Occurrence of Gossypol in Dried Bract of the Cotton Plant

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ABSTRACT

Gossypol has been identified as a component of bract (FDB) from frost-killed, field-grown cotton by its chromatographic behavior on films of silica and cellulose (four eluents and five methods of detection). In addition, a derivative of gossypol was detected, and it was shown chromatographically that gossypol was removed from extracts of FDB by a divalent metal and by aniline. The total gossypol content, by spectrophotometric determination of gossypol-aniline complex, of FDB was 0.93%, but for a sample of bract from a cotton plant that had been grown in a hothouse it was only 0.048%. However the free gossypol contents in both were similar (0.065% and 0.044%, respectively). Both bract samples were from glanded cotton varieties. It is worthwhile to investigate the effect of gossypol on lung tissue to see if it can contribute to the acute response of byssinosis. In addition, it is noted that the waste from ginned cotton which is sometimes used as livestock feed may contain gossypol.

Bract is a leaf-like tissue under the boll of the cotton plant and a reportedly prominent component of cotton dust that arises in many processing operations involved with cotton. Bract is thought to contain the chemical causative(s) of byssinosis (1-5), an industrial pulmonary disease. Polyphenolic plant pigments, in addition to many other substances, have been suggested (4) as a causative of byssinosis. In addition, bract is part of the trash associated with seed cotton removed during ginning; this waste is sometimes made into pellets and used as feed for livestock. Gossypol, a cotton plant pigment, when consumed in sufficient amounts, is toxic to many animals (6,7). Pulmonary edema and congestion, depression of appetite and body weight, and hemolytic anemia are some of the adverse effects attributed to gossypol after ingestion.

Gossypol-related pigments and gossypol (6,8) are indigenous in the genus *Gossypium*. In the cotton plant these polyphenolic pigments are concentrated in pigment glands (9), discrete morphological entities, ovoid in shape and 100-400μ in diameter (10), that occur throughout the heliotropic portion of the normal cotton plant (11). These pigments occur in greater quantity in cotton roots than in the seed and to a much smaller amount in other parts of the cotton plant (12). Gossypol-free or "glandless" cotton plants contain approximately the same amount of gossypol in the roots, stem bark and leaves as glanded plants, but ca. 15 times less in the seed (13); however the total amount of gossypol does vary from variety to variety. The green bract contains many pigment glands, and the glands are sometimes still visible in dried bract (Fig. 1). The predominant naturally occurring pigment, comprising up to 40% of the pigment gland, is the yellow pigment gossypol (6,8). The other naturally occurring pigments closely related to gossypol occur in much smaller quantities, each probably comprising less than 1% of the gland (8). These gossypol-like pigments revert to gossypol on hydrolysis or can be formed from gossypol. Gossypol, a highly reactive substance, can occur as "free gossypol" (that which can be extracted with aqueous acetone), or "bound gossypol" (that which can be extracted only after acid treatment). A measurement of total gossypol is obtained when the plant material is first heated with oxalic acid and then extracted (6). Gossypol forms a complex with phospholipids (14) and with iron (method used for detoxifying feed) (6,15-17), reacts with proteins (18-20), amines (6,8,18), carbohydrates (18), triglyceride esters (18,21), and forms compounds readily with acetic acid and aniline (6) (method by which it is isolated and quantitatively determined).

Smith (13) found that pigment glands exposed to light contain very little gossypol and Sadykov et al. (22) found that, while gossypol was in high concentration initially in the leaves, it had decreased to only traces in the mature plant. We were interested in determining if dried, field-grown, frost-killed bract still contained free and bound gossypol and if so, how much. Thin layer chromatography, a rapid and very sensitive analytical technique for detection compounds in trace amount, was mostly used for the detection.

EXPERIMENTAL PROCEDURES

Bracts from frost-killed, field-grown cotton (High Plains, Lubbock, Tex., area) were collected by hand with special care being taken that the sample contained only dried bract from the base of mature bolls (FDB). Dried bracts from cotton grown in a greenhouse were collected in the same manner (HHDB). Both samples of bract were from glanded varieties of cotton. All extractions and determinations were performed on dried bracts ground in a Wiley mill (room temperature, 20 mesh) and stored in a dry state (stock).

The content of free and total gossypol in FDB and

FIG. 1. Dried bract from field-grown cotton showing gossypol glands (black dots).

