Metabolism of Gossypol in the Chick

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Abstract

^{14}C labeled gossypol was administered to young chickens and the deposition of gossypol plus gossypol decomposition products in the various tissues was evaluated by determination of ^{14}C activity in a scintillation spectrometer. Most of the gossypol activity was recovered in the feces with relatively smaller amounts retained by the tissues. Most of the labeled compound retained was found in the liver, muscle, blood and kidneys, with the highest concentration in the liver. Increasing the protein content of the diet by the addition of fish meal reduced the amount of gossypol plus gossypol decomposition products found in the tissues.

Introduction

The unfavorable physiological effects of gossypol have been known for many years (1,2). More recently the literature has been reviewed by Eagle (3). However, little information is available concerning the metabolic fate of ingested gossypol.

Smith (4) isolated and identified gossypol from livers of pigs fed rations containing high levels of free gossypol and subsequently developed chemical methods for the determination of both free and bound gossypol in swine tissues (5). These methods were used to study factors which affect the deposition of gossypol in swine liver tissue (6).

No report was found in the literature concerning the extent to which gossypol present in poultry rations is absorbed from the digestive tract and retained in the tissues of chickens. The purpose of this communication is to present the results of an investigation of this problem by the use of formyl ^{14}C labeled gossypol. Since the measurement of ^{14}C activity was the parameter of the investigation, the results include both gossypol and metabolic products of gossypol.

Since the livers of chickens fed formyl-^{14}C-gossypol represented the tissue of highest ^{14}C activity, a fractionation scheme was devised which would extract free gossypol, gossypol metabolites of varying solubilities and finally protein bound gossypol or metabolites of protein bound gossypol. A silicic acid column chromatographic technique was used in the separation of compounds in each extract. ^{14}C activity was determined, and ultraviolet and visible absorption spectra were obtained for the various fractions.

Experimental Procedures

Formyl ^{14}C labeled gossypol was administered in gelatin capsules to 10 chickens weighing 500 to 600 g. Approximately 3.5 mg of gossypol (accurately weighed) was given on each of four consecutive days. The total activity of the four doses was approximately 1 μc. The feces were collected on the day following the administration of each capsule and on an additional day, totaling five collections for each chicken. The animals were killed on the sixth day and the organs removed for the determination of ^{14}C activity.

The chickens used in these tests were raised on a practical chick starter all mash ration. On the day that the first gossypol capsule was administered five chickens were changed to a ration prepared by mixing the practical ration, half and half, with fish meal giving a mixture which contained 42% protein. This was done in order to determine whether a high protein ration would affect gossypol retention by the tissues.

Preparation of Formyl ^{14}C Labeled Gossypol

The general procedure was to prepare apogossypol from purified gossypol by removal of the formyl group with NaOH. Clark (7). Apogossypol was then converted to labeled dianilino gossypol by reaction with N-N^1 diphenyl formamidine-1-^{14}C. The dianilino gossypol was hydrolyzed with H_2SO_4 and gossypol labeled in the formyl position was isolated as gossypol-acetic acid.

This synthesis without experimental details was originally reported by Edwards (8). The details of the preparation of the labeled gossypol used in this investigation were as follows:

Gossypol. The gossypol used as the starting material was isolated from cottonseed and purified as gossypol-acetic acid (9).

Conversion of Gossypol-Acetic Acid to Apogossypol. Five milliliters of 40% of NaOH were added to 1 g of gossypol-acetic acid in a broad test tube. One tenth to 0.15 g of sodium hydrosulfite were then added in such a way that part of it was spread on the sidewalls of the test tube and part was added to the reaction mixture. The hydrosulfite prevents oxidation which otherwise turns the preparation a dark purple color. When this occurs, low yields of an impure product are obtained.

