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Biological vs. Chemical Evaluation of Toxicity and Protein Quality of Cottonseed Meals[1]

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THE DESIRABILITY of rapid chemical tests for evaluating the toxicity and the protein quality of cottonseed meals in lieu of expensive and time-consuming biological methods can hardly be questioned. At the present time however, the chemical methods commonly used for such purposes have enjoyed questionable success.

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Toxicity. The earliest recorded statement on the harmful effect of cottonseed is attributed to Voelker in England in 1859 (1). In the intervening years many materials were blamed for the adverse findings in animals after cottonseed feeding until Withers and Carruth (2, 3, 4) and Carruth (5) published a series of papers between 1915 and 1918, attributing the toxicity of cottonseed to gossypol, a yellow polyphenolic pigment which originally had been isolated from cot-

tonseed oil "foots" by Longmore in 1886 (6) and which had been extracted, purified, and named by Marchlewski in 1899 (7). Details on detoxification procedures have been summarized by Eagle *et al.* (8). It should be emphasized however that successful detoxification of cottonseed by overcooking is in reality a failure if the favorable effects from decreased toxicity are offset by the unfavorable effects from protein damage in the process.

Protein Quality. The basic concept that, in determining its biological value, a protein must be fed at an appropriately low level developed from the work of many investigators, who found that the biological value of a protein decreases as the protein concentration in the diet is increased (9, 10, 11, 12, 13). Mitchell, in describing a method for determining the biological value of protein, set the condition that the amount of protein fed be adjusted to so low a level that the dietary protein would not be used as a source of energy (14). It is apparent that, in evaluating the true quality of a protein for growth and maintenance, the single protein source must be fed at a level sufficiently low to supply the nitrogen requirements of the tissues and not at a level high enough so that nitrogen is no longer stored by the animal and is excreted. In the latter instance the results obtained would be misleading.

Gossypol Analyses. Since the time of the series of papers by Withers and Carruth (2, 3, 4, 5) it has been generally accepted that gossypol is the sole toxic principle of cottonseed. Through the intervening 35 or 40 years the numerous methods for quantitative estimation of the gossypol content of cottonseed have undergone many radical changes and revisions from the original gravimetric determination of dianilinogossypol by Carruth (15). This may be noted from the reports of the various gossypol committees of the Amercian Oil Chemists' Society between 1946 and 1950. Careful collaborative evaluation of many different methods of extraction, and still more methods of gossypol determination over a period of several years left little doubt as to the unsatisfactory state of gossypol methodology (16). In 1950 the method of Pons and Guthrie (24) was adopted as the official A.O.C.S. method for determination of free gossypol.

Nitrogen Solubility. The solvent action of dilute solutions of salts on proteins was first described by Panum more than 100 years ago (17). According to Osborne (18), alkaline solutions were used extensively by Ritthausen during the 1860's for extraction of protein. Although heat is only one of the many physical and chemical agents that denature proteins, it is of particular interest in connection with cottonseed because all commercial cottonseed processing involves the use of heat, the adverse effect of which on cottonseed protein was shown by Osborne and Mendel (19), Gallup (20), and Olcott and Fontaine (21, 22). The last studied the solubility of the proteins of commercial cottonseed meals in 3% sodium chloride, reported that the nitrogen solubility decreases with autoclaving, and proposed a laboratory assay of commercial meals based on this solubility for use by mill laboratories as a means of controlling the cooking temperature. Lyman, Chang, and Couch (23) were unable to find a relationship between this nitrogen solubility in dilute sodium chloride solution and the protein quality of cottonseed meals. They did suggest a relationship however between the solubility of cottonseed protein in 0.02N sodium hydroxide and the protein quality of cottonseed meal as determined by chick feeding tests in which sufficiently high levels of meals (*ca.* 50%) were fed to supply 21% protein in the diet. These authors likewise proposed a chemical index based on the total gossypol content of cottonseed meal and its nitrogen solubility in 0.02N sodium hydroxide, for which they claimed a correlation with chick growth after feeding the physiologically optimum level of protein for the chick (21%) instead of feeding a sub-optimal level to insure maximum protein utilization.

The present investigations involve the comparison of biologically evaluated toxicity of cottonseed meals and their free and total gossypol content, and biologically evaluated protein quality of cottonseed meals *vs.* chemical analyses for their percentage of nitrogen solubility in 0.02N sodium hydroxide.

