Biological Studies with Cyclopropenoids Inactivated with Fatty Acids[1]

D.T. HOPKINS, R.R. DAHLGREN, D. DAVIS and A.W. MUNSON, Ralston Purina Company, St. Louis, Missouri 63188, and H.P. DUPUY, Southern Regional Research Laboratory, New Orleans, Louisiana 70119[2]

ABSTRACT

Cyclopropenoids inactivated by reacting *Sterculia foetida* oil with cottonseed oil fatty acids were fed at three dietary levels to growing rats and laying hens for 4 weeks. At the termination of the experiments, all animals were autopsied and examined microscopically for pathological lesions, but no pathology that could be related to dietary treatment was observed. Hemoglobin, packed cell volume and plasma cholesterol were similar in animals fed all of the diets. Growth rate of rats and egg production of hens fed the experimental diets were similar to those of animals fed the control diet. After 3 and 6 months of storage, eggs from hens fed the inactivated cyclopropenoids were normal and showed no evidence of the unusual characteristics of cyclopropenoid feeding. Lipids of heart, liver and adipose tissues of all the rats and hens varied little from the normal fatty acid composition. Small amounts of three unidentified fatty acids were found in the adipose tissues of rats fed the higher levels of inactivated cyclopropenoids. The results of these feeding studies suggest that inactivation of cyclopropenoids with fatty acids eliminates the unusual biological effects attributable to cyclopropenoids.

INTRODUCTION

Cyclopropenoid fatty acids, found in the triglycerides of certain oils, cause biological effects when included in the rations of animals. The anomalous effects, which were reviewed in detail by Phelps et al. (1), include reduced egg production and decreased hatchability of fertile eggs from laying hens. The albumin of eggs stored for 3 months from hens fed cyclopropenoids turns pink, and the pH of the albumin decreases while the pH of the yolk increases. Dietary cyclopropenoids decrease the growth and sexual development of rats.

Cottonseed oil, the only edible oil of commercial importance containing cyclopropenoids, has levels of up to 0.5% of these materials in the form of esters of malvalic and sterculic acids. Because of their unusual and undesirable effects, it is important that cyclopropenoids be inactivated or eliminated from cottonseed oils. Workers at the Southern Utilization Research and Development Division (2,3) showed that cyclopropenoids in cottonseed oil could be inactivated by heating the oil in the presence of cottonseed oil fatty acids. Presumably the fatty acids "inactivate" the cyclopropenoids by rupturing the ring and forming a variety of ester linkages (4,5).

The studies reported here were conducted to determine if animals fed high levels of cyclopropenoids inactivated with fatty acids were free of the symptoms associated with the feeding of cyclopropenoids and also free of other anomalous effects.

In order to test the biological effects of inactivated cyclopropenoids at high levels, *Sterculia foetida* oil was treated with cottonseed oil fatty acids, and the reaction products were fed to weanling rats and laying hens at levels of 0, 0.14, 0.71 and 3.56%.

EXPERIMENTAL PROCEDURES

Inactivation of Cyclopropenoids

Two parts of alkali-refined *Sterculia foetida* oil, which contained 52% combined sterculic and malvalic acids (6), were added to one part of cottonseed oil fatty acids in a laboratory-type deodorizer (2). The mixture was heated from room temperature to 235 C within 30 min and kept at 235 C for 1 hr while sparging with nitrogen. Heating was continued for an additional 30 min while sparging with steam and gradually applying a vacuum, until the final pressure was between 2 and 5 mm of mercury. The final product had a negative response to the Halphen test (7).

Analysis of Inactivated Cyclopropenoids

The Halphen-negative *Sterculia foetida* oil was interesterified with methanol, and the methyl esters of the normal fatty acids were separated from the methyl esters of the inactivated cyclopropenoid acids by alumina column chromatography. The esters of the normal fatty acids were eluted with 5% ethyl ether in pentane and the esters of the inactivated cyclopropenoid acids with chloroform. Both fractions were reduced to a suitable concentration, and an appropriate amount of an internal standard was added. The samples were analyzed by gas liquid chromatography on 3 ft x 0.125 in. stainless steel tubing packed with 15% stabilized DEGS on Anakrom A. The column was heated isothermally at 190 C (6).

