nickel on kieselguhr. The product boiled at 105-110°C. at 1 mm. and melted at 74–75°C.

Preparation of the Phenol. Trimethylene dioxyaniline (24 g.) dissolved in dilute sulfuric acid (25 g. in 250 cc.) was diazotized at 0–5°C. with sodium nitrite in water (9.2 g. in 50 cc.). The diazotized solution was run into boiling copper sulfate solution, saturated at 30°C., (250 cc.) with vigorous stirring. The product after extraction with ether boiled at 122–125°C. at 0.25 mm. and melted at 94–95°C.

Butylation. To 5 g. of trimethylene dioxyphenol in 50 g. of 85% phosphoric acid, 2.5 cc. of acetic anhydride, and 2.5 cc. of acetic acid at 70–80° were added 5 g. of tert-butyl alcohol with vigorous stirring. The product crystallizes from the liquid catalyst. Water was added, the product was filtered and crystallized from ether-petroleum ether, m.p. 133–135°C.

All of the other preparations are essentially similar.

Preparation of the 4,7-dihydroxyindane (2) and 1,4-dihydroxy-5,6,7,8-tetrahydronapthalene (10) are both described in the literature. The quinone of 1,4-dihydroxy-5,8-methano-5,6,7,8-tetrahydronaphthalene (5) reduced in methanol with hydrogen in the presence of a platinum catalyst was sublimed at 145°C. at 1 mm. and crystallized from benzene, m.p. 154–155°.

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Relation of Amount and Quality of Protein in the Diet to Free Gossypol Tolerance by the Rat

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A NEGATIVE CORRELATION between free and bound gossypol contents of cottonseed meals and nutritive value of the cottonseed protein as estimated by several biological methods has been shown in reports from this laboratory (1) and (2). Furthermore current studies of gossypol toxicity in swine by Earle and Stevenson (3) have frequently given anomalous or irregular results with respect to the maximum level of free gossypol which will be tolerated when different sources of supplementary protein have been used. In these swine tests the level of free gossypol has been varied in different diets without varying the level of total protein. This was done by supplementing different low gossypol protein concentrates, which included several cottonseed meals and soybean meal, with a calculated small proportion of high gossypol cottonseed meal. The results have indicated that a level of free gossypol which is toxic when fed with some protein supplements may be well tolerated with others.

Parallel with the swine feeding tests, cottonseed meals used in the swine diets (both the basic meals and the blended mixtures) have been assayed by the rat repletion method. Such assays have indicated that those meals on which toxic symptoms are most easily produced in swine also give lower weight responses in rats receiving a borderline level of free gossypol. This is true without there being any significant difference in the content of free gossypol as determined by chemical methods. These results with swine and rats, when considered together, suggest the presence, or absence, in these meals of other factors than free gossypol which are related to the development of toxicity in swine and to the rate of weight gain during repletion in protein depleted rats.

The variation in availability of amino acids in different meals brought about by differences in processing conditions in manufacture has been studied by Lyman *et al.* (4), K. A. Kuiken (5), and Boatner *et al.* (6). That such differences in the protein component of the diets may be related to variations in gossypol tolerance of both swine and rats has been considered as a possibility. Information regarding possible effects on gossypol tolerance of some changes in the amino acid composition or balance, and also of increases in the amount of total protein as supplied by various protein supplements, has been sought in some rat-feeding tests.

Data are reported here from a number of such feeding trials in which the Cannon (7) protein-repletion, rat-assay technique has been used. By the use of various combinations of several cottonseed meals, casein, soybean meal, and crystalline amino acids, it has been possible to vary either a) the total nitrogen intake or the probable amino acid balance while maintaining a constant intake of free gossypol, or b) the free gossypol intake without greatly changing the other factors. In this manner an attempt has been made to measure the effect of gossypol tolerance of changes in total protein intake and of some variations in the amino acid composition of the nitrogen component of the diet.