The reaction mixture was heated on a steam bath for 30 min with occasional stirring, after which it was cooled and ice added directly to the preparation. On acidifying with concentrated H_2SO_4 (just acid to litmus), apogossypol precipitated as a slightly yellowish-white solid. It was immediately taken up in ether (peroxide free). The ethereal solution was washed, dried over anhydrous sodium sulfate and evaporated at reduced pressure. The yellowish-brownish white solid which was obtained was dissolved in 2–3 ml of benzene and precipitated by the addition of petroleum ether. The precipitate was filtered in a Hirsh funnel and when almost all of the solvent was removed, the wet preparation was transferred to a flask, and the remaining solvent was evaporated under reduced pressure. This precaution is necessary in order to avoid oxidation. Yield 0.65–0.70 g.

Preparation of N-N^1-Diphenyl Formamidine 1-^{14}C. A mixture of 1-^{14}C-sodium formate (4 mc. Sp. Activity 3.5 mc/mM), 8 ml of formic acid (98–100%) and 0.06 ml of 85% phosphoric acid was allowed to stand at room temperature in a round bottom flask for 4 hr. Forty-six milliliters of aniline were then added. After fitting the flask with a distilling head and a condenser, the reaction mixture was heated at 150 C for 3 hr during which time the water produced by the reaction distilled off. At the end of this period the temperature was slowly raised to 240–250 C when about 20–22 ml of aniline distilled off. On refriger-

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TABLE I
Recovery of ^{14}C Activity From Labeled Gossypol After Combustion in the Schoniger Flask[a]

Sample No.	Added ^{14}C gossypol d/m	Recovered ^{14}C d/m	Recovery %
1	1811	1896	104.7
2	1811	1866	103.0
3	1811	1804	99.6
4	1811	1776	98.0
5	1811	1820	100.5
6	1811	1722	95.5
7	1811	1776	98.0
8	1811	1818	100.4
9	1811	1846	101.9
10	1811	1696	93.6
		Ave.	99.5

[a] Counting time: 30 min.

ating the resulting thick syrupy liquid overnight, yellowish white N-N^1-diphenyl formamidine 1-^{14}C was obtained. The product was recrystallized from ethanol. Light orange long needles were obtained. Yield 4.5 g mp 136–137 C. The original filtrate on further refrigeration for 24 hr gave an additional 0.9 g of N-N^1-diphenyl formamidine 1-^{14}C.

Preparation of Labeled Dianilinogossypol. Seven tenths of a gram of freshly prepared and thoroughly dried apogossypol were mixed with 1.8 g N-N^1-diphenyl formamidine-1-^{14}C and heated under reduced pressure (160–170 mm) at 170–175 C for 3 hr in a flask fitted with a water condenser. The reaction mixture was cooled, treated with 3–4 ml of acetone and allowed to stand overnight. Dianilinogossypol crystallized out as dark orange crystals. The product was filtered, washed with 2–3 ml of acetone and dried. For further purification, the crystals were finely powdered and treated with boiling acetone (5–7 ml) for 10 min. Yield 0.5 g (50% based on weight of apogossypol) mp 295–298 C. A mixed melting point with an authentic sample of dianilinogossypol mp 300–303 C (prepared from gossypol-acetic acid and aniline) showed no depression and a comparison of the infrared spectra showed complete identity of the compounds. (This purity of dianilinogossypol mp 295–298 C) was found to be satisfactory for further hydrolysis to gossypol-acetic acid.)

Hydrolysis of Labeled Dianilinogossypol: Isolation of Labeled Gossypol Acetic Acid. One and five tenths of a gram of labeled dianilinogossypol were suspended in a mixture of 22.5 ml of peroxide free ethyl ether and 22.5 ml of glacial acetic acid. The mixture was cooled to 18–20 C in an ice bath and 2.7 ml of concentrated H_2SO_4 were added with constant stirring. After 2 min 6 ml of distilled water were added and the mixture allowed to stand for 10–15 min when gossypol-acetic acid crystallized out. (At this stage the color of the precipitated gossypol-acetic acid was greenish rather than the expected deep yellow. Approximately 15 mg of sodium hydrosulfite were added to the reaction mixture when immediately the color changed to a deep yellow.) The product was filtered and washed well with distilled water.

