originally designed to dissociate and solubilize nucleoproteins (*2*). This resulted in the isolation of a fraction which was 15% higher than the average protein in lysine content.

This investigation, therefore, is concerned with the isolation and characterization of that portion of glandless cottonseed (*8*) which is soluble in high molarity

Glandless cottonseed meal fractionated by successive extraction with aqueous ethanol solutions of varying salt content yielded three fractions. Each is high in nitrogen and sulfur and low in ash and contains nonreducing carbohydrates. Each gives a single peak in the ultracentrifuge with an estimated S_{20} value of 2 but shows a multicomponent system when analyzed with cation exchange cellulose chromatography. Analysis showed the fractions to differ in content from whole meal and each other, particularly in such nutritionally important amino acids as lysine, leucine, cystine, and glutamic acid.

NaCl–50% aqueous ethanol solutions in terms of sedimentation constant, chromatographic fractionation, and amino acid content.

Experimental

Isolation. Cottonseed, devoid of gossypol-containing pigment glands (glandless cottonseed), was dehulled, flaked, exhaustively extracted with hexane at room temperature, and air-dried (CM 72). Portions of CM 72 (225 grams each) were extracted successively with 20 1-liter quantities of 80% aqueous ethanol, followed by three 1-liter quantities of diethyl ether (CM 72-A).

Portions of CM 72-A were extracted successively at 4° C. with 0.75*M* NaCl in 50% aqueous ethanol for 1 hour (ratio 2 grams to 100 ml.); 0.75*M* NaCl in 50% aqueous ethanol for ½ hour (ratio 4 grams to 100 ml.); and 1.5*M* NaCl in 50% aqueous ethanol for 1 hour (ratio 4 grams to 100 ml.). The centrifuged supernatants, fractions I, II, and III, respectively, were dialyzed (Visking 18/32) for 58 hours against 0.01*M* acetic acid pH 3.5 with six changes of acid (ratio 2 ml. to 30 ml.), lyophilized, and stored at −20°. The very slight but discernible precipitate which occurred upon dialysis was removed by centrifugation at 13,500 × g for 30 minutes before lyophilization.

Ultracentrifugation. All patterns were obtained with a Spinco ultracentrifuge Model E using the analytical rotor at a speed of 59,780 r.p.m. CM 72 and CM 72-A were twice extracted with 10% NaCl for 4 hours and dialyzed against phosphate-NaCl buffer (pH 7.8, Γ 0.5) for 2 days in preparation for analysis. The lyophilized fractions were also dialyzed against the appropriate buffer for 2 days.

Chromatography. Cation exchange chromatography of the three fractions was performed on carboxymethylcellulose (Brown Co., Lot 1008, 0.47 meq. per gram) at pH 4.68 with acetate buffer (0.05*M*) and a discontinuous LiCl elution scheme. Care was taken to keep conditions as identical as possible. CMC was suspended in water, made acid to Congo red paper with glacial acetic acid, allowed to settle, decanted, and resuspended in distilled water until the pH was neutral and the fines were removed. The cellulose was then washed exhaustively with absolute alcohol and dried in a vacuum oven at 40° overnight.

Each column, 1 cm. in diameter, was packed in sections to a height of 18 cm. under 10 pounds of nitrogen and washed with 1.5 liters of acetate buffer at a rate of 1 ml. per minute. Final height was 19 cm.

Each fraction was dissolved in the starting buffer (1 mg. per ml.), dialyzed against the buffer for 16 hours, and centrifuged for 30 minutes at 13,500 × g. Twenty milliliters of the centrifuged solution was placed on the column at a rate of 1 ml. per minute.

One liter of the starting buffer was made 1*M* with respect to LiCl, adjusted with 10% NaOH to pH 4.68, and diluted with the starting buffer to obtain the appropriate concentration of LiCl for elution.

The fractions were eluted at a rate of 1.2 ml. per minute, with the following elution schedule in the order of application: 35 ml. of starting buffer; 100-ml. portions of 0.05*M*, 0.10*M*, 0.18*M*, and 0.24*M* and 150-ml. portions of 0.32*M*, 0.4*M*, and 0.6*M* LiCl. Five-milliliter aliquots were collected. Protein content was determined by the method of Lowry (*7*) and pentose content by the method of Dische (*3*).

Amino Acid Analysis. Each fraction was hydrolyzed in duplicate in sealed ampoules under nitrogen at 117° for 16 hours with twice-distilled constant boiling HCl at a ratio of 1 ml. per mg. of protein. Performic acid oxidation (*5*) of each fraction was executed for the determination of cystine-cysteine content as cysteic acid. The amino acid determinations were obtained with an automatic Spinco amino acid analyzer (*11*).