Experimental

Determination of Toxicity. All cottonseed meals tested for toxicity in the present report were incorporated into the diets of rats at the usual level of 67%, the remaining 33% of the diet consisting of the following: dextrose 14%, commercial shortening 10%, Jones and Foster salt mixture 4%, Wilson 1:20 liver concentrate (NF IX) 3%, vitamin A and D oil (2250 U.S.P. units A, 300 U.S.P. units D_3 per gram) 1%, yeast (AB 300) 1%. In the soybean control diets the 67% level of cottonseed meal was replaced by 67% solvent extracted soybean oil meal having a protein content of 47.2%. The stock diet used as the laboratory control diet for all tests consisted of whole ground wheat 21%, meat and bone scraps 19.6%, non-fat dry milk solids 15%, soybean oil meal 15%, ground yellow corn 13%, commercial shortening 10%, salt and yeast mixture 2.5%, alfalfa leaf meal 2%, wheat germ oil 1%, vitamin A and D oil 0.5%, and Wilson 1:20 liver concentrate (NF IX) 0.4%.

Determination of Protein Quality. Test cottonseed meals were incorporated into otherwise protein-free, synthetic rat diets at levels supplying 9% protein. The diets were likewise isocaloric with respect to fat and consisted of:

	Percentage
Vitamin premix	20
Jones & Foster salt mixture	4
Agar	2
A and D oil (2250 A; 300 D_3/gram)	1
Wheat germ oil	1
Test cottonseed meal, sufficient to supply 9% protein	15.36–22.61%
Commercial shortening, 10% minus the fat from the protein source	9.53– 9.97%
Dextrose, sufficient to make 100%	39.56–46.67%

The 20 g. of vitamin premix added in 100 g. of diet consisted of the following: Wilson 1:20 liver concentrate (NF IX) 700 mg., choline chloride 100 mg., inositol 25 mg., para-aminobenzoic acid 10 mg., d-calcium pantothenate 5 mg., niacin 4 mg., 2 methyl-1,4-napthoquinone 1 mg., thiamine hydrochloride 0.8 mg., riboflavin 0.8 mg., pyridoxine hydrochloride 0.8 mg., folic acid 0.2 mg., biotin 0.03 mg., and sufficient dextrose to make a total of 20 g. of premix.

Analyses for free gossypol were made by the method of Pons and Guthrie (24), and those for total gossypol by the method of Pons, Hoffpauir, and O'Connor (25). In practically all cases at least duplicate analyses were made although in many instances 3, 4, 5, and sometimes 6 analyses for free gossypol were per-

