ADRENAL ESTROGENS IN PATIENTS WITH METASTATIC BREAST CANCER [1]

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Numerous studies in both patients and experimental animals have established that estrogens play an important role in the growth of some breast cancers. It is generally agreed that the beneficial effect of bilateral oophorectomy in some patients with metastatic breast cancer is due to the elimination of the principal source of estrogens. Inevitably, patients who obtain castration remissions relapse. Many will respond favorably to subsequent adrenalectomy or hypophysectomy (1–3). As one possible explanation for these observations, it has been suggested that the adrenal cortex might function as an extragonadal source of estrogens. Since certain adrenal cortical tumors cause feminization in men and since other hypercortical states are frequently associated with the excretion of excessive amounts of estrogens (4), it is conceivable that normal adrenal cortical tissue might function as a significant source of estrogens in patients with breast cancer. The demonstration of biologically active estrogens in the urine of ovariectomized women by Dao (5) and Smith and Emerson (6) supports this concept. That the pituitary gland might be involved in the production of adrenal estrogens is suggested by the work of Nathanson, Engel, and Kelley (and in the discussion of Reference 7 by Paschkis) who reported increased levels of urinary estrogens in patients given adrenocorticotrophic hormone (ACTH).

The purpose of this study was to investigate the possible role of adrenal estrogens in breast cancer by attempting to isolate and identify estrogens excreted by castrated patients and to determine the effects of adrenalectomy, hypophysectomy, and the administration of ACTH upon their excretion.

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ANALYTICAL METHODS

The analytical methods used in this investigation have been reported in detail elsewhere (8). Urine specimens were hydrolyzed with β-glucuronidase and extracted continuously with ether for 48 hours. The phenolic fractions were prepared from the ether extracts by partitioning between toluene and 1 N NaOH. Unless otherwise specified, the phenolic fractions were routinely fractionated as outlined in Figure 1. Estrone, estradiol and estriol were separated from the phenolic fractions by multiple sequential fractionations by countercurrent distribution and paper chromatography.

The technique used for countercurrent distribution was developed by Craig and Craig (9) and adapted to the analysis of estrogens by Engel (10). Countercurrent distribution served not only for fractionation, but also to provide information on the identity, purity and quantity of the isolated estrogens.

The fundamental techniques used for paper chromatography were developed by Bush (11) and adapted to the analysis of estrogens by Mitchell and Davies (12). The solvent systems, time of development and temperature for the paper chromatographies are indicated in Figure 1. The estrogens on the paper chromatograms were visualized by staining with an aqueous solution of 1 per cent ferric chloride and 1 per cent potassium ferricyanide as described by Axelrod (13).

Estrone, estradiol and estriol were measured by photofluorometry using Engel's modification (10) of the method of Bates and Cohen (14). To obtain biological confirmation of the chemical methods, the isolated estrogens were assayed for estrogenic activity by either the rat uterine weight or the rat vaginal cornification methods (15, 16).

To support the identification of the free estrogens by paper chromatography and countercurrent distribution, the acetate derivatives were prepared and analyzed by paper chromatography against authentic estrogen acetates. Acetylation was carried out in acetic anhydride and pyridine overnight at room temperature.

RESULTS

Urines from patients without ovaries were analyzed for estrogens by the methods described

