Effect of Free Gossypol on Tissue Uptake of Iron by Everted Segments of Rat Duodenum

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This study shows that gossypol reduces tissue uptake of iron by everted segments of rat duodenum when gossypol and iron are present simultaneously in the mucosal medium. This decreased uptake is probably due to the formation of a gossypol-iron complex. Iron uptake is not impaired in everted segments prepared from rats that are gavaged with gossypol or in everted segments that are incubated with sodium gossypolate prior to the addition of iron; however, the concentration of iron in the glycocalyx of the latter is significantly increased.

Dietary gossypol is toxic to nonruminant animals (Withers and Carruth, 1915; Alsberg and Schwartze, 1919; Smith, 1957), but the reason for the toxic effect of gossypol is not understood. Recent studies indicate that gossypol toxicity may be related to the availability of nonheme iron (Herman and Smith, 1973; Skutches et al., 1973, 1974). Skutches et al. (1973) have shown that intravenous injection of gossypol into swine results in an increase in the concentration of iron in the liver and bile but decreases hemoglobin and hematocrit levels. They postulated that gossypol was reacting with the liver iron, and the gossypol-iron complex thus formed was excreted via the bile. When a ration containing 600 ppm of free gossypol was fed to pigs, a decrease in the liver iron concentration was noted (Skutches et al., 1974). This decrease was attributed to the excretion of a gossypol-iron complex in the bile. However, the results of their study did not exclude the possibility that gossypol was interfering with the intestinal absorption of iron.

In a previous paper (Herman and Smith, 1973), it was shown that iron absorption was decreased by dietary bound gossypol. Absorption was measured as the total amount of dietary ^{59}Fe that was present in the body tissues at the end of a 7-day period. The reduced level of iron found in the animals consuming the bound gossypol diet was attributed to the formation of a gossypol-iron complex in the intestinal tract which was unavailable for absorption. Since iron absorption was measured as the amount of iron retained in the body over a 7-day period, the possibility existed that gossypol did not actually reduce the iron absorbed but did increase the excretion of iron via the bile. The present study was undertaken (1) to determine if free gossypol reduces iron uptake by everted segments of duodenum, (2) to determine if in vivo interaction of free gossypol with the intestinal mucosa affects the uptake of iron by everted duodenal segments, and (3) to determine the effect of in vitro incubation of everted segments of duodenum with gossypol immediately prior to measuring iron uptake.

MATERIALS AND METHODS

A modified method of Dowdle et al. (1960) was used to study the tissue uptake of iron. Male rats of the Wistar strain, fed a commercial rat chow from weaning, were fasted for 24 hr and then killed by decapitation. The intestine was surgically removed at the stomach and 15 cm distally. The segment was everted over a glass rod and chilled in ice-cold 0.146 M sodium chloride–0.004 M potassium chloride. A ligature was tied on the pylorus, and a hypodermic needle with a blunted tip was inserted into the lumen of the intestine from the opposite end. A second ligature was loosely tied 7 cm distal to the pylorus, and the sac was injected with 0.5 ml of a standard medium containing the following: 0.145 M sodium chloride, 0.02 M fructose, 0.05 M sodium ascorbate, and 0.004 M sodium phosphate buffer at pH 7.4. As the needle was withdrawn, the ligature was drawn tight and the excess segment was shortened just distal to the second ligature. The sac was suspended in a 25-ml erlenmeyer flask so the ligatures were held just above the surface of the mucosal medium. The mucosal medium consisted of 8 ml of the standard medium to which ferrous sulfate and ^{59}Fe had been added to yield an iron concentration of 10 μM and a specific activity of approximately 75,000 cpm/ml. The ^{59}Fe was obtained as $^{59}FeCl_3$. Enough 0.14 N H_2SO_4 was added to displace the chloride, and the ferric sulfate thus formed was reduced to ferrous sulfate by the sodium ascorbate in the medium. The flask was then sealed with a serum stopper, and oxygen was bubbled through the mucosal medium for 35 sec at a rate of 90 ml/min. Incubation of the flasks was carried out in a shaking water bath at 37° for 3 hr. At the end of this time, the intestinal sac was removed from the flask and blotted on paper towels, and the total amount of radioactive iron transported into the sac wall and serosal medium was assayed in a Nuclear-Chicago Model 8725 well-type γ scintillation counter. The serosal medium was allowed to drain from the sac, and the radioactive iron in the sac wall was determined. The iron in the serosal medium was calculated by subtracting the iron in the sac wall from the total amount of iron. A 5.0-ml aliquot of the mucosal medium was taken, and the amount of radioactive iron remaining in this medium was determined.