Material and Methods

Protein Assays by Rat Repletion Method. Adult male rats weighing between 200 and 300 g. were depleted by feeding a low protein diet containing 0.1% Protomone (iodinated casein) for 3 weeks. The diet and experimental design used in the trials have been described previously (1). During the repletion period the low protein diet without Protomone was fed *ad libitum* and supplemented daily with the protein or protein mitxure under test in an amount necessary to supply the desired levels of nitrogen and of free gossypol. The effects of variations in the protein supplements and in the intake of free gossypol were measured in terms of mean weight changes of the assay animals. A repletion period of 10 days was used for obtaining the weight changes. Further details of the procedure as applied to the study of the nutritional value of cottonseed meals, with some statistical treatment of data obtained in previous trials showing precision to be expected with the method, has been reported by Cabell and Earle (1).

Protein Supplements. Five cottonseed meals were used which were made by different manufacturing processes. Meal No. 1 was a specially prepared solvent-extracted product in which the pigment glands had not been ruptured. It had a high content of both total and free gossypol. Re-extraction of this meal in the laboratory with hexane and then with butanone containing 3% water resulted in a meal with a relatively high biological value and a low content of free gossypol. Meal No. 2 was a prepress solvent-extracted meal; No. 3 a hydraulic press meal; and No. 4 was a screw-press meal. The fifth meal, which was used in obtaining data presented in Table III, was a butanone extracted meal prepared at the Southern Utilization Research Branch.

Determinations of the gossypol content of these meals were made by the Oilseed Section of the Southern Utilization Research Branch before these tests were begun and again after they were completed. Analytical values were:

	Gossypol, %		
	At beginning	At end	
	Free	Total	Free
Meal No. 1, solvent-extracted	0.73	1.22	0.62
Meal No. 2, prepress solvent-extracted	.060	.992	.051
Meal No. 3, hydraulic press	.060	.948	.043
Meal No. 4, screw-press	.030	.856	.022
Meal No. 5, butanone-extracted	.01	—	.01

The losses in free gossypol which these analyses indicate as having occurred during the course of the tests are estimated to be responsible for a possible difference, not exceeding 15%, between the actual gossypol intake and the calculated intake. Other protein supplements used were commercial crude casein containing 13.7% nitrogen and solvent-extracted soybean meal containing 7.03% nitrogen.

Various mixtures of these protein meals were prepared to contain calculated amounts of free gossypol through the combination of appropriate proportions of the high gossypol meal No. 1 with the various low gossypol products.

In every series of these trials one group of rats receiving crude casein fed at a level of 160 mg. of nitrogen per rat per day was used as a standard. Since the mean weight gain of all these standard casein-fed groups was 30 g., for the sake of ease of comparisons the mean weight gains of all groups in all series have been adjusted to this value as a standard.

Results and Discussion

Free Gossypol Intake and Weight Gain of Rats. Results of feeding trials with two cottonseed meals and casein, each fed with two or more levels of gossypol, are given in Table I. The allowance of these supplements was restricted to 160 mg. of nitrogen per rat per day to emphasize the effect of different levels of gossypol intake.

TABLE I
Free Gossypol Intake and Weight Gain of Rats Fed 160 mg. Nitrogen Per Day

Rat group No.	Cottonseed meal No. and other protein supplement	Free gossypol intake	Ten-day adjusted mean wt. gain	
		mg./rat/day	*g.*	*S.E.*[a] ±
1	No. 1 (High gossypol solvent extracted meal)	17.5	3.9	1.3
2	No. 1 (Re-extracted in lab.)	0.4	26.8	2.5
3	No. 4 (Hydraulic meal)	0.7	20.8	2.0
4	No. 4 (97%) + No. 1 (3%)	1.2	19.7	1.6
5	No. 4 (97%) + No. 1 (3%)[b]	0.7	21.9	2.0
6	No. 4 (90%) + No. 1 (10%)	2.5	22.6	1.0
7	No. 4 (90%) + No. 1 (10%)[b]	0.7	22.2	1.6
8	No. 4 (47%) + No. 1 (53%)	9.5	11.3	1.8
9	No. 4 (47%) + No. 1 (53%)[b]	0.7	23.3	2.8
10	Casein only	0.0	30.0	1.1
11	Casein (45%) + No. 1 (55%)	6.5	31.3	1.5
12	Casein (28%) + No. 1 (72%)	9.5	22.7	3.8

[a] Standard error of the mean, 8 animals per group.
[b] No. 1 cottonseed meal fed in these mixtures was re-extracted in the laboratory to reduce free gossypol to 0.03%.