The crude gossypol-acetic acid was dissolved in 25 ml of ethyl ether and washed twice in a separatory funnel with 50 ml of water containing 10–15 mg of sodium hydrosulfite. The ether layer was filtered to remove a very small amount of unhydrolyzed dianilinogossypol. An equal volume of glacial acetic acid was added and the mixture allowed to stand for 1 hr. The gossypol-acetic acid which precipitated was washed with a few drops of glacial acetic acid and then thoroughly with Skellysolve F. After recrystallization a second time as indicated above, pure gossypol-acetic acid, mp 188–184 C was obtained. Yield 0.815 g. A mixed melting point with an authentic sample showed no depression of the melting point. The IR spectra showed complete identity. Specific activity 38 μc/mM.

Preparation of Samples and Determination of ^{14}C Activity

^{14}C activity was determined by the use of a Packard Tri-Carb liquid scintillation spectrometer after preparation of the samples as described below.

Tissues. Samples of fresh tissues (100 mg) were prepared for combustion by drying on small squares of cellophane under infrared lamps. The dried samples were burned in Schöniger flasks in at atmosphere of oxygen and the CO_2 was collected in 10 ml of hyamine added after the combustion, as described by Buyske et al. (10) with minor modifications. After absorption of the CO_2, 2 ml aliquots were transferred directly to the scintillation vials. Fifteen milliliters of scintillation solvent [4 gm, 2,5-diphenyloxazole, scintillation grade PPO, and 100 mg, 1,4-bis-2(5 phenyloxazolyl)-benzene, scintillation grade POPOP, per liter of toluene] and phosphor were added and the disintegrations were counted in the scintillation spectrometer for a minimum of 1 hr.

Quenching in the scintillation counter due to colored material and water was determined for all types of samples and the activity values were corrected accordingly. Quenching was constant at almost exactly 50%.

Feces. Each day's collection of feces for each animal was analyzed separately. The samples were dried under infrared lamps in a radiological hood, ground in a mortar and pestle and mixed thoroughly. Twenty-five milligrams of samples were placed in cellophane casings, moistened with 1 ml of 10% glucose solution, dried under a heat lamp and burned in Schöniger flasks.

Ether Extracts. In an attempt to determine the amount of lipid soluble gossypol (free gossypol), a simple ether extraction system was used. The tissue was homogenized in a Potter Elvehjem glass homogenizer and placed on a mat of powdered filter paper in an extraction thimble and extracted for 16 hr with anhydrous diethyl ether. After the extraction was complete the ether was evaporated over dry

TABLE II
Recovery of ^{14}C Activity in Tissues and Feces From Chicks Fed Formyl ^{14}C-Labeled Gossypol
Values Expressed as Per Cent of Administered Formyl-^{14}C-gossypol

Practical diet				Fish meal diet			
Chick no.	Recovered in tissues %	Recovered in feces %	Total activity recovered %	Chick no.	Recovered in tissues %	Recovered in feces %	Total activity recovered %
8[a]	8.06	91.77	99.8	10[a]	5.88	90.28	96.2
9[a]	3.64	97.50	101.1	11[a]	4.32	97.51	101.8
12	9.60	85.01	94.6	15	6.93	88.03	95.0
13	11.87	83.00	94.9	16	6.34	91.65	98.0
14	11.31	89.04	100.4	17	4.59	89.97	94.6
Average	8.90	89.26	98.2	Average	5.61	91.49	97.1

[a] Blood samples were not analyzed.