TABLE I

Biological Evaluation of Toxicity of Cottonseed Meals *vs.* Gossypol Analyses

Meal No.	Type Meal	Treatment	No. Rats Used	Av. Wt. Gain After 8 Wks.	Gossypol Content of Meal	
					Free	Total
				grams	%	%
1	Solvent extracted flakes	Untreated	10	7[a]	1.29	1.41
2	Solvent extracted flakes	Untreated	10	10[a]	0.90	.....
3	Solvent extracted flakes	Untreated	10	7[a]	0.64	.....
4	Meal No. 3	Dry-heated	10	9[a]	0.62	.....
5	Solvent extracted flakes	Untreated	5	8[a]	0.61	.....
6	Solvent extracted flakes	Untreated	8	10[a]	0.55	1.02
7	Solvent extracted flakes	Untreated	10	8[a]	0.51	1.09
8	Solvent extracted flakes	Untreated	10	6[a]	0.35	0.97
9	Solvent	Acid	10	153	0.32	0.88
10	Solvent	Carbonate	10	149	0.32	.....
11	Solvent	Carbonate	10	185	0.31	.....
12	Solvent	Alkali	10	186	0.30	.....
13	Meal No. 5	Moist-heated	10	87	0.30	.....
14	Solvent	Commercial	5	81	0.29	0.94
15	Solvent	Ammonium carbonate	10	169	0.28	.....
16	Solvent	Commercial	8	14[a]	0.28	.....
17	Solvent	Alkali	10	173[b]	0.26	.....
18	Solvent	Commercial	10	77	0.26	1.05
19	Solvent	Alkali	17	158	0.23	1.15
20	Solvent	Sodium chloride	10	99	0.25	.....
21	Solvent	Alkali	6	254	0.22	0.89
22	Solvent	Acid	10	250	0.22	0.54
23	Solvent	Alkali	6	245	0.22	1.19
24	Solvent	Trisodium phosphate	5	228	0.20	.....
25	Solvent	Acid	8	179	0.20	1.29
26	Solvent	Moist-heated	5	141	0.20	.....
27	Solvent	Commercial	15	89	0.20	1.15
28	Solvent	Alkali	5	248	0.19	.....
29	Solvent	Alkali	5	218	0.19	.....
30	Solvent	Alkali	8	210	0.19	1.36
31	Solvent	Urea	5	179	0.19	.....
32	Solvent	Alkali	8	280	0.18	0.93
33	Solvent	Alkali	5	239	0.18	.....
34	Solvent	Hydrolyzed protein	5	174	0.18	.....
35	Solvent	Alkali	6	202[c]	0.17	0.94
36	Solvent	Alkali	15	214	0.16	1.07
37	Solvent	Sodium hypochlorite	5	170	0.16	.....
38	Solvent	Alkali	10	259	0.15	0.90
39	Solvent	Alkali	10	250	0.15	0.97
40	Solvent	Alkali	10	247	0.15	0.96
41	Solvent	Commercial	10	235	0.15	0.76
42	Solvent	Bicarbonate	8	273	0.14	0.89
43	Solvent	Alkali	10	269	0.14	0.92
44	Solvent	Alkali	10	266	0.14	1.07
45	Solvent	Commercial	10	248	0.14	1.08
46	Solvent	Commercial	10	238	0.14	0.90
47	Solvent	Commercial	10	271	0.13	1.02
48	Solvent	Degossypolized	15	251	0.13	0.82
49	Solvent	Protein	10	160[b]	0.13	.....
50	Solvent	Alkali	10	273	0.12	0.90
51	Solvent	Alkali	10	266	0.11	0.90
52	Solvent	Alkali	10	265	0.11	1.02
53	Solvent	Moist-heated	8	255	0.11	0.91
54	Solvent	Alkali	10	274	0.10	1.02
55	Solvent	Commercial	15	262	0.10	0.99
56	Hydraulic	Commercial	8	239	0.10	0.74
57	Solvent	Carbonate	8	274	0.09	0.94
58	Solvent	Alkali	8	240	0.08	0.93
59	Solvent	Degossypolized	20	259	0.07	0.88
60	Solvent	Degossypolized	10	280	0.05	0.77
61	Pre-pressed solvent	Commercial	10	268	0.05	0.82
62	Meal No. 51	Extracted with butanone	10	260	0.05	0.80
63	Expeller	Commercial	5	115[d]	0.05	.....
64	Solvent	Commercial	5	231	0.04	1.00
65	Butanone-extracted	.....[e]	10	286	0.03	0.19
66	Expeller	Commercial	8	222	0.03	1.06
67	Expeller	Commercial	8	254	0.02	0.89
68	Butanone-extracted	.....[e]	15	264	0.01	0.03
	Soybean meal controls		118	258	0	0
	Stock diet controls		152	263	0	0

[a] 100% mortality within number of days indicated.
[b] Average weight gain after only 4 weeks on test.
[c] Average weight gain after only 5 weeks on test.
[d] Average weight gain after only 26 days on test.
[e] These butanone-extracted cottonseed meals were supplied by the Southern Regional Research Laboratory.

formed on the same sample, including those made by other laboratories for confirmation. The method of Olcott and Fontaine (22) was used for nitrogen solubility in 3% sodium chloride, and that described by Lyman, Chang, and Couch (23) was used for nitrogen solubility in 0.02N sodium hydroxide. Analyses on all samples for the latter were carried out not only in our own chemical laboratories but also in an outside commercial laboratory. Weanling male rats of the Holtzman strain were fed stock diet for a period of one to three days, after which they were distributed into various groups according to body weight, so that the average starting weights of all groups within a given experiment were the same. All rats were kept in individual wire-bottom cages in an air-conditioned room maintained at 79° ± 1°F., and *ca.* 45% relative humidity. Food and water were allowed *ad libitum*. All rats were weighed daily for the first 7 days and at least twice weekly thereafter. With few exceptions all rats were fed for a period of 8 weeks.

