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All-Vegetable Protein Mixtures for Human Feeding
XIII. Effect of Cooking Mixtures Containing Cottonseed Flour on Free Gossypol Content

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SUMMARY

INCAP Vegetable Mixture 9 (58% corn, 38% cottonseed flour, 3% torula yeast. 1% calcium carbonate) was cooked with water for several periods. Free gossypol decreased as a result of cooking, with no change in either total gossypol or available lysine. Biological trials in swine and dogs confirmed that the nutritive value of the protein of the mixture was not decreased.

The addition of water to the mixture followed by drying was found in 14 out of 18 trials to decrease free gossypol levels. The substitution of calcium carbonate by calcium hydroxide further decreased free gossypol, although calcium phosphate salts did not. Ferrous sulfate addition was also effective in decreasing free gossypol content, to almost 0 when added in the presence of $Ca(OH)_2$ or when the mixture was cooked at alkaline pH values. Alkaline pH of cooking, associated with calcium ions but not with sodium ions, was found important in reducing free and total gossypol. Ferrous sulfate alone was not as effective at low concentrations as at higher. Sugar also seemed to be important in the inactivation of free gossypol.

INTRODUCTION

During the last few years, several laboratories have developed vegetable protein mixtures for supplementary feeding of children and adults as a means of controlling protein malnutrition in underdeveloped areas (National Research Council, 1961). Experimental INCAP Vegetable Mixture 9 (28% ground corn, 28% ground sorghum, 38% cottonseed flour, 3% torula yeast, 3% dehydrated leaf meal) (Bressani *et al.*, 1961b) was tested extensively in chicks (Bressani *et al.*, 1961a), rats (Bressani *et al.*, 1962b; Bressani and Elías, 1962) and other experimental animals (Bressani *et al.*, 1962a) before it was fed to children (Scrimshaw *et al.*, 1961, 1962). In all cases the mixture was found to be of high nutritive value.

It is well known that cottonseed flour contains free gossypol, which reacts with the ε-amino group of lysine when heated (Altschul *et al.*, 1958). The amount present varies with variety, maturity, and processing conditions. Since INCAP Vegetable Mixture 9 is cooked for human consumption, it was desirable to determine the effects of this moist heat. Accordingly, changes in gossypol, lysine, thiamin, and riboflavin were measured after INCAP Vegetable Mixture 9 or its variations (Scrimshaw *et al.*, 1961) were cooked for various periods. Biological tests of protein quality of the cooked product with dogs and swine were included.

MATERIALS AND METHODS

After 75 g of Vegetable Mixture 9 were suspended in 500 ml of water and stirred, representative duplicate samples were withdrawn for analysis and the rest of the slurry cooked on a hot plate with constant stirring at 85–87°C. Duplicate samples were obtained after cooking for 5, 10, 15, and 25 min, from the moment the beaker was placed on the hot plate. Subsequent trials included: a) the use of cottonseed flour with a different content of free gossypol; b) the addition of 56 g of sucrose to the 75 g of the mixture; c) the addition of 0.30% L-lysine HCl to the mixture; d) the addition of 0.2% Rhozyme H-39; e) the addition of several levels of calcium salts and of ferrous sulfate, alone and in combination; f) and the effect of pH in the presence and absence of ferrous sulfate.

After the samples were cooked for the specified period, aliquots were analyzed for moisture by vacuum-oven method (AOAC, 1950). The remainder was lyophilized and stored at 4°C until analyzed. Riboflavin was determined fluorometrically by the method of Hodson and Norris (1939), and thiamin by the thiochrome method of Hennessey and Cerecedo (1939). The samples were also analyzed for free

Table 1. Effect of cooking on changes in several substances in Vegetable Mixture 9.[a]

Cooking time (min)	Thiamin (mg/100 g)	Riboflavin (mg/100 g)	Gossypol			Free ε NH_2 groups of lysine (g/100 g)
			Free (mg/100 g)	Total (g/100 g)	Free (mg/100 g)	
Raw[b]	2.18	1.21	15.8[c]	0.41[c]	21.3[d]	1.24
0	2.25	1.12	7.1	0.43	23.7	1.16
5	1.96	1.07	5.3	0.35	20.9	1.09
10	2.01	1.05	6.4	0.43	14.7	0.95
15	1.99	1.14	8.4	0.41	12.9	1.18
20	1.75	0.86	5.3	0.41	14.0	1.19
25	1.54	1.18	2.7	0.42	10.4	1.09

[a] Formula: corn, 58%; cottonseed flour, 38%; torula yeast, 3%; $CaCO_3$, 1%. All analytical values are on a dry-weight basis.
[b] Before water was added.
[c] Cottonseed flour containing 45 mg free gossypol per 100 g flour.
[d] Oottonseed flour containing 60 mg free gossypol per 100 g flour.

and total gossypol according to the AOCS official methods (1945–50), and for free epsilon-amino groups of lysine by the method of Conkerton and Frampton (1959).

