Isolation of Coumestrol and Other Phenolics from Alfalfa by Countercurrent Distribution

By E. M. BICKOFF, A. L. LIVINGSTON, S. C. WITT, B. E. KNUCKLES, JACK GUGGOLZ, and R. R. SPENCER

Countercurrent distribution (CCD) procedures are described for the isolation of coumestrol and 12 other phenolic compounds from alfalfa. Three of these were identified as trifoliol, tricin, and salicylic acid. Elemental analysis indicated empirical formulas for four of the other compounds. R_f values on paper chromatograms and fluorescent characteristics under ultraviolet light are presented for all the compounds isolated.

IN RECENT YEARS, a tremendous interest has developed in the use of synthetic hormones to stimulate rate and efficiency of gain in animals. Alfalfa meal, rich in the estrogenlike compound coumestrol (1), has been shown to produce increased growth rates when fed to sheep (2, 3) or cattle (4). This increased growth rate may be attributed either to coumestrol or to factors presently unidentified. Because of inadequate supplies of the pure plant estrogens, their effectiveness on animal growth has not yet been ascertained.

Work was recently undertaken to obtain enough coumestrol as a concentrate or in crystalline form to permit evaluation of its potential as a growth promoter. An earlier paper (5) reported the preparation from alfalfa of approximately 2500 Gm. of a crude crystalline product consisting of about 70% coumestrol. This paper reports the procedures employed to obtain the coumestrol in pure crystalline form.

It also seemed of interest to investigate the accompanying compounds, many of which fluoresced strongly under ultraviolet light. The techniques employed for the isolation of a number of them by countercurrent distribution are also presented.

EXPERIMENTAL

Equipment.—Two Craig countercurrent distribution (CCD) instruments were used for the separations. Both instruments are 100-tube models, robot-operated, and equipped with fraction collectors. The smaller (20-ml. tube capacity) was mainly used for exploratory development of solvent systems, while the larger (200-ml. tube capacity) was used for most of the isolation work. The single withdrawal procedure (6) was used.

Received August 7, 1964, from the Western Regional Research Laboratory, Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Albany, Calif.

Accepted for publication September 15, 1964.

The authors are indebted to Laurence M. White and Geraldine E. Secor for elemental analyses, to Saima Karp for infrared absorption spectral measurements, and to Kay Lee for X-ray diffraction measurements.

Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

Paper Chromatography.—The progress of each CCD run was followed by spotting solutions from selected tubes, both from the instrument and from the fraction collector on Whatman No. 1 paper. The system which gave maximum resolution of the compounds under investigation was isopropyl alcohol/ammonium hydroxide (28–30% NH_3) (2/1), descending (solvent system 1). The various components could be detected on the developed chromatogram as colored fluorescent spots under ultraviolet light. Exposure to ammonia vapor or spraying with dilute sodium carbonate solution usually intensified the fluorescence and often changed the color of the spot. A chromatogram made at the completion of each CCD run served as a permanent record of that separation (Fig. 1). The composition of the various fractions obtained during processing could be evaluated more accurately by two-dimensional paper chromatography. Solvent system 2, employed for the second dimension, consisted of 50% acetic acid, ascending.

Many compounds were present in minute amounts and were not detected by paper chromatography in the original crude extract or in the initial fractions. However, as the major components were removed by CCD, the minor ones became visible on the developed chromatograms. Table I gives approximate R_f values and fluorescent color for the compounds under investigation. The R_f values of the compounds are averages from a number of chromatograms of the various fractions. Since these compounds move a little differently when chromatographed as mixtures, these data only locate the relative position of each compound approximately.

Distribution of the Crude Crystalline Product.—One thousand grams of the coumestrol preparation, isolated in the large-scale processing of alfalfa (5), was the starting material for this study. Coumestrol and a number of the other compounds have only limited solubility in most of the common sol-

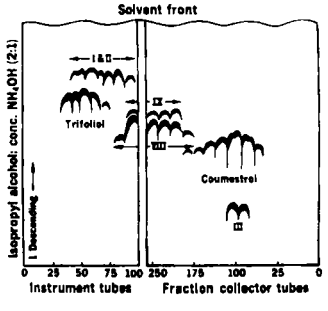


Fig. 1.—Schematic representation of the paper chromatogram of the CCD run for fraction *19* through solvent system D.

