Effect of Calcium and Phosphate Ions on the Formation of Soluble Iron-Gossypol Complex

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Spectral measurements and potentiometric titration of the gossypol-ferrous complex in 25% EtOH indicated that ferrous ion formed a soluble 2 to 1 chelate at the $6, 7, 6', 7'$ positions of the binaphthyl-aldehyde ring. Ferric ion formed an insoluble gossypol-iron hydroxide. When ferric ion was added to a gossypol solution containing more than 3 moles of phosphate per mole of iron, a soluble dark-brown complex having the characteristic spectrum of iron gossypol was obtained. Addition of Ca^{2+} into iron-gossypol complex in phosphate solution resulted in the removal of the soluble ferrous-gossypol complex from the solution. The formation of insoluble ferrous gossypol by calcium may explain the biological synergistic effect of calcium with iron in the inactivation of gossypol.

Cottonseed pigments, particularly gossypol (1, 1', 6, 6', 7, 7'-hexahydroxy-3, 3'-dimethyl-5, 5'-diisopropyl-2, 2'-binaphthyl-8, 8'-dialdehyde), are toxic to many species of animals, including dogs, guinea pigs, swine, and poultry (Boatner et al., 1948; Eagle et al., 1948; and Withers and Carruth, 1918). Because of the sensitivity of these monogastric animals to gossypol, the use of cottonseed oil meal as a protein concentrate has been limited in the diets of pigs and poultry (Smith, 1963; Southern Regional Research Laboratory, New Orleans, La., 1959).

The addition of ferrous salts to diets containing cottonseed meal has been shown to reduce partially the toxicity of gossypol (Clawson and Smith, 1966; Clawson et al., 1962; Gallup, 1928; Lyman, 1962; Smith and Clawson, 1965). Bressani et al. (1964) have shown that the addition of iron in the presence of calcium salts reduces the concentration of free gossypol in food mixtures containing cottonseed flour. Recently, Jarquin et al. (1966) have also shown that supplementation of calcium and iron protects swine against gossypol toxicity. The beneficial effects of the added iron and calcium salts are believed to be due to the formation of an insoluble metal complex of gossypol which decreases the concentration of free gossypol.

Although nutritional data have been published on the inactivation of gossypol toxicity by metals, no fundamental investigation of the formation of complexes between gossypol and minerals has been made. This report describes the interaction of iron with gossypol. Since calcium and inorganic phosphate normally are present in high concentration in common feed materials, the effects of calcium and phosphate ions on the formation of iron-gossypol complex were also studied.

MATERIALS AND METHODS

Purification of Gossypol Acetate. Pure gossypol acetate was obtained by repeated extraction and precipitation of crude gossypol acetate in acetone and acetic acid as described by Castillon et al. (1948). Gossypol that is free from acetic acid was obtained by first washing the gossypol acetate with toluene, and then suspending it in the form of an aqueous slurry in hot water for 1 hour to hydrolyze the gossypol–acetic acid complex.

Visible Absorption Spectra Measurement of Gossypol and Metal Chelates. Spectral measurements were made with a Beckman Model DB-G recording spectrophotometer. The pH measurements were made with a Leeds and Northrup pH meter. Samples for spectral measurements were prepared as follows: Two milliliters of freshly prepared stock solution of gossypol acetate containing 2.18×10^{-6} mole of gossypol in EtOH solution was mixed with 10 ml. of 100% EtOH and 27 ml. of H_2O and adjusted to pH 7.0. One milliliter of freshly prepared $FeSO_4 \cdot 7H_2O$ and various metal solutions of various concentrations were then added slowly to the gossypol solution with stirring. The pH of the solution was simultaneously readjusted to 7.0, and the final volume was adjusted to 50 ml. with water. If insoluble materials were formed after the addition of metals, the mixtures were centrifuged, and the spectra of the clear supernatants were recorded.

Titration of Iron-Gossypol Chelates. Twenty milliliters of absolute ethanol containing 2.3×10^{-6} mole of gossypol was mixed with 20 ml. of water containing 0, 2.31×10^{-6}, and 4.62×10^{-6} mole of $FeSO_4 \cdot 7H_2O$. To this mixture, various amounts of $0.01N$ NaOH were added, and the pH of each sample was recorded after 1 hour at room temperature. The titration curve was corrected for that of a blank solution containing 20 ml. of ethanol and 20 ml. of H_2O.