For the study with dogs, to learn whether the nutritive value of the mixture was affected by cooking, three dogs 10 months old and weighing 8.90, 11.98, and 8.44 kg were fed approximately 5.5 g of protein and 125 cal per kg body weight per day. The protein fed was exclusively from Vegetable Mixture 9, while the intake of calories was adjusted by feeding a nitrogen-free diet consisting of 20% cornstarch, 40% dextrose, 24% sugar, 3% cellulose, 10% hydrogenated vegetable fat, 2% mineral mixture (Hegsted *et al.,* 1941), and 1% cod liver oil, supplemented with 4 ml of a complete vitamin mixture (Manna and Hauge, 1953). The animals were fed once a day, at 8:00 A.M., and weighed daily.

The calculated quantity of Vegetable Mixture 9 for each dog was mixed with 800 ml of water, and 1% Rhozyme H-39 (kindly supplied by Rohm & Haas, Philadelphia, Pa.) with respect to the weight of Vegetable Mixture 9 used was added to make the gruel fluid after cooking. The suspension was cooked for 0, 8, 16 and 24 min, with constant stirring. The enzyme is resistant to the temperatures of cooking, which never exceeded 87°C. After cooling, the necessary quantity of the nitrogen-free diet was added and then fed to the animals. One balance period of 7 days was carried out for each cooking time. Feces and urine were collected twice daily and stored at 4°C, the urine in dark-colored bottles containing 2 cc of concentrated acetic acid. After each balance period, the feces were homogenized and weighed and the urine volume measured. The nitrogen content of the diet, feces, and urine was determined by the Kjeldahl method.

The experiment with swine, for detecting possible adverse physiological effects, was carried out with 3 animals (Duroc female × New Hampshire male cross) 5 weeks old, with initial weights of 4.90, 4.91, and 3.70 kg. The animals were placed in cages for metabolic studies and fed the cooked Vegetable Mixture 9 (Bressani *et al.,* 1961b) for 90 days. Two diets were used, one consisting of Vegetable Mixture 9 alone and the other of a mixture of 94.4 g of corn, 3.0 g minerals, 2.4 g Rovimix (Hoffmann-LaRoche), and 0.2 g Aurofac (Pfizer Corp.). The vegetable mixture alone was cooked for 20 min, and the supplement added before feeding. Records of food intake were kept daily. The intake of Vegetable Mixture 9 was increased from 116 g per pig daily in the first balance to 636 g per pig daily in the last. Every 10 days, feces and urine were collected for a 4-day period for nitrogen-balance analysis. The feces and urine collected were handled as described above. The changes in weight were measured every seven days.

RESULTS

The data of Table 1 show that when Vegetable Mixture 9 was cooked for various periods the thiamin content decreased but no change took place in riboflavin concentration. The free-gossypol content decreased with different initial free-gossypol content, in this case the decrease being larger for the sample containing less initial free-gossypol. A decrease in free gossypol occurred upon wetting of the samples made from the low free-gossypol cottonseed flour, but not in the other. Total gossypol and the free epsilon-amino groups of lysine were essentially unchanged by the cooking. The low value of 0.35 for total gossypol, 0.95 for lysine, and 0.86 for riboflavin was probably due to sampling.

Table 2 indicates that added sugar and enzyme did not alter the sequence of results. The lower initial figures are due to the dilution effect of the sugar added. The addition of ferrous sulfate in the presence of the sugar and enzyme caused a further decrease in free gossypol, with no effect on total gossypol and ε-amino groups of lysine. Cooking had no effect on added lysine. Cooking a sample of cottonseed flour alone, also caused a decrease in free gossypol, but no change in total gossypol and lysine.