TABLE I.—FLUORESCENCE AND R_f VALUES OF PHENOLIC COMPOUNDS OF ALFALFA ON TWO-DIMENSIONAL PAPER CHROMATOGRAMS

Compd.	R_f Values: First Dimension[a]	R_f Values: Second Dimension[b]	U.V. Fluorescence: Untreated	U.V. Fluorescence: plus NH_3 vapor
Coumestrol	0.50	0.52	Purple-white	Yellow-white
Salicylic acid	0.77	0.81	Blue-gray	Deep blue
Tricin	0.36	0.56	Black absorbing	Yellow
Trifoliol	0.66	0.57	Dull blue-gray	Blue-gray
I	0.75	0.60	Blue	Intense blue
II	0.75	0.60	Blue	Intense blue
III	0.36	0.36	Bluish-white	White
IV	0.44	0.69	Light tan	Yellow-green
V	0.17	0.55	Tan	Yellow
VI	0.16	0.34	Purple-gray	Yellow-gray
VII	0.26	0.43	Violet	Yellow-pink
VIII	0.58	0.42	Blue	Intense blue
IX	0.64	0.53	Blue	Intense blue

[a] Solvent system 1—isopropyl alcohol:concentrated ammonium hydroxide (2:1), descending. [b] Solvent system 2—50% glacial acetic acid, ascending.

TABLE II.—SOLVENT SYSTEMS EMPLOYED IN COUNTERCURRENT DISTRIBUTION OF ALFALFA PHENOLICS

Solvent System	Solvents and Proportions by Vol.	Starting Material	Total Solids, Gm.	Solids in Each Run, Gm.
A	Skellysolve B:ether:MeOH:H_2O:DMF[a] (2:5:3:1:0.4)	Crude coumestrol preparation	1000	40
B	Ethyl acetate:skellysolve B: Ether:MeOH:H_2O:DMF (12:12:10:5:4:8)	Fraction *2*	860	215
		Fraction *11*	56	28
		Fraction *12*	96	24
C	Acetone:skellysolve B:ether:H_2O (2:1:1:1)	Combined fractions *4* and *7*	200	10
D	H_2O:DMF:acetone:CCl_4 (2:4:3:5)	Fraction *19*	36	12

[a] DMF, dimethylformamide.

vents employed for the distributions. However, by using dimethylformamide as a carrier solvent, over 200 Gm. of material could be distributed in certain solvent systems, and adequate separations could be made.

Fifty-eight distributions employing five solvent systems (Table II) were required to separate coumestrol and 12 of the other phenolic compounds. Figure 2 presents the sequence of these distributions. The Roman numeral assigned to a particular compound depended upon its place in the sequence of purification. No number was assigned to a compound unless it was evident that it could be isolated. The first objective was to isolate the bulk of the coumestrol from the minor constituents in the mixture. This was necessary because the total amount of material to be processed greatly exceeded the normal capacity of the equipment.

Distribution in Solvent System A.—The material was first distributed in 40-Gm. batches (Fig. 2). Because of limited solubility, the material was introduced into the first 18 tubes of the instrument. After 260 transfers, the runs were divided into fractions *1–3*, determined by a paper chromatogram of selected tubes of the completed distribution. Comparable fractions from each of the 25 identical distributions were combined and taken to dryness *in vacuo*.