Animal Feeding Studies

Four groups of five male and five female weanling rats of the Wistar Strain (Manor Farms North, Staatsburg, N.Y.) were caged individually in a sanitary air-conditioned animal room. Each group of rats was fed different experimental rations ad libitum for 4 weeks. These rations were prepared by supplementing the basal ration with 0, 0.14, 0.71 and 3.57%, respectively, of Halphen-negative *Sterculia foetida* oil, which was added to the diet at the expense of an equal number of sucrose calories to maintain a constant ratio of

TABLE I

Fatty Acid Composition of Inactivated *Sterculia foetida* Oil Measured by Gas Liquid Chromatography of Methyl Esters

ECL[a]	Fatty acid	%
14.0	Myristic	0.6
16.0	Palmitic	25.4
18.0	Stearic	0.6
18.5	Linoleic	16.3
19.3	Linolenic	21.2
20.4	Arachidonic	2.1
21.6	Inactivated cyclopropenoids[b]	1.6
23.1	Inactivated cyclopropenoids[b]	1.9
24.1	Inactivated cyclopropenoids[b]	17.5
25.0	Inactivated cyclopropenoids[b]	0.4
26.4	Inactivated cyclopropenoids[b]	0.7
26.9	Inactivated cyclopropenoids[b]	1.7
26.9	Inactivated cyclopropenoids[b]	6.3

[a]Equivalent chain length.

[b]Total inactivated cyclopropenoids, 30.1%.

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[2]S. Util. Res. Dev. Div., ARS, USDA.

TABLE II

Effect of Feeding Growing Rats *Sterculia foetida* Oil Treated to Inactivate Cyclopropenoids

Measurement	Dietary level of oil, %				Standard error of mean
	0	0.14	0.71	3.57	
Rats started	10	10	10	10	
Rats died	0	1	0	0	
Weight gain, g	135	130	128	134	5.0
Total feed consumption, g	458	432	441	430	11.3
Blood hemoglobin, g/100 ml	14.2	14.2	14.0	13.6	0.26
Packed cell volume, %	46	46	44	44	0.7
Plasma cholesterol, mg/100 ml	214	185	175	256	15.3
Total liver fat[a]	12	17	15	16	0.5
Liver wt, %[b]	4.7	4.6	4.9	5.0	0.28
Heart wt, %[b]	0.38	0.39	0.40	0.38	0.024
Adrenals, wt %[c]	0.013	0.016	0.014	0.014	0.0010
Thyroids, wt %[c]	0.007	0.008	0.008	0.007	0.0004
Testes, wt %[c]	0.54	0.52	0.52	0.53	0.031
Ovaries, wt %[c]	0.037	0.038	0.035	0.036	0.0030

[a]% liver dry matter.

[b]Fresh organ weight as % live weight.

[c]Fixed organ weight as % live weight.

calories to all other nutrients.

The basal ration had the following percentage composition: sucrose, 21.02; ground yellow corn, 38.35; soybean meal (50% protein), 36.86; vitamin mixture, 0.78; mineral mixture, 2.71; DL-methionine, 0.18; and ethoxyquin (Santoquin, Monsanto Chemical Co., St. Louis, Mo.), 0.01. Each 10 g of vitamin mixture contained the following: vitamin A, 20,000 IU; vitamin D_3, 2000 ICU; vitamin E, 100 IU; menadione sodium bisulfite, 2 mg; thiamine HCl, 10 mg; riboflavin, 10 mg; niacin, 50 mg; pyridoxine HCl, 10 mg; calcium D-pantothenate, 20 mg; vitamin B_{12}, 10 mcg; folic acid, 2 mg; biotin, 0.5 mg; choline Cl, 1.5 g; and BHT, 20 mg. The composition of the mineral mixture was as follows: $CaHPO_4 \cdot 2H_2O$, 2.5 g; NaCl, 0.5 g; Fe citrate, 0.03 g; $MnCO_3$, 0.05 g; $ZnCO_3 \cdot Cu(OH)_2$, 2 mg; $NaIO_3$, 100 mcg; Na_2SeO_3, 10 mcg.

