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All-vegetable Protein Mixtures for Human Feeding

XV. STUDIES IN DOGS ON THE ABSORPTION OF GOSSYPOL FROM COTTONSEED FLOUR-CONTAINING VEGETABLE PROTEIN MIXTURES [1,2]

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ABSTRACT The results of the present study indicate that in the dog and under the conditions used, the cottonseed pigment, gossypol, is excreted in the feces almost quantitatively, particularly for total gossypol. Fecal free gossypol is about 3.5 times greater than intake, suggesting that some of the bound gossypol is liberated during passage through the gastrointestinal tract. The pigment was identified by maximal absorption curves, for the original compound and as the dianilido gossypol and phloroglucinol derivatives. Other chemical and biological tests also indicated that the fecal pigment is gossypol. Furthermore, typical gossypol reactions could not be demonstrated in fecal extracts of dogs fed cottonseed-free diets, and upon gossypol additions to the diet gossypol appeared in the feces. Several possible dietary factors were studied to learn the conditions under which gossypol is excreted with the feces. None of the factors studied, such as type and intake of calories, and different fats, altered the pattern of gossypol excretion in feces.

The use of cottonseed flour in the feeding of monogastric animals, including man, has been limited by the presence of the pigment gossypol in the cottonseed. Although during processing of cottonseed for oil extraction, most of the gossypol is either extracted or inactivated, the amounts remaining following one type of cottonseed processing are high and could produce toxic symptoms if fed in sufficient amounts. The toxicity, however, is variable since chicks, rats and other experimental animals grow at normal rates when consuming the amounts of gossypol found to be toxic to swine, making this animal the most sensitive to the toxicity of the pigment. Furthermore, reduced growth is not always a good indication of gossypol toxicity since much of the reduced growth encountered with cottonseed meal rations now appears to have been the result of a lysine deficiency (2). No explanation concerning the variability in toxicity has been given (1, 2).

During the development of INCAP Vegetable Mixture 9, which contains 38% cottonseed flour (3), extensive nutritional tests were carried out in several experimental animals (4, 5) including dogs (6) and humans (7). Since no adverse effects were noticed during prolonged feeding of the mixture, it became important to determine the fate of the pigment once it was consumed. The results reported by Smith (8, 10) and Clawson et al. (9) have indicated that gossypol is present in the tissues of swine. In a recent study by Smith (8), the pigment from swine tissue was isolated and identified chemically. In the present study, results will be presented showing that in dogs the gossypol consumed is excreted, to a very large extent, in the feces. Some possible factors affecting the absorption were also studied.

MATERIALS AND METHODS

In the first experiment, 3 dogs 10 months of age and weighing 8.90, 11.98 and 8.44 kg were fed approximately 5.5 g of protein and 125 kcal/kg body weight/day. The protein fed was exclusively from Vegetable Mixture 9,[3] and the intake of calories was adjusted by feeding a nitrogen-free diet consisting of cornstarch, 20;

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[2] INCAP Publication I-310.
[3] Ground corn, 58; cottonseed flour, 38; torula yeast, 3; calcium hydroxide, 1%; vitamin A, 4500 IU/100 g.

dextrose, 40; sugar, 24; cellulose, 3; hydrogenated vegetable fat, 10; mineral mixture, 2; and cod liver oil, 1%, supplemented with 4 ml of a complete vitamin mixture (11). The animals were fed once a day, at 8:00 A.M.

The calculated quantity of Vegetable Mixture 9 for each dog was mixed with 800 ml of water, and 1% Rhozyme H-39 [4] was added to make the cooked mixture more fluid. The suspension was cooked for 0, 8, 16 and 24 minutes, with constant stirring. After cooling, an amount of the nitrogen-free diet was added to bring the intake of calories to the desired level and then fed to the animals. One balance period of 7 days' duration was carried out for each cooking time. The animals were weighed daily and feces and urine were collected twice daily and stored at 4°C. The urine was collected in dark-colored bottles containing 2 cm^3 of concentrated acetic acid. The nitrogen balance results have been reported previously.[5] A representative aliquot of homogenized feces was freeze-dried for estimation of free and total gossypol (12) and another sample was taken for moisture analysis. The entire collection of feces of each dog was dried for studies on the extraction and identification of gossypol.

The dry feces were extracted with either acetone-water (80:20) or chloroform containing 10% concentrated hydrochloric acid with constant shaking for one hour. After filtering, the chloroform extract was concentrated in a current of air and then chromatographed through either a Florosil[6] or a silicic acid column. The extract was eluted first with chloroform, then with chloroform containing 10% ethyl ether and then with chloroform containing 80% methanol. The maximal absorption of the different fractions and their aniline and phloroglucinol derivatives was determined in a DU Beckman spectrophotometer.