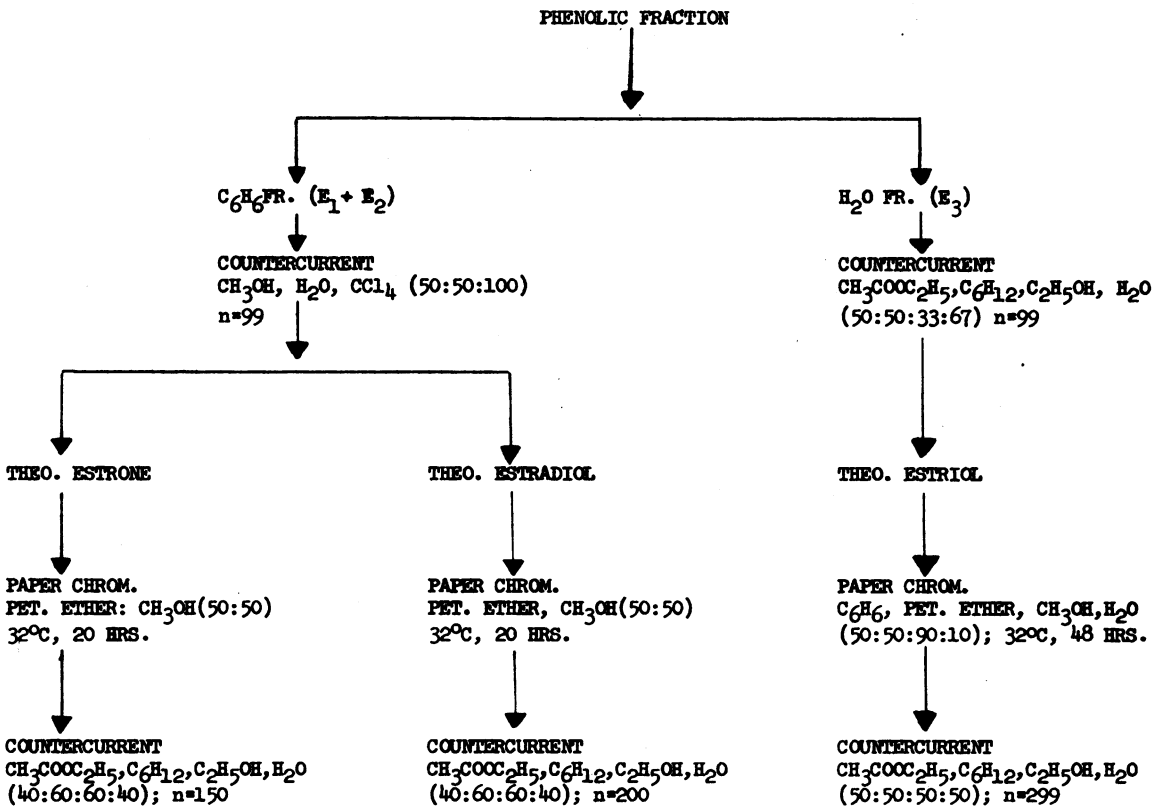


FIG. 1. OUTLINE OF METHOD OF FRACTIONATION FOR URINARY ESTROGENS

E_1, estrone; E_2, estradiol-17β; E_3, estriol. Solvent ratios are by volume; n equals number of transfers.

both during control periods and while the patients were on ACTH, in an attempt to increase the excretion of urinary estrogens by stimulating the adrenal cortex. The results obtained in one patient will be presented in detail to afford an opportunity for critical evaluation of the methods used.

This 66 year old patient (A.S.) had a radical mastectomy for carcinoma of the breast six years prior to this study, at which time she was 12 years postmenopausal. She developed osseous metastases two years later and was treated with both estrogens and androgens without improvement. In April, 1956, a hypophysectomy was performed and the patient had a remission lasting six months. Upon relapse, the metastatic osseous disease was treated with X-ray and a radiation dose of 2,000 r was delivered to both ovaries. The present estrogen excretion study was done three months later during the time the patient's neoplastic disease was advancing rapidly.

In order to stimulate the adrenal cortices of this hypophysectomized patient to maximal secretory activity, 50 mg. of ACTH was administered intramuscularly every six hours. After one week of ACTH therapy the adrenal cortices were actively secreting, as evidenced by a striking increase in urinary 17-ketosteroids and 17-hydroxycorticoids and the development of sodium retention and hypokalemic alkalosis. At this time a three day urine specimen was collected for estrogen analyses.

The phenolic fractions were prepared from the urine samples and analyzed for estrone, estradiol and estriol as outlined in Figure 1. The results of the initial countercurrent distribution of the benzene soluble material in the phenolic fraction are shown in Figure 2A. The distribution curve exhibited two nonhomogeneous peaks composed of mixtures of fluorescent phenols. It was theoretically possible that estrone could have been one of the fluorescent phenols making up the first