TABLE III

Fatty Acid Ratios of Lipids from Rats Fed *Sterculia foetida* Oil Treated to Inactivate Cyclopropenoids

Tissue lipid	Dietary level of oil, %			
	0	0.14	0.71	3.57
	Saturated-unsaturated			
Liver				
Liver	0.89	1.02	0.94	0.90
Abdominal adipose tissue	0.54	0.56	0.51	0.52
Epididymal fat pad	0.48	0.45	0.50	0.49
Heart	0.67	0.70	0.68	0.77
	Stearic-oleic			
Liver	1.29	1.56	1.72	2.13
Abdominal adipose tissue	0.09	0.10	0.10	0.13
Epididymal fat pad[a]	0.08	0.09	0.10	0.13
Heart	0.78	0.80	0.94	1.09
	Linoleic-oleic			
Liver	0.55	0.62	0.71	1.00
Abdominal adipose tissue	0.40	0.47	0.51	0.66

[a]Males only.

The basal ration had the following percentage composition: corn starch, 55.71; soybean meal (50% protein), 29.05; dehydrated alfalfa meal, 2.20; dried fish solubles, 0.44; mineral mixture, 8.06; vitamin mixture, 0.88; ground cellulose, 3.52; DL-methionine, 0.14; and ethoxyquin, 0.01. Each 10 g of vitamin mixture contained the fol-

Four groups of five White Leghorn pullets were caged individually in a sanitary air-conditioned animal room. Each group of hens was fed different experimental rations ad libitum for 4 weeks. These rations were prepared by supplementing the basal ration with 0, 0.14, 0.71, and 3.57%, respectively, of Halphen-negative *Sterculia foetida* oil, which was added to the diet at the expense of an equal number of starch calories.

TABLE IV

Fatty Acid Composition of Lipids of Selected Tissues of Rats Fed *Sterculia foetida* Oil Treated to Inactivate Cyclopropenoids

Organ	Fatty acid	Dietary level of oil, %			
		0.0	0.14	0.71	3.57
Liver	Myristic	0.9	0.8	0.4	0.4
	Palmitic	22.4	23.4	21.8	19.4
	Palmitoleic	4.0	3.6	2.5	1.9
	Stearic	23.7	26.3	26.4	27.7
	Oleic	18.4	16.8	15.3	13.0
	Linoleic	10.2	10.5	10.8	13.0
	Linolenic	0.05	Trace	0.05	0.2
	Arachidonic	20.5	18.6	22.8	24.4
Abdominal adipose tissue	Myristic	1.8	2.6	1.8	1.8
	Palmitic	29.6	29.9	28.0	28.4
	Palmitoleic	10.8	12.2	9.6	8.4
	Stearic	3.6	3.3	3.8	4.0
	Oleic	38.0	34.6	35.9	30.9
	Linoleic	15.3	16.4	18.5	20.4
	Linolenic	0.7	0.8	1.2	2.4
	Arachidonic	0.2	0.2	0.5	0.8
	Unknown	—	—	Trace	0.2
	Unknown	—	Trace	0.4	2.2
	Unknown	—	—	0.2	0.2

TABLE V

Effect of Feeding Laying Hens *Sterculia foetida* Oil Treated to Inactivate Cyclopropenoids

