Preparation of ^{14}C-Gossypol by Incorporation of Acetate-1-^{14}C and Acetate-2-^{14}C by Biosynthesis[1]

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ABSTRACT

Details for the germination of cotton seedlings and their utilization for incorporating ^{14}C-labeled acetate into gossypol are presented. Methods for the isolation and purification of the biosynthesized gossypol are given. The specific activity of the gossypol obtained from root tissue and cotyledons had mean values for 8 preparations of 428.6 x 10^3 and 6.3 x 10^3 dpm/mg, respectively. Corresponding incorporations were 5.42 and 0.32%.

INTRODUCTION

Gossypol (2,2′-binaphthalene-8,8′-dicarboxaldehyde-1,1′,6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl) is a toxic polyphenol which is synthesized in the roots (1) and other parts of the cotton plant (2,3). The enzyme systems found in the 105,000 x g supernatant from cotton root homogenates stereospecifically incorporate ^{14}C-acetate into the binaphthalene ring and substituent groups of gossypol (4,5). When acetate-1-^{14}C is incorporated into gossypol, the molecule is labeled at positions 1, 3, 6, 8, 10, and 12; and when acetate-2-^{14}C is incorporated, the label is in positions 2, 4, 5, 7, 9, 11, 13 or 14, and 15 (4). Biosynthetically prepared ^{14}C-labeled gossypol would be highly desirable in metabolic studies involving this compound.

This article reports a procedure for the biosynthetic preparation of labeled gossypol from either sodium acetate-1-^{14}C or sodium acetate-2-^{14}C and a method for the isolation and purification of the gossypol as gossypol acetic acid.

EXPERIMENTAL PROCEDURES

Materials required are: cottonseed of good quality, concentrated sulfuric acid, calcium hydroxide, germination paper (Anchorage Germination Paper Co., St. Paul, Minn.), sodium acetate-1-^{14}C, sodium acetate-2-^{14}C, ethanol, diethyl ether, aniline, and hexane. The concentrations (mg/liter) of the chemicals that were included in a modified Robbins nutrient solution (6) were as follows: calcium nitrate anhydrous, 40.4; potassium nitrate, 83; potassium hydrogen phosphate, 83; potassium chloride, 42; and ferric sulfate, 2.5. To a liter solution of these chemicals was added 1 ml stock solution that contained in mg/ml the following micronutrients: boric acid, 0.29; cupric sulfate·$5H_2O$, 0.008; manganese sulfate·H_2O, 0.154; ammonium molybdate, 0.01; zinc sulfate·$5H_2O$, 0.012; and potassium iodide, 0.006. Immediately before using the nutrient solution, the following was added in mg/liter: thiamine hydrochloride, 3; niacin, 13; pyridoxine hydrochloride, 20; penicillin G potassium, 4; and streptomycin sulfate, 15.

The cottonseeds were delinted by placing ca. 1500 ml seeds in a 2000 ml beaker and stirring with sufficient sulfuric acid to coat the seeds. When the fiber was disintegrated, ice water was poured into the beaker while the seeds were stirred vigorously. A screen was placed over the beaker, and the acid was decanted. After washing the seeds with several changes of tap water, an excess of calcium hydroxide was added to neutralize any residual acid. After ca. 15 min with occasional stirring, the solution was drained off, and the seeds were either air-dried or used immediately. The seeds were treated with 0.5% sodium hypochlorite just before using to prevent mold growth. Some lots of seeds developed excessive mold growth in spite of the hypochlorite treatment. These were discarded, and other lots of seeds were tested until a satisfactory lot was found.