The total amount of iron in each sample was calculated from the average of five repetitive 1-min counts. The average was corrected for background and decay of the isotope, and the result was divided by the specific activity of the iron in the original mucosal medium to yield the micrograms of iron in each sample.

Experiment 1. The method of Smith (1960) was used to prepare gossypolacetic acid (99.28% pure) from which the sodium salt of gossypol was prepared in water (Skutches et al., 1973) to yield a stock solution containing 10 mg of gossypol/ml of solution. Dilutions of this stock solution were made so the addition of 40 μl to 8 ml of the mucosal medium would yield gossypol concentrations of 100, 50, 10, 5, and 1 μM. These concentrations of gossypol were equivalent to the following molar ratios of gossypol to iron in the mucosal medium: 10:1, 5:1, 1:1, 1:2, and 1:10, respectively. The gossypol was added to the mucosal medium immediately prior to putting the everted sac in the medium.

Forty-eight rats, weighing approximately 300 g each, were randomly divided into groups of 12 animals. One everted sac, prepared as previously described, was made from each animal. Duplicate incubations were made at each gossypol level. Similarly, sacs incubated in the same medium lacking gossypol served as controls. Identical ex-

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Table I. Effects of Various Levels of Gossypol on Tissue Uptake of Iron by Everted Segments of Rat Duodenum

Gossypol concn in mucosal medium, μM	Fe concn in mucosal medium, μM	Fe in sac wall, $\mu g \times 10^2$	Fe in serosal medium, $\mu g \times 10^2$
0		45.73[a]	21.92
1	10	51.68	16.05
5	10	33.10	10.54
10	10	11.83	5.04
50	10	8.53	4.23
100	10	8.05	3.42
LSD[b] ($P < 0.05$)		13.46	6.38
LSD ($P < 0.01$)		18.06	8.57

[a] Each value is the mean for eight samples. [b] When the difference between any two means equals or exceeds the least significant difference (LSD), then the comparison is judged significant at the stated probability level.

Table II. Effect of Gossypol Gavaged to Rats on Tissue Uptake of Iron by Everted Segments of Duodenum

Treatment	Fe concn in mucosal medium, μM	Fe in sac wall, $\mu g \times 10^2$	Fe in serosal medium, $\mu g \times 10^2$
Control	10	29.92[a]	17.91
Gossypol	10	38.67	15.63
LSD[b] ($P < 0.05$)		14.35	6.52

[a] Each value represents the mean for six samples. [b] See footnote *b* to Table I.

periments performed on four consecutive days resulted in eight observations for the control and each gossypol level.

Experiment 2. A volume of Wesson oil was added to a diethyl ether solution of gossypol; then the ether was evaporated under a stream of nitrogen to yield a solution containing 16.5 mg of gossypol/ml of oil. Six rats weighing approximately 150 g each were gavaged with 0.2 ml of this solution per 100 g of body weight (33 mg of gossypol/kg body weight) once each day for three consecutive days. Twenty-four hours after the third gossypol feeding the rats were killed, and everted sacs were prepared and incubated as previously described. Gossypol was not added to the mucosal incubation medium. Everted segments prepared from six rats of the same size as those intubated with gossypol served as controls.

Experiment 3. Twenty-three rats weighing approximately 175 g each were used to prepare everted sacs as previously described. Eleven sacs were individually incubated in 25-ml erlenmeyer flasks containing 8 ml of the standard medium, lacking iron, to which 100 μM gossypol had been added. After 1 hr, the sacs were removed from the medium and the residual gossypol was removed by dipping the sacs into three separate beakers containing the standard medium at room temperature. The sacs were then transferred to prepared erlenmeyer flasks containing 8 ml of the mucosal medium to which 10 μM iron and no gossypol had been added and were handled as previously described. The remaining 12 sacs were handled in the same manner except the mucosal medium during the first hour of incubation contained neither iron nor gossypol.

