Preparation of Pure Gossypol from Dianilinogossypol[1]

**F. H. SMITH, Animal Nutrition Section, Department of Animal Industry,
North Carolina Agricultural Experiment Station, Raleigh, North Carolina**

Studies on the chemistry and toxicology of gossypol and the development of methods for its estimation have necessitated the preparation of a supply of the pure compound. To obtain this supply, gossypol has been extracted from cottonseed, isolated from the extract, and purified by recrystallization.

Gossypol has usually been prepared by extracting it with ether from flaked cottonseed meats, which either have (2, 3, 4) or have not been defatted with a petroleum solvent (6, 11). After removal of the ether, the gossypol can be precipitated with glacial acetic acid as gossypol-acetic acid. The yield of gossypol by this procedure however has frequently been low when compared to the amount known to have been present in the cottonseed meats (3, 9). Furthermore the filtrates from the acetic acid precipitation yield additional gossypol when treated with aniline (9), indicating incomplete precipitation as the acetic acid derivative.

Aniline precipitates gossypol as the dianilino derivative almost quantitatively even in the presence of large amounts of oil (4), thus making this reaction very satisfactory for isolating gossypol from extracts of cottonseed. The stability of dianilinogossypol however presents a problem in the separation of this compound into gossypol and aniline. Small amounts of dianilinogossypol have been separated by heating with concentrated H_2SO_4 (1, 5). Murty *et al.* (9) found this method unsatisfactory for 10 g. and subsequently hydrolyzed dianilinogossypol by boiling with acetic anhydride. King and Thurber (8) noted that neither of the methods cited was completely satisfactory for hydrolyzing large amounts of dianilinogossypol.

Carruth (4) hydrolyzed dianilinogossypol with hot ethanolic KOH, which contained sodium hydrosulfite to prevent the oxidation of the liberated gossypol; steam-distilled the aniline; and extracted the gossypol from the acidified solution with ether. King and Thurber (8) modified Carruth's method for the separation of the liberated gossypol from the aniline and the unhydrolyzed dianilinogossypol. They then crystallized the gossypol, in good yields, from a mixture of ether and xylene.

In the study reported herein, a method has been developed for the acid hydrolysis of dianilinogossypol in large amounts and from which pure gossypol can be readily prepared. The hydrolyzed gossypol is purified first by crystallizing it as gossypol-acetic acid and then as gossypol from either a mixture of ether and petroleum ether or a mixture of ether, ethanol, and water.

Procedure

Extraction of Gossypol and Precipitation of Dianilinogossypol. Place 8–9 kg. flaked cottonseed meats with slight packing into a glass percolator, an inverted 5-gal. bottle from which the bottom has been removed. A 9-in. porcelain desiccator plate with ⅜-in. perforations, covered with a layer of cotton, serves as a bottom for the percolator. Place in the mouth of the bottle a cork stopper through which passes a glass tube with a piece of rubber tubing attached. Pour peroxide-free ether over the meats, and maintain the level above their surface during the extraction. Regulate the rate of percolation to 3 to 4 gal. per hour by means of a screw clamp on the rubber tubing at the bottom of the percolator. Discontinue the extraction after 6 to 7 gal. of ether have passed through the meats and the extract shows little color. Transfer the extract, collected in gallon bottles, to a 12-liter flask placed in a steam-heated water bath and remove the ether by distillation. Filter the residual extract through glass wool into a 5-liter flask.

To avoid the formation of very fine crystals, at first add only 15 ml. of aniline and mix. When the precipitation subsides, add an additional 20 ml. of aniline and heat the mixture to 60°C. Decant the supernatant solution through a paper in a Buchner funnel after the mixture has stood over-night at room temperature. Finally transfer the precipitate to the Buchner funnel, and wash well with light petroleum ether (Skellysolve F). The dried dianilinogossypol had a purity of 97.8%.

Hydrolysis of Dianilinogossypol and Recrystallization of Gossypol-Acetic Acid. Suspend 50 g. of dianilinogossypol in a mixture of 750 ml. of peroxide-free ethyl ether and 750 ml. of glacial acetic acid in a 2-liter beaker. Cool the mixture to 18–20°C. in an ice-water bath. With constant stirring, add 90 ml. of concentrated H_2SO_4 and, after 1.5 min., add 200 ml. of distilled water. Let the mixture stand for about 10 min. for the gossypol-acetic acid to precipitate. Filter under vacuum through paper in a Buchner funnel, wash well with water, and suck dry.