After the seeds were treated with hypochlorite for 5 min, 75-100 seeds were placed on 25 x 38 cm germination paper ca. 5 cm from the top edge. (The paper was previously autoclaved for 30 min at 121 C.). Another sheet of germination paper was placed over the seeds to hold them in place. Both sheets were folded upward ca. 3.5 cm from the bottom edge and then from the left edge into ca. 6 cm folds. A rubber band was placed around the folded paper to keep the seeds in place between the papers. After removal of the rubber bands, the folded papers with the seed at the top were packed in a wire basket with sufficient pressure to prevent movement of seeds. The basket was placed in a tray that contained water ca. 2 cm deep. The tray was transferred to a plastic-lined cabinet or a germination chamber previously sprayed with lysol to inhibit mold contamination. It was maintained at ca. 30 C and at a relatively high humidity.

The seeds germinated in 36-48 hr, and the roots grew to ca. 8 cm in length in 4-5 days. The seedlings were bunched in groups of ca. 75 and were held together with a small rubber band placed just below the cotyledons. The bunched seedlings were covered with a wet germination paper to prevent drying. Any seedlings growing near a mold colony were discarded.

To 800 ml beakers were added 300 ml prepared nutrient solution, and as many bunches of the seedlings as could be squeezed into the beaker without damage were grouped together and lowered into the nutrient solution so that the roots barely touched the bottom of the beaker. Then 200-400 μCi ^{14}C-labeled acetate was added by means of a pipette. The beakers were placed in a tray in a hood with the roots protected from light by surrounding the beaker with cardboard ca. 10 in. high, over which was placed clear plastic. Air was bubbled slowly through the solution during the incubation.

After ca. a week, the residual nutrient solution in the beaker was drained off, and the seedlings were washed with several changes of water and were allowed to drain thoroughly on paper towels in a tray. The rubber bands were removed, and the roots were cut from the seedlings at the middle of the hypocotyl. Ca. 75 g roots were thoroughly homogenized, first at low speed and then at high speed, in a Waring blender with 150 ml ethanol containing 0.2 ml acetic acid/liter. To the homogenate, 150 ml diethyl ether was added, and the homogenization was continued for 1.5 min. The extract was filtered through a filter paper in a Buchner funnel using vacuum. The residue was washed with several portions of diethyl ether. The filtrate and washings were transferred to a 500 ml separatory funnel, and 150 ml ice water was added and mixed. The lower phase was transferred to a second separatory funnel and was washed with ether which was returned to the first separatory funnel. The extract was washed with 100 ml ice water that contained 25-50 mg sodium hydrosulfite and then with 100 ml ice water. The ether phase was filtered through a filter paper disc in a Gooch crucible and was transferred to a 500 ml

[1]Paper no. 4190, North Carolina State University Experiment Station, Raleigh, N.C.

TABLE I

Preparation of ^{14}C-Labeled Gossypol from Acetate-1-^{14}C and from Acetate-2-^{14}C

	Acetate		Tissue		Gossypol			
Preparation[a]	Supplies μCi	Label	Type	Weight %	Isolated[b] mg	In tissue %	dpm x 10^{-3}/mg[c]	Incorporation %
1 A	200	1-^{14}C	Root	90	50.3	0.055	738.7	8.37
B	200	1-^{14}C	Root	89	38.4	0.043	494.1	4.27
A	200	1-^{14}C	Cotyledon	125	105.7	0.085	5.2	0.12
B	200	1-^{14}C	Cotyledon	132	136.9	0.078	3.8	0.12
2 A	300	1-^{14}C	Root	150	84.0	0.077	356.2	4.49
B	300	1-^{14}C	Root	138	89.0	0.104	204.8	2.74
A	300	1-^{14}C	Cotyledon	210	236.7	0.126	3.5	0.14
B	300	1-^{14}C	Cotyledon	230	374.5	0.163	2.2	0.12
3 A	410	2-^{14}C	Root	152	51.6	0.034	538.2	3.05
B	410	2-^{14}C	Root	155	100.9	0.065	443.0	4.91
A	410	2-^{14}C	Cotyledon	201	217.4	0.108	19.0	0.45
B	410	2-^{14}C	Cotyledon	225	256.8	0.114	1.6	0.04
4 A	200	2-^{14}C	Root	150	101.7	0.068	381.6	8.74
B	200	2-^{14}C	Root	131	111.3	0.085	271.9	6.81
A	200	2-^{14}C	Cotyledon	162	485.4	0.230	12.9	1.41
B	200	2-^{14}C	Cotyledon	147	273.6	0.186	1.8	0.11
			Mean for root		78.4	0.062	428.6	5.42
			Mean for cotyledon		264.3	0.136	6.3	0.315
			Ratio of roots:leaves				17:1	