Reaction with hydrogen peroxide. The gossypol extract was treated with a 10% sodium hydroxide solution; hydrogen peroxide (20 volumes) was added drop by drop, in the presence of gossypol, a violet color developed.

Reaction with phloroglucinol. The chloroform elution was treated with a phloroglucinol solution in ethyl alcohol and with concentrated hydrochloric acid. On standing, a purple color developed. The maximal absorption of the developed color was determined in a DU spectrophotometer.

Balance test in dogs. In another experiment, 4 dogs weighing at the start of the experiment 14.0, 10.1, 12.0 and 9.5 kg were used. Six grams of protein from Vegetable Mixture 9 and 120 kcal/kg body weight/day were fed. To adjust the intake of calories, a nitrogen-free diet of the composition previously described was used. The fats added to this diet at a level of 10% were: a) hydrogenated vegetable fat; b) butter fat, and c) purified cottonseed oil. Two balance periods of 4 days' duration were carried out with each type of fat and gossypol analysis as well as nitrogen balance were determined with each fat.

Three additional experiments were carried out to learn whether certain dietary factors affect gossypol absorption. Four dogs were used to study the effect of intake of 2 levels of calories, 140 and 85 kcal/kg body weight/day. In each case, one dietary treatment consisted of providing all calories as carbohydrate compared with a second treatment in which 10% of the calories were provided by fat. The average weight of the dogs, when fed the higher level of calories was 7.79 kg and when fed the lower level, the average weight was 11.2 kg. Protein intake was from Vegetable Mixture 9 and was held at a constant rate of 6 g/kg/day.

In another experiment using 3 dogs with an average weight of 13.5 kg, the effect of cooking Vegetable Mixture 9 in a glass container, as compared with cooking it in a metal container, on gossypol absorption was studied. The intake of calories and of protein were 110 kcal and 6.0 g/kg body weight/day, respectively.

In a third experiment 3 dogs with an average weight of 9.90 kg were fed 6 g protein/kg/day from Vegetable Mixture 9 and 140 kcal/kg/day for 8 days in 2 four-day balance periods. During the first 3 days, of the first 4-day balance, the dogs were given 50 mg of free gossypol/day, in

[4] Rohm and Haas, Philadelphia, Pa.

[5] Bressani, R., L. G. Elías, R. Jarquin and J. E. Braham 1964 All-vegetable protein mixtures for human feeding. XIII. Effect of cooking mixtures containing cottonseed flour on free gossypol content. J. Food Sci., in press.

[6] Floridin Company, Tallahasee, Florida.

capsules, and no additional gossypol was fed for the last 4-day balance period. Collection, treatment and analysis of feces in these experiments was performed as previously described.

RESULTS

Table 1 presents the results of the first test in dogs. Free gossypol intake varied from 22 to 34 mg/dog/day, and the intake of total gossypol ranged from 689 to 780 mg/dog daily. The excretion of free gossypol in the feces averaged approximately 3.3 times higher than the intake. However, total gossypol in the feces was essentially the same as the intake. A decrease in free gossypol and in fat content in the feces was observed with respect to feed cooking times.

The possibility existed that the determination for free gossypol was giving high values because of contamination with other fecal pigments. Therefore, studies were carried out to identify gossypol. Figure 1 shows the absorption curves of acetone-water extracts of the feces and of a solution of pure gossypol. The upper part of the figure shows the derivative of gossypol