Table 3 reveals that, of several cal-

Table 2. Effect of cooking Vegetable Mixture 9 and cottonseed flour for different periods on the free and total gossypol and free-amino groups of lysine content.[a]

Vegetable Mixture 9[b]	Gossypol analysis (mg/100 g)	Cooking time (min)					
		0	5	10	15	20	25
+ sugar + 0.2% enzyme	Free	12.5	11.9	8.1	7.1	7.3	7.1
	Total	262	305	394	254	267	267
	ε-HN_2-Lys.	658	676	658	664	682	626
+ sugar + 0.2% enzyme + 0.1% $FeSO_4$	Free	4.6	4.0	2.6	4.0	3.2	2.6
	Total	275	266	255	234	266	258
	ε-NH_2-Lys.	626	664	684	658	696	746
+ 0.30% L-Lys. HCl + 0.2% enzyme	Free	16.7	15.7	11.3	9.8	10.1	9.7
	Total	411	422	378	381	390	380
	ε-NH_2-Lys.	932	911	900	888	862	944
Cottonseed flour alone	Free	46.3	41.5	36.1	33.1	33.0	27.5
	Total	941	905	947	909	951	858
	ε-NH_2-Lys.	1558	1529	1570	1427	1448	1547

[a] Analyses were carried out on freeze-dried samples. Results are expressed on a dry-weight basis.
[a] Formula: Corn, 58%; cottonseed flour, 38%; torula yeast, 3%; and calcium carbonate, 1%.

cium salts tested for their property of reducing free gossypol during cooking, only calcium hydroxide had a marked effect. The addition of 0.1% ferrous sulfate in the presence of $Ca(OH)_2$ reduced free gossypol, even when no cooking was done. With various concentrations of ferrous sulfate alone, the decrease of free gossypol was not as large as with the addition of calcium hydroxide. Wetting of the sample with no cooking reduced free gossypol content.

Table 4 presents the results when 1% $Ca(OH)_2$, 0.1% $FeSO_4$, the combination of both, or 1.8% $Ca(OH)_2$ and 0.5% $FeSO_4$ were added independently and in combination. The addition of 1% $Ca(OH)_2$ and 0.1% $FeSO_4$ reduced free gossypol, but the decreases were small. When higher amounts of salts were added, 1.85% $Ca(OH)_2$ and five times more $FeSO_4$ together, free gossypol disappeared and even total gossypol decreased. Cooking is not required for the effect of $FeSO_4$ alone in reducing free gossypol. As in previous cases wetting of the sample caused a reduction in free gossypol concentration.

Table 5 shows that cooking the mixture at a pH of 6.35 decreased free gossypol as much as at an alkaline pH. Alkaline pH that was due to calcium hydroxide alone and was in the presence of ferrous sulfate, decreased both free and total gossypol, whereas alkalinity associated with sodium ion and ferrous sulfate decreased only free gossypol. As in previous examples wetting of the sample resulted in a decrease in free gossypol content of Vegetable Mixture 9.

Biological tests with dogs. No significant alterations of nitrogen balance were found in 3 dogs fed Vegetable Mixture 9 cooked for different periods. Average retentions for each cooking time were as follows: 0 min, 17.5%; 8 min, 16.2%; 16 min, 16.4%; 24 min, 16.3%; and again 0 min, 13.6%. Nitrogen intakes varied little, from 844 to 865 mg/kg body weight per day, and nitrogen absorption averaged 71.2% of nitrogen intake.

Biological tests with swine. Fig. 1 shows the results when swine were fed the cooked mixture. Nitrogen retention remained high and proportional to nitrogen intake. The animals gained weight slowly at first, but as the experiment progressed they reached daily gains similar to those of swine fed an adequate diet under standard conditions. The average weight gain for the experimental period was 20 kg. The figure also shows the calculated free-gossypol intake of the swine assuming no destruction during cooking. No adverse physiological effects of the gossypol ingested were detected.