[a]Two batches of seedlings were incubated for each preparation and were designated A for root and cotyledon for the first batch and B for the root and cotyledon of the second batch.

[b]Calculated from the dianilino derivative.

[c]Calculated from gossypol acetic acid.

Erlenmeyer flask to which was added 2 ml aniline. After the volume was reduced under reduced pressure, a second batch was added and the concentration continued. To the extract, 10 ml ethanol and 2 ml water were added, and the removal of ether was continued until precipitation began. After standing at room temperature for 1 hr, 2-3 ml water was added in 1 ml portions at ca. 5 min intervals. The flask was allowed to set overnight for completion of the precipitation of the dianilinogossypol. The precipitate was collected on a paper disc in a tared Gooch crucible and was washed with 50% aqueous ethanol, a small amount of ethanol, and finally with ethanol-hexane 50/50, v/v. The dianilinogossypol was dried at 110 C or in a vacuum oven at 60 C.

The dianilinogossypol was purified by hydrolyzing 100-600 mg dianilinogossypol suspended in 20 ml glacial acetic acid in a 250 ml Erlenmeyer flask with 3 ml sulfuric acid (23 ml H_2SO_4 + 2 ml H_2O) for 4 min, while swirling the flask and keeping it cold by occasionally dipping it in ice water. After adding 100 ml ethyl ether and 100 ml ice water, the mixture was transferred to a 500 ml separatory funnel. The water phase was transferred to another separatory funnel and washed with a small amount of ether which was transferred to the first separatory funnel. The ether layer was washed once with 100 ml ice water (all water washings were ice cold) containing 1 ml of the above H_2SO_4 mixture, twice with 100 ml water containing 0.5 ml H_2SO_4, once with 100 ml water, once with 100 ml water containing 25-30 mg sodium hydrosulfite, and finally with 100 ml water. The ether layer was filtered through a paper disc in a Gooch crucible and then transferred to a 250 ml Erlenmeyer flask. The gossypol was reprecipitated as the dianilinogossypol using 1 ml aniline and 10 ml ethanol, as previously described.

Conversion of Dianilinogossypol to Gossypol Acetic Acid

The reprecipitated dianilinogossypol was hydrolyzed as described above. The ether phase was filtered and transferred to a 250 ml Erlenmeyer flask and then was taken to dryness under reduced pressure, while swirling the flask in hot water. After the dry residue was dissolved in 30 ml glacial acetic acid and 25 ml 95% ethanol, water was added (usually 40-45 ml) until the solution became slightly turbid. The mixture was heated by swirling the flask in hot water (70-75 C) until the turbidity disappeared or until crystals began to grow. After standing for ca. 2 hr, an additional 10 ml water was added, and the gossypol acetic acid was allowed to precipitate overnight. The gossypol acetic acid was filtered in a tared Gooch crucible containing a paper disc and was washed with a mixture of ethanol-water-acetic acid, 5:5:1, respectively, then with a small amount of ethanol-hexane-acetic acid, 5:5:1, and finally with a small amount of hexane. The gossypol acetic acid was either dried in an oven at 105 C or 30 min or for 2 hr in a vacuum oven at 60 C.

The purity of either the gossypol or the gossypol-acetic acid was determined by preparing the dianilino derivative in a solvent mixture of ethanol-water-diethyl ether-acetic acid (715:285:200:0.2) and determining the absorbance of a known amount diluted to 25 ml on a Beckman DU spectrophotometer at 445 nm and a light path of 10 mm (3). Purity was calculated from the molar extinction coefficient of 39,706 for the equivalent dianilino derivative of these compounds derived from an absorbance-concentration standardization curve (3).