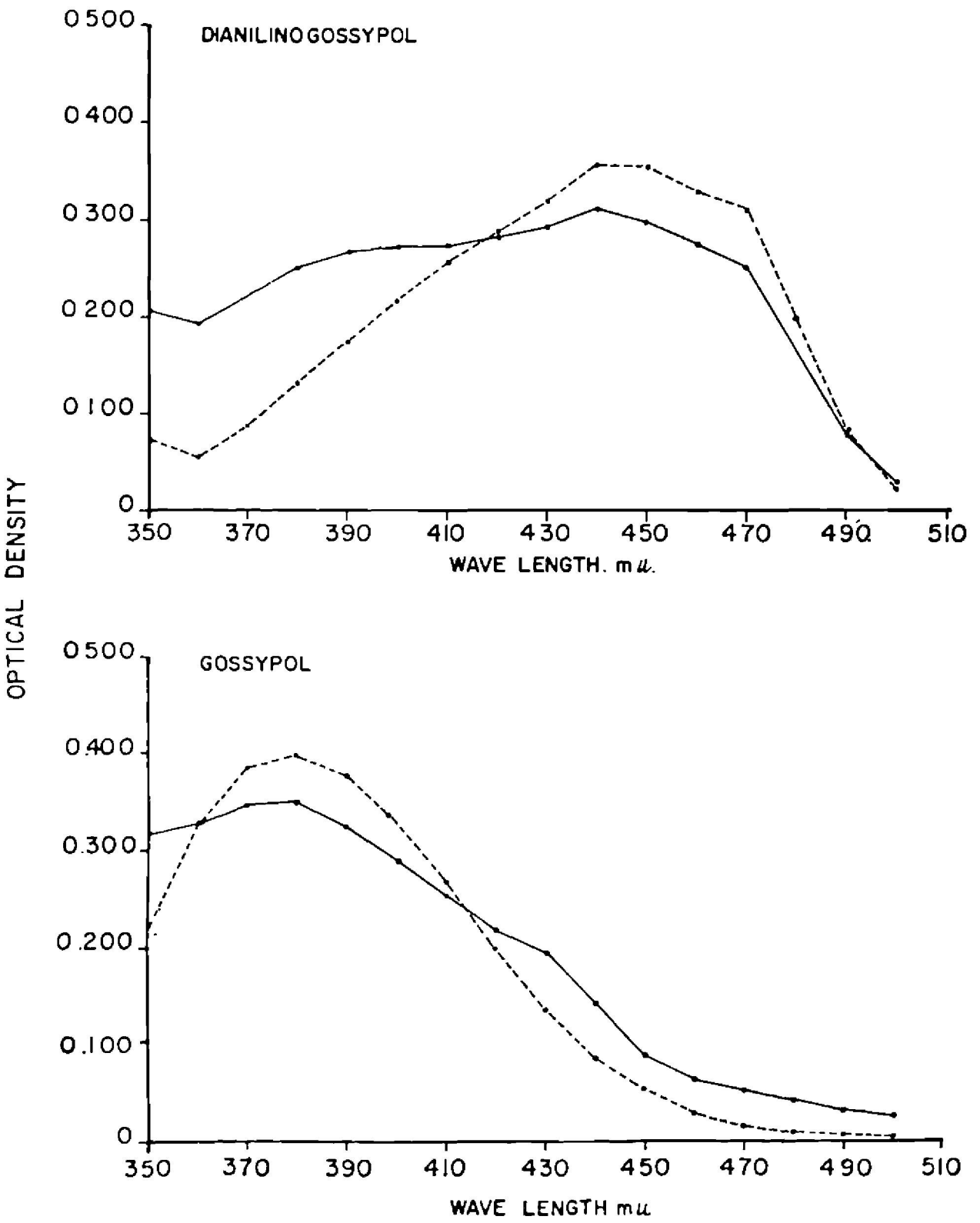

Fig. 1 Acetone-water extracts of dog feces. —— feces extract; - - - - pure gossypol.

TABLE 1

Gossypol intake and fecal excretion of dogs fed a cottonseed-corn mixture cooked for several periods of time [1]

Gossypol [2]	Cooking time,[3] min				
	0	8	16	24	0
Intake					
Free (A)	31	30	26	22	34
Total	709	689	704	715	780
Fecal					
Free (B)	108	91	86	83	103
Total	703	687	757	735	805
Free B/A	3.48	3.04	3.31	3.78	3.03

[1] Average of 3 dogs.
[2] Mg/dog/day.
[3] Each cooking period was tested in feeding tests lasting 7 days.

with aniline. Both curves show a peak of maximum absorption at 440 mμ. The lower part of the figure shows the curves of the acetone-water extracts read directly in the spectrophotometer. Both curves show a peak of maximal absorption at 370 to 380 mμ. Figure 2 shows the absorption curve of gossypol extract chromatographed on a silicic acid column. Curves A and B corresponding to the chloroform eluates and curve D corresponding to pure gossypol, gave a peak of maximal absorption of 370 to 380 mμ. Figure 3 shows the absorption curves of the feces extract chromatographed on Florisil, eluted with different solvents and reacted with aniline. All the solutions studied gave a maximal peak of absorption at 440 mμ. Figure 4 shows the absorption curves of the phloroglucinol derivatives of pure gossypol, and of the feces extract. The maximal absorption for both solutions was at 550 mμ. Further evidence showing that the largest amount of gossypol ingested is present in