To date, no further work has been done on fraction *1*, which weighed about 60 Gm. and contained most of the dark brown waxy impurities and few fluorescing compounds. Fraction *2* weighed 860 Gm. and contained most of the coumestrol. Fraction *3* contained a small amount of coumestrol and three other compounds which were difficult to separate from one another. However, they could be separated more readily as their acetates. Repeated crystallization of the acetylated mixture from acetone yielded a pure preparation of the acetate of a compound identified as the coumestan, trifoliol. The latter compound was recently isolated from ladino clover (7). The other two compounds (compounds I and II) had similar solubility characteristics and were difficult to separate from one another. About 1 mg. of each as the acetate has been obtained by thin-layer chromatography (TLC) on silica gel using ether/skellysolve B (4/1) as the developer. However, they could not be separated in larger quantities by CCD, column chromatography, or recrystallization.

Distribution of Fraction 2.—This fraction was distributed in solvent system B (Table II) in four 215-Gm. batches. For each distribution, the starting material was introduced into the first 22 tubes of the instrument. After 400 transfers, three fractions (*4–6*,) were made as determined by a paper chromatogram of the completed run. The corresponding fractions from each distribution were combined and concentrated *in vacuo*. Fraction *4* (160 Gm.) contained most of the minor constituents and only a small amount of coumestrol. Fraction *6*, which amounted to about 10 Gm. and was mostly compounds I, II, and trifoliol, was acetylated and combined with fraction *3*.

Fraction *5* contained 80% of the weight of the starting material. When analyzed by a fluorometric method (8), this fraction contained about 90% coumestrol. The other compounds could not be

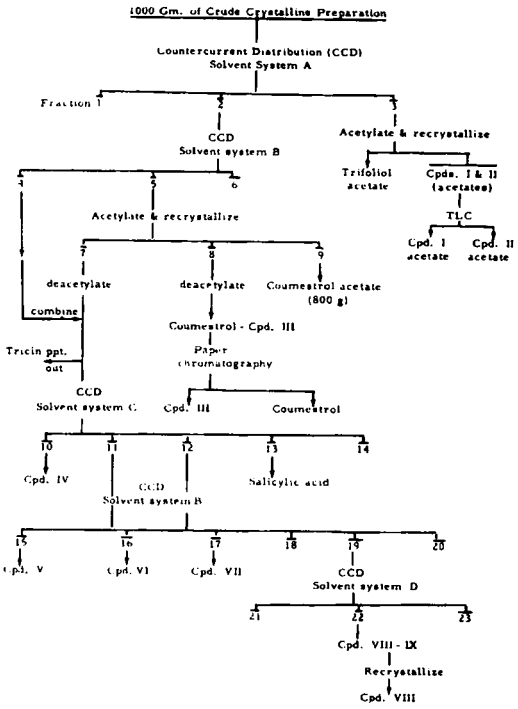


Fig. 2.—Flow sheet for separation procedures.

separated from the coumestrol by recrystallization. However, acetylation and repeated careful recrystallization from chloroform gave pure coumestrol acetate (fraction *9*) and two additional fractions, *7* and *8*. Fraction *7* appeared to be similar to fraction *4* in composition, except that it contained a small amount of the acetates of compounds I, II, and trifoliol. Therefore, fraction *7* was deacetylated, combined with fraction *4*, and these served as the starting material for the next series of distributions. Fraction *8*, after deacetylation, weighed about 55 Gm. and consisted of only two compounds (coumestrol and compound III). Compound III could be separated from coumestrol by paper chromatography employing solvent system I. To date, we have not succeeded in separating it by CCD or recrystallization.

Isolation of Coumestrol.—Fraction *9* consisted of approximately 800 Gm. of pure coumestrol acetate. It was deacetylated to yield 560 Gm. of coumestrol. For deacetylation, the coumestrol acetate was hydrolyzed in 40-Gm. batches. To a cold 1/2% potassium hydroxide solution in methanol (2 L.) was added 40 Gm. of the acetate. The mixture was stirred in an ice-bath for 1 hour; then the ice-bath was removed, and the reaction was slowly allowed to come to room temperature with application of heat. The stirring was continued for 5 to 6 hours until almost complete solution had occurred. The mixture was filtered and added to 6 L. of ice-water. The pH of the yellow solution was adjusted to about 5.5 with dilute hydrochloric acid to precipitate coumestrol. The heavy slurry was cooled for several hours; then the pH was adjusted to 2–4, and the coumestrol (30 Gm.) was collected. A whiter product could be obtained when the final pH adjustment was made just before filtering the product.