Table 3. Effect of several calcium salts and of level of ferrous sulfate on the gossypol and available lysine content of INCAP Vegetable Mixture 9 when cooked.[a]

Treatment	Gossypol (mg/100 g)	Raw[b]	Cooking time (min) 0	15	25
None[c]	Free	....	12.8	11.6	12.4
	Total	....	320	342	364
+ 1% $Ca(OH)_2$	Free	....	11.6	5.9	1.5
	Total	....	311	329	325
+ 1% $CaH_2(PO_4)_2$	Free	....	15.7	13.8	12.9
	Total	....	398	327	311
+ 1% $Ca_2H(PO_4)_2$	Free	....	16.9	13.5	12.0
	Total	....	374	321	291
+ 1% $Ca_3(PO_4)_2$	Free	....	17.2	12.2	11.7
	Total	....	364	247	327
+ 1% $Ca(OH)_2$ + 0.1% $FeSO_4$	Free	....	7.8	7.6	1.3
	Total	....	351	334	320
+ 0.1% $FeSO_4$	Free	....	8.7	8.6	11.5
	Total	....	367	336	351
None[d]	Free	35.1	33.3	15.4	13.9
	Total	384	469	399	432
	ϵ-NH_2-Lys.	1049	1049	1037	1098
+ 0.025% $FeSO_4$	Free	35.9	31.3	16.8	14.7
	Total	409	469	372	420
	ϵ-NH_2-Lys.	1025	1025	1049	1005
+ 0.050% $FeSO_4$	Free	33.2	27.4	18.8	14.9
	Total	406	437	405	378
	ϵ-NH_2-Lys.	999	999	1019	955
+ 0.10% $FeSO_4$	Free	31.8	25.0	19.7	17.5
	Total	394	410	421	405
	ϵ-NH_2-Lys.	1025	1025	944	1019

[a] Composition of basal mixture: Corn, 58%; cottonseed flour, 38%; torula yeast, 3%; starch, 1%. The calcium salts and ferrous sulfate added replaced 1% starch.
[b] Before water was added.
[c] The cottonseed flour in the first set contained 32 mg free gossypol per 100 g flour.
[d] The cottonseed flour in the second set contained 96 mg free gossypol per 100 g flour.

DISCUSSION

One factor limiting the use of cottonseed flour in the nutrition of both nonruminant domestic animals and man, is the presence of free gossypol, which has been found to have adverse physiological effects, particularly with swine (Altschul *et al.*, 1958; Clawson *et al.*, 1962; Hale and Lyman, 1957). Free-gossypol concentration in cottonseed is decreased during processing of the seed for oil extraction in at least three ways: elimination with oil, reaction with sugars, and reaction with free amino groups of amino acids, of which lysine appears to be affected the most (Altschul *et al.*, 1958; Frampton, 1960). Because of this last the protein quality of cottonseed meal may be significantly reduced.

The present study shows that simple cooking in water significantly reduces

Table 4. Effect of different concentrations of calcium and ferrous ion on the free and total gossypol content of Vegetable Mixture 9 when cooked.[a]

Treatment	Gossypol[b] (mg/100 g)	Raw[c]	Cooking time (min) 0	15	25[d]
None	Free	15.0	15.7	10.2	11.0
	Total	291	317	280	292
+ 1% $Ca(OH)_2$	Free	15.1	15.3	12.1	12.6
	Total	295	295	284	276
+ 0.1% $FeSo_4$	Free	14.6	10.0	9.7	9.9
	Total	274	304	292	317
+ 1% $Ca(OH)_2$ + 0.1% $FeSO_4$	Free	14.3	9.9	8.7	10.4
	Total	285	317	296	281
None	Free	15.8	15.4	11.3	12.2
	Total	292	305	258	265
+ 1.8% $Ca(OH)_2$	Free	16.0	9.1	7.8	7.5
	Total	288	245	237	252
+ 0.5% $FeSO_4$	Free	7.2	8.5	8.8	8.0
	Total	261	295	271	211
+ 1.8% $Ca(OH)_2$ + 0.5% $FeSO_4$	Free	6.2	0	0	0
	Total	258	177	173	205

[a] Composition of basal mixture: Corn, 58%; cottonseed flour, 38%; torula yeast, 3%; starch, 1%; the calcium salts and ferrous sulfate added replaced 1% starch.
[b] Free gossypol content in cottonseed flour: 50 mg/100 g.
[c] Before water was added.
[d] In the second experiment, cooking period was 30 min.