Experiment 4. The experiment was conducted the same as the preceding experiment except for using six everted

Table III. Iron Uptake by Everted Segments of Rat Duodenum Incubated with 100 μM Gossypol Prior to Incubation with 10 μM Iron

Treatment	Fe in sac wall, $\mu g \times 10^2$	Fe in serosal medium, $\mu g \times 10^2$	Fe in mucosal medium following incubation, $\mu g \times 10^2$	Fe in sac blottings, $\mu g \times 10^2$
Control[a]	51.58	14.04	200.47	100.88
Gossypol[b]	21.10	12.55	149.19	221.19
LSD[c] ($P < 0.05$)	10.83	6.16	17.00	15.61
LSD ($P < 0.01$)	14.74	8.38	23.14	22.44

[a] Each value represents the mean for 12 samples except the value for the sac blottings which is the mean for 6 samples. [b] Each value represents the mean for 11 samples except the value for the sac blottings which is the mean for 5 samples. [c] See footnote *b* to Table I.

sacs per treatment from rats weighing approximately 300 g each and two additional modifications. The modifications were the use of [^{14}C]gossypol prepared by the method of Smith (1974), and the iron concentration was increased to 100 μM. These modifications were made to determine the amount of gossypol in the samples, particularly the sac blot. The amount of [^{14}C]gossypol was determined in each sampling as previously mentioned for iron, except for the final mucosal medium and the full sac. This was done by placing the samples and sac blots respectively on 5-cm Whatman No. 1 filter paper. All the samples were then prepared for oxidation in a Packard Tri-Carb sample oxidizer (Packard Instruction Manual, 1970).

Prior to oxidation, the samples were counted for ^{59}Fe content in the γ counter. The samples were then oxidized to separate the ^{14}C from the ^{59}Fe as the iron remains as ash after oxidation. The ^{14}C from the gossypol was trapped as $^{14}CO_2$ in 4 ml of ethanolamine to which was added 9 ml of methanol and 6 ml of scintillator containing 15 g of 2,5-diphenyloxazole and 1 g of *p*-bis(*o*-methylstyryl)benzene made to a liter with toluene. The ^{14}C was counted in a Packard Tri-Carb liquid scintillation spectrometer, and the counts were corrected for quench and background. The amount of gossypol was calculated by dividing the disintegrations in each sample by the specific activity of the gossypol.

Statistical Analysis. Statistical analyses were performed on each experiment using the analysis of variance appropriate for the completely randomized design (Snedecor and Cochran, 1968). The results are summarized as tables of means for each treatment. The experimental error for each variable in each experiment is expressed as the least significant difference (LSD) for making comparisons between any two treatments.

RESULTS

Data showing the amounts of iron absorbed by everted duodenal sacs incubated in the presence of various levels of gossypol from experiment 1 are presented in Table I. At 1:1 and higher molar ratios of gossypol to iron, the amount of iron taken up by the segments was significantly decreased from the control segments ($P < 0.01$). At the gossypol to iron ratio of 1:2, the iron found in the sac wall was not significantly different from the control probably due to the large variations within the groups; but the iron in the serosal medium was decreased ($P < 0.01$). When the gossypol to iron ratio was 1:10, the iron uptake was not different from that of the control.

Table IV. Iron Uptake by Everted Segments of Rat Duodenum Incubated with Gossypol Prior to Incubation with Iron

Treatment	Sac wall, nmol		Serosal medium, nmol		Sac blottings, nmol	
	Iron	Gossypol	Iron	Gossypol	Iron	Gossypol
Control[a]	80.94		31.99		71.38	
Gossypol	66.97	33.21 ± 14.26[c]	33.28	18.86 ± 4.33	146.05	100.39 ± 18.82
LSD[b] ($P < 0.05$)	18.06		13.53		16.89	
LSD ($P < 0.01$)	25.69		19.25		24.02	

[a] Each value in the table is the mean value for six samples except the values for the gossypol treatment serosal medium which is the mean value for five samples. [b] See footnote *b* to Table I. [c] Standard deviation of mean.

The data presented in Table II show that the iron absorbed by everted segments of duodenum prepared from rats which were gavaged with gossypol for 3 days preceding the measurement of iron absorption (experiment 2) was not significantly affected by the gossypol treatment.

