Long-Term Rat Feeding Studies with Vegetable Mixtures Containing Cottonseed Flour Produced by Different Methods

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A long-term feeding test with rats was carried out for 420 days. Both male and female rats were fed four different samples of cottonseed flour prepared by different processing techniques, prepress solvent and screw press. The free gossypol content of the four samples ranged from 0.028 to 0.084%, which provided from 0.011 to 0.028% in the diet. The small intakes of free gossypol did not interfere with the normal growth of males and females nor with their breeding and lactation performance. The animals showed no pathological changes and the weight of the organs examined was normal. Weaning performance was similar to that of the control group.

A well-known limitation to the use of cottonseed flour in nutrition is the presence of small amounts of the pigment gossypol (Altschul *et al.*, 1958; Braham *et al.*, 1965; Bressani *et al.*, 1964; Danke *et al.*, 1965), which is toxic in varying degrees to all monogastric animals. The swine is probably the animal least resistant to dietary gossypol, while higher levels also affect rats, chicks, dogs, and other animals (Altschul *et al.*, 1958).

As with many other toxic substances that are ingested daily in small amounts, gossypol accumulation may reach a level in important tissues sufficient to initiate the appearance of signs of toxicity culminating in a relatively prompt death (Clawson and Smith, 1966; Sharma *et al.*, 1966). Most studies with gossypol-containing cottonseed flours have been carried out for relatively short periods. It was, therefore, of practical interest to conduct long-term feeding experiments with rats for the purpose of determining if any deleterious effects were due to the constant intake of gossypol in cottonseed flour. The results of such a study are also important in human nutrition, in view of the fact that, recently, cottonseed flour is being used in human food supplements (Bressani *et al.*, 1966).

MATERIAL AND METHODS

Cottonseed. Material was brought to the laboratory from four cottonseed oil mills, three located in Central America. One of the Central American samples was obtained by the prepress solvent extraction procedure (sample B), and the other two by screw pressing (samples C and K). The fourth sample, a human grade cottonseed flour which is commercially available in the United States, served as control (sample P). The samples were analyzed for proximate chemical composition (Association of Official Agricultural Chemists, 1950), free and total gossypol (American Oil Chemists' Society, 1945–1950), and epsilon-aminolysine (Conkerton and Frampton, 1959). Their chemical description is presented in Table I.

Experimental Animals. Sixty weanling rats of the Wistar strain from the INCAP colony were used. One half of the rats were male and the other half female, and they were divided into five groups of six males and six females each.

Experimental Diets. The cottonseed flour samples were included at the 40% level in the diet. They were supplemented with 2.0% torula yeast, 4.0% mineral mixture (Hegsted *et al.*, 1941), 5.0% crude casein, 1.0% cod liver oil, 4.0% vegetable fat, 22.0% yellow corn, 21.7% whole wheat flour, and 0.3% aurofac. Torula yeast was included as a source of vitamins of the B-complex group. Crude casein was used to supply the diet with extra protein as well as to correct the lysine deficiency of all the flours used, therefore increasing the protein quality of the experimental diets. This eliminated a possible lysine deficiency which would leave gossypol as the only significant variable to be studied. A control diet not containing cottonseed flour was prepared by mixing a commercial rat-feed base (40.0%) with ground yellow corn (57.0%) and mineral mixture (3.0%). The composition and gossypol content of the diets are shown in Table II, obtained by chemical analysis using the methods already described. Although protein content, as well as other chemical components, varied between diets, their levels are all above the minimum needed by the rat. The epsilon-aminolysine content of the diets was determined following the method used on the cottonseed flour samples.

Study Plan. All animals were placed in individual all-wire screen cages, with food and water provided *ad libitum*. They were weighed once every 7 days for a total period of 420

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Table I. Description of Cottonseed Flour Samples

Cottonseed Flour	Industrial Preparation	Nitrogen, %	Fat, %	Crude Fiber, %	Gossypol, %		ϵ-NH_2 Lysine, %
					Free	Total	
B	Prepress solvent	8.32	1.70	4.40	0.071	0.98	1.74
C	Screw-press	6.53	10.40	8.70	0.084	1.06	1.23
K	Screw-press	6.76	6.35	8.95	0.028	1.04	0.89
P	Control human grade flour[a]	8.48	5.56	2.87	0.036	0.72	1.74

[a] Process not known.

