tion. Above that level, conductivity was relatively constant.

Conclusion

Data reported in this paper show that a conductivity method may be used for the computation of fatty acid contents of soap in the range of 78 to 83%. The method is adaptable to the control of soap dryers.

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Preliminary Report on the Nutritional Significance of Bound Gossypol in Cottonseed Meal

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A NUMBER OF INVESTIGATORS have shown that the nutritional value of different samples of cottonseed meal varies over a wide range (5, 12, 14, 20). The work of Lyman *et al.* (12, 13), Haddon *et al.* (7), and Chang *et al.* (5) indicates that processing conditions are of major importance in connection with these variations. Two distinct nutritional factors are involved. The first is the necessity of reducing the content of free gossypol to low levels in order to avoid the unfavorable physiological effects of this compound. The second factor concerns the quality of the protein as modified by processing methods and other variables. During an investigation designed to develop laboratory methods for estimating protein quality in cottonseed meal Lyman, Chang, and Couch (12) found a relationship between protein quality and bound or inactivated gossypol. This relationship between protein quality and bound gossypol was given further consideration during the course of a cooperative survey of the nutritional value of prepress solvent cottonseed meals (5). Four different investigators studied the same group of meals in chick growth tests. The correlation coefficients between chick growth rate and total gossypol (essentially bound gossypol) were —0.899, —0.791, —0.805, and —0.140. All of these coefficients were significant at the 1% level except the last. In a rat protein-repletion test the correlation between protein quality and total gossypol was —0.682. This value is significant at the 1% level.

Such a relationship between the nutritional value of the protein in cottonseed meal and bound gossypol would be predicted on the basis of previous postulates concerning the chemical nature of bound gossypol. A number of years ago Clark (6) proposed the concept that, during the processing of cottonseed meal, free gossypol, which is toxic in guinea pigs, rabbits, and pigs, is converted into an insoluble, inert gossypol-protein complex. It is known that gossypol in this form has lost its harmful properties. In the laboratory gossypol-protein complexes have been prepared by Hale and Lyman (8) and Castillon and Altschul (3). Gossypol is known to combine with amino groups. Chang (4) prepared a lysine-gossypol compound and fed this to rats. Essentially all of the lysine as well as the gossypol were recovered in the feces. These findings and others suggest that gossypol may be bound to protein through an amino group.

Kuiken and Lyman (11) reported that the availability of lysine in a sample of cottonseed flour was only 64.5% whereas in wheat and peanut flour it was about 95%. The formation of a gossypol-protein complex could account for this decreased lysine availability and resulting lowered nutritional value of the cottonseed protein. The importance of lysine availability in cottonseed meal is further indicated by the reports of Sherwood and Couch (19) and Richardson and Blaylock (16, 17), who showed that the addition of lysine to the diet increased the growth of chicks when cottonseed meal was used as the source of protein.

As a part of an evaluation of the general concept concerning the nature of bound gossypol and its significance in the utilization of cottonseed meal protein, the purpose of the present communication is 1) to describe a procedure by which bound gossypol can be removed from samples of cottonseed meal without subjecting the material to heat; 2) to report the results of tests designed to determine whether the removal of bound gossypol results in improvement of the nutritional value of the protein; and 3) to report the change in nutritional value of purified cottonseed protein which takes place when the protein combines with gossypol.

Experimental

Description of Cottonseed Meal Sample. The meal used in this study was a commercial prepress solvent meal. It was selected for study solely on the basis of its high bound gossypol content.

Treatment to Remove Free Gossypol. Three liters of 70% acetone were added to 2,000 g. of meal, and the mixture was stirred vigorously with a mechanical stirrer for 6 hrs. The mixture was allowed to stand for 24 hrs. at room temperature, then filtered, and washed three times with acetone and finally once with ethyl ether. The treated meal was spread on paper in thin layers to dry.

Treatment to Remove Bound Gossypol. a) To 2,000 g. of cottonseed meal 450 g. of redistilled aniline

and 2 liters of acetone were added. The mixture was stirred vigorously for 24 hrs., then filtered and washed three times with acetone and then finally twice with ethyl ether. The meal was spread out on paper in thin layers and allowed to dry. Meal prepared in this manner was used in the feeding tests.

b) As an alternate procedure about 2,000 g. of cottonseed meal were stirred for 6 hrs. with 450 g. of a fatty acid amine, either octylamine or decylamine, dissolved in 2 liters of hexane. The mixture was allowed to stand at room temperature over-night, then filtered, and washed with hexane. After filtration with suction, the residual hexane was removed by spreading the material in a thin layer on paper. Decylamine proved to be somewhat more satisfactory for removing bound gossypol than octylamine.