The method for the hydrolysis and purification was worked out using nonradioactive dianilinogossypol.

Although several preparations of ^{14}C-labeled gossypol have been prepared and converted to gossypol acetic acid, only representative data will be presented to show the specific activities that were obtained for the root and cotyledon portions of the seedlings. The ^{14}C-gossypol was more readily purified by precipitation as gossypol acetic acid in which the molar ratio of gossypol to acetic acid is 1 to 1 (7). The radiochemical purity of the purified gossypol acetic acid was determined by radioautograph of paper chromatograms prepared by the method of Schramm and Benedict (8).

Determination of Specific Activity of ^{14}C-Labeled Gossypol

The radioactivity of some of the samples was determined by oxidation with the Van Slyke reagent in an apparatus for static combustion (9) and absorbing the $^{14}CO_2$ in ethanolamine, an aliquot of which was added to 15 ml scintillation mixture consisting of 4 g 2,5-diphenyloxazole and 0.1 g 1,4-bis-[2-(5-phenyloxazolyl)] benzene in 1 liter of

toluene-cellosolve (50:50). The samples, for the most part, were oxidized to $^{14}CO_2$ on a Packard model 305 Tri-Carb sample oxidizer. After weighing, the dianilinogossypol was dissolved in chloroform and made to 25 ml; the gossypol-acetic acid was dissolved in a small amount of ether and then diluted to 25 ml with a mixture of ethanol-water-ether-acetic acid (715:285:200:0.2). Aliquots (1 ml) of these solutions were dried on 5.5 cm filter papers which were made into rolls that were inserted into a 10 cm section of inflated dialysis tubing previously closed at one end. The tubes were folded and pressed into pellets in a pelleting press. The pellets were burned in the Packard Tri-Carb sample oxidizer, and the $^{14}CO_2$ was collected in 4 ml ethanolamine. The ethanolamine was washed into the scintillation vial with 9 ml methanol and 6 ml scintillator consisting of 15 g 2,5-diphenyloxazole and 1 g p-bis-(o-methylstyryl) benzene diluted to 1 liter with toluene. All samples were counted in a Packard Tri-Carb liquid scintillation spectrometer, and the counts were corrected for background and quench. The activity is reported as dpm/mg of gossypol (Table I), since all of the ^{14}C in the product was derived from the gossypol molecule.

RESULTS AND DISCUSSION

Pure gossypol acetic acid was obtained most readily by conversion of reprecipitated dianilinogossypol. The recovery in this step was ca. 90%. The results from the conversion of 5 portions of 100 mg reprecipitated dianilinogossypol to gossypol acetic acid showed an average recovery of 89.2 ± 2.5% of the gossypol calculated from an average of 77.1 ± 2.2 mg gossypol acetic acid recovered. The purity of the gossypol acetic acid average 101.0 ± 0.80%. Radioautographs of paper chromatograms of the purified ^{14}C-gossypol acetic acid showed only one spot indicating a high radiochemical purity.

The data from the preparation of ^{14}C-labeled gossypol from sodium acetate-1-^{14}C and sodium acetate-2-^{14}C are shown in Table I. The data indicate that the root tissue of the cotton seedlings very actively incorporated acetate-1-^{14}C and acetate-2-^{14}C into gossypol. It is surmised from the low specific activity of the gossypol in the cotyledons compared to that in the roots, mean dpm values of 6.8×10^3 vs. 428.6×10^3, respectively (Table I), that very little gossypol was synthesized in the cotyledons. The ratio of incorporation for the respective tissues was 1:17. Much of the gossypol found in the cotyledons was probably from gossypol stored in the original seeds.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service Grant AM-07039.

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[Received October 11, 1973]