TABLE III
^{14}C Activity in Tissues of Chicks Fed Formyl-^{14}C-Gossypol
Values Expressed as mμ c/g Fresh Tissue

Tissue	Practical diet						Fish meal diet					
	Chick no.					Average	Chick no.					Average
	8	9	12	13	14		10	11	15	16	17	
Heart	0.344	0.197	0.132	0.167	0.220	0.212	0.169	0.115	0.092	0.122	0.092	0.118
Brain	0.062	0.025	0.164	0.187	0.171	0.122	0.155	0.006	0.173	0.065	0.038	0.087
Lung	0.308	0.151	0.280	0.387	0.267	0.279	0.384	0.468	0.208	0.217	0.200	0.295
Spleen	0.404	0.187	0.275	0.175	0.288	0.266	0.212	0.136	0.104	0.214	0.158	0.165
Kidney	0.302	0.465	0.541	0.443	0.510	0.452	0.176	0.117	0.385	0.644	0.345	0.333
Muscle[a]	0.181	0.036	0.139	0.263	0.167	0.157	0.126	0.077	0.123	0.069	0.038	0.087
Liver	2.637	2.240	5.104	3.620	7.050	4.130	2.703	2.266	1.610	3.240	1.580	2.280
Blood[b]			0.265	0.300	0.496	0.354			0.358	0.271	0.241	0.290

[a] The amount of muscle tissues was estimated at 30% of the total body wt.
[b] The amount of blood was estimated as 8% of body wt.

nitrogen. The extract was then taken up directly with the scintillation solvent and phosphor.

Evaluation of Analytical Procedures

The recovery of the ^{14}C activity of pure labeled gossypol by the combustion technique is shown in Table I. In preliminary tests attempts had been made to measure ^{14}C activity in the tissues by homogenizing the samples, placing aliquots on paper strips, air drying and placing the paper strips directly in the scintillation counter vials. With this technique the per cent quenching was in most cases too high and the method was judged to be inadequate for this investigation.

Fractionation of Liver Metabolites

The livers of chickens 12 through 17 were freeze dried to about 2% moisture content. This dried tissue was then extracted with three solvents of increasing polarity; diethyl ether, 5% water in acetone and 1.2% oxalic acid in butanone-2. The combined extracts from three extractions of the tissue with each type of solvent were dried at low temperature under reduced pressure, slurried with chloroform and placed on a 2 cm i.d. × 30 cm silicic acid column. The silicic acid was 100 mesh analytical grade supplied by Mallinckrodt. The column was prepared from a slurry of the silicic acid in chloroform. The developing solvents were chosen to suit the extract (Table V). Individual colored bands were collected and a ^{14}C assay was obtained for the major metabolites found in this study.

Results and Discussion

The combined recovery of ^{14}C activity from the feces and tissues was considered good (Table II). These results indicate that only a small part of the ingested gossypol was retained in the tissues. It will be noted that the average amount of activity found in the tissues was less with the high protein diet containing fish meal. Although these results suggest that high protein in the diet reduces the deposit of gossypol in the tissues, five chicks in each group scarcely justify an attempt at statistical evaluation of the data and a firm conclusion cannot be drawn. However, it is pointed out that the gossypol found in the tissues of four out of five chicks receiving the practical ration was higher than that found in every one of the five chicks which received the high protein fish meal ration.

In experiments with swine, Hale et al. (11,12) found that gossypol toxicity could be prevented by increasing the level of protein in the diet. Narain et al. (13) reported that increasing the protein content of chick rations increased gossypol tolerance. These findings are consistent with the concept that when gossypol is converted to bound gossypol, it combines with the free amino groups of proteins (14) to form a complex with the protein which is largely indigestible and inert with respect to toxicity effects. It is conceivable that such a reaction might take place in the digestive tract if enough protein were present. Fish meal is generally high in iron content and this may have been a contributing factor in reducing the deposit of gossypol or gossypol metabolic products, or both, in the tissues.

Table III shows the results of the determination of ^{14}C activity in the tissues of the individual chickens. It is of interest that more gossypol was deposited in the liver than in any other organ. Although the concentration in the muscle was not particularly high, the total accounted for, based on the estimated weight of muscle tissue, was high. As compared to a number of other tissues, the ^{14}C activity was relatively high in the kidneys. These findings suggest that the liver and kidneys may be important sites for the metabolic breakdown of gossypol.