HHDB was determined by AOCS Official Methods Ba 7-58 (23) and Ba 8-55 (24), respectively. These standard methods are for "gossypol and gossypol derivatives or gossypol-like pigments," rather than being specific for gossypol, and are based only on the spectrophotometric determination of the gossypol-aniline complex (25). For this reason, additional experiments were designed to prove that gossypol is a component of FDB.

To obtain chromatographic evidence for the presence of gossypol, extracts of FDB were prepared by extracting separate stock samples of FDB (5 g) with *n*-hexane, chloroform, ether and petroleum ether (bp 30-60 C) in a Soxhlet apparatus until the extracting solvent was colorless in the extraction vessel (~2 hr). The extracts were separately evaporated to a small volume (~1 ml) by flash evaporation followed by a stream of nitrogen gas. Samples (1-2 µl) of the concentrated solutions were examined separately on Silica Gel G (Merck) and cellulose MN300 (Macherey Nagel) by one dimensional thin layer chromatography in lined tanks at room temperature in several solvent systems: eluent I, acetone-benzene 2:8 w/w; eluent II, acetone-benzene-water 7:2:1 v/v; eluent III, *n*-heptane-chloroform-acetic acid 8:1:0.5 v/v; a fourth solvent system, chloroform-acetic acid 16:3 v/v (26) (eluent IV) was used only with silica. In addition, eluent I, which moves interfering pigments, and eluent II, which moves gossypol, were used as a two dimensional eluent pair on silica only. A sample of isolated gossypol (received from the Plains Cooperative Oil Mill, Lubbock, Tex. and prepared at USDA Southern Regional Research Laboratory, New Orleans, La.), which had been recrystallized from benzene-light petroleum ether (bp 30-60 C) (27), was applied to every chromatogram, prior to elution, and used as a standard (as a side marker and for overspotting). Gossypol was detected by: (a) examination of the chromatogram in visible light-yellow color; (b) examination in UV light-brown fluorescence; (c) spraying the chromatogram with sulfuric acid (6)-red color; (d) spraying with phloroglucinol solution (28) (positive reaction for aromatic aldehydes [29])- purple color; (e) spraying with ferric solution, saturated solution of Fe $(NH_4)(SO_4)_2 \cdot 12H_2O$ in water (positive reaction for phenols [30])-green color. The extraction and chromatographic procedures were performed with a minimum exposure to light to prevent degradation.

To show the presence of gossypol in different extracts of FDB, we also attempted to obtain evidence for its removal by specific reagents. Two samples of bract (5 g), to one of which gossypol (1 mg) had been added, were extracted with *n*-hexane in a Soxhlet apparatus for 2 hr. Each sample was divided into three equal portions. To one portion of each sample was added aniline, to a second, aqueous $CuSO_4$ solution (0.5 M), both of which form characteristic complexes with gossypol, and the solutions were stirred overnight. The third portion served as a control. The extracts were examined by one dimensional thin layer chromatography on silica.

To isolate a derivative of gossypol, the standard AOCS method for determination of total gossypol (24) was used in an attempt to obtain a solution containing the gossypol-aniline complex. Using this method a large sample of FDB (10 g) was digested (overnight) in a solution of oxalic acid (70 ml; 1 M in methyl ethyl ketone-water azeotrope), to increase the content of free gossypol. A portion of the solution obtained after reaction with aniline was chromatographed on silica using eluent I as the solvent and ferric solution as the chromogenic reagent. A standard gossypol-aniline complex, which was used for comparison, was prepared by dissolving gossypol (1 mg) in benzene (5 ml), adding aniline (5 ml) and heating (90 C) the solution overnight. After chromatography on silica, gossypol-aniline complex appears as a yellow-orange spot with a green ring if ferric solution is used as the chromogenic reagent. The standard contained an additional compound with a higher R_f value, which appeared blue with ferric solution, and was likely to be aniline-Fe^{+3} complex (31).

RESULTS

The total gossypol content of FDB was 0.93%, but of HHDB, only 0.048%. However the free gossypol content in FDB was 0.065%, and in HHDB, 0.044%. This information indicated spectroscopic evidence for the presence of a compound with similar absorption properties as the gossypol-aniline complex.