Preparation of Cottonseed Protein. The oil was removed from rolled cottonseed meats by extraction with hexane in the cold. The meats were then suspended in petroleum ether (33°–60°) by means of a high-speed mixer. After allowing the preparation to settle for 10 minutes, the suspension was decanted from the glands and heavier particles, which had settled to the bottom. The gland-free solids were collected by centrifugation or by allowing the preparation to settle for several hours.

To 50 g. of the solids 200 ml. of 0.5 *N* sodium chloride solution were added, and after adjusting the pH to 7.6, the mixture was stirred with a magnetic stirrer for three hours in the refrigerator. The undissolved residue was removed by centrifugation and extracted a second time. The protein was precipitated from the combined extracts by adjusting to pH 4.6 and diluting with two volumes of distilled water. The precipitated protein was dried by treatment with acetone and ether in the cold.

Preparation of a Gossypol-Cottonseed Protein Complex. One hundred and fifty grams of purified cottonseed protein were added to 750 ml. of redistilled water, and while the fine suspension was stirred with a magnetic stirrer, 75 g. of gossypol dissolved in 750 ml. of methanol were added. The preparation was placed in the refrigerator, and the stirring was continued overnight. The gossypol-protein complex was separated by centrifugation and washed several times with acetone and finally with ether to remove all free gossypol.

A yield of 135 g. of a gossypol-protein complex containing 3.25% bound gossypol was obtained. Preparations containing less bound gossypol were obtained by treating the protein in the same manner with smaller amounts of gossypol.

Determination of Gossypol. Gossypol, free and total, was determined according to the official A.O.C.S. methods. Dianilino gossypol was determined by an unpublished method supplied by the Buckeye Cotton Oil Company. The results were later checked by the procedure of Pons *et al.* (15).

Nitrogen Solubility. Nitrogen solubility in 0.02 *N* NaOH was determined by the procedure described by Lyman *et al.* (12).

Rat Repletion Test. A modification of the Cannon (2) rat protein-repletion test described by Cabell and Earle (1) was used to determine the relative nutritive value of the cottonseed meal protein.

Lysine Availability. A new and simplified method was developed for the determination of lysine availability in foods based on a combination of the procedure of Kuiken and Lyman (11) and the chromic oxide marking technique described by Schurch, Lloyd, and Crampton (18).

The meal samples to be tested were fed to young rats as the sole source of protein in a 12% protein diet of the composition shown in Table I.

TABLE I

Composition of Basal Diet for Rat Feeding Tests

	g.
Salts IV (9)	44
Stock vitamin mix [a]	30
Cottonseed oil	40
Cod liver oil	10
Choline	5
Dried liver fraction L	3
Cottonseed meal to give a 12% protein diet	
Cerelose to make up to 1 kg.	

[a] The stock vitamin mix contained the following vitamins mixed with 1 kg. of starch: calcium pantothenate, 0.5 g.; pyridoxine·HCl, 0.2 g.; riboflavin, 0.2 g.; thiamin·HCl, 0.1 g.; niacin, 1.0 g.; inositol, 1.6 g.; para-amino benzoic acid, 1.6 g.; menadione, 30 mg.; folic acid, 7 mg.; vitamin B_{12}, 1 mg.

The diet and water were supplied *ad libitum* to the rats in individual cages for a preliminary period of five days followed by a six-day collection period. Representative samples of the feces were collected, taking care to avoid any spilled feed. The feces were dried in a vacuum-drying oven and ground in a mortar. Both feed and feces were analyzed for chromic oxide as described by Schurch *et al.* (18) and for lysine by microbiological assay. The apparent lysine availability was calculated as follows:

$$\text{Apparent percentage lysine availability} = \frac{\text{lysine in 1 g. of food—lysine in feces from 1 g. of food}}{\text{lysine in 1 g. of food}} \times 100$$

$$\text{Lysine in feces from 1 g. of food} = \text{lysine in 1 g. of feces} \times \frac{Cr_2O_3 \text{ in food}}{Cr_2O_3 \text{ in feces}}$$

Chick Feeding Test. Day-old New Hampshire chicks were fed in batteries with raised screen floors. Food and water were supplied *ad libitum*. The birds were weighed initially and at weekly intervals over an experimental period of four weeks. Groups of 25 chicks were used for testing the different meals. The tests were repeated with White Leghorn chicks.