Isolation of Tricin.—When combined fractions *4* and *7*, weighing 225 Gm., were dissolved in acetone and mixed with about a half volume of skellysolve B, a copious precipitate was formed. Recrystallization from methanol gave 25 Gm. of pure tricin. This flavone has been previously isolated from alfalfa by another process (9).

Distribution of Combined Fractions 4 and 7.—The above filtrate was evaporated to dryness, then distributed in 10-Gm. batches in solvent system C (Table II) for a total of 200 transfers. Comparable tubes from each of the runs were combined and divided into five additional fractions (fractions *10–14*). Recrystallization of the solids obtained from fraction *10* gave 5 Gm. of compound IV. Fraction *14* consisted of a mixture of compounds I, II, and trifoliol and was combined with fraction *3*.

Isolation of Salicylic Acid.—Crystals were obtained from fraction *13* by extraction with hot skellysolve B, followed by recrystallization from water. About 5 Gm. of colorless needles, identified as salicylic acid, were obtained.

Distribution of Fractions 11 and 12.—Fractions *11* and *12* contained essentially the same group of compounds, although their relative proportions differed greatly. Therefore, each fraction was separately distributed in solvent system B for a total of 900 transfers. Based on paper chromatograms of the completed runs, comparable tubes were combined to give six fractions (fractions *15–20*). Although several of the fractions consisted predominantly of one compound, small amounts of other compounds were also present. However, by recrystallization of the solids in each fraction from methanol, the compound present in largest amount was obtained. In this way, compounds V, VI, VII were obtained from fractions *15*, *16*, and *17*, respectively.

Distribution of Fraction 19.—Fraction 19 was a mixture of six compounds. Distribution of this fraction in solvent system D for 365 transfers gave three additional fractions (fractions *21–23*) (Fig. 2). Fraction *22* contained only compounds VIII and IX. Compound VIII was obtained from this mixture by recrystallization from ethyl acetate.

TABLE III.—ANALYTICAL AND PHYSICAL DATA OF PHENOLIC COMPOUNDS ISOLATED FROM ALFALFA

Compd.	Empirical Formula	Anal. C Calcd., %	Anal. C Found, %	Anal. H Calcd., %	Anal. H Found, %	M.p., °C.
Coumestrol	$C_{15}H_8O_5$	67.1	66.8	3.09	3.10	385° dec.
Trifoliol	$C_{16}H_{10}O_6$	64.4	64.5	3.36	3.42	327° dec.
Tricin	$C_{17}H_{14}O_7$	61.8	61.8	4.24	4.38	291–294°
Salicylic acid	$C_7H_6O_3$	60.9	61.1	4.35	4.41	158°
IV	$C_{15}H_{10}O_4$	70.9	70.8	3.93	3.99	315–316°
VI	$C_{15}H_8O_6$	63.4	62.8	2.84	3.20	>350° (darkens)
VII	$C_{16}H_{10}O_6$	64.4	64.3	3.38	3.46	303° dec.
VIII	$C_{17}H_{12}O_6$	65.4	65.5	3.85	3.85	306°

Characterization.—For all 13 compounds, two-dimensional paper chromatograms were prepared, and the R_f values and fluorescence of each compound were ascertained (Table I). In addition, for eight of the compounds, melting points (uncorrected) were determined and the compounds subjected to elemental analysis. From the elemental analyses, the most probable empirical formulas have been calculated (Table III).

Identification of coumestrol, tricin, trifoliol, and salicylic acid was accomplished by comparison of their X-ray diffraction patterns and infrared spectra with those of authentic samples. In addition, mixed melting point determinations and two-dimensional paper chromatograms further confirmed their identity.