The data presented in Table III (experiment 3) show that the iron found in the serosal medium of the gossypol-treated segments was not statistically different from the control segments; however, the iron found in the sac wall of the gossypol-treated segments was significantly lower than that in the controls ($P < 0.01$). The iron in the mucosal medium from the gossypol-treated sacs following the incubation was significantly decreased from the control medium ($P < 0.01$), and the iron in the sac blottings of the gossypol-treated sacs was significantly elevated ($P < 0.01$) over the controls.

The data obtained from experiment 4, presented in Table IV, show that the nanomoles of iron found in the serosal medium and the sac wall of the gossypol-treated sacs was not significantly different from that found in the control sacs. The nanomoles of iron found in the sac blottings of the gossypol-treated sacs was very highly significant ($P < 0.01$), as in the preceding experiment. The average nanomoles of gossypol present in the aliquot of serosal medium corrected back to original volume, sac wall, and sac blottings were 18.86, 33.21, and 100.39, respectively.

DISCUSSION

Using a purified medium, this study has shown that 1:1 and higher molar ratios of gossypol to iron significantly decrease iron uptake by everted segments of duodenum (Table I). This decreased uptake can be due either to the formation of a gossypol–iron complex or to gossypol inhibiting the intestinal iron transport mechanism (Dowdle et al., 1960; Hahn et al., 1943; Granich, 1954; Wheby et al., 1964; Saltman, 1965). Gossypol has been shown to form a complex with iron in a 1:1 molar ratio (Jonassen and Demint, 1955; Muzaffaruddin and Saxena, 1966; Ramaswamy and O'Connor, 1968). A gossypol–iron complex could have two effects on iron absorption. First, the complex could be stabilizing the iron in the ferric state which is not efficiently absorbed physiologically, or second, the complex may have the iron more tightly bound than those which are used physiologically for absorption of iron. The stability constants for ferric–gossypol and ferrous–gossypol complexes are 6.75 (Muzaffaruddin and Saxena, 1966) and 7.6 (Jonassen and Demint, 1955), respectively. This indicates that the ferric–gossypol complex is slightly less stable than the ferrous–gossypol complex. The ascorbate in the medium should tend to keep the iron reduced to the ferrous state and thus favor the formation of the ferrous–gossypol complex. The data show reduced iron uptake by the intestinal mucosa, thus suggesting that the iron is more tightly bound in the ferrous–gossypol complex than those used physiologically for iron absorption. It has been shown, however, that nonruminant animals are protected against gossypol toxicity when a divalent iron salt is added to their diet (Withers and Brewster, 1913; Gallup, 1928; Olcott, 1948; Eagle, 1949; Clawson and Smith, 1966; Smith and Clawson, 1970). Some complexes of iron are poorly absorbed by the intestinal mucosa (Benjamin et al., 1967; Somers, 1947; Moore, 1961). Decreased absorption of a gossypol–iron complex could explain the reduced iron uptake observed in this study and also explain how the addition of iron salts to the diet decreases the toxicity of gossypol.

However, since gossypol readily binds with the ϵ-amino group of lysine (Lyman et al., 1959), any protein involved in the transport of iron could be inactivated by reacting with gossypol. Iron uptake was not impaired in intestinal sacs prepared from rats that were gavaged with gossypol for 3 days (Table II). This indicates that gossypol was either not affecting the iron transport mechanism or that the rapid turnover of the intestinal epithelium was ridding itself of inactive gossypol-bound protein before iron uptake was measured. To study this, everted segments were treated with a gossypol solution immediately prior to measuring iron uptake (experiment 3; Table III). The iron in the serosal medium of the gossypol-treated sacs was not different from the control; however, the iron found in the sac wall of the gossypol-treated segments was significantly reduced from that in the controls ($P < 0.01$), and the final mucosal medium of the gossypol-treated sacs contained significantly less iron than the control medium. A measurement of the iron in the sac blottings showed that those from the gossypol-treated sacs were significantly higher in iron than were those of the control. From these data, it is postulated that the gossypol was absorbed into the glycocalyx (intestinal fuzzy coat) from which it was not absorbed by the intestinal wall before the sac was transferred to the medium containing iron. As the iron entered the glycocalyx, it complexed with the gossypol and this complex was unavailable for absorption by the intestinal wall. The result was an increase of iron in this cell coating and a reduction of iron in the sac wall of the gossypol-treated sacs vs. the controls. Once enough iron entered the glycocalyx to complex all or most of the gossypol, the remainder was free to be absorbed normally.