Results

Toxicity vs. Gossypol Content. Table I lists a total of 68 cottonseed meals covering a wide range of free

and total gossypol levels in the order of decreasing free gossypol content. Although a group of rats fed the soybean control diet and a group fed control stock diet were included in every experiment on a series of test meals, the results obtained were so consistent that all of these controls were combined into two large groups as shown at the bottom of the table. Since toxicological evaluations in rats of all of the cottonseed meals were made at the usual 67% level in the diet, the free gossypol content of all diets would be 67% of the levels indicated in the table. If the free gossypol content is a true criterion of the toxicity of cottonseed meal, as has been commonly believed for so many years, the average body weight gains afforded by the various cottonseed meals in rats after eight weeks on test should likewise have some semblance of being arranged in the order of increasing amount as the free gossypol content of the meal (and of the diet) decreases. That such is not the case is quite obvious from Table I.

The first eight samples of cottonseed meal listed in Table I were highly toxic. The eight diets containing these meals caused immediate weight loss in weanling rats, and the 73 rats fed diets containing these samples showed 100% mortality within 10 days. Samples 9 to 13, on the other hand, despite such high free gossypol contents as 0.32, 0.32, 0.31, 0.30, and 0.30% not only caused no mortality but led to some good weight gains; there were such wide variations in final average weight gains, however, as 153, 149, 185, 186, and 87 g., respectively, almost 100 g. difference between the performance of two cottonseed meals whose free gossypol contents were the same (0.30%). Sample 17 with a free gossypol content of 0.26% resulted in an average weight gain of 173 g. after only four weeks on test, better than six other samples with the same or lower free gossypol content showed in twice that time. Samples 18 and 20 with free gossypol levels of 0.26 and 0.25% gave average body weight gains of only 77 and 99 g. in eight weeks, poorer performances than those of six other samples having still higher free gossypol content.

Sample 21 had a free gossypol content of 0.22%, yet when fed to rats for eight weeks at the 67% level in the diet, it yielded an average body weight gain per rat of 254 g., a result better than was obtained from 22 other samples with considerably lower free gossypol content (0.03 to 0.20%). Meal No. 27 (free gossypol content 0.20%) manifested a poorer average weight gain than 11 samples with still higher free gossypol levels (0.22 to 0.32%). Cottonseed meals No. 24, 25, 26, and 27 all had the same free gossypol content (0.20%), yet they produced average body weight gains, respectively, of 228, 179, 141, and 89 g., a difference of 139 g., between the two extremes. Again, samples No. 28, 29, 30, and 31 gave such divergent average body weight gains as 248, 218, 210, and 179 g., although all four had the same free gossypol content (0.19%). Similar observations can be made for samples No. 32, 33, and 34, all of which had the same free gossypol content (0.18%); here the maximum difference between the average weight gain of two of the groups was 106 g. The worst performance of these three was that of sample No. 34, which led to an average body weight gain of 174 g. after eight weeks, not a bad performance for a cottonseed meal containing 0.18% free gossypol, but still a lower body weight gain than was obtained from 14 other cottonseed meals having the same or even higher free gossypol content.

Samples No. 36 and 37, which had the same free gossypol content (0.16%), yielded average body weight gains of 214 and 170 g. One of these (No. 37) showed lower final average weight gain than 16 other samples which had still higher free gossypol levels (0.17 to 0.31%). Similarly, sample No. 31 containing 0.19% free gossypol displayed worse performance than 10 samples with the same or higher free gossypol levels (0.19 to 0.31%). Twelve of the cottonseed meals tested yielded average body weight gains per group, respectively, of 280, 266, 269, 273, 271, 273, 265, 266, 274, 274, 268, and 280 g., *i.e.,* better weight gains than were given by a butanone-extracted cottonseed meal control which contained only 0.01% free gossypol, notwithstanding the fact that these 12 samples had such free gossypol levels as 0.18, 0.14, 0.14, 0.14, 0.13, 0.12, 0.11, 0.11, 0.10, 0.09, 0.05, and 0.05%. Sixteen samples containing 0.18 to 0.05% free gossypol yielded better average body weight gains per group than the standard solvent extracted soybean meal (47.2% protein) which has been used as a control in these and many other cottonseed studies.