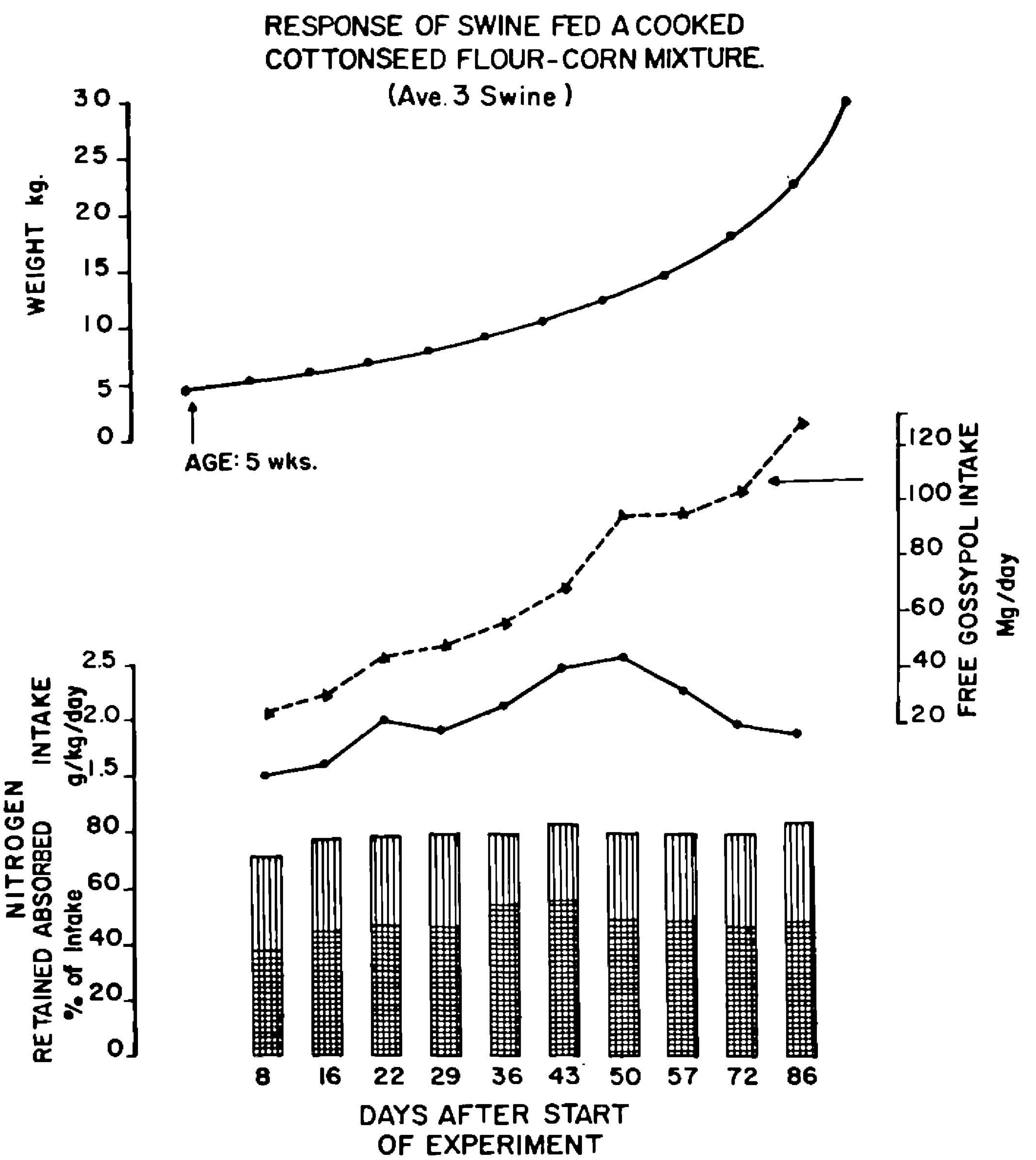

Fig. 1. Response of swine fed a cooked cottonseed flour-corn mixture. Free-gossypol intake was calculated from concentration in mixture fed, and assumed no destruction upon cooking.

