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Identification of Estrogens by Paper Chromatography

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In animals, estrogenic steroids occur almost invariably in mixture, but in spite of the great differences in their biological activity, chemical and biological assays are usually reported in terms of a single estrogen, owing to the technical difficulties in their separation and identification. Partition chromatography on paper can be used for separating closely related compounds, provided they show a difference in distribution coefficients and can be detected in small amounts. This technique has been applied successfully to the identification of ketosteroids by Zaffaroni *et al.* (*3*). A simple and rapid procedure for the identification of minute amounts of estrone, estradiol, and estriol by paper chromatography is presented here, which may be applicable to differential determination of estrogen content of biological specimens.

Colored derivatives of the estrogens are prepared by coupling with diazotized *p*-nitrobenzeneazodimethoxyaniline (Fast Black Salt K[1]). This reagent, proposed by Marx and Sobotka (*1*) for the determination of equilenin and dihydroequilenin, was reported as not coupling readily with estrone, estradiol, or estriol. However, K salt can be made to react with these steroids in a strongly alkaline medium, either by heating the mixture or by allowing it to stand overnight. A saturated aqueous solution of K salt is prepared freshly and filtered.[2] The estrogens are dissolved in absolute ethanol and 0.1 ml of each solution, containing a minimum of 3 μg of any steroid, is placed in a test tube, and a tube containing 0.1 ml alcohol is used as a blank. Then 0.2 ml of the diazo reagent and 0.1 ml of 20% Na_2CO_3 solution are added and the contents of the tubes are mixed and heated in a boiling water bath for 10 min and later cooled in water. To check evaporation, a glass bulb is placed in the mouth of each tube. Coupling also takes place if the mixture is allowed to stand overnight. The color deepens on standing and, once developed, it is stable for weeks. To extract the dyes, 0.2 ml benzene is added, and the tubes are shaken vigorously. The contents of each tube are drawn up into a medicine dropper with a fine tip, allowed to separate, the aqueous phase is expelled, and the entire benzene solution is applied to the filter paper.

[1] Emil Greiner Co., New York City.

[2] The solution keeps at least a day at room temperature and several days at 5° C, but it should be filtered before use to minimize the blank.

TABLE 1

COLOR AND LOCATION OF SPOTS IN THE PAPER CHROMATOGRAM OF ESTROGENS AND RELATED COMPOUNDS

Compounds	Color of spots	R_f values
Estrone*	purple	0.95 ± .03
Estradiol*	"	0.81 ± .06
Estriol*	"	0.07 ± .03
Estrone and estradiol†	"	0.96 and 0.85
Estrone and estriol†	"	0.96 and 0.09
Estradiol and estriol†	"	0.85 and 0.09
Estrone, estradiol, and estriol†	"	0.96, 0.85 and 0.09
Estrone sulfate‡	"	0.95
Equilin§	"	0.96
Phenol	red	0.50
Catechol	yellow	0.00
Diethylstilbesterol‖	none	...
1-Methylestradiol diacetate§	purple	0.81 and 0.96
Dehydrocorticosterone acetate‖	yellow	0.95
Progesterone*	"	0.74
Pregnanediol¶	none	...
Dehydroisoandrosterone acetate*	"	...
cis-Testosterone**	yellow	0.21
Isoandrosterone††	none	...
Δ^4-Androstene-3,17-dione††	yellow	0.60 and 0.97
Δ^1-Androstene-3,17-dione§	none	...
$\Delta^{1,4}$-Androstadiene-3,17-dione§	"	...

* Courtesy of Schering Co.
† Equal parts.
‡ Courtesy of Ayerst, McKenna, and Harrison, Ltd.
§ Courtesy of Dr. Martin I. Rubin, Georgetown University.
‖ Merck and Co., Inc.
¶ Bios Laboratories, Inc.
** Courtesy of Ciba Pharmaceutical Products, Inc.
†† Courtesy of Organon, Inc.

The solvent mixture found most suitable for the separation of the three dyes is prepared by mixing 200 ml toluene, 100 ml petroleum ether (boiling range 35°–60° C), and 30 ml ethanol with 70 ml water in a separatory funnel. After overnight equilibration, the water layer is drawn off into an open vessel and used in keeping the atmosphere of the chromatographic chamber saturated with respect to this phase. Equilibration and chromatography are carried out in an air-conditioned laboratory kept at 75° ± 3° F. The ascending method of chromatography (*2*) is used. The solvent mixture is placed directly on the bottom of a cylindrical glass jar, 12 in. high and 8 in. inside diam. The vessel, a so-called museum jar, has a flat glass cover with a rubber sealing ring and can be made airtight by means of a screw clamp. The bottom is raised in the center, which results in a saving of solvent. Sheets of Whatman filter paper No. 1, 18½ × 22½ in., are cut in half, and subsequently either cut into strips, which are suspended from hooks inside the lid of the jar, or, for up to 15 chromatograms, stapled together as cylinders 11¼ in. high and 18½ in. in circumference.