Measurement	Dietary level of oil, %				Standard error of mean
	0.0	0.14	0.71	3.57	
Hens started	5	5	5	5	
Feed consumed, g	2480	2620	2700	2470	163.9
Weight change, g	-51	-26	-1	-13	33.9
Eggs produced	21.6	21.6	20.8	19.4	1.7
Ave. egg wt, g	56	55	57	55	1.5
Yolk wt, g fresh	15.9	16.3	16.1	16.1	0.45
3 months	17.3	17.5	17.5	16.7	0.51
6 months	19.0	19.4	18.6	18.3	0.71
Yolk pH, fresh	6.2	6.1	6.1	6.2	0.06
3 months	6.5	6.4	6.5	6.4	0.06
6 months	6.5	6.6	6.6	6.7	0.10
Albumin pH, fresh	8.2	8.5	8.5	8.4	0.11
3 months	9.0	9.0	9.0	9.0	0.03
6 months	8.8	8.8	8.7	8.7	0.02
Blood hemoglobin, g/100 ml	8.2	8.4	8.0	8.3	0.38
Packed cell volume %	29	29	29	28	1.3
Plasma cholesterol, mg/100 ml	98	79	72	84	12.5
Total liver fat[a]	23	26	36	27	---
Liver wt, %[b]	2.2	2.1	2.3	2.2	0.12
Heart wt, %[b]	0.52	0.44	0.43	0.53	0.056
Adrenal wt, %[c]	0.006	0.007	0.005	0.006	0.0005
Thyroid wt, %[c]	0.005	0.005	0.005	0.005	0.0007

[a]Fat as % liver dry matter.

[b]Fresh organ weight as % live weight.

[c]Fixed organ weight as % live weight.

lowing: vitamin A, 15,000 IU; vitamin D_3, 1500 ICU; vitamin E, 20 IU; menadione sodium bisulfite, 2 mg; thiamine HCl, 10 mg; riboflavin, 10 mg; calcium D-pantothenate, 40 mg; niacin, 50 mg; pyridoxine HCl, 10 mg; biotin, 0.4 mg; folic acid, 2.0 mg; vitamin B_{12}, 50 mcg; choline Cl, 1.5 g; BHT, 20 mg. The composition of the mineral mixture was as follows: ground limestone, 6 kg; feed grade dibasic calcium phosphate, 2.5 kg; iodized salt, 0.4 kg; feed grade $MnSO_4$, 27 g; $ZnCO_3$, 10 g; $MgSO_4$, 200 g; $FeSO_4 \cdot 7H_2O$, 20 g; $CuSO_4 \cdot 5H_2O$, 2 g; $CoCl_2 \cdot 6H_2O$, 0.2 g; $Na_2MoO_4 \cdot H_2O$, 0.8 g.

Biological Studies

At the end of 4 weeks, the weight gain and feed consumption of each group of rats and the weight change, the feed consumption and egg production for each group of hens was determined. The average egg weight, yolk weight when fresh and after storage, and albumin pH when fresh and after storage were also determined. Blood samples were taken from the animals, and hemoglobin, packed cell volume and plasma cholesterol was determined. The cholesterol was determined by the method of Abell et al. (8). The animals were sacrificed and examined for gross pathological lesions. Heart and Liver weights were recorded. Portions of the liver, heart, aorta, kidney, adrenal gland, brain, adipose tissue, thyroid, lungs, spleen, pancreas, small intestine, stomach, reproductive organs, bladder and skin were saved. The adrenal glands, thyroids and reproductive organs (rats only) were weighed after fixing in neutral formalin. Samples of liver, heart, abdominal and epididymal fat pads from rats, and liver, heart and abdominal fat from hens were collected for determination of fatty acid composition. The samples were frozen in liquid nitrogen immediately after collection and stored in a freezer. At a convenient time, the tissues were lyophilized prior to biochemical analysis. Liver fat was determined by extracting lyophilized liver for 24 hr on a Goldfisch extraction apparatus with chloroform-methanol 2:1 v/v. The solvent was removed under vacuum and the residue weighed. The methyl esters of the lipids were prepared and analyzed by gas liquid chromatography after separation by alumina column chromatography as described above (6).