The high gossypol meal No. 1, when fed alone to supply the 160 mg. of nitrogen, provided also an intake of 17.5 mg. of free gossypol. This amount was definitely toxic as evidenced by the low mean weight gain of 3.9 g., by poor feed consumption, and by general appearance of low vitality and debility. That these effects may be attributed to the free gossypol, or perhaps some other factor extracted therewith, is indicated by results obtained on the rats in group 2 which were fed the same meal after extraction of gossypol.

No effect was obtained with cottonseed meal No. 4 when gossypol intake was increased from 0.7 mg. to 2.5 mg., but at 9.5 mg. a marked depression of weight gain was shown (group 8). That this depression in rate of gain was not the result of changed amino acid balance in this mixture is demonstrated by the weight gain of group 9 which was fed the same combination of meals with most of the free gossypol extracted. When a combination of casein and No. 1 meal was fed at the 160-mg. nitrogen level, again a daily intake of 9.6 mg. of free gossypol appeared to depress weight gain although no effect was observed at an intake of 6.5 mg. However an intake of 9.5 mg. of free gossypol was not effective with all protein mixtures as is shown in the following section.

TABLE II
Effect of Different Protein Supplements on Tolerance by Rats for a Daily Intake of 9.5 mg. Free Gossypol When Nitrogen Is Restricted to 160 mg.

Rat group No.	Cottonseed meal No. and other protein supplements	Free gossypol intake	Ten-day adjusted mean wt. gain	
		mg./rat/day	*g.*	*S.E.*[a] ±
13	Soybean meal	0.0	24.9	1.4
14	Soybean meal (45%) + No. 1 (55%)	9.5	23.9	1.7
15	Casein	0.0	30.0	1.1
16	Casein (28%) + No. 1 (72%)	9.5	22.7	3.8
17	No. 2	1.4	17.7	0.9
18	No. 2 (49%) + No. 1 (51%)	9.5	11.9	1.7
19	No. 3	1.7	17.6	1.6
20	No. 3 (49%) + No. 1 (51%)	9.5	16.7	1.2
21	No. 4	0.7	20.8	2.0
22	No. 4 (47%) + No. 1 (53%)	9.5	11.3	1.8

[a] Standard error of the mean.

Effect of Different Protein Supplements on Tolerance for Free Gossypol. Results of assays of five supplements with and without the inclusion of enough cottonseed meal No. 1 to provide a free gossypol intake of 9.5 mg. are presented in Table II. In the cases of two of these five supplements, namely, soybean meal and cottonseed meal No. 3, this intake of free gossypol had no effect. With the other three, *i.e.*, casein, cottonseed meals No. 2 and No. 4, there was a definite depressing effect on weight gain.

The difference in this observed effect of free gossypol in the instances of these oil meal samples is of special significance when considered in the light of results obtained with swine. Meals No. 2 and No. 4, when supplemented with meal No. 1 to supply graded levels of free gossypol, produced toxicity when fed to swine at the same levels of free gossypol which were well tolerated if the meal supplements were mixtures of No. 1 with No. 3 or soybean meal. It is suggested that the same factors producing toxicity in swine also interfere with weight gains of these rats. The small difference in bound gossypol content of meals 2, 3, and 4 appears to eliminate bound gossypol as the factor responsible for differences in response to this group of meals.

Effect of Increasing Total Protein Intake. Data from experiments to test the effect of increased protein intake on free gossypol tolerance are presented in Table III. Differences in tolerance to a borderline level (9.5 mg.) of gossypol were determined at three levels of nitrogen intake, namely: 160, 240, and 320 mg. These three levels were tested with each of two protein supplements, casein and butanone-extracted meal No. 5. Cottonseed meal No. 1 was used to supply the required amount of gossypol, and the rest of the protein allowance consisted of the basic protein supplement. Butanone-extracted meal No. 5 has been found to be indistinguishable in rat repletion studies from the No. 1 meal extracted with hexane and aqueous butanone in this laboratory. Hence No. 5 meal was substituted for No. 1 in all low gossypol combinations.