The basal diet contained 5% of salts IV, 4% of Wesson Oil, cottonseed meal to give a 21% protein diet (approximately 50% of the diet), and cerelose to make 100%. The diet was supplemented with the following vitamins, in milligrams per kilogram: riboflavin 6, calcium pantothenate 15, niacin 100, vitamin B_6 3, thiamine 4, biotin 0.2, folacin 2, inositol 1,000, para-aminobenzoic acid acid 100, menadione 0.5, choline 2,000, and alpha tocopherol 6. Vitamin B_{12} was also added (50 mg. per kilogram). Some 600 I.U. of vitamin A were added per kilogram of diet.

Results and Discussion

The results of the chemical and biological tests before and after the removal of bound gossypol are given in Tables II and III. In order to eliminate the possibility that the tests might be affected by the presence of small amounts of free gossypol, the first step was to reduce the content of free gossypol to a very low level by extraction with acetone. This procedure resulted in a slight improvement in protein quality as indicated by the rat protein-repletion test and also a small increase in the growth of chicks during a four-week test period. After the removal of free

TABLE II

Effect of Removing Bound Gossypol on the Nutritional Value of Cottonseed Meal

Group no.	Meal description	Gossypol content		Nitrogen solubility in 0.02 *N* NaOH	Lysine availability[b]	Chick growth,[c] av. gain for 4 weeks
		Free	Bound			
		%	%	%	%	*g.*
1	Original meal	0.033	1.32	61.0	54.9	121.7
2	Treated to remove free gossypol	0.003	1.10	69.0	54.1	157.8
3	Treated to remove bound gossypol	0.004	0.49[a]	77.0	70.4	222.4
4	Standard meal (butanone-extracted)	0.006	0.37	89.0	87.5	233.2

[a] Includes 0.42% dianilino-gossypol. [b] Three rats in each group. [c] Twenty-five chicks in each group.

gossypol a part of the same preparation was further treated to remove bound gossypol.

The results of the biological tests in this case were much more striking. Lysine availability increased from 54.1% to 70.4%. The protein-repletion value, expressed as grams gain in weight in 10 days, increased from 29.0 to 46.0 (Table III). The gain in weight of chicks during a four-week period increased from 157.8 g. to 222.4 g. All three of these types of tests indicate that the nutritional value of the protein in this sample of cottonseed meal was markedly improved by the removal of bound gossypol.

TABLE III

The Effect of Removing Bound Gossypol on Protein Quality as Determined by Rat Protein-Repletion Test [a]

Description of meal	Av. initial wt.	Av. final wt.	Av. gain in wt. in 10 days
	g.	*g.*	*g.*
Original cottonseed meal	113.8	139.6	25.8
Treated to remove free gossypol	114.4	143.6	29.2
Treated to remove bound gossypol	118.0	163.8	45.8
Standard meal (butanone-extracted)	116.6	171.6	55.0

[a] Five rats in each group.

A standard reference meal (Group 4), prepared by removing both oil and gossypol without heat, was included in the tests for comparison with the commercial meal with bound gossypol removed. It will be noted that the removal of bound gossypol did not raise the nutritional values completely up to those of the reference standard. The difference in these two meals may well be due to heat damage, by a mechanism not involving gossypol.

Consideration of the hypothesis that gossypol combines with protein through amino groups and that the removal of gossypol by aniline treatment liberates these groups with a resulting improvement in nutritive value prompts the suggestion that aniline may also free amino groups tied up by reaction with carbohydrates or other constituents of the meal. Evidence presented here is not sufficient to eliminate completely this possibility.

Since nitrogen solubility in 0.02 *N* NaOH has proven to be a useful practical guide in the production of cottonseed meals of high protein quality (5), it is of interest to note that the removal of bound gossypol results in higher nitrogen solubility.

Table II shows the results obtained with a single commercial meal chosen for its high content of bound gossypol. In a second series of tests results comparable to those given in Table II were obtained with a meal from a different source.

Since hexane is generally the solvent of choice for the solvent extraction of cottonseed, experiments were conducted to determine whether bound gossypol might be removed by extraction with hexane containing other chemicals. It was found that this could be accomplished by adding fatty acid amines containing eight or nine carbon atoms to hexane. (Further experimentation will be required to determine the commercial possibilities of a process based on this finding.)