RESULTS AND DISCUSSION

The cyclopropenoid moiety in *Sterculia foetida* oil was readily inactivated by heating in the presence of cottonseed oil fatty acids as indicated by a negative response to the

TABLE VI

Fatty Acid Ratios of Lipids from Hens Fed *Sterculia foetida* Oil Treated to Inactivate Cyclopropenoids

Source of lipid	Level of treated *Sterculia foetida* oil, %			
	0	0.14	0.71	3.57
	Saturated-unsaturated			
Liver	0.42	0.46	0.43	0.43
Eggs	0.61	0.59	0.63	0.62
Abdominal adipose	0.42	0.42	0.41	0.41
Heart	0.74	0.69	0.72	0.77
	Stearic-oleic			
Liver	0.17	0.18	0.16	0.17
Eggs	0.19	0.19	0.22	0.30
Abdominal adipose	0.17	0.15	0.13	0.15
Heart	0.37	0.28	0.35	0.37

TABLE VII

Fatty Acid Composition of Lipids of Eggs and Livers from Hens Fed *Sterculia foetida* Oil Treated to Inactivate Cyclopropenoids

Lipid source	Fatty acid	Dietary level of oil, %			
		0.0	0.14	0.71	3.57
Liver	Myristic	0.8	0.9	0.8	0.8
	Palmitic	20.9	22.4	21.8	21.8
	Palmitoleic	5.8	5.7	6.2	6.2
	Stearic	7.7	8.2	7.6	7.6
	Oleic	44.2	44.6	46.3	45.1
	Linoleic	19.4	17.3	16.4	17.9
	Linolenic	0.3	0.3	0.4	Trace
	Arachidonic	0.9	0.6	0.5	0.6
Egg	Myristic	0.4	0.4	0.4	0.6
	Palmitic	28.7	27.5	28.8	28.1
	Palmitoleic	5.8	5.4	4.3	2.6
	Stearic	8.7	9.3	9.6	9.6
	Oleic	46.4	47.9	42.6	31.7
	Linoleic	8.0	7.4	12.3	24.9
	Linolenic	0.4	0.4	0.5	0.5
	Arachidonic	1.6	1.7	1.5	2.0

Halphen test. After methanolysis, the esters of the normal fatty acids were separated from the esters of the inactivated cyclopropenoid acids by alumina column chromatography and analyzed by gas liquid chromatography. As shown in Table I, the inactivated cyclopropenoid acids comprised ca. 30% of the Halphen-negative *Sterculia foetida* oil. Use of an internal standard indicated that only 88% of the material injected into the chromatograph was being measured by the detector. Furthermore calculations based on the original cyclopropenoid content of the oil indicated that the amount of inactivated material should be 35%. The inactivated oil was fed to four groups of weanling rats and four groups of laying hens at levels of 0, 0.14, 0.71 and 3.57%, respectively. Since cyclopropenoid acids are found in refined cottonseed oil at levels of up to 0.5%, the dietary levels of these inactivated cyclopropenoid acids represent the equivalent of feeding inactivated cottonseed oil (cyclopropenoid moiety inactivated by heating in the presence of cottonseed acids) at levels of 0, 10, 50 and 250%, respectively.

As shown in Table II, the feeding of these high levels of inactivated cyclopropenoid material had no effect on weight gain, feed consumption or mortality of growing rats. Measurements of blood hemoglobin, packed cell volume and plasma cholesterol revealed no differences that could be attributed to dietary treatment. The rats fed the control diet had lower levels of liver fat than the rats fed any of the levels of inactivated *Sterculia foetida* oil. However, since the liver fat did not increase as the dietary level of inactivated cyclopropenoid material increased, this observation appears to be a reflection of normal biological variation. None of the dietary treatments affected the weights of the livers, hearts, adrenals, thyroids, testes or ovaries. Necropsy and microscopic examination of tissues revealed that there were no pathological lesions relating to the dietary treatments. Male and female rats responded similarly to the dietary treatments.