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Complex Formation of Various Metal Ions with Gossypol. The characteristics of the complexes formed by gossypol acetate and various metals at a 1-to-1 mole ratio in 24% ethanol solution are summarized in Table I.

The yellow color of gossypol acetate has a maximum absorption peak at 380 mμ. Upon addition of ferrous ion at various pH levels, a dark, brownish complex is formed which gives a pH-dependent absorption band in the region of 500 to 550 mμ.

No other ions, among those tested, chelated gossypol in the same way as iron, although some chelating activity seemed evident. Copper and aluminum ions proved to be strong chelating agents which completely precipitated gossypol from solution. Zinc gave a considerable change in maximum absorption peak, but had only a weak spectral band at 500 to 550 mμ, where ferrous ion gives a characteristic strong band. Calcium, magnesium, nickel, and manganese ions showed little chelating activity, since little or no change occurred in the spectral bands in the 500-mμ region, no change in maximum absorbance occurred at 390 mμ, and the color and solubility characteristics of gossypol remained constant in their presence.

Absorption Spectra. A characteristic increase in absorption in the 500-mμ region due to the formation of the gossypol-Fe chelate occurs as the concentration of ferrous ion is increased (Figure 1). This may indicate the existence of a single chelate species for which the gossypol-Fe complex formation may be determined by the absorbance change at 500 mμ. As shown in Figure 2, the absorbance at 500 mμ reaches its maximum when the iron to gossypol ratio is 2 to 1. A further increase in the Fe^{2+} to gossypol ratio at a constant gossypol concentration results in no further change in the spectrum. This indicates that no colored complex ion containing more than 2 moles of iron per mole of gossypol is produced.

Titration of Iron-Gossypol Chelates. The existence of the 2-to-1 ferrous-gossypol chelate is supported by potentiometric titration curves of solutions containing various ratios of ferrous iron to gossypol (Figure 3).

In the absence of iron, only 2 moles of protons per mole of gossypol were titrated. This indicates that the dissociation of one of the protons of an —OH group in the naphthylaldehyde ring (probably at the 7, 7' position)
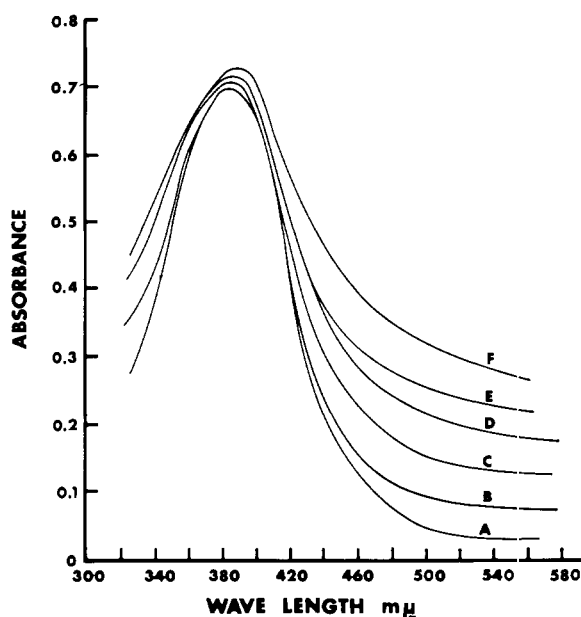

Figure 1. Spectra of ferrous-gossypol complexes for various Fe^{2+} to gossypol molar ratios

A.	Fe^{2+} : Gossypol	=	0
B.	Fe^{2+} : Gossypol	=	0.30
C.	Fe^{2+} : Gossypol	=	0.45
D.	Fe^{2+} : Gossypol	=	0.60
E.	Fe^{2+} : Gossypol	=	1.20
F.	Fe^{2+} : Gossypol	=	1.80

causes the induction of electrons into the aromatic ring system, which in turn reduces the acidity of the other —OH groups at the 1, 6 and 1', 6' positions of the binaphthylaldehyde ring.

Upon addition of 1 mole of ferrous iron, a total of 4 moles of protons were neutralized. This probably corresponds to the titration of protons at the 1, 6, 7, and 7' position for a 1-to-1 chelate. For the titration of a 2-to-1 chelate, all the theoretical amount of six hydroxyl groups at the 1, 1', 6, 6', and 7, 7' positions were neutralized. Only a single strong inflection, corresponding to the complete neutralization of an even number of protons, is observed in the presence of iron. Thus, the neutralization of —OH groups in a single step indicates that the affinity constants of various oxygen atoms for the formation of coordinate bonds with iron are fairly comparable in magnitude. The possibility of a metal chelate at the 1, 1' —OH groups (Jonassen and Demint, 1955) is ruled out based on the following observations.