Table IV shows the results of the estimation of lipid soluble and lipid insoluble gossypol which may be considered to represent free and bound gossypol, although in this case free gossypol may include gossypol bound to ether soluble substances containing free

TABLE IV
Total and Ethyl Ether Soluble ^{14}C Activity in Tissues From Chicks Fed Formyl-^{14}C-Gossypol

Tissue	Practical diet				Fish meal diet			
	Total radioactivity		Ethyl ether soluble activity		Total radioactivity		Ethyl ether soluble activity	
	mμ c/g	Activity recovered[a] %	mμ c/g	Activity recovered[a] %	mμ c/g	Activity recovered[a] %	mμ c/g	Activity recovered[a] %
Heart	0.212	0.079	0.017	0.007	0.118	0.046	0.016	0.007
Brain	0.122	0.028	0.026	0.002	0.087	0.020	0.008	>0.002
Lung	0.279	0.105	0.022	0.015	0.295	0.137	0.024	0.011
Spleen	0.266	0.017	0.051	0.005	0.165	0.016	0.040	0.003
Kidney	0.452	0.259	0.031	0.023	0.333	0.231	0.033	0.012
Muscle	0.157	3.079	0.011	0.086	0.087	1.420	0.011	0.169
Liver	4.130	4.377	0.394	0.732	2.280	3.042	0.577	0.777
Blood	0.354	1.590			0.290	1.160		
Total		9.53				6.07		

[a] Expressed as per cent of administered activity found in tissues.

TABLE V
Fractionations of Gossypol Metabolites From Chick Liver

Extract	Fraction no.	Developing solvents	Description of fraction	^{14}C Activity of fraction
				mμ c
Ethyl ether	1	1:1Hexane-chloroform		
	2	Chloroform	Colorless-lipid	0.051
	3	Chloroform:5% methanol	Light yellow band	0.003
	4	Chloroform:10% methanol	Colorless, Colorless } Combined	0.015
5% Water in acetone	1	Chloroform	Light yellow	0.044
	2	Chloroform:5% methanol	Light brown	0.032
	3	Chloroform:20% methanol	Yellow	0.028
	4	Chloroform:50% methanol	Dark brown	0.046
Oxalic acid in butanone-2	1	Chloroform	Colorless	0.053
	2	Chloroform:10% methanol	Wide yellow band	32.140
	3	Chloroform:50% methanol	Narrow yellow band	0.498
	4	Methanol	Narrow yellow brown band	0.578
Total				33.480[a]

[a] Represents 58% of the activity of the original liver sample.

NH_2 groups. The values given for bound gossypol are calculated values representing the difference between free and total gossypol.

It will be noted that only a small part of the ^{14}C activity was extracted from the tissues by ethyl ether. The reaction of the carbonyl groups of gossypol with the free amino groups of different types of organic compounds to form Schiff bases constitutes one of the most characteristic reactions of gossypol. It has been shown that this reactivity toward amino groups includes the free amino groups of protein molecules and the formation of gossyopl protein complexes in buffered solution at physiological hydrogen ion concentration (14,15). Gossypol protein complexes were prepared using semi-purified cottonseed protein, crystalline blood albumen and crystalline insulin. All of these proved to be insoluble in ether. The free amino groups of structural proteins and enzymes account for a major portion of the free amino groups in body tissues. These observations suggest, but do not prove that the portion of gossypol found in body tissues which cannot be extracted with ether may be bound to proteins.

Fractionation of Liver Metabolites. A typical set of data obtained in the fractionation of gossypol liver metabolites is shown in Table V. Butanone-2 containing oxalic acid was included as one of the solvents for extracting the tissue because it is known that oxalic acid liberates gossypol from its bond form under rather mild conditions. The determination of total gossypol both in cottonseed meal (16) and in animal tissues (5) is dependent on this reaction. The major metabolite extracted with 1.2% oxalic acid in butanone-2 separates as a yellow band on a silicic acid column when the column is treated with 10% methanol in chloroform. Gossypol may be readily eluted from a silicic acid column with pure chloroform whereas this procedure leaves the gossypol chick liver metabolite on the column.

The ultraviolet absorption spectrum of the fraction containing the gossypol metabolite is shown in Figure 1. There is a plateau or peak in the region of 370 mμ where gossypol shows a maxima, but the spectrum in the ultraviolet region is quite different from that of gossypol. From these data the pigment appears to be a metabolite which may have had its origin in bound gossypol. A total recovery of 58.28% of the ^{14}C activity in the liver by the three extraction procedures leaves a considerable portion of the labeled compound in the liver protein residue.

In their review of comparative biochemistry of drug metabolism, Brodie and Maickel (17) come to some interesting conclusions concerning the mechanism by which foreign organic compounds including drugs are eliminated from the body. They point out that organic compounds which are weak electrolytes are partly in the dissociated and partly in the associated form at physiological pH. If the associated form is lipid soluble, tubular reabsorption by the kidney is virtually complete. These authors further point out that one of the major pathways for the metabolism of drugs is oxidation to more polar compounds which are not rapidly reabsorbed in the kidneys. The chromatographic behavior of the gossypol metabolite found in the chick liver indicates an increase in polarity over gossypol.

Although gossypol is by no means a drug, its metabolism would be expected to follow the general pathways of drug metabolism. The finding of a gossypol metabolite which is apparently more polar than gossypol is in agreement with this concept. The most reactive groups in the gossypol molecule are the two carbonyl groups. Oxidation of these groups to carboxyl groups would be expected to result in increased polarity. However, the carbonyl groups are the sites of reaction between gossypol and the free amino

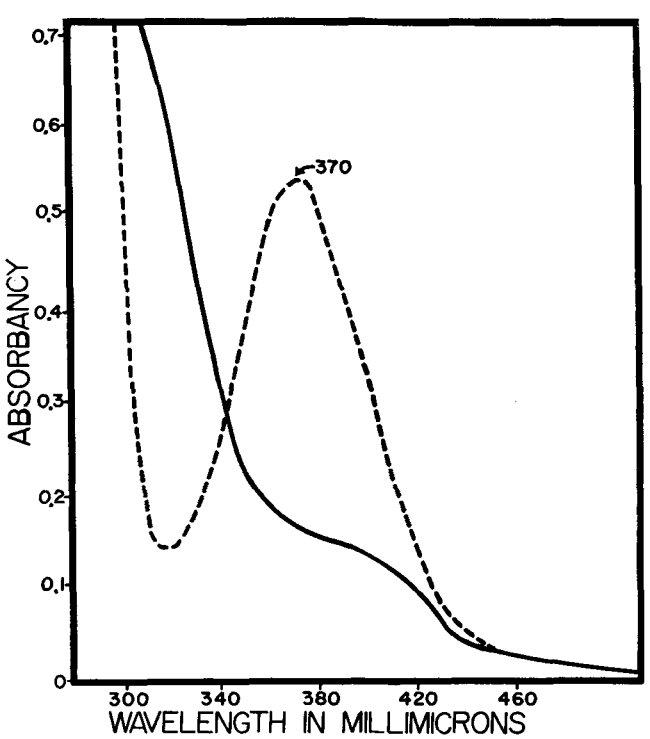

FIG. 1. Absorption spectra of gossypol and a chick liver metabolite of gossypol. - - - Gossypol in methanol; — Chick liver metabolite extracted in 1.2% oxalic acid in butanone-2 chromatographed and redissolved in methanol.

groups of proteins (13) and solubility relationships indicate that much of the gossypol metabolite in the liver is in some bound form. The probability is that it is combined with protein. If this is true, then the hydroxyl groups attached to the napthalene rings would be the likely sites of oxidation.

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