In our earlier experiments we did not do total gossypol analyses on our samples for little interest and still less significance had been attached to this analytical finding. The use of the total gossypol level in the chemical index proposed by Lyman, Chang, and Couch (23) however prompted us to see if there was any connection between the total gossypol content of a meal and its toxicological performance. When the meals listed in Table I are arranged in the order of decreasing total gossypol content, it may be seen that there is likewise no apparent correlation between the total gossypol content and biologically evaluated toxicity of these cottonseed meals, as is also the case for combined gossypol content *vs.* toxicity of these cottonseed meals.

Protein Quality vs. Nitrogen Solubility. Table II lists, in the order of increasing nitrogen solubility in 0.02N sodium hydroxide, the results obtained in protein quality tests on male weanling rats fed various cottonseed meals at the 9% protein level in an otherwise protein-free, synthetic diet. It may be seen that there is poor correlation between the average body weight gains and the percentage of nitrogen solubility in either 3% sodium chloride or 0.02N sodium hydroxide. Since the nitrogen solubility in the latter solvent is of current interest in the cottonseed industry, we shall confine ourselves to this chemical finding as it might relate to biologically evaluated protein quality.

Specifically, cottonseed meal No. 36, which gave the poorest average body weight gain per group after eight weeks on test (only 50 g.), had close to the highest nitrogen solubility in 0.02N sodium hydroxide of all the cottonseed meals evaluated (74.2%). Contrariwise, meal No. 44, which showed the greatest average weight gain per rat (158 g.), had a nitrogen solubility value of only 69.5%. Within the range of from 61.7 to 74.9% nitrogen solubility, a difference of only 13.2%, there were 19 cottonseed meals which varied from poor to excellent in protein quality, which gave eight-week average body weight gains of from 50 to 158 g., and which had protein efficiency values of from 1.13 to 1.78. Sample No. 1, made from hexane-extracted, air-dried cottonseed flakes with no heat

TABLE II

Protein Quality of Cottonseed Meals Evaluated at the 9% Protein Level in the Diets of Rats *vs.* Nitrogen Solubility

Meal No.[a]	Type and Treatment	No. Rats Used	Av. Wt. Gain After 8 Wks.	Protein Efficiency[b]	Nitrogen Solubility		Gossypol Content of Meal	
					In 0.02 N NaOH	In 3% NaCl	Free	Total
			grams		%	%	%	%
66	Expeller–commercial	8	61	1.00	36.5	.....	0.03	1.06
A	Solvent–expeller cake[c]	8	63	1.13	39.4	.....	0.03	0.89
67	Expeller–commercial	8	80	1.26	41.1	.....	0.02	0.89
23	Solvent–alkali	8	88	1.56	61.7	.....	0.22	1.19
21	Solvent–alkali	8	99	1.46	64.6	.....	0.22	0.89
1	Solvent–raw flakes	8	.....[d]	.....	65.1	43.8	1.29	1.41
64	Solvent–commercial	8	81	1.13	65.2	.....	0.04	1.00
62	Solvent–alkali[e]	8	90	1.28	67.9	.....	0.05	0.80
58	Solvent–alkali	8	140	1.78	68.0	.....	0.08	0.93
44	Solvent–alkali	8	158	1.31	69.5	.....	0.14	1.07
51	Solvent–alkali	8	127	1.43	69.8	.....	0.11	0.90
45	Solvent–commercial	8	107	1.27	70.0	.....	0.14	1.08
55	Solvent–commercial	8	55	1.42	70.4	42.9	0.10	0.99
54	Solvent–alkali	16	105	1.55	70.6	.....	0.10	1.02
18	Solvent–commercial	8	84	1.16	71.5	43.1	0.26	1.05
19	Solvent–alkali	8	92	1.42	71.9	44.7	0.23	1.15
27	Solvent–commercial	8	59	1.29	72.0	45.5	0.20	1.15
48	Solvent–degossypolized	8	83	1.54	72.7	34.9	0.13	0.82
52	Solvent–alkali	8	86	1.46	72.8	.....	0.11	1.02
B	Solvent–cooked flakes	8	78	1.23	73.4	.....	0.16	1.18
36	Solvent–alkali	8	50	1.26	74.2	48.1	0.16	1.07
47	Solvent–commercial	16	123	1.66	74.9	.....	0.13	1.02
68	Butanone extracted (SRRL)	16	112	1.82	86.5	69.0	0.01	0.03
65	Butanone extracted (SRRL)	8	160	1.74	87.3	.....	0.03	0.19
C	Solvent–raw flakes	8	14	0.47	89.8	.....	0.94	1.11
—	Soybean meal controls	47	141	1.88	.....[f]	.....	.....	.....