Chelation of iron at the 1, 1' positions would be unstable because of the formation of a seven-membered ring. The methyl groups at 3, 3' limit the freedom of rotation of

Table I. Characteristics of Gossypol-Metal Ion Complexes

| Complex[a]	λ_{Max}	Color	Solubility	Spectral Band at 500-550 Mμ
Fe^{2+}	390	Dark brown	Soluble	Strong
Ca^{2+}	390	Yellow	Soluble	None
Mg^{2+}	390	Yellow	Soluble	Very weak
Ni^{2+}	390	Yellow	Soluble	Very weak
Mn^{2+}	390	Yellow	Soluble	Very weak
Zn^{2+}	425	Yellow	Soluble	Very weak
Cu^{2+}	. . .	Yellow	Insoluble	. . .
Al^{3+}	. . .	Yellow	Insoluble	. . .
Gossypol	390	Yellow	Soluble	None

[a] Gossypol acetate:metal = 1 to 1 molar ratio.
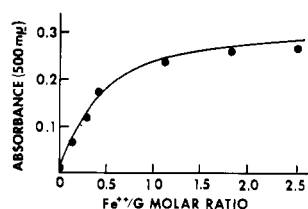
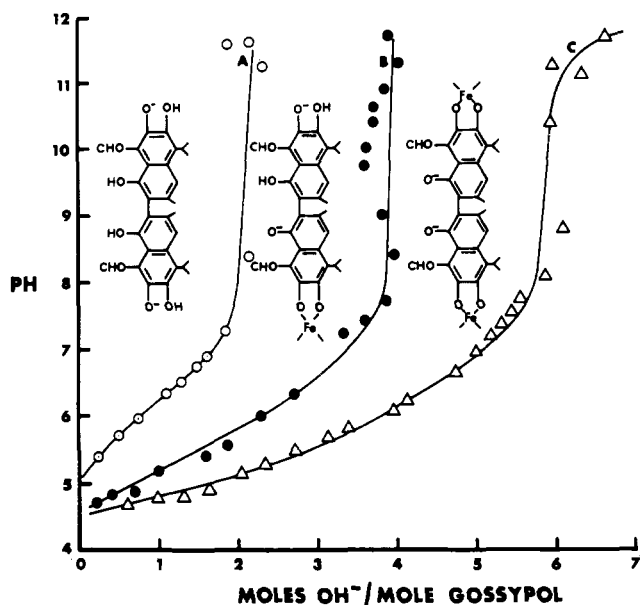Figure 2. Measurement of absorbancy of various ratios of ferrous ion to gossypol

Figure 3. Titration of iron-gossypol chelates

A. Gossypol alone
B. Ferrous gossypol, 1-to-1 ratio
C. Ferrous gossypol, 2-to-1 ratio

Figure 4. Formation of insoluble ferric-gossypol complex in presence or absence of phosphate

the carbons at 2, 2' of the naphthyl rings so that two naphthyl rings cannot exist in a planar position. The nearly perpendicular position of the two naphthyl rings would limit the spacing of the metal ion for the formation of proper coordination bonds with oxygen. Examination of a molecular model of gossypol supports the above hypotheses.

The possibility exists that the o-hydroxyaldehyde moiety of the naphthyl ring may also furnish the site for formation of metal-ion complex (Scheiffele and Shirley, 1964). However, the stability constant reported for a metal chelate of salicylaldehyde and catechol (Schwarzenbach, 1953; Schwarzenbach and Moser, 1953) indicates that iron has much greater preference to combine with a vicinal hydroxy group rather than with a hydroxy aldehyde moiety.

Effect of Phosphate on Formation of the Ferric-Gossypol Complex. The soluble ferrous-gossypol complex became a dark-brown precipitate during a 24-hour period. This is probably due to the oxidation of ferrous gossypol to ferric gossypol, which is insoluble in 24% EtOH solution at pH 7.0. This is evidenced by the instantaneous formation of an insoluble ferric-gossypol complex when more than 1 mole of ferric iron is added per mole of gossypol (Figure 4). This insoluble ferric gossypol is probably due to the polymerization of the 1-to-1 ferric-gossypol complex.