TABLE III

Effect of Increasing Protein Intake on Depression of Weight Gain Due to Free Gossypol

Rat group No.	Protein supplement[a]	Nitrogen intake	Free bossypol intake	Ten-day adjusted mean wt. gain	
		mg./rat/day	*mg./rat/day*	*g.*	*S.E.*[b] ±
23	C.S. meal only	160	9.5	18.5	1.6
24	C.S. meal only	160	0.2	25.8	1.3
25	C.S. meal + casein	160	9.5	22.9	2.3
26	C.S. meal + casein	160	0.1	34.4	2.6
27	C.S. meal only	240	9.5	37.8	2.9
28	C.S. meal only	240	0.3	37.4	1.9
29	C.S. meal + casein	240	9.5	47.1	3.4
30	C.S. meal + casein	240	0.1	49.4	3.1
31	C.S. meal only	360	9.5	53.3	3.5
32	C.S. meal only	360	0.3	54.0	1.6
33	C.S. meal + casein	360	9.5	64.4	4.3
34	C.S. meal + casein	360	0.1	58.0	2.2

[a] The cottonseed meal used was butanone-extracted meal or mixtures of this and No. 1 high gossypol meal.
[b] Standard error of the mean.

The results recorded in Table III indicate that with both butanone—extracted meal and casein there is depression of weight gain due to free gossypol when nitrogen intake is restricted to 160 mg. per day. This effect does not occur at either of the higher levels of nitrogen. The differences between groups 23 and 24 and 25 and 26 are significant while weight gains in the other comparative groups 27 and 28, 29 and 30, 31 and 32, and 33 and 34 show no significant differences.

Although the experimental animals used here are protein-depleted and the levels of nitrogen fed are below those normally required for maximum repletion, these observations still suggest that, even under practical conditions of feeding, protein intake may affect gossypol tolerance.

TABLE IV

Effect of Crystalline Amino Acids on Tolerance of Free Gossypol Fed at 9.5 mg./Day to Rats Receiving 160 mg. of Total Nitrogen per Day

Rat group No.	Cottonseed meal No. and other protein supplements	Crystalline amino acid added	Level of amino acid	Ten-day adjusted mean wt. gain	
			mg./rat/day	*g.*	*S.E.*[a] ±
				mg./rat/day	
35	No. 1 + No. 2[b]	None	—	11.9	1.7
36	No. 1 + No. 3	None	—	16.7	1.2
37	No. 1 + No. 2	Lysine	5	10.7	3.2
38	No. 1 + No. 2	Lysine	10	13.0	2.1
39	No. 1 + No. 3	Lysine	5	13.2	2.5
40	No. 1 + No. 2	Isoleucine	5	16.2	1.5
41	No. 1 + No. 2	Isoleucine	10	15.0	2.4
42	No. 1 + No. 2	Glycine	5	9.2	2.7
43	No. 1 + No. 2	Glutamic acid	5	15.5	2.2
44	No. 1 + No. 2	Five essential	30	15.7	2.2
45	No. 1 only	All nine essential[d]	770	57.6	3.3
46	No. 1 only	All nine essential	580	40.1	2.3
47	No. 1 re-extracted[c]	All nine essential	580	49.2	2.4
48	Casein (crude)[c]	All nine essential	580	48.5	2.1

[a] Standard error of the mean.
[b] Groups 35 through 44 were fed meal mixtures of approximately 50% each of No. 1 and No. 2 or No. 1 and No. 3 meals.
[c] None or very low level of free gossypol fed these groups.
[d] Fed on a basis of 160 mg./day as L amino acid N but included an additional 30 mg. N as D amino acids.

Effects on Gossypol Tolerance of Substitution of Crystalline Amino Acids for a Part of the Nitrogen Allowance. Many reports including those of Lyman *et al.* (4) and K. A. Kuiken (5) indicate that destruction of lysine occurs in some cottonseed meals as a result of high processing temperatures. Improvement of cottonseed meal growing-fattening diets for swine by addition of lysine has been shown by Miner *et al.* (8). These and other reports suggested that addition of small amounts of lysine would improve weight gain when added to the meal mixtures that gave depressed growth with these depleted rats. In addition to their role in protein repletion, there seems a possibility that some amino acids may also serve as detoxifying agents in the presence of gossypol. However, data in Table IV show that addition of lysine or glycine, at least in the amounts used here, failed to increase weight gains. Some improvement was obtained with isoleucine and glutamic acid though the weight gains are not as high as the value of 26.8 g. shown by group 2, Table I, for re-extracted No. 1 meal.