[a] These numbers correspond to the numbers of the meals listed in Table I.
[b] Protein efficiency = grams gained ÷ grams of protein eaten.
[c] The flakes were cooked and then passed through a regular expeller before solvent-extraction.
[d] 100% mortality within 16 days.
[e] Meal No. 62 is meal No. 51 which had been extracted with butanone.
[f] The average figure was 85.6%; we do not know the significance of this value for soybean meal.

treatment, whose protein quality (ignoring toxicity for the moment) should have been superior to any heat-treated samples, had a nitrogen solubility value of only 65.1%. A similar preparation (sample C) had a value of 89.8%.

Nine samples of cottonseed meal which showed good to excellent final average body weight gains had low nitrogen solubility values, namely, 41.1, 61.7, 64.6, 65.2, 67.9, 68.0, 69.5, 69.8, and 70.0%. Each of three samples with such low nitrogen solubility figures as 36.5, 39.4, and 41.1% gave better protein quality results than did three other samples (Nos. 55, 27, and 36) with such nitrogen solubility values as 70.4, 72.0, and 74.2%. Five samples with essentially the same values for nitrogen solubility in 0.02N sodium hydroxide (69.5, 69.8, 70.0, 70.4, and 70.6%) displayed such wide variations in average final body weight gains in the protein quality evaluations as 158, 127, 107, 55, and 105 g. respectively, a difference of 103 g. between the extremes whose nitrogen solubilities differed by only 0.9%. Five other samples whose nitrogen solubilities were likewise quite close (71.5, 71.9, 72.0, 72.7, 72.8%) yielded final average body weight gains, respectively, of 84, 92, 59, 83, and 86 g. There was a difference of 33 g. between the performances of two samples whose nitrogen solubility values differed by only 0.1%. It should be emphasized that in biological evaluation of protein quality at threshold levels of protein in a synthetic diet, minor variations in the average final body weight gain are of considerably more significance than much larger average body weight gain differences between groups in the toxicological evaluations in which 67% levels of cottonseed meal are incorporated in diets that are more than nutritionally adequate.

Two samples of butanone-extracted cottonseed meal, which should represent maximally attainable protein quality and minimal toxicity for cottonseed meal differed considerably in their final average weight gain results (112 and 160 g.) although their nitrogen solubility figures differed by only 0.8%. A portion of sample No. 51, which had a nitrogen solubility value of 69.8% and yielded an average body weight gain of 127 g. in the rat after 8 weeks in the protein quality test, had been extracted with butanone and re-evaluated (sample No. 62) for protein quality in the same experiment. Although the nitrogen solubility in 0.02N sodium hydroxide was hardly affected by this extraction procedure (changed from 69.8 to 67.9%), the resulting average body weight gain was only 90 g., 37 g. less in a type of test where such a difference cannot be ignored. This observation plus the finding that different butanone-extracted cottonseed meals may vary considerably in their protein quality despite absence of heat in their preparation raises the question of accuracy in the practice now commonly used of assigning a standard value of 100 to all butanone-extracted meals against which the performance of test cottonseed meals are graded. Perhaps the variations between the different "standard butanone extracted meals" themselves help to account for the many unusual and contradictory results encountered in the evaluation of cottonseed meals.

The data presented here fail to show a direct correlation between biologically evaluated protein quality of cottonseed meals in the rat and their nitrogen solubility in either 3% sodium chloride or 0.02N sodium hydroxide. It will be noted that several of the samples with unusually high bound gossypol (total gossypol minus free gossypol) content had good protein quality, contrary to the prevailing opinion that cottonseed meals with high bound gossypol content have low protein quality.