Recrystallize the precipitate immediately as gossypol-acetic acid, as follows. Transfer the precipitate to a 2-liter beaker, add about 1,200 ml. of distilled water, 0.3 to 0.4 g. of sodium hydrosulfite, and 600 ml. of peroxide-free ether. Stir until the gossypol has dissolved, then transfer the mixture to a 2-liter separatory funnel and shake. When the layers have separated, withdraw the water layer and wash the ether layer with an additional 400 ml. of water containing about 0.1 g. of sodium hydrosulfite. Filter the ether layer through paper in a small Buchner funnel to remove the small amount of unhydrolyzed dianilinogossypol (about 5%). Add an equal volume of glacial acetic acid, and allow the mixture to stand about 10 min. for the precipitation of gossypol-acetic acid. Filter and wash the precipitate with either low-boiling petroleum ether (Skellysolve F) or hexane. The yield of gossypol at this stage is about 86%, based on the starting material.

Further purify the gossypol by twice dissolving the gossypol-acetic acid, and recrystallize it as gossypol-acetic acid, as directed above. A recovery of approximately 95% results from each recrystallization as gossypol-acetic acid, based on the amount of dry gossypol-acetic acid dissolved.

---

[1] Published with the approval of the Director of Research, North Carolina Agricultural Experiment Station, as Paper No. 1086 of the Journal Series.

Pure Gossypol from Gossypol-Acetic Acid. Dissolve 50 g. of gossypol-acetic acid in 600 ml. of peroxide-free ether over 1,200 ml. of water containing 0.3 to 0.4 g. of sodium hydrosulfite in a separatory funnel and shake. Withdraw the water layer, and wash twice with 400 ml. of water, omitting the sodium hydrosulfite from the last wash. Transfer the ether solution to a beaker, dry it with anhydrous sodium sulfate, and filter under vacuum through paper on a small Buchner funnel.

The gossypol may be recovered from the filtrate by either crystallizing it from a mixture of ether and low-boiling petroleum ether and then from ether over water according to a method previously described (11) or from a mixture of ether, alcohol, and water. Although both methods were used in this study, only the latter is considered in detail.

To crystallize the gossypol from an ether-alcohol-water mixture, concentrate the filtrate to 200 ml. under reduced pressure by placing the flask into a pan of warm water. Transfer the concentrated ether solution to a 1-liter beaker, and add 400 ml. of 95% ethanol previously used to remove the residual gossypol from the suction flask. Cool the mixture to 15 to 20°C. in an ice bath. Mix 300 g. of crushed ice, prepared from distilled water, with the solution. After about 10 min. add 100 ml. of ice water with stirring, and allow to stand for 10 to 15 min. before filtering through paper in a Buchner funnel. Wash the precipitate first with aqueous ethanol (1:1 by volume), then with an ethanol-hexane mixture (1:3 respectively), and finally with hexane. Dry the precipitate at room temperature and then in a vacuum oven at 70°C. for about 16 hrs. Store the gossypol in tightly closed bottles under refrigeration.

The addition of 400 ml. of 95% ethanol to 200 ml. of ether solution of gossypol gives a solvent mixture containing about 31.4% ether, 65.2% ethanol, and 3.4% water. To this mixture water can be added in relatively large amounts without separating the solution into two phases. Since gossypol is relatively insoluble in water, the addition of 300 g. of crushed ice to the cold solution starts the crystallization, which is carried to completion by adding the final 100 ml. of ice water. The final composition of the solvent mixture is approximately 17% ether, 35% ethanol, and 48% water. Cooling the solution before adding the crushed ice prevents the formation of a scum of non-crystalline gossypol on the surface of the solvent mixture caused by a momentary rise in temperature. If all of the ice and water are added at once, the crystals are so fine that filtering and washing may become a problem.

The yield from the ether-alcohol-water crystallization is almost quantitative. The over-all yield of pure gossypol from dianilinogossypol is about 75%.

Purity of Gossypol

Spectral absorption curves were made, using a Beckman DU spectrophotometer, on solutions of the pure gossypol in spectro-grade cyclohexane. A representative curve is shown in Figure 1. This curve is in good agreement with that shown by Pons *et al.* (10) on gossypol purified by numerous recrystallizations. It is also in agreement with the data obtained by King and Thurber (7) on a highly purified sample prepared by an improved procedure. Absorption maxima were observed at wavelengths of 236, 286, and 358 mμ. These maxima agree with those reported by other investigators (7, 10), who consider the extinction coefficient at 358 mμ as a measure of the purity of the gossypol. The extinction coefficients, $E^{g./l./1\,cm.}$, at 236, 286, and 358 mμ for various preparations of gossypol prepared from dianilinogossypol are shown in Table I. The purity of each preparation is also indicated in Table I and was calculated from the extinction coefficient observed at 358 mμ by assuming that the value of 39.9 observed by Pons *et al.* (10) is the correct value. These data indicate that the purity of gossypol prepared from dianilinogossypol is equal to that of gossypol prepared by other methods (7, 10).