Examination of the solvent extracts by thin layer chromatography on silica, using all four eluents, and all the methods used for detection (particularly with phloroglucinol and ferric solution, which can be regarded as specific reagents for the functional substituents of gossypol), indicated that all of the extracts contained a compound that had the same mobility as a standard sample of gossypol. On silica with all eluents there was streaking (the degree varied in different eluents) of the compound that corresponded to gossypol as well as with the gossypol standard, and the R_f values of gossypol were concentration dependent. Chromatography on cellulose using eluent III showed, for the *n*-hexane extract and for the *n*-hexane extract to which gossypol had been added prior to extraction but not for gossypol, some streaking from the origin to about half way up the chromatogram, which appeared purple after the chromatogram had been sprayed with phloroglucinol solution. Also on cellulose, using eluents II and III, each sample contained a compound that had a similar mobility to the gossypol standard with all of the methods of detection with the exception of sulfuric acid, which was not used. Such an observation suggests that some gossypol was destroyed by the isolation procedure but not by chromatography on cellulose. In addition, some chemical alteration of the gossypol may have taken place on the silica as well as during the isolation procedure.

Chromatographic examination indicated that gossypol was removed by $CuSO_4$ and aniline treatments, from *n*-hexane extracts of plain bract and bract to which gossypol had been added prior to extraction, since the compound that corresponded to gossypol as well as the gossypol standard were missing after these treatments. No compound that had a similar mobility to gossypol-aniline complex was detected in these samples, possibly because of the low concentration of free gossypol. However, after the bracts had been hydrolyzed and treated with aniline, chromatography showed that hydrolyzed bracts contained a compound that had an R_f value identical to that of a known sample of gossypol-aniline complex if ferric solution was used as the chromogenic reagent.

The isolation of gossypol in bract was as reported for cottonseed—"tedious and time consuming" (8). In fact we were not able to isolate gossypol as its acetic acid or aniline complex from the various extracts of FDB, but only as the aniline complex after hydrolysis, probably because of its very low concentration, its high reactivity with other compounds, its ease of oxidation and alteration by light, and the interference of lipid. The concentration of lipid in these extracts is high, compared to that of free gossypol and bound gossypol that can be released, and the lipid tends to precipitate with these gossypol complexes. Even when FDB was extracted with *n*-hexane followed by extraction with ether, the same analytical difficulties were encountered.

DISCUSSION

Pigment glands were observable in dried bract (FDB and HHDB). Gossypol has been identified in bract (FDB) by

spectrophotometric determination of the gossypol-aniline complex and by its chromatographic behavior on films of silica and cellulose. In four eluents using four methods of detection, its R_f values were identical with those of a standard sample. In addition, it was shown chromatographically that gossypol was removed from extracts of FDB by a divalent metal and by aniline, and that a derivative of gossypol, the aniline-gossypol complex, was formed after the bracts had been hydrolyzed and treated with aniline. All of these observations indicate that gossypol is a component of FDB.

That the bound gossypol is much less abundant in HHDB may be due to FDB being killed rapidly by frost before translocation or chemical alteration of the gossypol, whereas in a hothouse under humid conditions these processes may have taken place by the time the bract becomes dried material (13). This suggestion is supported by the observation by Sadykov et al. (22) who reported that the initially high concentration of gossypol in the leaves decreased to traces in the mature plant.

The free gossypol and some of the bound gossypol (32) may be available for reaction. The surface lining of lung alveoli consists mainly of one or more monolayers of phospholipid (pulmonary surfactant) directly overlying the epithelial tissue (33-35). Phospholipid is a substance with which gossypol can form complexes (14). It is possible that substances which cause pulmonary edema (liquid on the lung surface, the liquid consisting mainly of transudates from the blood [34]) by ingestion, as does gossypol (6,7), may also cause a pulmonary problem by inhalation. In addition, polyphenolic compounds have been reported as a causative of byssinosis (4). Therefore, in view of the observation that gossypol, a polyphenolic compound, has been found in FDB (from the High Plains Area of Texas) and that gossypol can cause pulmonary edema when ingested, we suggest that the effect of gossypol on lung tissue should be investigated to see if it can contribute to the acute response of byssinosis. As far as the authors are aware, nothing has been published on the effect of gossypol on lung tissue, although some work in this area may have been done.

In addition, since the waste obtained from ginning cotton is sometimes used as livestock feed and this waste contains bract, it may therefore contain gossypol, a toxic substance to many monogastric animals (6,7). It is worth noting that an increased amount of gin waste may be used as animal feed, since ginners are now hard-pressed to dispose of gin waste with the restrictions on burning imposed by air pollution laws (36). Up to now gin waste was usually used only for ruminants which are not affected by gossypol (37). However, if gin waste is fed to monogastric animals, then gossypol could conceivably be a problem if present as free gossypol at levels greater than 0.05% (17).

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