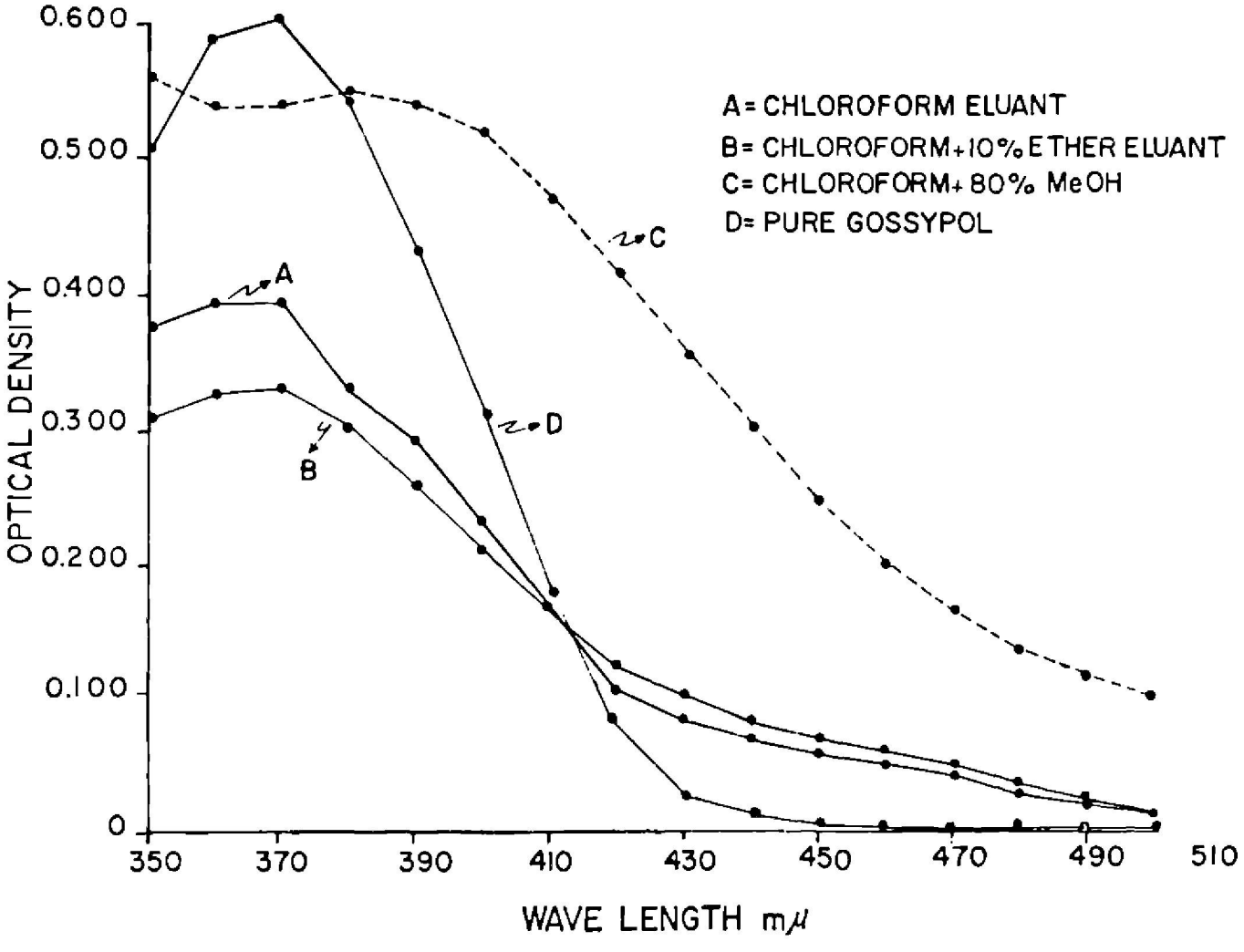

Fig. 2 Gossypol extracts from dog feces. A = chloroform eluant; B = chloroform + 10% ether eluant; C = chloroform + 80% MeOH; D = pure gossypol.