In a second phase of this investigation a gossypol-cottonseed protein complex was prepared without the use of heat or extremes of pH which might destroy gossypol or denature the protein. The preparation of the complex presented a problem because of the insolubility of gossypol in water at neutral pH and the insolubility of proteins in organic solvents which will dissolve gossypol. The problem was solved by suspending the protein in water and adding the gossypol in a methanol solution; the mixture was stirred in the cold for a number of hours. Preparations containing various percentages of gossypol were made in this way.

The nutritional value of the original protein and of a gossypol-protein complex containing 3.25% gossypol was determined by the rat protein-repletion and the lysine availability tests (Table IV). Because of the possibility that the protein might have been altered by the methanol water solution, a sample treated in the same way, except for the omission of gossypol, was included in the nutritional tests.

The changes in lysine availability and protein-repletion value resulting from the methanol treatment alone were extremely small. The rat protein-repletion value for the gossypol-protein complex was 16.6 as compared to 51.0 for the original protein. The apparent lysine availability value for the complex was 48.7 as compared to 82.9 for the original protein. Along with the reduction in the rat protein-repletion value and lysine availability, nitrogen solubility dropped from 89.0 to 48.3%.

It is sometimes assumed, when a sample of cottonseed meal gives a low value in a nutritional test, that this results from heat damage to the protein during processing. The experiment given above shows that the nutritive value of cottonseed proteins can be reduced to a low level by reaction with gossypol even though no heat is involved. At the same time it is logical to assume that during the processing of cottonseed meal the gossypol-protein reaction is promoted by heat. Also heat may be detrimental to the nutritional value of cottonseed meal in other ways than by promoting the reaction between protein and gossypol.

A study of relative importance of bound gossypol content and heat damage not involving gossypol in determining protein quality in cottonseed meal made by various commercial methods will be reported in a later communication.

TABLE IV

Change in the Nutritional Value of Cottonseed Protein on Reaction with Gossypol

Sample description	Bound gossypol	Nitrogen solubility in 0.02 *N* NaOH	Lysine availability [b]	Rat protein repletion value [a]		
				Av. initial wt.	Av. final wt.	Av. gain in wt. in 10 days
	%	%	%	*g.*	*g.*	*g.*
Original protein	0	89.0	82.9	116.0	167.0	51.0
Methanol-treated protein	0	85.5	86.0	115.8	165.2	49.4
Gossypol-protein complex	3.25	48.3	48.7	116.6	133.2	16.6

[a] Five rats in each group. [b] Three rats in each group.

Summary

A procedure is described by which bound or inactivated gossypol can be removed from cottonseed meal without the application of heat which might damage the protein. The removal of bound gossypol increased the nutritional value of the protein as determined by chick feeding tests, rat protein-repletion tests, and lysine availability tests. A procedure is described for the preparation of a gossypol-cottonseed protein complex without heating the materials. As a result of the combination of the protein with gossypol, marked reduction in nutritional value occurred. The nitrogen solubility of the complex was only about half that of the original protein. The results are in accord with the concept that the inactivation of gossypol during the processing of cottonseed meal is accomplished through the formation of an insoluble, inert gossypol-protein complex which results not only in rendering the gossypol harmless but also in the loss of part of the nutritional value of the protein.

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Determination of Rosin Acids in Mixtures With Fatty Acids

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IN THE UNITED STATES the most widely used methods for analytical determination of rosin acids in mixture with fatty acids, especially in tall oil products, are the methods according to Wolff (1, 2, 8), McNicoll (6), and Herrlinger-Compeau (3). The first two of these give satisfactory accuracy only when the rosin content in the products is about 20–50%. The agreement between results from different analysts is also, in general, rather poor. The Herrlinger-Compeau method gives excellent results in the range of 0–15%.

The authors described in 1949 (4) a new method, applicable to all compositions. Like almost all other methods, this one was based upon the difference in esterification velocity of the fatty acids and the rosin acids. But to make the esterification of the fatty acids more complete, the water formed during the treatment was removed by azeotropic distillation.

To hold back the esterifications of the rosin acids as far as possible, benzene sulphonic acid was used as catalyst. As a small amount of catalyst was used, the esterification took quite a long time.

Olavi-Ivermark (7) have developed a method, similar to the Herrlinger-Compeau method but with corrections for all rosin acids concentrations. The method seems to give reliable results in the whole range of compositions. At high rosin acids concentrations benzene has to be added as solvent, which has an influence on the corrections. The analysis requires a lot of laborious work through repeated extractions with ethyl ether. Therefore the possibility of using potentiometric titration directly on the reaction mixture has been investigated, but the agreement was not as good as for extraction.

To eliminate the disadvantages of our original method the following changes have been made: