^{14}C-GOSSYPOL: OPTIMUM CONDITIONS FOR SYNTHESIS BY COTTON SEEDLINGS

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Abstract The biosynthesis of ^{14}C-gossypol from sodium [1-^{14}C]-acetate has been studied in 6 day old seedlings of the cotton plant, *Gossypium hirsutum* L. c.v. ST-506. The highest specific activity gossypol (0.8 μCi/m mole) was obtained when seedlings were incubated for 96 hr in the presence of 6 mg/200 seedlings of sodium [1-^{14}C]-acetate (0.4 mCi/m mole) in a defined nutrient medium.

Key words Gossypol; *Gossypium hirsutum*; Cotton seedlings; Biosynthesis; [1-^{14}C]-Acetate; Exogenous acetate; ^{14}C-Gossypol

The discovery and use of gossypol in China as an effective agent for the regulation of male fertility[1,2] has stimulated interest in the study of its mechanism of action and its metabolism in humans and in animals. Studies in animal models can be facilitated by using radioactively labeled gossypol; such studies require gossypol of high specific activity.

A considerable amount is known regarding the biosynthesis of gossypol. It is well known that the carbon skeleton is entirely derived from acetate via mevalonate. The incorporation of acetate into gossypol (I) was shown by Smith et al[3~5] using excised roots of cotton seedlings and on whole seedlings of several varieties of *Gossypium hirsutum*. More recent studies by Heinstein have been aimed at the elucidation of the biosynthesis of gossypol at the enzymic level[6] and its formation by callus tissue culture[7]. In spite of all that is known on gossypol biosynthesis, the conditions for producing gossypol of the highest specific activity have never been optimized. The present study defines these conditions more precisely.

EXPERIMENTAL PROCEDURE

Materials: Cotton seeds (*Gossypium hirsutum* c. v. ST-506)[a], vermiculite(Tera-lite of size 234), sodium acetate [1-^{14}C], modified Robbin's nutrient solution[8], which contained in mg/1 the following chemicals: calcium nitrate, 41; potassium chloride, 45; potassium nitrate, 83.8; potassium hydrogen phosphate, 85, and ferric sulfate 2.5. To each liter of this solution was added 1 ml stock solution containing in mg/

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[a]Stonville Pedigreed Seed Co., Stoneville, Miss.

100 ml the following inorganic materials: boric acid, 30; cupric sulfate·5 H_2O, 1.2; potassium iodide, 0.6; manganese sulfate·H_2O, 16; ammonium molybdate, 1.2; zinc sulfate·5 H_2O, 1.2. Immediately prior to use, vitamins and antibiotics were added to the nutrient solution as follows: thiamine hydrochloride, 4 mg; niacin, 14 mg; pyridoxine hydrochloride, 20 mg and penicillin 10,000 U-streptomycin 10 mg. All chemicals used were reagent grade and solvents were either reagent or HPLC grade.

Procedures: *G. hirsutum* seeds were treated with conc. sulfuric acid and 5% sodium hypochlorite solution according to the method of Smith[5], prior to being placed in trays of vermiculite at room temperature (22～25°C) for germination. Approximately 400 seeds were spread evenly on top of a 12 cm depth of vermiculite in a tray(36×36×18 cm). The seeds were then covered with a 3 cm layer of moist vermiculite and watered daily. An 8% solution of captan[b] was added on the fourth day. On the sixth day, the seedlings, which had attained a size of *ca* 12 cm were collected, cleaned with water and grouped together in bundles of 100 with a rubber band placed just below the cotyledon. After immersing in captan solution for 5 min, these seedlings were used in feeding experiments. Thus, two bundles of seedlings were gently placed in each 800 ml beakers (200 seedlings per beaker), which contained 300 ml of modified Robbin's solution[8] and a known amount of [1-^{14}C] acetate. The roots of the seedlings were covered by the nutrient solution and were protected from light by surrounding the beakers with aluminium foil. The top of the beakers were covered with weighing papers. A stream of filtered air was allowed to bubble through the solution continuously.

At the times indicated in the tables and figures, the seedlings were removed from a beaker, the cotyledons excised and the roots washed in distilled water and dried between two sheets of filter paper in a stream of air at 30°C. The dried roots were ground and percolated with 75 ml portions (6×) of petroleum-ether, each percolation requiring 4 hrs of maceration.

A 20 μl aliquot from each extract was subjected to HPLC analysis[9] using a solvent system of acetonitrile: 33% acetic acid (3:2) and a combination instrument consisting of a Beckman Model 421 System Controller, Beckman Model 100 A and Model 110 A pumps with an added stop flow valve, a Perkin-Elmer LC-85 variable wavelength spectrophotometer detector with LC-75 Autocontrol, and Altex Model C-RIA recorder and a Waters Associates reversed phase, 10 microns, μ-Bondapak C_{18} column (30 cm×3.9 mm ID) with a guard column packed with the same stationary phase.