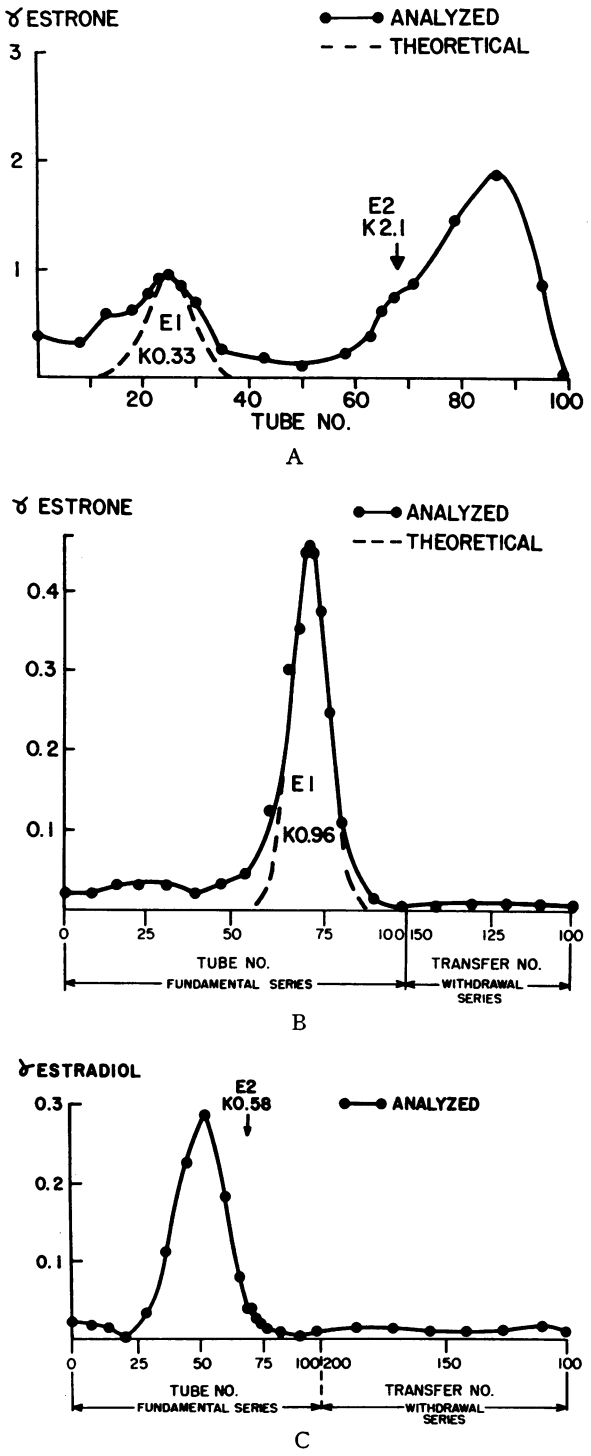


FIG. 2. COUNTERCURRENT DISTRIBUTION CURVES FOR ESTRONE (E_1) AND ESTRADIOL (E_2) ON PATIENT A.S.

●——●, analyzed curves; – – – –, theoretical curves.

A. Initial countercurrent distribution of the benzene fraction of the phenolic extract in CH_3OH, H_2O, CCl_4 (50:50:100); n equals 99; partition coefficient (K) for E_1 equals 0.33; K_{E_2} equals 2.1.

B. Countercurrent distribution for estrone following paper chromatography in $CH_3COOC_2H_5$, C_6H_{12}, C_2H_5OH, H_2O (40:60:60:40); n equals 150; K_{E_1} equals 0.96.

C. Countercurrent distribution for estradiol following paper chromatography in $CH_3COOC_2H_5$, C_6H_{12}, C_2H_5OH, H_2O (40:60:60:40); n equals 200; K_{E_2} equals 0.58. Single withdrawal procedure used in B and C.

peak, and estradiol one of those in the more polar peak.

To explore these possibilities further the tubes which would theoretically have contained estrone were combined and chromatographed on paper as indicated in Figure 1. The fraction from the paper chromatogram corresponding in mobility to reference standards of authentic estrone was eluted and analyzed by countercurrent distribution for a second time in a different solvent system. This time a single fluorescent compound was obtained, and the distribution curve agreed with the theoretical curve for estrone (Figure 2B). The 24 hour excretion of estrone was calculated from this countercurrent distribution curve to be 10 micrograms.

An aliquot of this material was analyzed by paper chromatography for the second time. As can be seen by the photograph of the finished paper chromatogram (Figure 3), the isolated material had the same mobility as the two reference standards of authentic estrone on each side.

The acetate derivative of the isolated material was prepared and analyzed simultaneously with reference standards of estrone acetate and free estrone by paper chromatography in petroleum ether and methanol (1:1) at 24° C. The chromatographic behavior of the acetate derivative was identical with that of estrone acetate, providing additional evidence for the identification of the isolated material as estrone.

The tubes from the initial countercurrent distribution of the benzene fraction (Figure 2A), which theoretically would have contained estradiol, were combined and analyzed by paper chromatography and again by countercurrent distribution. The final distribution curve did not exhibit a peak in the region expected for estradiol. A more polar fluorescent phenolic compound was observed but unidentified (Figure 2C).