The dark-brown precipitate was dissolved in acidic ethanol, and a study of the spectrum indicated that nearly 100% of the gossypol was recovered. When ferric ion was added to gossypol solution containing more than 3 moles of phosphate per mole of iron, a soluble dark-brown complex having the characteristic spectrum of iron gossypol was obtained.

Formation of a soluble ferric-gossypol complex in the presence of phosphate suggests that one or more ligands of a metal-ion sphere of the chelate is occupied by the phos-
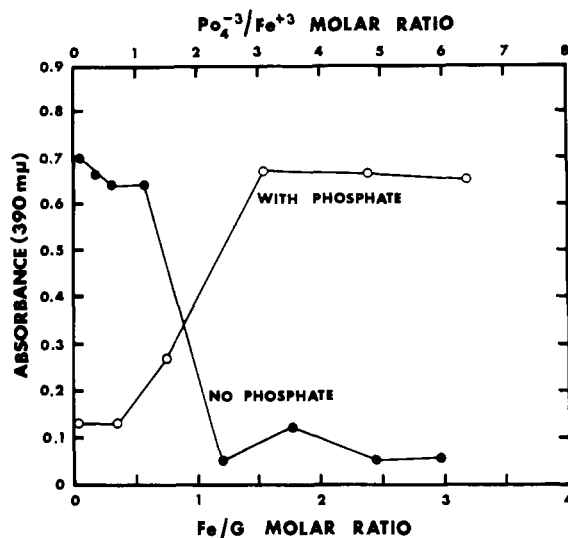
phate anion. This prevents the polymerization of the ferric-gossypol complex.

Effect of Calcium on the Solubility of the Iron-Gossypol Complex. Nutritional data suggest that calcium compounds may have a synergistic effect with iron in the inactivation of gossypol. However, calcium itself shows little chelating activity with gossypol (Table I). In an effort to explain the effect of calcium on iron-gossypol chelation, experiments were conducted to test the chelation of gossypol by Fe^{2+} and Fe^{3+} in the presence of calcium. Since both ferrous and ferric gossypol have been shown to be soluble in a solution containing 5 moles of phosphate
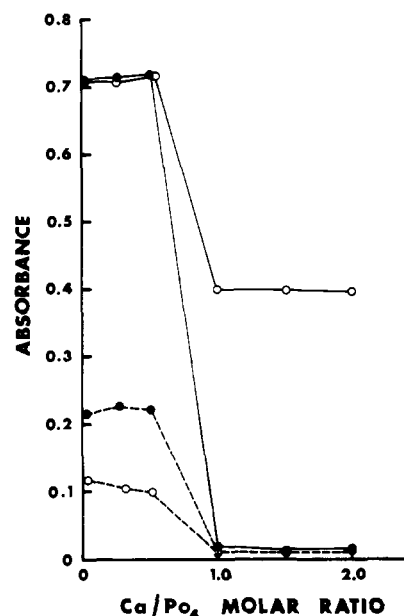

Figure 5. Effect of Ca^{2+} on solubility of iron-gossypol complex in the presence of phosphate

—●— Fe^{2+}-G, 390 mμ; —O— Fe^{3+}-G, 390 mμ
-- ● -- Fe^{2+}-G, 580 mμ; -- O -- Fe^{3+}-G, 580 mμ

per mole of iron, ferrous or ferric ions were first allowed to chelate gossypol in the presence of phosphate, and then various amounts of calcium were added.

In the case of Fe^{2+}, the ferrous gossypol was completely removed from solution when Ca^{2+} was added at a Ca/PO_4 molar ratio of 1.0 or greater; in the case of Fe^{3+}, about half of the ferric-gossypol complex was precipitated under similar conditions. Also, the characteristic absorption band at 500 mμ for the ferric-gossypol complex disappeared (Figure 5). This indicates that free gossypol is formed from the ferric-phosphate-gossypol complex when calcium is added to the solution at a molar ratio of 1.0 or above.

At the present time, the chemistry of the formation of an insoluble iron-gossypol complex by calcium is not known. However, the present data may explain the reported synergistic effect of calcium with iron in the inactivation of gossypol, since reduced solubility of iron gossypol by calcium would also reduce the absorption of such a compound in the animal intestine.

ACKNOWLEDGMENT

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