Under these conditions, gossypol has a retention time(rt) of 7.79 min. A standard curve was set up for amounts of gossypol ranging from 16 ng to 32 μg, Beer's Law was followed over this range. The slope (0.29), Y axis intercept (the peak height)

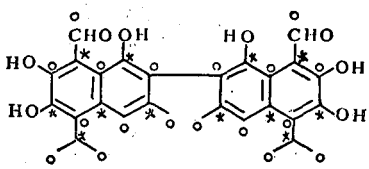
[b] Captan, an all purpose commercial fungicide and seed protectant.

(0.14) and the correlation coefficient (0.999) were calculated by linear regression analysis.

The effluent from the HPLC was collected in scintillation vials at 30 sec intervals for 10 min. Ten ml of Aquozoil II[c] scintillant was added to each vial and, the activity was counted in a liquid scintillation counter[d].

RESULTS AND DISCUSSION

In designing experiments to optimize the incorporation of ^{14}C-acetate into gossypol four parameters were considered: the age of the seedlings, the position of the radioactive label, the amount of exogenous acetate and the length of incubation. The large variation observed in the rate of germination made the measurement of the first parameter unfeasible. Instead, seedlings of the same size(12 cm, total length) on day 6 after germination were used for all experiments. The variation in germination was probably related to the time the seeds were treated with sulfuric acid. The expected labeling patterns of gossypol derived from [1-^{14}C] and [2-^{14}C]-acetate are indicated on the structure for gossypol. Of the 30 carbon atoms in gossypol, 12 can be derived from [1-^{14}C]-acetate and the remaining 18 can be formed from [2-^{14}C]-acetate. However, the known metabolic lability of the two formyl groups (10～12) made the use of [1-^{14}C-]-acetate preferable in spite of the potentially lower specific activity of the gossypol. The results obtained are the variation in specific activity of gossypol using [1-^{14}C]-acetate with the concentration of exogenous acetate and the time of incubation being variables.



(I)

* From[1-^{14}C]-acetate ° From[2-^{14}C]-acetate

The validity of the methods used for determining gossypol by HPLC and radioactivity in the gossypol can be seen in Figs 1 and 2 respectively. In normal seedlings gossypol is the major compound absorbing at 254 nm. The other compounds are unidentified. The coincidence of the major peak in the radioactivity profile (Figure 2) with gosspyol in Fig 1 can be clearly seen.

In the absence of exogenous acetate, the seedlings produced gossypol at a constant rate over 196 hr (Figure 3). The rate increased rapidly with the addition of exo-

[c]Aquozoil II: scintillation cocktail purchased from New England Nuclear Company, Boston, Massachusetts.

[d]Liquid Scintillation Counter: Parckard Model C 2421

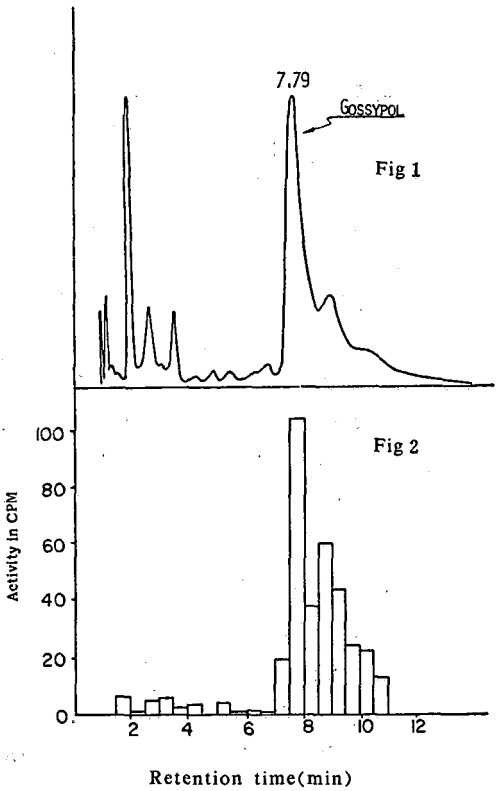


Figure 1. HPLC chromatogram of cotton seedling root extract
HPLC system: μ-Bondapak C_{18} column(with a guard column)
Acetonitrile: 33% acetic acid(3:2); 20 μl sample;
RT=7.79 min
Figure 2. Profile of radioactivity VS retention time

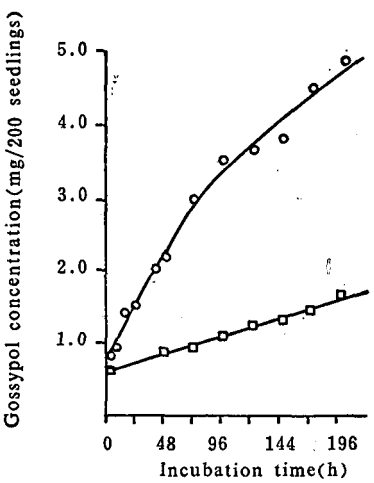


Figure 3. Production of gossypol
□——□ In absence of exogenous acetate
○——○ In presence of exogenous acetate

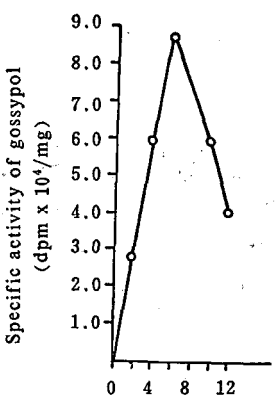


Figure 4. Specific activity of gossypol vs exogenous acetate concentration

*Specific activity of exogenous acetate=1.1×10^7 dpm/mg

genous acetate. Even with the addition of as little as 2 mg/200 seedlings the rate increased 7 fold over the first 72 hr but decreased somewhat thereafter (Figure 3). At a constant incubation time of 168 hr, the amount of gossypol synthesized increased with increasing exogenous acetate to give a maxium yield of 7.2 mg/200 seedlings when the seedlings were grown in the presence of 6 mg of acetate (Table 1). Higher amounts of acetate resulted in a sharply decreased production of gossypol presumably through an inhibitory mechanism.

TABLE 1 EFFECT OF EXOGENOUS ACETATE ON GOSSYPOL BIOSYNTHESIS IN *G. HIRSUTUM* SEEDLINGS

Acetate Added[a] (mg/200seedlings)	Wt. of Gossypol (mg)	Total Activity (dpm)	Specific Activity (dpm/mg)	% of Incorporation
0	1.5	0	0	0
2	4.3	119627	27821	0.54
4	6.3	367529	58805	0.84
6	7.2	621176	86274	0.94
10	6.2	358235	58249	0.33
12	4.9	194706	39736	0.15

[a]The specific activity of the added [1-^{14}C] acetate was 1.1×10^{7}dpm/mg and the time of incubation was 168 hrs

[1-^{14}C]-Acetate is rapidly taken up by the seedlings. Within 6 hr, 65 per cent is taken up when 2 mg of acetate was used. The uptake is the difference between the amount of radioactivity added and that remaining in the nutrient solution. Radioactivity can be detected in the gossypol produced by cotton seedlings incubated for 5 hr in [1-^{14}C]-acetate (Table 2). The incorporation rose almost linearly to a maximum of 0.54 per cent after 96 hr. Thereafter, there was no further incorporation. However, the specific incorporation slowly declined until at 192 hr, when it was 74 per cent of the maximum value at 96 hr, suggesting that after 96 hr, the exogenous acetate is

TABLE 2 EFFECT OF INCUBATION TIME ON GOSSYPOL BIOSYNTHESIS IN *G. HIRSUTUM* SEEDLINGS

Time of Incubation (hrs)	Gossypol Isolated (mg)	Total Activity (dpm)	Specific Activity (dpm/mg)	% of Incorporation[a]
5	0.8	18920	23649	0.09
10	0.9	23946	26606	0.11
15	1.4	35898	25641	0.16
24	1.5	42122	28081	0.19
39	2.0	58824	29412	0.27
48	2.2	68329	31059	0.31
72	3.0	97720	32573	0.45
96	3.5	118585	33881	0.54
120	3.7	121076	33172	0.55
144	3.8	124211	32687	0.56
168	4.5	121178	26928	0.55
192	4.9	123031	25108	0.56

[a]Calculated on the basis of [1-^{14}C]-acetate of activity 2.2×10^{7} dpm added at time zero

no longer being utilized and gossypol is being made primarily from endogenous acetate. The percentage incorporation increased with increasing exogenous acetate until there was a maximum incorporation of 0.94% at an acetate concentration of 6 mg/200 seedlings (Table 1). The specific activity at the maximum incorporation was 8.6×10^4 dpm/mg (Figure 4).

From these results it can be concluded that if 200 cotton seedlings are incubated with 6 mg of exogenous acetate for 96 hours in a defined nutrient medium then gossypol of high specific activity will be formed. Gossypol is present in most varieties of cotton and its biosynthesis has been easily demonstrated in several of these (3～6). The results presented here should be applicable to any variety of cotton seedlings producing gossypol.

CONCLUSIONS

In the present study, four days (96 hrs) of incubation in [1-^{14}C]-acetate medium was found to provide gossypol with the highest specific activity. Prolongation of incubation time did not increase the uptake of labeled acetate into the compound. It was also noted that prolonged incubation time subjected the seedlings to fungal infection. The addition of exogenous acetate was found to have a stimulatory effect on the biosynthesis of gossypol. The highest percent incorporation of labeled precursor occurred at a concentration of 6 mg of acetate per 200 cotton seedlings. Therefore, we recommend that cotton seedling be incubated for 96 hrs in a culture medium enriched with 6 mg of exogenous acetate per 200 seedlings for the production of labeled gossypol with high specific activity to be used in metabolic studies in animals.

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^{14}C-棉酚：棉花幼苗生物合成的最佳条件

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提 要

将[1-^{14}C]-乙酸钠加入棉花（*Gossypium hirsutum* c. v. ST-506）幼苗的培养液中，经四天的培育，得到最高放射活性的棉酚。实验中亦显示体外加乙酸钠具有激发棉酚生物合成的特性。每两百株棉花幼苗加入 6 mg 乙酸钠可得最高量的棉酚。

关键词 棉酚；棉花；棉花幼苗；生物合成；[1-^{14}C]-乙酸盐；外加乙酸盐；^{14}C-棉酚