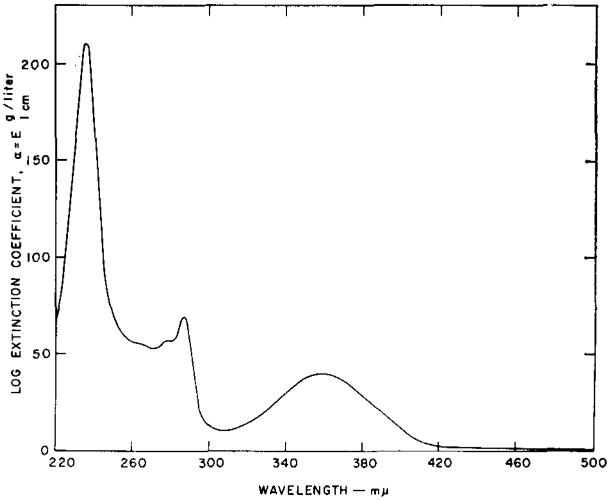

FIG. 1. Spectrophotometric curve for gossypol, prepared from dianilinogossypol, in cyclohexane.

The purity of each preparation was measured also by a second procedure. Samples of the gossypol were placed in a 250-ml., wide-mouth flask and dissolved in 10 ml. of peroxide-free ether. Fifteen milliliters of 0.05 N NaOH were added, and the ether was removed under reduced pressure while rotating the flask in warm water. Then 100 ml. of boiled and cooled distilled water were added, and the excess NaOH was titrated electrometrically, using glass-calomel electrodes, with standard 0.05 N HCl. The titration was continued through the second inflection of the curve, indicating that the disodium salt was converted to gossypol. A typical titration curve is shown in Figure 2.

The percentages of purity calculated from the titration data are shown in Table I. These values substantiate those derived spectrophotometrically and are in agreement with values obtained on a sample prepared

TABLE I

Characteristics of Pure Gossypol Prepared from Dianilinogossypol

Preparation No.	Extinction coefficient in cyclohexane			Purity calculated from	
	236 mμ	286 mμ	358 mμ	Extinction coefficient at 358 mμ	Alkalimetric titration
				%	%
1 [a]	206.0	70.4	39.9	100.0	99.5
2 [a]	210.0	70.0	39.8	99.7	99.1
3 [b]	205.0	70.0	39.8	99.7	99.9
4 [b]	210.0	69.0	39.8	99.7	100.0
5 [a]	212.0	69.4	40.2	100.5	97.8
Average	208.6	69.8	39.9	99.9	99.3

[a] Crystallized from an ether-alcohol-water mixture.
[b] Crystallized two times from the ether-alcohol-water mixture.

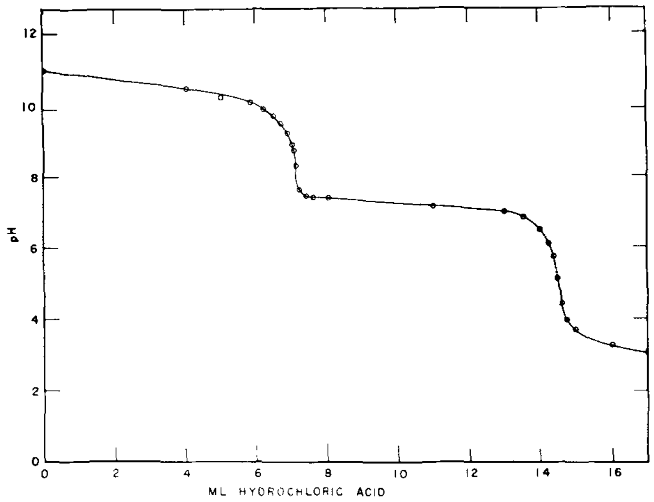

FIG. 2. Electrometric titration curve of 0.1013 g. of gossypol, prepared from dianilinogossypol, dissolved in an excess of NaOH (15 ml., 0.0503 N) with 0.0516 N HCl.

from gossypol-acetic acid by an earlier method (11). The nitrogen content of representative samples Nos. 2, 4, and 5 were respectively 0.093, 0.008, and 0.040% by the Macro-Kjeldahl method.

Discussion

Considerably more gossypol can be precipitated from an ether extract of cottonseed as dianilinogossypol than as gossypol-acetic acid. For example, 16 kg. of flaked cottonseed meats were extracted with ether, and the resulting oil was divided into two equal parts. The gossypol isolated as dianilinogossypol from one-half of the oil was 1.2%, based on the weight of the seed, while the gossypol isolated as gossypol-acetic acid from the other half of the oil was only 0.51%. An additional 0.56% gossypol precipitated from the filtrate of the latter by adding aniline.