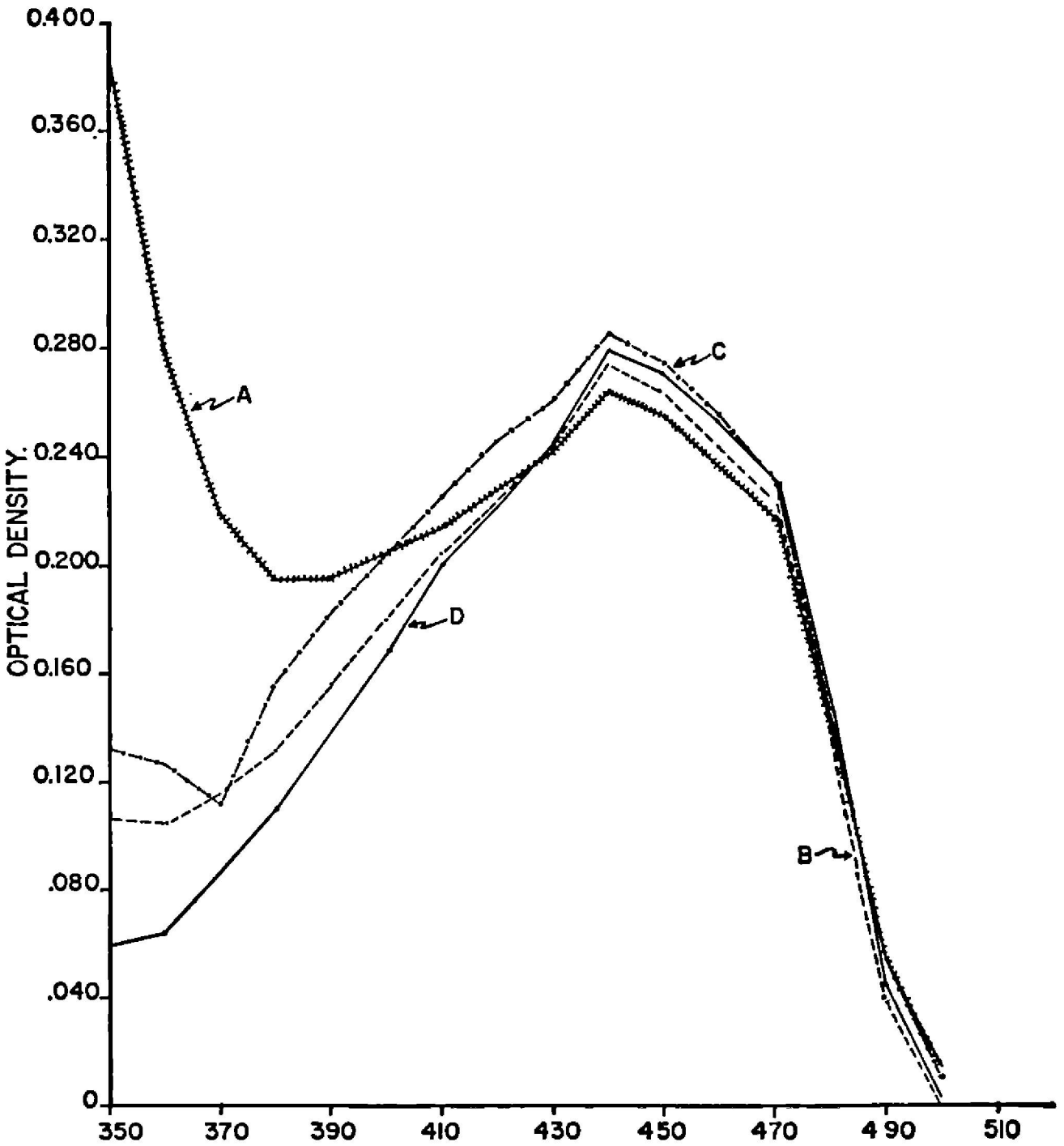

Fig. 3 Dianilino gossypol derivatives of $CHCl_3$ feces extract absorbed on Florisil. -|-|-|-|- Florisil eluant $CHCl_3$ (A); – – – – Florisil eluant $CHCl_3$ (B); –·–·–·– original extract in $CHCl_3$ (C); ——— pure gossypol in $CHCl_3$ (D).

the feces is indicated in figure 5. In this experiment, 4 dogs used previously were fed a casein diet for 4 days, followed by the casein diet plus 100 mg of gossypol daily, to 2 dogs from crude cottonseed oil and to the other two from ground cottonseed, for 4 days and finally, the casein diet. The feces were collected daily and analyses for gossypol were performed on freeze-dried samples from 2-day pools. The graph shows that when no gossypol was fed, little gossypol was present in the feces. A peak excretion of gossypol occurred 2 to 4 days after initial gossypol feeding and then decreased to low values when gossypol was withdrawn. On the basis of 400 mg of gossypol, about 75 to 80% was recovered in feces over the entire period. Only one dog gave zero fecal gossypol values at the end of the study. The results suggest a lag in gossypol excretion.

Table 2 shows the results of qualitative reactions of gossypol. All reactions carried out in the fecal extract gave positive results. The extract of the feces, and the feces were fed to hens and the eggs collected. After storage, eggs from hens fed pure gossypol, the fecal extract, a fraction from the silicic acid column and the dry feces showed discoloration of the egg yolk.