REFERENCES

(1) Bickoff, E. M., *et al.*, *Science*, 126, 969(1957).
(2) Fox, C. W., and Oldfield, J. E., *Proc. Western Sec., Am. Soc. Animal Prod.*, 13, (No. XLVII) (1962).
(3) Oldfield, J. E., Fox, C. W., and Bickoff, E. M., *J. Animal Sci.*, 19, 1281(1960).
(4) Matsushima, J., *Nebraska Univ. Agr. Exptl. Sta. Bull.*, 29 (April 17, 1959).
(5) Bickoff, E. M., *et al.*, *J. Agr. Food Chem.*, in press.
(6) Craig, L. C., and Post, O., *Anal. Chem.*, 21, 500(1949).
(7) Livingston, *et al.*, *Tetrahedron*, 20, 1963(1964).
(8) Livingston, A. L., *et al.*, *Anal. Chem.*, 32, 1620(1960).
(9) Fergerson, W. S., Ashworth, de B., and Terry, R. A., *Nature*, 163, 606(1949).

Synthesis of Some Hexamine Derivatives as Potential Antispasmodics

By GARY OMODT

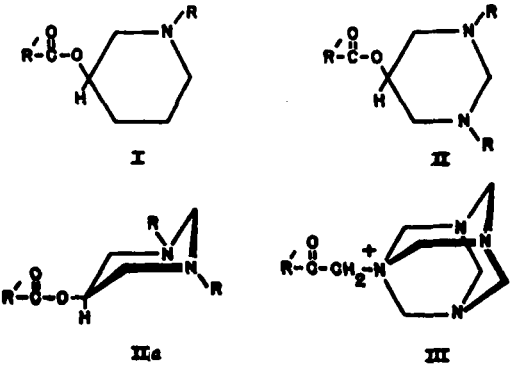
The activity of an antispasmodic of the atropine type appears to be related to the rigidity of the nitrogen-containing moiety. Hexamine contains a very rigid ring system, and it would seem that hexamine would furnish a promising starting point in the synthesis of a good antispasmodic. Some hexamine derivatives were synthesized by reacting the appropriate halomethyl amide or ketone with an excess of hexamine in refluxing chloroform. Preliminary pharmacological evaluation indicates that one of the hexamine derivatives possesses good antispasmodic activity.

THE LITERATURE indicates that a parasympatholytic antispasmodic containing a rigid nitrogen ring system is more active than when the nitrogen is not held in a conformation so rigid (1, 2). Perhaps the reason for this is due to a more or less fixed nitrogen-carbonyl distance *versus* a variable distance in the nonrigid compound. The foregoing hypothesis presumes that the fixed distance is optimum for combination with the potential acetylcholine receptor. Recent research has been directed along the lines of incorporating very rigid bridged nitrogen systems into synthetic antispasmodics (3–8).

Compounds with the general structure of I are on the market as useful antispasmodics (piperidolate, pipenzolate methylbromide, mepenzolate methylbromide) in which the R group is a small alkyl, and the R′ is a large, bulky blocking group. Considering only receptor combination and disregarding effects due to solubility and distribution, compounds relative to structure II might possibly possess activity equal to or better than compounds relative to structure I. This is the case because of the additional nitrogen located in an identical position (with relation to the carbonyl) to the first nitrogen, and thus providing an additional receptor combination site; *i.e.*, the chances of the compound combining with the potential acetylcholine receptor would be increased. This is illustrated better by structure IIa. This is actually structure II from a "45° side view," whereas structure II is a view from the "top."

In reference to compound III, three nitrogens of equal distance from the carbonyl are present, the distance being approximately equal to the nitrogen-carbonyl distance in structure I. In addition, structure III is very symmetrical with respect to the nitrogen ring system. Since all three nitrogens are of equal distance from the carbonyl (this distance being rigidly fixed) and the ring system is quite symmetrical, it would appear that a compound with a structure similar to that of III would be apt to combine with the potential acetylcholine receptor.

The quaternized nitrogen in compounds possessing type III structure may affect the ability to



I II IIa III

Received August 7, 1963, from the Department of Pharmaceutical Chemistry, College of Pharmacy, South Dakota State University, Brookings.
Accepted for publication June 22, 1964.