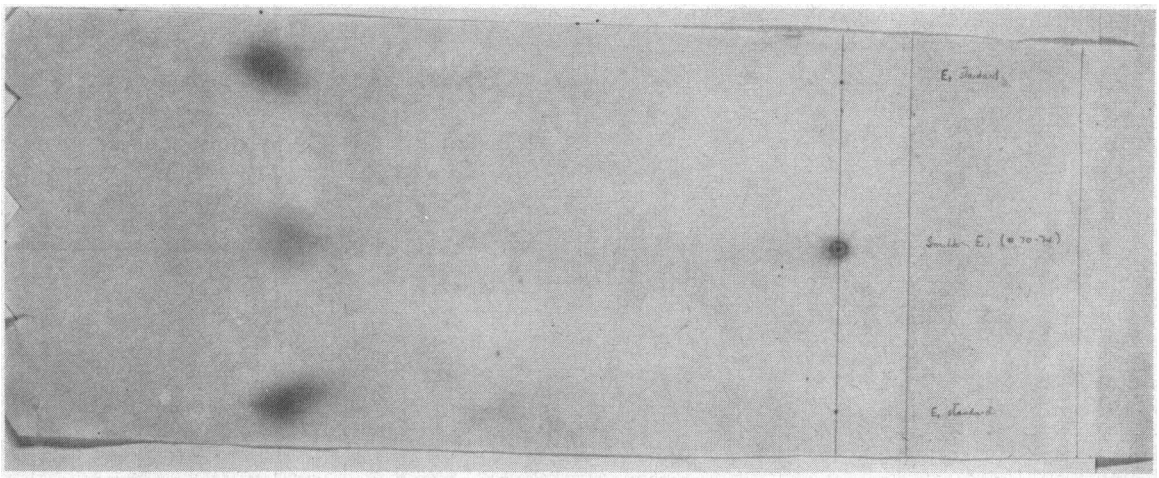


FIG. 3. PHOTOGRAPHS OF THE PAPER CHROMATOGRAM OBTAINED ON THE ESTRONE FRACTION FROM THE FINAL COUNTERCURRENT DISTRIBUTION, PATIENT A.S.

The middle spot is the isolated estrone with two reference standards of authentic estrone on each side. Developed in methanol: petroleum ether (1:1 v/v) for nineteen and one-half hours at 32° C.

An attempt to identify estriol was successful. The initial countercurrent distribution of the water soluble material in the phenolic fraction revealed a mixture of fluorescent phenols which could have contained estriol (Figure 4A). The tubes which theoretically would have contained any estriol present were combined, fractionated further by paper chromatography, and analyzed again by countercurrent distribution. This time a single fluorescent compound was obtained whose distribution curve fit the theoretical distribution curve for estriol exactly (Figure 4B). From these countercurrent data it was calculated that 51 micrograms of estriol was excreted daily by the patient.

One aliquot of the isolated material was again chromatographed on paper and identified as estriol (Figure 5A). A second aliquot was acetyl-