Table I. Composition of Isolated Fractions

Fraction	Nitrogen, %	Sulfur, %	Phosphorus, %	Ash, %	Moisture, %
I	14.5	2.3	0.00	0.53	6.9
II	15.3	2.2	0.00	1.38	6.8
III	15.9	1.6	0.08	1.38	6.6

Results and Discussion

The 80% alcohol extraction procedure effectively removed the major carbohydrate, raffinose, the phosphatides, 20% of the meal by weight, and 4% of the total nitrogen (*9*).

Naismith (*10*) has observed that a 10% salt extract of cottonseed meal in phosphate buffer showed three components having estimated S_{20} values of 13, 8, and ~2. The patterns obtained under the same conditions from extracts of CM 72 (Figure 1, *A*) and CM 72-A (Figure 1, *B*) showed the same three components with no observable difference between the hexane - extracted and the hexane-alcohol–extracted meals. The major portion of the protein extracted seems to be distributed between the 8_S and 2_S components.

Analysis of the initial meal (CM 72-A) and the final residue established that the alcohol-salt procedure extracted 20% of the meal solids and 20% of the meal nitrogen. However, only 80% of this nitrogen and 50% of these solids could be accounted for in the isolated fractions. When the extracts were dialyzed against distilled water, still more solids and nitrogen were lost. The function of the acetic acid is not known—that is, whether the retention of solids and nitrogen is due to pH or some complexing ability of the acetate ion. No further loss was noted when the isolated fractions were dialyzed against either an acetate or a phosphate buffer in preparation for analysis.

Fontaine noted that the carbohydrate-rich protein nitrogen of cottonseed meal is insoluble at pH 3.5 in the absence of added salts (*4*). One possible explanation for the apparent contradiction between this statement and the present work may be coprecipitation of the phosphatide fraction with the pentose protein. We have found the phosphatide fraction to precipitate copiously at pH 3.5. Meal CM 72-A, however, has been rendered essentially phosphatide-free by the ethanol-ether extractions. The high phosphorus content of the pentose protein of Jones also lends support to this explanation.

Table I gives the compositional analysis of the fractions. Each fraction was positive to the Molisch and negative to the Benedict tests.

Ultracentrifugal studies of the isolated fractions I, II, and III, in both phosphate-NaCl buffer (pH 7.8, Γ 0.5) and acetate buffer (pH 4.68, 0.05*M*), showed only a single component with an esti-

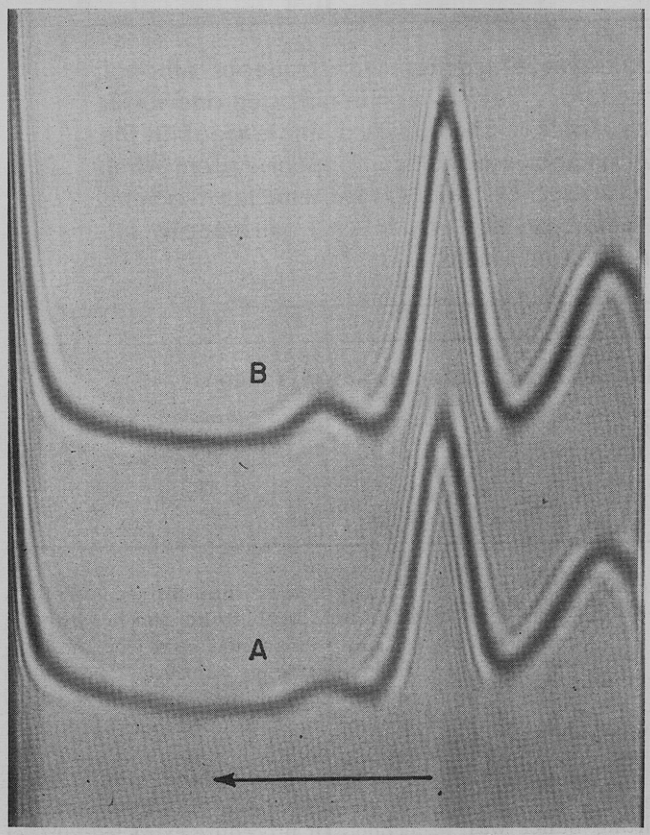

Figure 1. Ultracentrifuge patterns of 10% salt extracts of glandless cottonseed meal

A. CM 72, standard cell, 2.2 mg. N per ml.
B. CM 72-A, wedge quartz cell, 2.4 mg. N per ml., 32 minutes past maximum speed