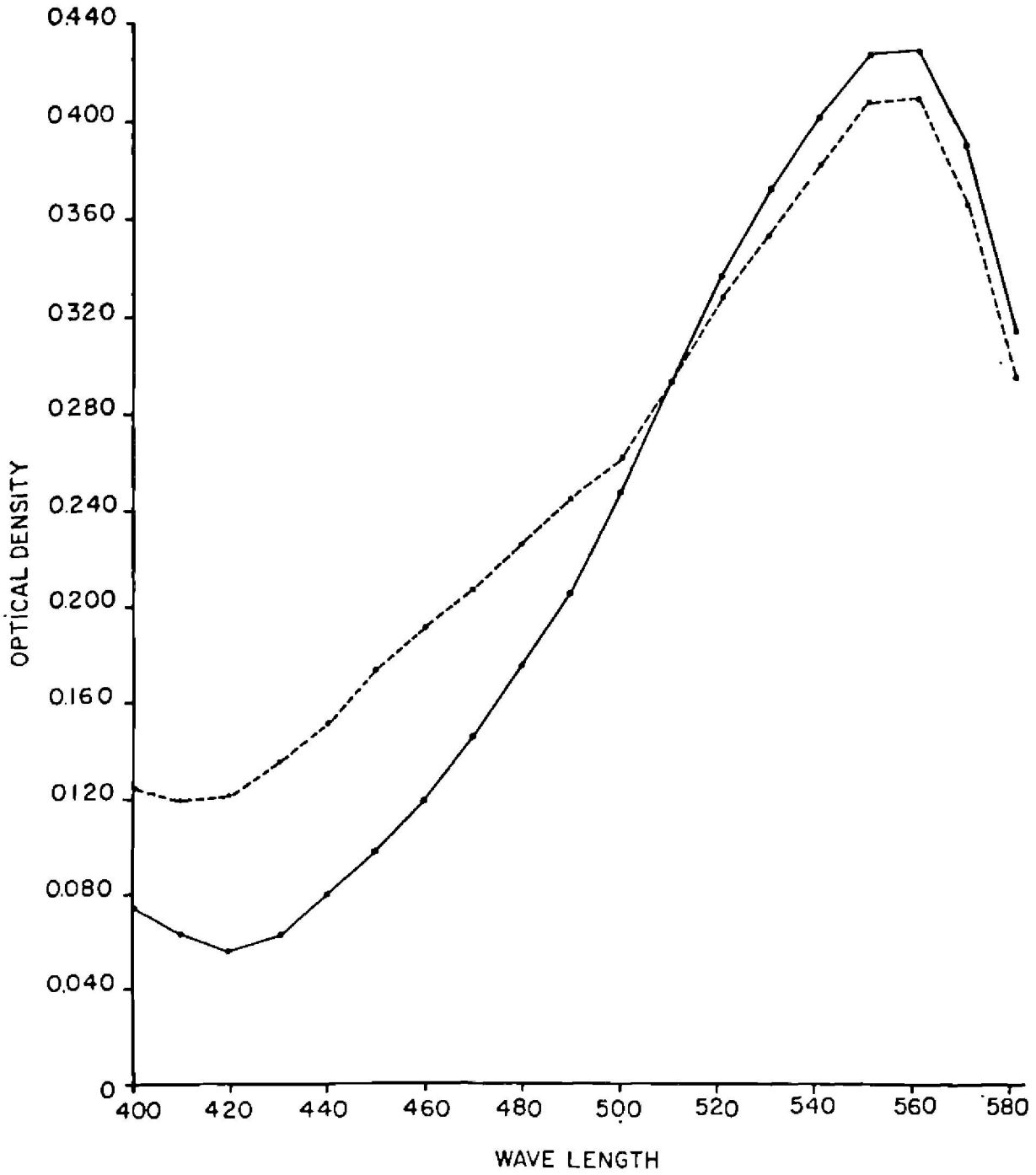

Fig. 4 Phloroglucinol derivative of feces extract and pure gossypol. —— pure gossypol; – – – – feces extract $CHCl_3$ eluant.

TABLE 2

Qualitative reactions on feces and fecal extracts of dogs fed cottonseed flour

Test	Reaction
Fecal extract	
$+NaOH+H_2O_2$	+
+ aniline	+
+ phloroglucinol	+
$+SbCl_3$	+
fed to hen	egg yolk discoloration
Feces fed to hen	egg yolk discoloration

Table 3 presents the results of the experiment where 3 types of fat were tested to learn their effect on gossypol absorption. The excretion of free gossypol was essentially the same with any of the fat used and it was, as in previous studies, about 3.4 times greater than the intake. Total gossypol excretion was 83% of the intake when hydrogenated vegetable fat was fed. It was, however, totally excreted when butter fat or refined cottonseed oil were fed. Nitrogen retention was better when the fat used was purified cottonseed oil.