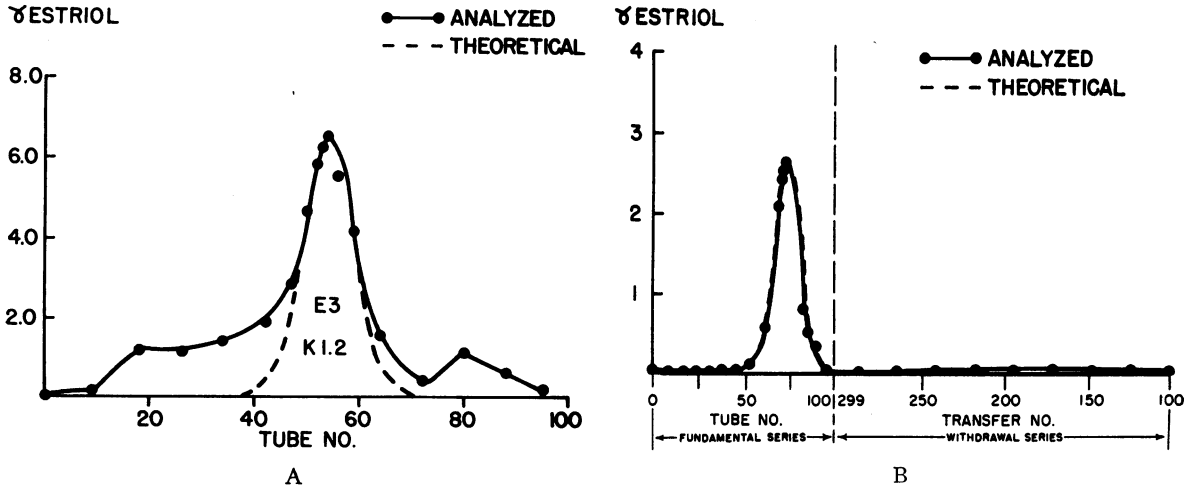
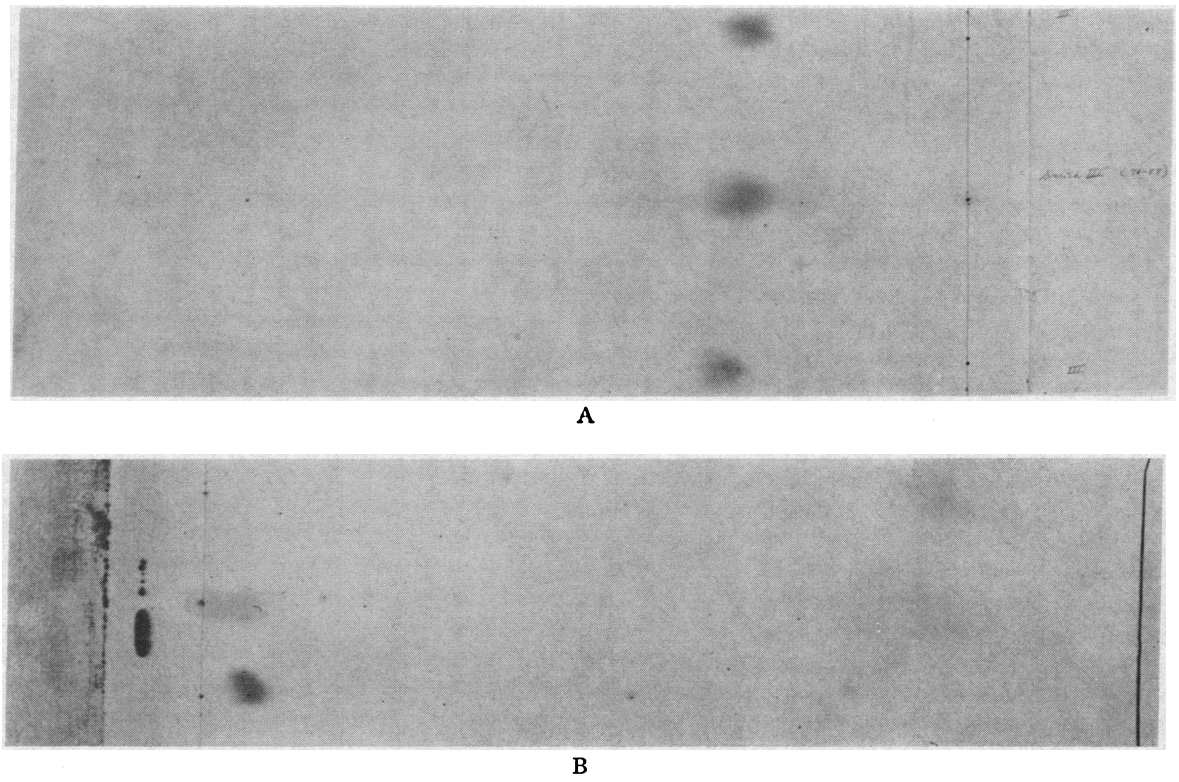


FIG. 4. COUNTERCURRENT DISTRIBUTION CURVES FOR ESTRIOL (E_3) ON PATIENT A.S.

A. Initial distribution of aqueous fraction of the phenolic extract in $CH_3COOC_2H_5$, C_6H_{12}, C_2H_5OH, H_2O (50:50:33:67); n equals 99; K_{E_3} equals 1.2.

B. Countercurrent distribution for estriol following paper chromatography in $CH_3COOC_2H_5$, C_6H_{12}, C_2H_5OH, H_2O (50:50:50:50); n equals 299; K_{E_3} equals 0.35. Single withdrawal procedure used.



A

B

FIG. 5. PHOTOGRAPHS OF PAPER CHROMATOGRAMS OBTAINED ON THE ESTRIOL FRACTION FROM THE FINAL COUNTERCURRENT DISTRIBUTION, PATIENT A.S.

A. Middle spot is the isolated estriol with estriol reference standards on each side, developed in petroleum ether, benzene, methanol, H_2O (50:50:90:10) for 48 hours at 32° C.