Table II. Chemical Composition of Diets

	Diet				
	B	C	K	P	Control
Protein, %	30.4	25.9	24.7	31.1	21.5
Fat, %	9.1	11.8	9.3	9.8	6.2
Crude fiber, %	3.0	5.1	7.6	2.9	3.7
Ash, %	6.7	6.3	6.2	6.4	6.6
Gossypol, %					
Total	0.37	0.45	0.39	0.30	...
Free	0.022	0.028	0.011	0.014	...
ϵ-NH_2 lysine, g./16 g. N	1.02	0.88	0.77	1.04	...

days. When the females reached the required age, they were bred with the males fed the same diet. A total of six breedings took place for every female during the entire experimental period. After breeding, the animals were separated. When the females gave birth, the number of offsprings, their weight per litter, and sex were recorded. Each female rat was left with 10 or 11 newborns during lactation, and the young rats were weaned at the age of 24 days, and weighed individually. After a rest period, the females were bred again with males from the same dietary group.

The experiment was terminated after 420 days because the supply of cottonseed flour purchased at the start of the experiment was exhausted.

At the end of the experiment all animals were sacrificed, their organs were weighed and examined for major pathological changes, and sections of the different organs were studied in detail.

RESULTS

Certain important chemical constituents of the cottonseed flours are shown in Table I, including a free gossypol content ranging from 28 to 84 mg. per 100 grams of flour. The screw press flour had the lowest available lysine values.

One male from the control group and one female from the cottonseed flour P group died at 198 and 96 days, respectively, after the initiation of the study. Necropsy was performed in both animals, but it was not possible to find the cause of death. The female rat had mammary tumors.

All other animals were healthy, as indicated by the weight gains shown in Table III for males and females. The irregularities in the growth curves of the females resulted from pregnancies.

The reproduction performance is shown in Table IV. The number of young weaned at 24 days of age and their average weight are shown in Table V.

The average weights of certain organs are shown in Table VI. Examination of the entire animal and of the organs collected revealed no pathological alterations, nor did microscopic examination of sections of the same organs indicate any damage to the tissues.

DISCUSSION

The rate of growth as measured by weight gain (Table III) was not different for either male or female rats among the four samples of cottonseed flour and the control group, although available lysine values of the flours were different. In many feeding studies with cottonseed flour, lysine deficiency in the diet could have been an important factor in increasing the susceptibility of the animal to gossypol. Because casein was included in the diets (5%), it provided enough lysine to meet the deficiency of this amino acid. Therefore, neither gossypol nor amino acid deficiencies in cottonseed protein affected growth or performance.

The results of the present study indicate that even though male and female rats consumed small amounts of free gossypol for 420 days, the cottonseed pigment did not interfere with the growth of the animals and their breeding, reproduction, or lactation (Tables III, IV, and V). Furthermore, visible or microscopic alterations were manifest neither in the different tissues studied nor in the organ weight of the adult animals (Table VI).

The rat was able to eliminate or detoxify the ingested gossypol, since the amounts consumed daily were relatively small. It has been indicated that rats are not as sensitive to gossypol as swine (Altschul *et al.*, 1958). However, they do not resist the toxic effects of high levels of the pigment, as previously reported (Bressani and Elías, 1968). It is possible also that the small intakes of free gossypol had no undesirable effects because the concentration of total protein in the diet was relatively high, and it has been demonstrated that high protein level counteracts gossypol toxicity (Hale and Lyman, 1957; Sharma *et al.*, 1966).

Table III. Monthly Mean Weight Gain of Male and Female Rats Fed Four Samples of Cottonseed Flour for 14 Months

(6 animals per group)

	Male					Female				
Months	B, g.	C, g.	K, g.	P, g.	Control, g.	B, g.	C, g.	K, g.	P, g.	Control, g.
0	52	52	52	52	52	48	48	48	47	47
1	226	235	244	234	236	169	162	166	170	162
2	308	329	327	331	331	215	210	219	216	208
3	384	402	397	400	411	267	259	259	261	245
4	402	425	432	433	439	269	268	273	261	266
5	450	478	480	463	493	311	309	312	308	315
6	443	475	468	464	479	286	292	299	284	276
7	479	507	520	466	525	328	345	338	338	302
8	493	538	521	482	552	317	328	331	306	306
9	494	523	541	516	536	331	356	329	332	313
10	499	531	549	524	534	332	337	344	324	309
11	505	541	553	537	533	358	359	369	361	308
12	506	538	542	520	534	341	338	355	336	305
13	522	543	548	520	545	381	384	389	376	327
14	522	538	555	546	547	343	342	355	331	314