As shown in Table III, the dietary treatments had some effects on the fatty acid composition of the abdominal adipose tissues, epididymal fat pads, liver and heart lipids. The ratio of total saturated to total unsaturated fatty acids was essentially the same, but there was some increase in the ratio of stearic acid to oleic acid as the dietary level of inactivated *Sterculia foetida* oil increased. As shown in Table IV, however, this shift in fatty acid ratio appears to result chiefly from linoleic acid replacing oleic acid rather than stearic acid replacing oleic acid, as has generally been shown to occur with the feeding of cyclopropenoids. Lipid analysis by gas liquid chromatography revealed that long chain fatty acids with equivalent chain lengths (ECL) of 23.1, 24.1 and 26.1 were found only in the lipids of the abdominal adipose tissues and the epididymal fat pads of the rats fed the two higher dietary levels of inactivated *Sterculia foetida* oil. Similar levels of the long chain fatty acids were found in both abdominal and epididymal fat pads. The concentration of the long chain fatty acids with ECL of 23.1 and 24.1 appeared to increase with the higher dietary level of inactivated *Sterculia foetida* oil, and their ECL was similar to the ECL of two of the long chain fatty acids found in the inactivated *Sterculia foetida* oil. The unusual long chain fatty acids were not found in the lipids of the heart or liver, the only other organs examined for their fatty acid composition.

As shown in Table V, the feeding of high levels of inactivated cyclopropenoid material to laying hens had no effect on feed consumption, weight gain, egg production or average egg weight. Some of the eggs collected during the fourth week were examined fresh and others were examined after storage at 2 C for 3 or 6 months. None of the dietary treatments appeared to produce any unusual biological effects commonly associated with the feeding of cyclopropenoid material to laying hens. Even after storage for 6 months, the eggs appeared normal. There were no changes in the weight of the yolks or in the pH of the yolks or albumin. No pink discoloration of the whites or mottled yolks were observed.

Gross and microscopic examination of tissues revealed that there were no pathological lesions relating to the dietary treatments. The different dietary treatments did not affect organ weights, hemoglobin, packed cell volume, plasma cholesterol and liver fat.

As shown in Table VI, the dietary treatments did not appear to affect the fatty acid composition of abdominal adipose tissues, liver and heart lipids, but the fatty acid composition of the egg lipids was slightly affected. The ratio of total saturated to total unsaturated fatty acids was similar, but there was an increase in the ratio of stearic acid to oleic acid at the higher dietary levels of inactivated *Sterculia foetida* oil. As shown in Table VII, however, this shift in fatty acid ratio appears to result from linoleic acid replacing oleic acid rather than stearic acid replacing oleic acid. Lipid analysis revealed mere traces of unidentified fatty acids in adipose tissues and egg lipids of the hens fed the higher dietary levels of inactivated *Sterculia foetida* oil.

As little as 1% of *Sterculia foetida* oil in the diet of growing rats resulted in reduced growth rate and a significant increase of saturated fatty acids in the epididymal fat pads (9). Even as little as 0.02% of *Sterculia foetida* oil in the diet of laying hens produced biological effects—increased saturated fatty acids in egg yolk lipids, pink-white discoloration and mottled yolk in stored eggs as well as pH changes of stored egg yolks and whites (1,10). As described above, however, as much as 3.57% of inactivated *Sterculia foetida* oil in the diets of growing rats and laying hens did not result in these biological effects. Consequently the above studies would indicate that it should be safe to inactivate the small concentration of cyclopropenoid acids in cottonseed oil with cottonseed acids during or prior to deodorization. The practicality of such a method of treating cottonseed oil has been demonstrated by Eaves et al. (3), who pointed out that cyclopropenoid deactivation can be carried out by in situ reaction during deodorization of the oil.

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