B. Middle spot is the acetate derivative of the isolated estriol with reference standards of estriol triacetate and estriol on each side. Developed in methanol, H_2O, petroleum ether (80:20:100 v/v) for three hours at 32° C.

ated and identified as estriol triacetate by paper chromatography (Figure 5B).

A third aliquot was assayed quantitatively for estrogenic activity by the rat uterine weight method. Doses of 0.2, 0.4, and 1.0 microgram of isolated estriol, as determined fluorometrically, were injected into each of three castrated 21 day old female rats at each dose level, in six divided doses on three successive days. An estriol standard dose response curve was run simultaneously, as well as controls. The isolated material gave a positive bioassay equivalent to 41 micrograms of estriol excreted daily, in comparison with a value of 51 micrograms per 24 hours which was calculated from the final countercurrent distribution data, thus providing biological confirmation for the chemical identification.

Under the influence of ACTH administration the amounts of estrone and estriol excreted by this 66 year old patient (A.S.) were within normal limits for an actively menstruating woman. Perhaps of significance is the fact that she excreted no measurable amounts of estradiol. Since the patient was 12 years postmenopausal, had undergone X-ray castration and hypophysectomy, and excreted the estrogens under the influence of ACTH, it would appear highly probable that these urinary estrogens or their precursors originated from the adrenal cortex. Nevertheless, the ovaries cannot be absolutely ruled out in this patient as the site of origin, since they were still *in situ* and X-ray castration and hypophysectomy may not have destroyed their hormonal function.

Similar estrogen excretion studies were carried out in two other patients with metastatic breast cancer who had been surgically castrated (M.H. and E.Z.) and one other patient who had been castrated and hypophysectomized (M.G.), both

TABLE I

Estrogen excretion by castrated patients with metastatic breast cancer and the effect of adrenocorticotrophic hormone (ACTH) *

Pt.	Age	Endocrine status	Control			ACTH treatment		
			E_1 (μg./24 hrs.)	E_2 (μg./24 hrs.)	E_3 (μg./24 hrs.)	E_1 (μg./24 hrs.)	E_2 (μg./24 hrs.)	E_3 (μg./24 hrs.)
M. H.	62	Bilat. oophorect.	2	0	1	19	0	38 (25)†
E. Z.	42	Bilat. oophorect.	0	0	0	0	0	6 (3)
A. S.	66	Spontaneous menopause X-ray cast. Hypophysectomy				10	0	51 (41)
M. G.	29	Bilat. oophorect. Hypophysectomy	0	0	0	0	0	5 (6)
M. M.	43	Bilat. oophorect.	0	0	0			
L. S.	48	Bilat. oophorect.	0	0	(35)			
C. M.	35	Bilat. oophorect.	0	0	(17)			

* E_1, estrone; E_2, estradiol; E_3, estriol.
† Estrogen values in parentheses determined by bioassay and the other values by photofluorometry.

before and while on ACTH therapy. The results are summarized in Table I.

One castrated patient (M.H.) excreted 2 micrograms of estrone and 1 microgram of estriol daily during the control period. The estrone was isolated as a pure fluorescent compound by a combination of countercurrent distribution and paper chromatography and measured quantitatively by fluorometry as previously described. The isolated estrone gave a positive biological assay for estrogens by causing complete cornification of the vaginal epithelium in 15 out of 15 castrated adult female rats in doses of 0.035 microgram per rat administered intravaginally. Appropriate controls and standards were run simultaneously. The estriol was also isolated in a homogeneous fluorescent form and measured by fluorometry. It gave a positive test for estrogenic activity by the vaginal cornification method in doses of 0.03 microgram per rat.

With the administration of ACTH the excretion of estrone by patient M.H. increased from 2 to 19 micrograms per 24 hours; and estriol, from 1 to 38 micrograms per 24 hours.

None of the three classical estrogens could be identified in the urine of the other castrated patient (E.Z.) during the control period, but upon the administration of ACTH she excreted 6 micrograms of estriol daily. Neither estrone nor estradiol were found in the urine during the ACTH period.

No identifiable estrogens could be detected in the control urine of patient M.G. who had undergone bilateral oophorectomy and hypophysectomy. Upon the administration of ACTH, she excreted 5 micrograms of estriol daily, but no estrone or estradiol. The results obtained on patient A.S. which were presented above in detail are included in Table I.

None of the four castrated patients (M.H., E.Z., A.S. and M.G.) excreted detectable amounts of estradiol either during the control period or on ACTH. However, all of them excreted a fluorescent phenol which did not separate from estradiol on paper chromatography but did on subsequent countercurrent distribution. This compound is slightly more polar than estradiol and is so far unidentified.