The isolation of gossypol as the aniline derivative eliminates the necessity for removing most of the oil in order to precipitate gossypol-acetic acid more completely. The reddish color associated with gossypol appears to be more readily removed by the hydrolysis of the dianilinogossypol than it is when the gossypol is isolated from the extract as gossypol-acetic acid.

The conditions of hydrolysis described apparently circumvent the decomposition of gossypol as observed by Murty *et al.* (9) when large amounts of dianilinogossypol were hydrolyzed with hot concentrated H_2SO_4. Moreover the recombination of the hydrolyzed gossypol and aniline is not a problem, as was noted by King and Thurber (8), when the hydrolysis was carried out with ethanolic KOH. This recombination was apparently suppressed by the presence of the strong H_2SO_4 with which the aniline was probably combined as the aniline salt.

In purification, the yield is improved because of the low solubility of gossypol in the ether-ethanol-water mixture. The yield of pure gossypol was approximately 75%. Additional gossypol could be recovered by treating the filtrates obtained in the purification process with aniline.

Summary

A simple method has been developed for the acid hydrolysis of dianilinogossypol, resulting in gossypol yields of about 86%. The gossypol may be readily purified by recrystallization as gossypol-acetic acid from a mixture of ethyl ether and acetic acid. After removal of the acetic acid the gossypol may be crystallized either from a mixture of ether and light petroleum ether or from a mixture of ether, ethanol, and water. Crystallization from the latter mixture results in a yield of about 75% of gossypol, based on the amount of dianilinogossypol hydrolyzed, with a high degree of purity as is indicated by spectrophotometric and titrimetric methods.

Acknowledgment

The author wishes to express his appreciation to the Buckeye Cotton Oil Division of the Buckeye Cellulose Corporation for the flaked cottonseed used in this study.

REFERENCES

1. Adams, Roger, Price, C. C., and Dial, W. R., J. Am. Chem. Soc., *60*, 2158–2160 (1938).
2. Boatner, Charlotte H., Oil and Soap, *21*, 10–15 (1944).
3. Campbell, K. N., Morris, R. C., and Adams, Roger, J. Am. Chem. Soc., *59*, 1723–1728 (1937).
4. Carruth, F. E., J. Am. Chem. Soc., *40*, 647–663 (1918).
5. Clark, E. P., J. Biol. Chem., *76*, 229–235 (1928).
6. Clark, E. P., Oil and Fat Ind., *6*, No. 7, 15–19 (1929).
7. King, W. H., and Thurber, F. H., J. Am. Oil Chemists' Soc., *30*, 70–74 (1953).
8. *Ibid.*, *33*, 169–171 (1956).
9. Murty, V., Krishna, Murty, K. Satyanarayana, and Seshadri, T. R., Proc. Indian Acad Sci., *16a*, 54–61 (1942).
10. Pons, Walter A. Jr., Murray, Mildred D., O'Connor, Robert T., and Guthrie, John D., J. Am. Oil Chemists' Soc., *25*, 308–313 (1948).
11. Smith, F. H., and Halverson, J. O., Oil and Soap, *23*, 361–363 (1946).

[Received October 28, 1959]

Synthesis of Amino Acid Derivatives of Ethanolamine[1,2]

ELIZABETH C. SMITH, P. M. ALTHOUSE, and JAMES W. SHIGLEY, The Department of Agricultural and Biological Chemistry, The Pennsylvania State University, University Park, Pennsylvania

UNTIL QUITE RECENTLY little interest has been shown in the amino acid derivatives of ethanolamine. Although Fränkel and Cornelius (1) synthesized N,O-diglycylaminoethanol in 1918, it remained for James and Synge (2) in 1951 to detect an amino acid-ethanolamine linkage in a naturally-occurring material, the cyclic peptide gramicidin. Cheftel and co-workers (3, 4) have since been puzzled by the existence of what appear to be phosphatidyl ethanolamine-amino acid combinations of either a physical or chemical nature in blood. These materials, called "peptidic cephalins," behave in a peculiar manner during the process of purification. Their behavior pattern resembles the instability of the simple fatty acid esters and amides of ethanolamine under certain conditions of acidity and basicity (5).

This paper describes several attempts to find suit-

---

[1] Portion of a thesis presented by Elizabeth C. Smith as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] Authorized for publication as Paper No. 2399 on Sept. 1, 1959, in the Journal Series of the Pennsylvania Agricultural Experiment Station.