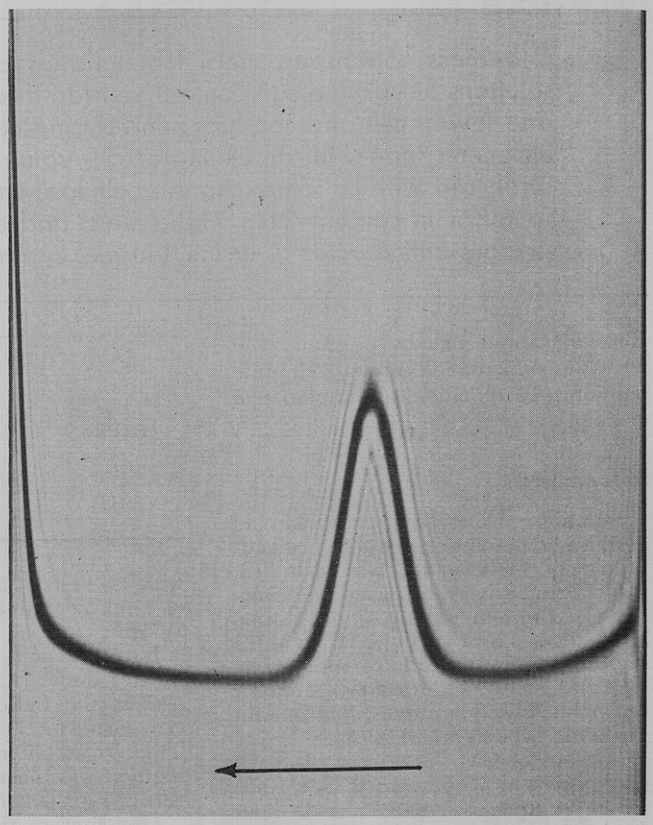

Figure 2. Ultracentrifuge pattern of fraction I

Synthetic boundary cell, 1.2 mg. N per ml., 12 minutes past maximum speed

mated S_{20} value of ~2. A typical pattern is illustrated by Figure 2.

Preliminary attempts at chromatography of the fractions of DEAE cellulose at pH 7.9 failed because of the strong affinity of the protein for the cellulose. Chromatography at pH 6 on CM cellulose was found inadequate because of the partial insolubility of the fractions at pH 6, the lack of resolution in the pentose portion of the sample, and the incomplete recovery of the protein from the column. There was also an indication of an association-dissociation mechanism occurring when the ionic strength of the starting buffer was varied from 0.006 to 0.06.

The chromatographic profiles of Figure 3 are the diagrams obtained with CM cellulose chromatography at pH 4.68 acetate buffer (0.05*M*), and represent 100% recovery of the pentose and protein of the eluted sample as estimated by the methods of analysis used. These profiles are reproducible in every detail. LiCl was found to be a far more effective eluting agent in this instance; the sodium ion gave broad ill-defined peaks. These diagrams have been divided arbitrarily into five sections. Though there exists a striking similarity between the profiles, there are certain specific differences. In section A there is a notable decrease in the pentose peak from fraction I through fraction III as well as a variation in the concentration and symmetry of the protein peaks. In section B there is a virtual disappearance of the pentose components in proceeding from fraction I and fraction III. This would occur with successive extractions of a particular material. Also in section B two of the major peaks of fraction I disappear, with the immergence of a sharp well defined peak in fraction II and a broad new peak in fraction III. The decrease in pentose protein concentration might possibly bring about these changes. In sections C, D, and E not only is there a variation in the weight distribution of the sections but there is in all three sections proceeding from fraction I through fraction III a very definite progression of the apex of the peak toward the left of the graph.

Table II. Amino Acid Composition of Cottonseed Proteins

Amino Acid	Cottonseed Meal, G./16 G. N	Fraction I G./100 g.	Fraction I G./16 g. N	Fraction II G./100 g.	Fraction II G./16 g. N	Fraction III G./100 g.	Fraction III G./16 g. N
Lysine	4.3	5.6	6.2	6.0	6.0	4.4	4.4
Histidine	2.7	2.3	2.6	2.8	2.8	2.5	2.5
Ammonia	2.2	2.2	2.5	2.3	2.3	2.1	2.1
Arginine	12.3	13.4	14.8	14.4	14.5	15.2	15.3
1/2 Cystine	1.9	6.2	6.8	6.6	6.9	3.5	3.5
Aspartic acid	9.9	5.7	6.3	6.7	7.1	7.7	7.8
Threonine	3.4	1.5	1.6	1.8	1.8	1.8	1.8
Serine	4.4	1.8	2.0	2.6	2.7	3.0	3.0
Glutamic acid	20.0	29.5	32.6	32.5	34.0	27.0	27.1
Proline	3.6	3.1	3.4	3.1	3.2	2.5	2.5
Glycine	4.4	2.7	3.0	3.5	3.7	5.0	5.1
Alanine	4.4	1.1	1.2	1.5	1.5	2.4	2.4
Valine	5.0	1.0	1.1	1.2	1.3	2.1	2.1
Methionine	0.7	1.1	1.3	1.9	2.0	1.7	1.7
Isoleucine	3.2	0.8	0.9	1.3	1.3	2.1	2.1
Leucine	5.8	2.0	2.2	2.7	2.9	4.5	4.5
Tyrosine	3.1	3.0	3.3	3.4	3.6	2.4	2.4
Phenylalanine	5.8	1.6	1.8	1.6	1.6	2.3	2.3