Using a less rigorous method estrogen excretion studies were carried out in three additional ovariectomized patients with metastatic breast cancer (M.M., L.S. and C.M.). With this method estrone, estradiol and estriol were separated from each other by a single countercurrent

distribution of the phenolic fraction using 100 transfers in a solvent system made up of methanol, water, $CHCl_3$ and CCl_4 (70:30:40:60). If the distribution curve, as determined fluorometrically, was consistent with the presence of any of the three estrogens, the total amount of the individual estrogen was measured by assaying the appropriate peak tubes for estrogenic activity by the rat uterine weight method. Although estrone, estradiol and estriol can be separated from each other by the single countercurrent distribution used, they cannot be isolated in a fluorogenically homogeneous form and accurate quantitative measurement by fluorometry is impossible. In addition the three classical estrogens may not necessarily separate from other unknown estrogenic materials in the urinary extracts which would affect the results obtained by bioassay. Nevertheless, the results obtained by this less rigorous technique in the three castrated patients (C.M., M.M. and L.S.) are included in Table I to demonstrate further that patients without ovaries can excrete estrogens.

None of the three patients excreted estrone or estradiol in amounts which were detectable by countercurrent distribution. However, the distribution curves were compatible with the excretion of estriol in two of the patients (C.M. and L.S.). Therefore, the single peak tubes which theoretically contained the maximum amount of estriol were assayed by the rat uterine weight method. In this way it was determined that patient C.M. excreted 17 microgram equivalents of estriol in 24 hours; and patient L.S., 35 micrograms in 24 hours. The comparatively high values for estriol in these two patients could have been due to contaminating estrogenic materials which were not separated from estriol by the single countercurrent distribution.

No estrogens were demonstrated in the urine of the third castrated patient (M.M.) by this method. It may have been significant that this patient differed from all the rest of the castrated patients in that her neoplastic disease was in remission at the time the estrogen analyses were done.

The demonstration of estrone and estriol in the urine of castrated patients and the increased excretion upon ACTH administration strongly suggest that the adrenal cortex is involved in the production of these urinary estrogens or their precursors. If this interpretation is correct, patients without both ovaries and adrenals should not excrete estrogens.

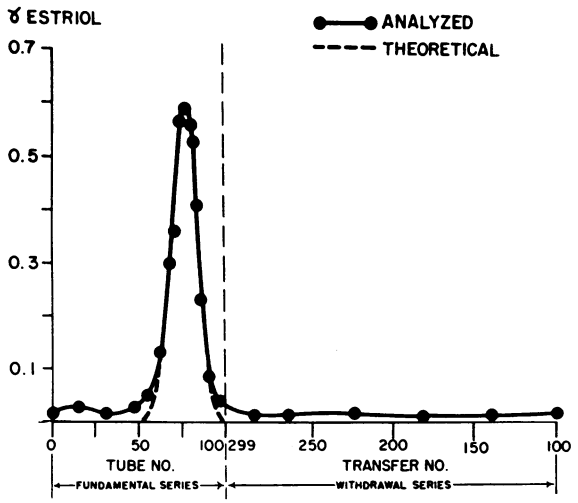


FIG. 6. COUNTERCURRENT DISTRIBUTION CURVE OBTAINED FOR ESTRIOL FOLLOWING AN INITIAL DISTRIBUTION AND PAPER CHROMATOGRAPHY IN A CASTRATED, ADRENALECTOMIZED, HYPOPHYSECTOMIZED PATIENT

●——●, analyzed curve; – – – –, theoretical curve for estriol; K_{E_3} equals 0.35; n equals 299; solvent system, $CH_3COOC_2H_5$, C_6H_{12}, C_2H_5OH, H_2O (50:50:50:50). Single withdrawal procedure used.

To test this theory, urines from two castrated, adrenalectomized patients and two other patients who had been surgically castrated, adrenalectomized and hypophysectomized (for a total of four patients without either ovaries or adrenals) were analyzed for estrogens by the complete procedure outlined in Figure 1. None of these four patients excreted estrone or estradiol, and estriol could not be demonstrated in the urine of three of these patients. However good evidence for the excretion of estriol by the fourth patient, who had undergone bilateral oophorectomy, adrenalectomy, and hypophysectomy, was obtained. By the procedure outlined in Figure 1, a homogeneous fluorescent compound was isolated whose distribution curve coincided with the theoretical curve for estriol (Figure 6). The amount of estriol excreted daily was calculated from the final countercurrent distribution curve to be 11 micrograms. The acetate derivative of this material was prepared and identified as estriol triacetate by paper chromatography. In addition, it was shown that

the isolated material had estrogenic biological activity.