Discussion

It was found that many cottonseed samples with rather high free gossypol content are not necessarily toxic to the rat, a test animal which is responsive to

the toxic factor(s) in cottonseed, and that these samples are indeed far less toxic than many other cottonseed meals of considerably lower free gossypol content. These data confirm the findings of Eagle *et al.* that, in acute oral toxicity studies with cottonseed pigment glands, there was no good correlation between the toxicity (oral LD50 values) of pigment glands and their extractable gossypol content (26, 27), that mortality and body weight depressions caused by adding various levels of cottonseed pigment glands to the diets of rats cannot be attributed to the gossypol content alone (28), and that the residual toxicity of treated cottonseed meals cannot be explained on the basis of the free gossypol content as analyzed (8). In three instances (26, 27, 28) the lack of correlation between toxicity and gossypol content was determined on cottonseed pigment glands which had not been chemically treated, a point difficult to explain if one were to take the point of view that methods for determining the free gossypol content might not be applicable to cottonseed products which have been chemically treated.

Just what is "free gossypol"? Free gossypol has been defined as gossypol and gossypol-like compounds which dissolve in 70% acetone under special conditions (24). This "free gossypol" is certainly not the "free gossypol" of Carruth (5), or that of Schwartze and Alsberg (29), or that of Clark (30, 31), or that of many of the more recent investigators whose methods were evaluated by the gossypol committees (16). Correlations between the toxicity of cottonseed and the gossypol content had been made by Schwartze and Alsberg (32), Clark *et al.* (33), Nelson and Jones (34), and Lillie and Bird (35), and it has been commonly accepted that the free gossypol content of cottonseed meal is the *sine qua non* of its toxicity. Within recent years however several groups of investigators who have questioned the reliability of gossypol analyses as true indicators of toxicity are listed in chronological order (36, 37, 26, 27, 38, 28, 8). The results reported in the present paper confirm and amplify the conclusion that the free gossypol content of a cottonseed meal is not a true measure of its toxicity. The fact that a "free gossypol" value obtained by gravimetric estimation of the aniline-gossypol complex was linked with the toxicity of cottonseed 38 years ago (15) is insufficient evidence that the "free gossypol" value obtained by modern gossypol methodology (24) has the same or greater biological signifiicance, or that it is the sole factor involved. It is hardly necessary to point out that processing procedures or treatments which cause reductions in the free gossypol content of cottonseed meals could hardly be so selective as to have no effect on any other toxic factor(s) present.

Must cottonseed meals be considered toxic merely because their free gossypol content may be as high as 0.1 to 0.3% in defiance of the fact that these meals can be successfully fed to rats at the unusually high level of 67% of the diet, resulting in body weight gains comparable to and at times better than those afforded by butanone-extracted cottonseed meal, by soybean meal of excellent quality, or by stock diet? Our data show no good correlation between the free, total, or combined gossypol content of cottonseed meals and their biologically evaluated toxicity in rats. Similar observations have been made in poultry feeding tests (39).

We have been unable to find a positive correlation between the nitrogen solubility in 0.02N sodium hydroxide and the biologically evaluated protein quality of cottonseed meals fed to rats at the 9% protein level and cannot consider meals whose nitrogen solubility values are below a limit of 75% or less to be of poor protein quality, particularly when some have been proved by biological evaluation to be superior to many other cottonseed meals, superior to soybean meal, and superior to butanone-extracted cottonseed meal.

Summary

1. Toxicological evaluation of 68 cottonseed meals in rats failed to show a direct correlation between their toxicity and their free, total or combined gossypol content. The common practice of considering the free gossypol content of cottonseed meal as a yardstick for its toxicity is questioned.

2. There was poor correlation between biologically evaluated protein quality of cottonseed meals and their nitrogen solubility in 0.02N sodium hydroxide. Application of this chemical test for indicating the protein quality of cottonseed meals is likewise questioned on the basis of existing evidence.

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Seed Oils from Cassia Fistula, C. Occidentalis, and C. Tora (Indian Varieties)[1]

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THE CHEMICAL CHARACTERISTICS of the seed oils of three Cassia varieties have been previously recorded. The oil of *C. occidentalis* from the Islands of St. Martins was partly analyzed by Steger and van Loon (17), that of *C. tora* from Indian sources by Manjunath and Jois (14), and that of *C. fistula* of the Sudanese variety by Grindley (5). Chrysophanic acid (4,5-dihydroxy-2-methyl anthraquinone), which has medicinal value, was found by Elborne (4) in the oil from *C. tora* seeds.