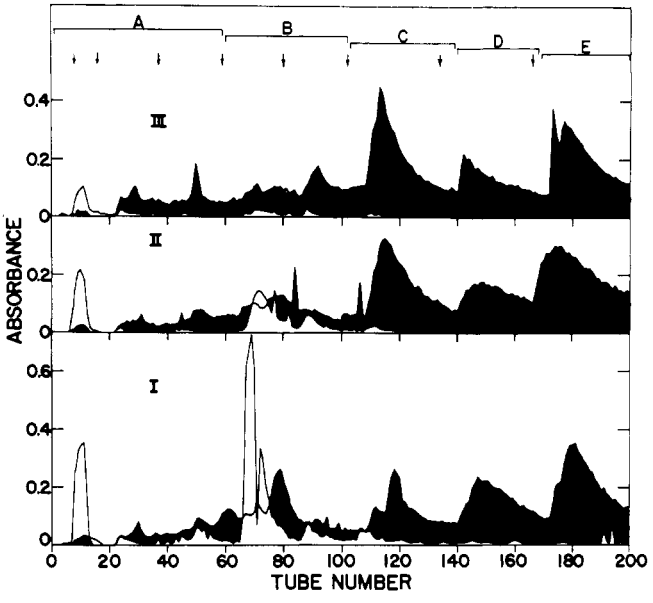

Figure 3. Elution diagram of protein (shaded area) and pentose

0.05M acetate buffer, pH 4.68, on CM cellulose
Stepwise increase of LiCl concentration indicated by arrows. Column eluted successively with 35 ml. of starting buffer, 100-ml. portions of 0.05M, 0.10M, 0.18M, and 0.24M and 150-ml. portions of 0.32M, 0.4M, and 0.6M LiCl

This could be interpreted as a decrease in positive charge or NH_3^+ groups which is supported by the lower lysine and glutamic acid (assuming glutamine) and ammonia content of fraction III.

The hydrolyzate of each of the three fractions contained the complete protein amino acid spectrum. The difference in humin formation between fraction I and fraction III was notable. Fraction I had considerably more than fraction III, with fraction II falling between.

Comparison of the amino acid composition of the fractions with that of whole glanded meal (*1*) (Table II) showed certain definitive differences. Fraction I is high in the basic amino acids, lysine and arginine; high in the acidic amino acid, glutamic acid; and exceptionally high in the sulfur amino acids. There is a sharp decrease in the neutral amino acids content as well as a complete inversion of the tyrosine-phenylalanine ratio. Fraction II shows some increase in the neutral amino acids content, but the pertinent characteristics are similar to fraction I. The neutral amino acid content of fraction III shows a definite increase and in some instances is double that of fraction I. The sulfur amino acids content is lower than that of fractions I and II but still greater than that of the whole meal; the tyrosine-phenylalanine ratio is now 1.

The sulfur content cannot be completely accounted for in any of the fractions by the amino acid analysis. It is possible that the pentose moiety contains sulfur.

The sum of the amino acid residues (similar to conditions of protein linkage) in grams per 100 grams of material shows that 77% of fraction I, 87% of fraction II, and 84% of fraction III can be accounted for as protein.

The combined weights of the isolated fractions (9% by weight and 16% of the nitrogen of CM 72-A) contain 38% of the cystine-cysteine, 23% of the methionine, 15% of the lysine, 14% of the arginine, and 18% of the glutamic acid present in the whole glanded meal. The combined fractions (9% of CM 72-A), therefore, contain a substantial part of certain nutritionally important amino acids in cottonseed.

These fractions are uniquely different in amino acid composition from that of the whole meal protein. However, neither the homogeneity expected from the ultracentrifuge patterns nor the gross differences which might be suspect from the amino acid analyses are found in the chromatographic profiles. The precise difference between the series of proteins studied here remains to be elucidated.

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