Experiment 4 was designed to study this hypothesis further. At the present time, a quantitative method has not been developed to measure the amount of gossypol–iron complex in tissues. Radioactive gossypol was used to determine the amount of gossypol in the sac blottings. It was observed that the mucosal surface of the sacs incubated with gossypol had a dark brown or black appearance when they were removed from the iron-containing medium. This dark subtance remained in the blot. The observed color is characteristic of the gossypol–iron complex (Shieh et al., 1968; Jonassen and Demint, 1955) and was not observed in the control sacs. The average gossypol content of the sac blottings was 100.4 nmol. This seems to be a high content, since the sac was removed from the gossypol-containing mucosal medium 3 hr prior to ending the incubation in the iron-containing mucosal medium. If one assumes that the blots of the gossypol-treated sacs

would contain the same amount of "free" iron as the controls and that the remainder of the iron is in the gossypol-iron complex, then this yields a gossypol to iron ratio of 1.36 to 1. This approaches a 1:1 ratio which seems to be the most quoted value. If, on the other hand, it is assumed that all the gossypol in the blot is tied up in a 1:1 ratio complex with iron and that the remaining iron in the blot is "free," then statistical analysis shows the gossypol-treated sacs have a significantly lower "free" iron content ($P < 0.01$).

From the data presented herein, it is believed that gossypol reduced iron uptake of everted intestinal sacs by the formation of a gossypol-iron complex which is not readily absorbed.

Although gossypol forms a 1:1 molar complex with iron and this paper shows reduced tissue uptake of iron at a 1:1 molar ratio, feeding studies have shown that a 9.3:1 molar ratio of iron to gossypol is required to protect non-ruminants from gossypol toxicity (Smith and Clawson, 1970). It is felt that this is probably due to the heterogeneous nature of the chyme interfering with the intimate contact between iron and gossypol required for the formation of the complex.

ACKNOWLEDGMENTS

The authors wish to thank John F. Roberts for the use of the γ scintillation counter.

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Received for review August 27, 1973. Accepted January 2, 1975. Paper No. 3987 of the Journal Series of the North Carolina Agricultural Experiment Station, Raleigh, N.C. Use of trade names in this publication does not imply endorsement of the products mentioned.

Effects of Several Vegetable Oils on Lipid Classes and Very Long Chain Polyenoic Fatty Acid Content of Rat Liver and Heart

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Weanling rats were fed diets containing 10% peanut, safflower, soybean, or linseed oil for 8 weeks to determine the effects of feeding natural fats varying widely in linoleic and linolenic acid content on the lipid classes and very long chain polyenoic fatty acid (VLCPFA) content of the liver and heart. Total liver lipid was reduced in the animals fed peanut oil and increased in the animals fed soybean oil compared to the other treatments, with these alterations being confined to the neutral lipids. The animals receiving the soybean and linseed oils accumulated more cephalin in relation to lecithin in the heart than the animals receiving the peanut and safflower oils. Fatty acid analyses indicated that the VLCPFA of the linolenic series increased in the phospholipids of the liver and heart with reductions in VLCPFA of the linoleic series as the dietary linolenic acid increased from trace amounts, and in the rats receiving linseed oil the VLCPFA of the linoleic series essentially disappeared with the exception of arachidonic acid which was greatly reduced.

Brain phospholipids of many species contain a relatively high concentration of docosahexaenoic acid (22:6, *n*-3), a metabolite of linolenic acid (Crawford, 1970). Fatty acids of the *n*-3 series have also been found in significant amounts in the lipids of the liver and heart (Egwim and Kummerow, 1972; Sinclair and Crawford, 1973). In studies conducted in our laboratory, it has been observed that when diets contained trace amounts of linolenic acid, docosahexaenoic acid was the only *n*-3 acid found in the heart and liver lipids with the remainder of the very long chain polyenoic fatty acids (VLCPFA), 20 or more carbons in the chain, being members of the linoleic acid (*n*-6) family. However, when oils containing more than trace amounts of linolenic acid were fed, docosapentaenoic (22:5, *n*-3) and eicosapentaenoic (20:5, *n*-3) acids were also present in these lipids.

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