Several possible factors studied to learn the mechanism by which gossypol was being excreted, are summarized in table 4. One variable studied was a diet with all calories coming from carbohydrate as against one in which part of the calories was contributed by fat. These treatments were tested at 2 levels of intake of calories, 140 and 85 kcal/kg body wt/day, respectively. The results, however, indi-

TABLE 3

Effect of type of fat on nitrogen balance and gossypol excretion in dogs

Type of fat in diet	Nitrogen balance [1]				
	Intake	Fecal	Urine	Absorption	Retention
	mg/kg/day		*mg/kg/day*	*mg/kg/day*	
Hydrogenated vegetable fat	973	230	490	743	253
Butter fat	977	230	498	747	249
Refined cottonseed oil	976	216	477	760	283

Type of fat in diet	Period	Gossypol balance [2]				
		Free		Total		Fecal fat
		Intake	Excreted	Intake	Excreted	
		mg/kg/day		*mg/kg/day*		*mg/kg/day*
Hydrogenated vegetable fat	1	2.8	9.1	59.8	50.0	0.15
	2	2.8	9.2	60.1	49.8	0.18
Butter fat	1	2.7	8.9	46.8	47.9	0.16
	2	2.7	10.0	47.0	49.3	0.14
Cottonseed oil	1	2.8	8.8	47.2	44.8	0.11
	2	2.8	10.6	47.1	48.9	0.13

[1] Average of 4 dogs and 2 four-day balance periods.
[2] Average of 4 dogs.

TABLE 4

Effect of type and intake of calories and of gossypol feeding on fecal content of free and total gossypol [1]

Treatment	Intake of calories	Free gossypol		Total gossypol	
		Intake	Fecal	Intake	Fecal
	kcal	*mg/kg/day*		*mg/kg/day*	
All calories from CHO	140	3.3 [2]	14.0	87.3	77.9
10% Calories from fat	140	3.3 [2]	14.0	87.6	73.9
All calories from CHO	85	2.7 [2]	11.3	65.7	50.4
10% Calories from fat	85	2.7 [2]	11.9	65.9	49.3
Cooking in metal container	110	1.9 [3]	4.6	63.4	45.4
Cooking in glass container	110	2.1 [3]	6.2	60.7	49.5
With gossypol	140	6.5 [3]	16.6	89.3	84.5
Without gossypol	140	2.7 [3]	14.8	85.2	70.7

[1] Protein intake: 6 g/kg/day.
[2] Average of 4 dogs and 2 four-day balance periods.
[3] Average of 3 dogs and 2 four-day balance periods.

cated no difference in excretion of free and total gossypol between the 2 treatments within intake of calories. The recoveries of ingested total gossypol at the 140- and 85-kcal intake levels was 87 and 76%, respectively. In another study, the food mixture containing cottonseed flour was cooked for 10 minutes in a glass container in one case, and in a metal container in another. This was done in view of previous results[7] which indicated that iron decreases gossypol content in cottonseed flour-containing mixtures. The results were again not different between treatments and about 78% of the ingested total gossypol was recovered in the feces. As in previous experiments, the amount of free gossypol in feces was about 4 times the amount ingested. The table also shows that addition of gossypol to the diet resulted in an increase of gossypol in the feces.

[7] See footnote 5.