Table 5. Effect of pH on the free and total gossypol upon cooking Vegetable Mixture 9.[a]

Treatment	pH	Gossypol	Raw	Cooking time (min) 0	25
None[b]	6.35	Free	33.2	24.5	4.5
		Total	424	432	434
+ $Ca(OH)_2$[c]	9.15	Free	28.7	13.8	0.5
		Total	424	404	351
+ $FeSO_4$[d]	6.07	Free	21.8	12.0	3.5
		Total	398	413	367
+ $Ca(OH)_2$ + $FeSO_4$[e]	8.85	Free	15.4	0.7	0
		Total	363	175	216
+ $FeSO_4$[f]	9.15	Free	22.1	3.4	0
		Total	408	360	384
None[g]	9.18	Free	31.5	20.4	3.1
		Total	433	463	420

[a] Gossypol in cottonseed flour used: free, 84 mg/100 g; total, 1037 mg/100 g.
[b] Formula of basal mixture: corn, 58%; cottonseed flour, 38%; torula yeast, 3%; starch, 1%. The calcium and ferrous salts replaced the starch.
[c] Amount added: 1.85%.
[d] Amount added: 0.496%.
[e] Amount of $Ca(OH)_2$ 1.85%; $FeSO_4$, 0.5%.
[f] Amount added: 0.5% $FeSO_4$; pH adjusted with 10*N* NaOH.
[g] pH adjusted with 10*N* NaOH.

the free gossypol content of Vegetable Protein Mixture 9, which contains 38% cottonseed flour, but causes no change in either the total gossypol concentration or, more significantly, in the free ϵ-amino groups of lysine. Furthermore, in 14 out of 18 trials free gosypol in the mixture decreased merely by wetting the sample. Since the analyses of gossypol were carried out on the freeze-dried material, the decrease was not due to moisture content or to the initial concentration of gossypol. The effect of wetting was more apparent when the calcium and iron salts were added. The rather small decrease in free gossypol content in the experiments of Table 4 presents the possibility that some batches of cottonseed flour will not show as large a decrease in free gossypol upon cooking. The mechanism by which free gossypol decreases with wetting with no cooking and with cooking of the mixture is apparently not the same as that proposed for the decreases of the free pigment during the processing of cottonseed, which has been shown to occur mainly by reaction with lysine (Altschul *et al.*, 1958; Frampton, 1960; Lyman, 1960).

An attempt was made to learn if gossypol was reacting with the nonprotein nitrogen in corn. After repeated dialysis to remove the free nitrogen, the residual material was mixed with cottonseed and cooked. Free gossypol decreased as much as before.

Eagle (1959) reported that certain salts added to cottonseed flour decreased its free-gossypol content. Kemmerer *et al.* (1961) demonstrated that an alkaline pH was necessary for the formation of the iron-gossypol complex responsible for the discoloration of egg yolks of eggs laid by hens fed cottonseed meal in the ration. The present results indicate that alkaline pH is an important condition necessary for reducing free gossypol in the presence or absence of iron. The effectiveness of calcium salts seems to be related to their alkalinity.

Ferrous sulfate has been used as an additive to swine rations that contain cottonseed flour in order to reduce gossypol toxicity (Gallup, 1928; Withers and Carruth, 1917). The present results indicate that it is effective in reducing the free-gossypol content, particularly in the presence of $Ca(OH)_2$. The mechanism is not well understood. Since it is a reducing agent, it may reduce gossypol or perhaps act as a chelating agent. The addition of $FeSO_4$ alone is not as effective as when $Ca(OH)_2$ is present, and the presence of sugars alone apparently increases its effectiveness in reducing the concentration of free gossypol. The decrease in free gossypol with or without additives when the mixture was cooked in relatively large amounts of water was apparently not related to initial gosspol content of the vegetable mixture.

Although free-gossypol levels are decreased, the chemical results also show that available lysine is not affected by $FeSO_4$ and $Ca(OH)_2$ addition. If lysine had been made unavailable, nitrogen balance would have decreased in the dogs fed the cooked mixture since the vegetable mixture is already slightly deficient in lysine (Bressani and Elías, 1962). Furthermore, swine are so sensitive to gossypol toxicity (Altschul *et al.*, 1958; Clawson *et al.*, 1962) that the amount of free gossypol present in swine rations should not exceed 0.01–0.03%, according to the protein content of the diet (Hale and Lyman, 1957). Cooking Vegetable Mixture 9 obviously inactivated the gossypol sufficiently without decreasing protein quality. The content of thiamin decreased, but less than would be expected from the alkalinity due to calcium carbonate. Biological testing for thiamin activity would be desirable to assure the accuracy of these observations. Slightly greater losses were found when $Ca(OH)_2$ replaced the carbonate.

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