The paper is held over a hot plate and the benzene solution of the dye is placed on a pencil line, 1½ in. from the bottom edge. By repeatedly delivering small amounts of solution from the medicine dropper the entire benzene solution can be evaporated without making the spot un-

duly large. The solvent is allowed to rise to a prescored pencil line, 10 in. from the bottom edge of the filter paper. This takes 1½ hr. The blank produces a yellow spot on the finish line, and estrogens give in addition a purple[3] spot.

Table 1 gives the average R_f values of spots obtained by chromatographing estrone, estradiol, and estriol, singly and in mixture. As expected from the theory of partition chromatography, the R_f values of the three compounds decrease with increasing number of hydroxyl groups. Mixtures are readily resolved and identified. Only steroids containing a phenolic group give purple spots; this includes the equine estrogens. Diethylstilbestrol does not give the test. Double spots indicate a chemical change during the test, e.g., hydrolysis, or an impurity in the compound tested. Certain 3-ketosteroids, having a double bond in ring A, give yellow spots.

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[3] Absorption maximum around 540 mμ.

Enzymatic Activities of Isolated Normal and Mutant Mitochondria and Plastids of Higher Plants

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The chloroplasts of higher plants derive from the mitochondrial elements of the cell (*4*, *6*), and possess extranuclear hereditary entities (chondriogenes [*1*]). Although it has been shown that isolated chloroplasts carry oxidizing enzymes (*7*), and that the activity of these enzymes may be deranged following chondriogene mutation (*9*), we are not aware of any reports dealing with the enzymic properties of the undifferentiated mitochondria in comparison with the chloroplasts. Furthermore, it is important to know whether plant plastids and mitochondria carry all or the major part of certain respiratory enzymes, as do animal mitochondria (*3*, *5*). It has been demonstrated that certain animal neoplasms are characterized by peculiarities in mitochondrial enzyme systems (*2*). Besides providing more information on extent of homology between plant and animal mitochondria, such data should also be of value in determining the role of mutant mitochondria in cell pathology, particularly in relation to disturbed metabolism and growth.

The genetically controlled plant material consisted of freshly harvested leaves of *Nicotiana tabacum* L. and *Lonicera japonica* Thunb.[1] The variegated (plastid mutant) *Nicotiana* material was secured by crossing an emasculated plant carrying the desired line of mutant mitochondria with a normal male parent. The F_1 plants were of the Maryland Medium Broadleaf type and, by virtue of somatic segregation of mitochondria, the leaves of the same plant often contained mesophyll cells with either normal, mutant, or normal and mutant mitochondrial elements. These mutant mitochondria are sexually inherited in non-Mendelian fashion through the female gametes and develop into chlorophyll-deficient plastids with cytological characteristics similar to type IIc variegations (*8*). The enzymic activity of centrifugally purified plastids and mitochondria of this material was compared with similar elements of normal cells.

Extractions were carried out at 5° C by grinding fresh leaf tissues in a Waring Blendor, in approximately four parts by weight of unbuffered sucrose solution (0.15 to 0.5 molar), and filtering through glass wool on a Buchner funnel. The filtrate (a cell-free homogenate) was compared with respect to its activity with fractions of two aliquots, fractionated by differential centrifugation in a Servall Superspeed angle-head centrifuge. One aliquot, subjected to 19,000 *G* for 20 min, yielded a "total pellet" and a supernatant devoid of microscopically visible particulates. The other aliquot, subjected to fractional centrifugations in order to remove nuclei, etc., yielded on sedimentation at 120 *G* for 10 min a pellet composed almost entirely of plastids, or plastids and starch. The plastids were sometimes subjected to additional cycles of suspension and sedimentation in fresh sucrose solution. The mitochondrial fraction was obtained by centrifugation of the supernatant at 19,000 *G* for 20 min following removal of the plastids. The mitochondria formed a compact brownish-gray pellet. Microscopically, they appeared mainly as colorless granules varying from 0.3 to 1 μ in diam. Some plastid fragments were always present in this fraction.

Considerable variability in enzymic activity, determined by Warburg technique, was encountered in leaves from plants of different ages or growth conditions. This variability was diminished by making comparisons between normal and mutant tissues derived either from equal areas of mutant and normal tissue of each leaf used, or from different leaves of approximately the same physiological age. Table 1 presents the results of some representative experiments in which composite tissue samples of normal and variegated leaves of approximately the same age were compared. Since preliminary data showed that the tissue extracts decreased auto-oxidation of the substrates, corrections were not made for auto-oxidation blanks. It was also determined that addition of cytochrome C to PPDA (paraphenylene diamine) did not stimulate oxidation in the absence of tissue fractions.

There is little difference between normal and mutant fractions with respect to the oxidation of PPDA with and without cytochrome C. Pronounced inhibition of oxygen uptake following addition of azide occurred mainly in the fractions containing particulates, indicating that the particulates are the main carriers of the cytochrome system. As would be expected, there was some decrease

[1] Acknowledgment is due to the Department of Botany of the University of Maryland for furnishing greenhouse facilities.