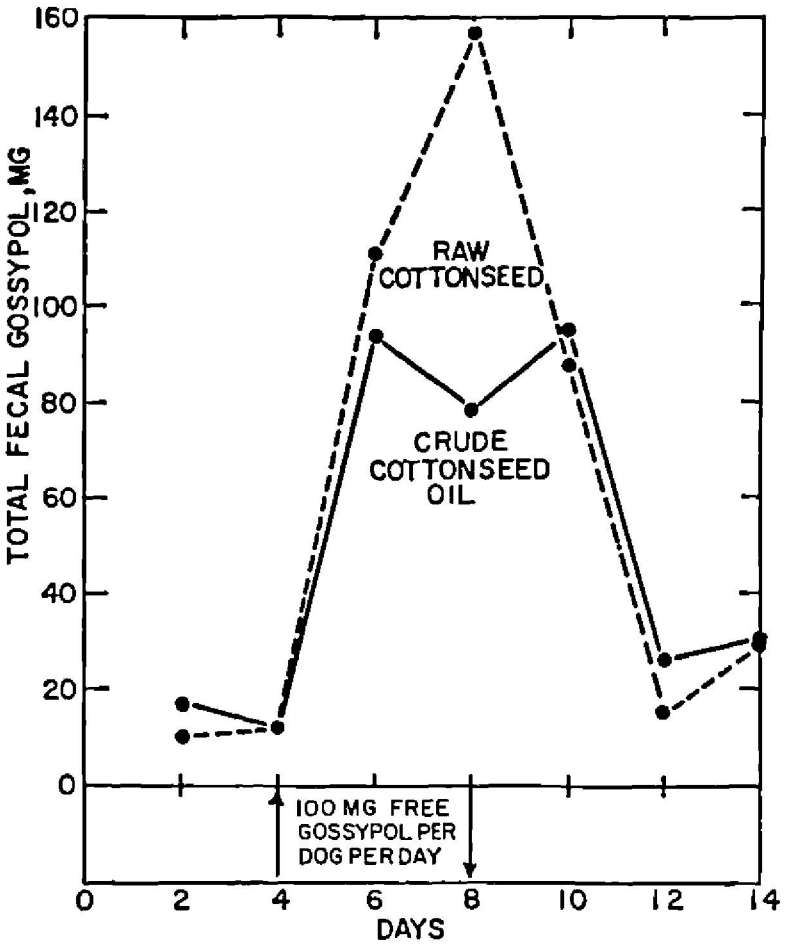

Fig. 5 Fecal excretion of gossypol in dogs fed a casein diet and free gossypol (average 2 dogs/group). —— Crude cottonseed oil; – – – – raw cottonseed.

DISCUSSION

During the time the problem of cottonseed and gossypol has been studied, it became apparent that not all monogastric animals have the same degree of susceptibility towards gossypol (1, 2). Although different animals may have different detoxification mechanisms, it is possible that a reason for differences in resistance to gossypol toxicity is the amount of gossypol absorbed by the animal through the intestinal tract.

The results of the present study indicate that in the dog and under the conditions used, the cottonseed pigment gossypol is excreted in the feces. The excretion is in the majority of the cases quantitative for total gossypol, whereas more free gossypol appears in the feces when compared with intake. At present, the only available explanation may be that certain compounds of gossypol are susceptible of being hydrolyzed in the intestinal tract, therefore resulting in higher values for free gossypol. That the higher values are not entirely due to an error in the method of analysis due to contamination is indicated by: a) the almost quantitative relationship between the amounts of total gossypol in food and feces, b) by low optical density readings of fecal extracts of dogs fed diets without cottonseed and subsequent increases when either cottonseed flour, crude cottonseed oil and pure gossypol are given, and c) by the maximal absorption curves of the fecal extracts and derivatives which were the same as those from pure gossypol and its derivatives. Furthermore, the pigment isolated from the feces is gossypol as indicated from the last point mentioned, as well as from the qualitative and biological tests which were positive for gossypol.

Holley et al. (13) reported on the toxicity of cottonseed flour and of gossypol in rabbits. It was indicated that gossypol was absorbed slowly and that the habit of coprophagy apparently increased, by some unknown mechanism, the sensitivity of the rabbit of gossypol.

The results presented on some of the possible factors which could affect excretion of gossypol indicated that no difference was found between treatments and that most of the gossypol ingested was in the feces. Therefore, it is not possible at this time to suggest a mechanism or conditions which would cause gossypol to be totally excreted. At this time, 3 possibilities can be offered. One is that the amounts of gossypol ingested are small, thus easily not absorbed. A second one is that during cooking, gossypol reacts with organic compounds or iron salts forming high molecular weight substances or chelates which, because of their size, are not easily absorbed through the intestinal tract. These high molecular weight compounds or chelates are decomposed later in the large intestine, resulting in fecal levels of free gossypol in food slightly larger than intakes. In all studies so far carried out, the relationship between free gossypol in food and feces is about 3.3 to 3.7 times greater in feces, and that in several studies the difference between the amount of total gossypol in feces is about 10 to 15 mg less in feces than in intake. And the third possibility, which is now being studied is that the gossypol is absorbed, transported to the liver and excreted with the bile into the intestinal tract. This mechanism could explain the higher free gossypol as well as

the slightly lower total gossypol values observed in feces as compared with the intakes of the 2 forms of the pigment. The results presented here are of practical interest, particularly if the conditions favoring excretion of gossypol are defined, so that they can be applied to other animals and increase the use of cottonseed flour in both human and animal feeding.

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