Table IV. Number per Litter and Average Rat Weight at Birth during Six Breedings

Cottonseed Flour	Number Bred	Number Pregnant	Litter		Av. Wt. per Rat, G.	Av. No. of Rats/ Mother
			Females	Males		
			Breeding 1			
B	6	5	32	29	5.8	12.2
C	6	6	36	41	5.6	12.8
K	6	5	26	28	5.8	10.8
P	6	6	29	38	6.1	11.2
Control	6	5	27	35	5.8	12.4
			Breeding 2			
B	6	6	27	40	6.1	11.2
C	6	6	37	40	6.1	12.8
K	6	6	46	26	6.1	12.0
P	5	4	20	28	6.2	12.0
Control	6	5	28	27	6.8	11.0
			Breeding 3			
B	6	6	26	40	6.4	11.0
C	6	6	44	31	6.3	12.5
K	6	6	35	42	6.4	12.8
P	5	5	25	29	6.3	10.8
Control	6	5	20	26	6.6	9.2
			Breeding 4			
B	6	6	30	36	5.9	11.0
C	6	6	35	41	6.4	12.7
K	6	6	32	33	6.3	10.8
P	5	4	14	27	6.4	10.2
Control	6	5	21	25	6.4	9.2
			Breeding 5			
B	6	5	27	28	6.0	11.0
C	6	5	23	32	6.4	11.0
K	6	6	30	26	6.3	9.3
P	5	5	32	27	6.5	11.8
Control	6	4	20	22	6.5	10.5
			Breeding 6			
B	6	5	27	30	6.3	11.4
C	6	5	30	26	6.1	11.2
K	6	6	32	37	6.3	11.5
P	5	5	21	34	6.5	11.0
Control	6	4	17	21	7.1	9.5

Table V. Average Weaning Weight and Percentage of Live Rats from Mothers Fed Different Cottonseed Flour Samples

Cottonseed Flour	No. of Rats Left with Mother	No. of Rats Weaned	Av. Wt., G.	Mortality, %
	Breeding 1			
B	50	41	38.3	18.1
C	60	55	44.3	8.3
K	50	49	39.5	2.0
P	60	56	38.4	6.7
Control	50	39	41.8	22.0
	Breeding 2			
B	66	65	41.9	1.5
C	66	64	45.6	3.0
K	66	64	44.4	3.0
P	44	43	42.7	2.3
Control	55	52	41.9	5.4
	Breeding 3			
B	60	56	36.7	6.7
C	60	55	39.9	8.3
K	60	60	32.8	0
P	50	49	34.2	2.0
Control	50	30	36.6	40.0
	Breeding 4			
B	60	54	41.8	10.0
C	60	56	39.3	6.7
K	60	44	47.9	26.7
P	40	32	41.3	20.0
Control	50	45	33.3	10.0
	Breeding 5			
B	50	48	45.3	4.0
C	50	50	40.5	0
K	60	60	43.2	0
P	50	45	42.0	10.0
Control	40	40	36.0	0
	Breeding 6			
B	55	51	41.2	7.3
C	55	48	38.3	12.7
K	66	63	40.0	4.5
P	55	52	33.7	5.4
Control	44	36	33.5	18.2

Table VI. Mean Organ Weight of Rats Fed Different Cottonseed Flours for 14 Months

Cottonseed Flour Sample	Sex of Rat	Heart, G.	Liver, G.	Kidney, G.	Lungs, G.	Spleen, G.	Adrenals, G.
B	F	1.24	9.83	1.43	0.75	0.64	0.070
	M	1.70	13.71	1.80	1.10	1.00	0.065
C	F	1.22	9.94	1.36	0.83	0.65	0.110
	M	1.45	14.21	1.75	1.16	0.80	0.061
K	F	1.32	9.65	1.36	0.77	0.72	0.085
	M	1.99	15.64	2.04	1.20	1.05	0.070
P	F	1.35	10.05	1.39	0.94	0.69	0.098
	M	1.70	14.02	1.88	1.27	0.84	0.084
Control							
F		1.21	9.15	1.19	0.80	0.52	0.078
M		1.75	15.21	1.89	1.26	0.87	0.076

The intake of gossypol by the mothers did not interfere with pregnancy, since the number of animals per litter and their average weight at birth (Table IV), particularly after the first breeding, are similar to those reported by other workers (Dymsza *et al.*, 1964; Goyco and Asenjo, 1965; Schultze, 1953; Schultze *et al.*, 1952; Venkatachalam and Ramanathan, 1964). On the other hand, weaning weight appears to be slightly low (Table V). However, this can be attributed to the strain of rats used or to common environmental factors and not to the gossypol, the members of the control group having behaved in a like manner.

Lactation performance has been measured by counting the number of young which survive weaning (Goyco and Asenjo, 1965; Schultze, 1953; Schultze *et al.*, 1952). On this basis, the performance was not affected by the intake of free gossypol or the type of cottonseed flour used, since mortality was relatively small except in the fourth breeding. In general, mortality of the young appears higher in the control group. The high mortality during the fourth breeding could be attributed to environmental conditions in the colony during that part of the year, as it is a time of relatively humid and hot weather.

The results of the over-all study indicate that, at least for the rat, small daily intakes of free gossypol do not interfere with growth and reproduction performance of this animal. It is important, however, to correct for other nutritional abnormalities in cottonseed flour, so that they do not predispose the animal to gossypol toxicity.

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