DISCUSSION

In this investigation it was demonstrated that castrated patients with metastatic breast cancer may excrete significant amounts of estrone and estriol in their urine, but not estradiol. With the additional observation that the administration of ACTH increased excretion, it seems highly probable that these urinary estrogens or their precursors were produced by the adrenal cortex. The observation that patients without both ovaries and adrenals usually failed to excrete these estrogens lends support to this conclusion.

Whether the isolated urinary estrogens were actually secreted by the adrenal cortex or whether they represent metabolic products of some other adrenal precursor could not be ascertained from this study. There is indirect evidence to support both of these possibilities. With the isolation of estrone from adrenal cortical tissue by Beall (17) and the demonstration by Meyer (18) that 19-hydroxy-Δ^4 androstene-3, 17-dione can be converted to estrone by adrenal tissue, it would appear that adrenal cortical tissue has the necessary enzyme systems for synthesizing estrogens and that secretion of estrogens by the adrenal gland is a real possibility. On the other hand, it has been demonstrated that certain C_{19} neutral steroids of the androgenic series, some of which are produced by the adrenal cortex, are converted to estrogens both *in vivo* and *in vitro* by a variety of tissues (18–21). Under the conditions of the present experiments the adrenal cortex could have secreted these C_{19} neutral steroids or other unknown precursors which were metabolized in part to estrogens elsewhere in the body and excreted in the urine.

The possibility that corticosteroids, such as cortisol and cortisone, might be converted in part to estrogens was considered since this conversion would play an important role in the management of adrenalectomized and hypophysectomized patients with estrogen-dependent breast cancer. Since the adrenalectomized and hypophysectomized patients in this study failed to excrete measurable amounts of estrogens even though they were receiving maintenance doses of cortisone throughout the study, it would appear that physiological levels of cortisone are not metabolized to estrogens to any great extent. To examine the possibility of this conversion more rigorously 600 mg. of cortisol was given intravenously to a castrated, adrenalectomized, hypophysectomized patient, and the urine analyzed for estrogens. Estrone, estradiol and estriol could not be demonstrated in the urine. These observations suggest that corticosteroids are not converted to estrogens in any significant amounts.

It is of interest that estriol was found more consistently in the urine of castrated patients than any other estrogen. Estriol is usually considered to be an estrogen metabolite rather than a secretory product. It cannot be ascertained from this study whether the estriol isolated from urine was a secretory product of the adrenal cortex or a metabolic end product of some other precursor.

The demonstration that the adrenal cortex in patients with metastaic breast cancer can be a source of significant amounts of estrogens is consistent with the theory that relapses following castration remissions are due to adrenal estrogens and that subsequent remissions following adrenalectomy are due to the removal of this source of estrogens, but does not prove it. Proof of this theory will depend upon whether the growth of the metastatic cancer in these patients can be correlated with the level of urinary estrogens during relapses and remissions.

The increased excretion of urinary estrogens by castrated patients upon the administration of ACTH and the absence of estrogens in the urine of castrated, hypophysectomized patients suggest that endogenous ACTH might be involved in the control of estrogen production by the adrenal cortex. If this is the case, one of the possible mechanisms by which hypophysectomy could cause remission in castrated patients is through secondary suppression of estrogen production by the adrenal gland.

In view of the fact that one of the castrated, adrenalectomized, hypophysectomized patients excreted appreciable amounts of estriol, other possible sources of estrogens besides the ovaries and adrenal glands, such as the diet or accessory ovarian or adrenal tissue, must be considered to explain relapses occurring in some patients with estrogen-dependent tumors.

SUMMARY

Significant amounts of estrone and estriol have been demonstrated in the urine of castrated patients with metastatic breast cancer by means of countercurrent distribution, paper chromatography, and bioassay methods. The excretion of these estrogens by castrated patients increased strikingly upon the administration of ACTH. With one exception, castrated and adrenalectomized patients failed to excrete identifiable amounts of estrogens. These observations suggest that estrogens or their precursors are produced by the adrenal cortex and that endogenous ACTH might be involved in the control of this estrogen production. Estriol was demonstrated in the urine of one patient who had been castrated, adrenalectomized, and hypophysectomized, raising the possibility of additional sources of estrogens besides the ovaries and adrenals, such as diet and accessory ovarian or adrenal tissue.

The possible pathophysiological significance of these findings in breast cancer was discussed.

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