The seeds of this family are not known as oil-bearing; they contain mainly carbohydrate as reserve material besides some protein whereas their oil content is small. Some of these oils are used in medicine. Because the recorded data about these oils were fragmentary and incomplete, the present study using methyl-ester fractionation technique was undertaken.

Experimental

The seeds for the present investigations were collected a) *C. fistula* in the vicinity of the Aligarh University and b) *C. occidentalis* and c) *C. tora* from the Barabanki District, U. P. Extraction of the dried and finely crushed seeds with petroleum ether (b.p. 60–80°) in a specially modified Soxhlet apparatus gave 3%, 2.8%, and 5.0% brownish yellow oils, respectively.

In Table I are recorded the physical and chemical constants found for these oils as compared to values reported by other workers.

Fatty acids were isolated in yields of 81.2%, 87%, and 81.4% by successively saponifying the oil with alcoholic potassium hydroxide, removing the unsaponifiable matter by extraction with ether, and acidifying with sulphuric acid. Resins were removed from the acids according to the method described by Lewkowitsch (11) in yields of 1.3%, 1.0%, and 1.5%.

The fatty acids were separated into liquid and solid fraction by the lead salt alcohol method (6).

[1] The work described in this paper was carried out in the Prince of Wales Chemical Laboratories, Muslim University, Aligarh, India.

Yields and compositions of the fractions are summarized in Table II.

TABLE II

Fatty Acids from Cassia Oils

Seed	*C. fistula*	*C. occidentalis*	*C. tora*
Total fatty acids			
Iodine value	115.5	114.5	106.1
Thiocyanogen value	74.6	74.0	66.7
Saponification equivalent	289.3	283.0	287.3
Liquid fatty acids			
Yield, % of total	79.1	74.9	73.8
Iodine value	143.7	151.3	142.6
Thiocyanogen value	84.0	.....	.....
Saponification equivalent	280.5	280.5	284.8
Solid fatty acids			
Yield, % of total	20.9	25.1	26.2
Iodine value	4.6	3.8	3.2
Saponification equivalent	289.4	287.8	274.4
Saturated acids (Bertram method) (2)			
Yield, % of total	19.4	24.0	25.4

The total fatty acid fractions were brominated by the method of Eibner and Mugganthaler (13). Bromo-derivatives having the following melting points were obtained:

Fatty acids	Hexabromo- m.p.	Tetrabromo- m.p.	Dibromo-
C. fistula	.....	114–15°	liquid
C. occidentalis	178–80°	115–16°	liquid
C. tora	.....	114–15°	liquid

The same products were obtained by bromination of liquid fatty acid fractions.

Methyl esters were prepared from the liquid and solid fatty acid fractions by the method of Hilditch (7) and were fractionally distilled under reduced pressure from a Claisen's flask attached to a Kon-type vacuum fractionation receiver (18).

Iodine values and saponification equivalents of the ester fractions were determined. These data were used in calculating fatty acid compositions of the esters (8).

Identities of the fatty acids were established 1) by isolating the saturated acids from samples used for

TABLE I

Physical and Chemical Constants of Cassia Oils

Seed	*C. fistula*		*C. occidentalis*		*C. tora*	
Origin	India	Africa (5)	India	St. Martins (17)	India	India (14)
Oil yield, %	3.0	2.04	2.8	2.3	5.0	5.0
Specific gravity	0.9112^{20}	....	0.9166^{32}	....	0.9012^{32}	0.8969^{25}
Refractive index	1.4672^{40}	1.4668^{40}	1.4714^{32}	1.477^{15}	1.4672^{32}	1.4669^{25}
Saponification value	184.2	184.4	176.1	178.7	163.4	154.2
Acetyl value	9.2	....	....	....	11.2	9.6
Acid value	2.9	....	5.4	10.2	4.2	10.8
Iodine value (Hanus)	109.3	94.5	110.3	113.9	91.3	90.7
Thiocyanogen value	66.6	63.2	72.6	78.2	58.2	....
Unsaponifiable, %	5.7	5.4	8.3	7.35	5.7	5.4
Protein, %	2.7	....	14.72	....	19.84	....