In group 44 an attempt was made to balance the essential amino acid intake from calculations based on amino acid analysis of cottonseed meal as given in (5) and minimum essential amino acid requirements of depleted rats as measured by Steffee *et al.* (9). Available analyses showed that cottonseed meal was relatively low in five essential acids including isoleucine, lysine, methionine, threonine, and valine. A mixture of these was added to the meal mixture in proportions calculated to balance deficiencies. Results of mean weight gains of group 44 show some increase but again not equal to re-extracted No. 1 meal.

Rats in group 45 were fed No. 1 meal sufficient to provide 9.5 mg. of free gossypol per day with the remainder of the 160 g. of nitrogen provided as a mixture of essential amino acids in the proportions described by Steffee *et al.* (9). In this calculation it was assumed that the D enantiomorphs were not available, and thus nitrogen from this source was not included as part of the 160-mg. allowance. Actually the total daily nitrogen consumption increased to 190 mg. From the high weight gain of the animals in this group and in view of the work of Phillips and Berg (10), it is believed that nitrogen from the D isomers probably was converted or in some way utilized for growth by these depleted animals.

In the last three groups in Table IV the D amino acid nitrogen was all included as part of the 160-mg. daily allowance. Weight gains in these groups were relatively high as compared with group 2 fed re-extracted No. 1 meal. However a comparison of groups 46 and 47 indicates that the free gossypol causes a depression of weight gain even in the presence of a combination of amino acids which will support this high rate of gain. If a balance of amino acids is involved in counteracting effects of free gossypol, apparently the proper balance is not attained by this combination of amino acids and cottonseed meal. It appears that further study is needed on possible detoxifying effects resulting from mixed proteins or amino acids and proteins.

It is recognized that the differences in mean weight gain of rat groups may not differentiate between nutritional deficiency and toxicity effects. However comparisons which are permitted by the data offered here seem to allow a distinction between depression of weight gain due to intake of gossypol and that due to amino acid differences.

More work is necessary for better understanding of these apparent effects of total nitrogen intake and of difference in kind of protein on free gossypol toxicity. But the results described in the experiments reported here are significant in any attempt to establish dependable toxic levels of free gossypol for farm animals.

Summary and Conclusions

The rat-repletion method for protein assay has been used to estimate effects on tolerance for free gossypol of a) difference in source or kind of protein; b) level of total nitrogen or protein intake; and c) changes in amino acid content or balance in the nitrogen allowance. The data demonstrate that the first two of these factors at least influence the depression in weight repletion of these protein depleted rats which may be brought about by an intake of free gossypol. It has been shown that a daily intake of 9.5 mg. of free gossypol, when total daily protein intake is limited to 160 mg. of nitrogen, is a borderline level which depresses weight gain when the protein is supplied by some meal mixtures but is without effect with some others.

It has further been demonstrated that the toxic or weight-depressing effect of 9.5 mg. of free gossypol which has been obtained with a nitrogen intake of 160 mg. may be nullified by increasing the protein intake 50% or more. Attempts to render ineffective an intake of 9.5 mg. gossypol at the lower nitrogen level by replacing a part of the nitrogen allowance with different amounts of some amino acid or amino acid combinations have not been markedly successful. The data do not identify the factor or factors in these protein supplements which influence gossypol toxicity. They do however point to the importance of considering both the amount and kind of dietary protein in attempts to establish a minimum level of free gossypol which is toxic for farm species, or a maximum level which will be tolerated.

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Isomerization During Hydrogenation. IV. Methyl Eleostearate[1]

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THE PROCESS of hydrogenation of unsaturated fatty acids causes isomerization of the double bonds in the molecule even in monoenes (3, 13). As the number of double bonds in the molecule increases, the number of possible isomers increases because there are more positions at which the hydrogen can attack and also more possibilities for migration of the remaining bonds. The type of polyene system also affects the isomerizations that take place. The methylene-interrupted diene is believed to undergo isomerization, by a process of half hydrogenation-dehydrogenation, which can lead to isomeric monoenes (4), while the conjugated dienes show no evidence of dehydrogenation during the process but add hydrogen with equal ease to the 1,2, 1,4, and 3,4 positions of the diene (2).

To extend the study of isomerization during hydrogenation, the conjugated triene system is a logical subject. Fortunately this system occurs in nature in eleostearic acid (9,11,13-octadecatrienoic acid), which can be isolated from tung oil.

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