A relationship is generally recognized between the in vivo activity of estrogenic hormones (either of endogenous or exogenous origin) and the growth of mammary carcinoma in humans. Ablation of the hypophysis, the endocrine glands producing estrogens, or the administration of estrogenic hormones may induce objective regression of tumor in approximately 30% of the patients so treated (18, 19). The basic nature of this relationship between carcinoma of the breast and the metabolism of estrogens in humans is obscure. As part of a broad study of this phenomenon, estrogen metabolism, as reflected by the urinary excretion of the several metabolites in the hydrolyzed fraction. Wide subject-to-subject variations were found. The metabolites other than estradiol, estrone, and estriol comprised a large portion of the total estrogens excreted. A significantly lower amount of estradiol was noted in the group of women with mammary carcinoma as compared to the amount found in the group of women with benign mammary dysplasia, suggesting that the former group metabolizes more rapidly the administered estradiol. Although the excretion patterns of patients who failed to respond to estrogen treatment (nonresponders) differed the greatest from the patterns associated with benign disease, distinctive patterns of the urinary excretion of isotopic estrogens which allowed a statistically significant or clinically useful separation between responders and nonresponders to subsequent estrogen therapy were not discovered.

SUMMARY

A study of the excretion patterns of the urinary metabolites of estradiol-4-C\textsuperscript{14} administered to a group of 43 postmenopausal women, 38 with advanced mammary carcinoma prior to endocrine therapy and 5 with benign mammary dysplasia, is reported. A chromatographic method was employed for the determination of estradiol, estrone, and estriol and other estrogen metabolites. The urinary excretion pattern is defined by (a) the extent of excretion of isotope in each of 3 successive 24-hr. collection periods, (b) the percentage of the urinary radiometabolites enzymatically hydrolyzed, (c) the relative concentration of the several metabolites in the hydrolyzed fraction. Wide subject-to-subject variations were found. The metabolites other than estradiol, estrone, and estriol comprised a large portion of the total estrogens excreted. A significantly lower amount of estradiol was noted in the group of women with mammary carcinoma as compared to the amount found in the group of women with benign mammary dysplasia, suggesting that the former group metabolizes more rapidly the administered estradiol. Although the excretion patterns of patients who failed to respond to estrogen treatment (nonresponders) differed the greatest from the patterns associated with benign disease, distinctive patterns of the urinary excretion of isotopic estrogens which allowed a statistically significant or clinically useful separation between responders and nonresponders to subsequent estrogen therapy were not discovered.

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SUMMARY

A study of the excretion patterns of the urinary metabolites of estradiol-4-C\textsuperscript{14} administered to a group of 43 postmenopausal women, 38 with advanced mammary carcinoma prior to endocrine therapy and 5 with benign mammary dysplasia, is reported. A chromatographic method was employed for the determination of estradiol, estrone, and estriol and other estrogen metabolites. The urinary excretion pattern is defined by (a) the extent of excretion of isotope in each of 3 successive 24-hr. collection periods, (b) the percentage of the urinary radiometabolites enzymatically hydrolyzed, (c) the relative concentration of the several metabolites in the hydrolyzed fraction. Wide subject-to-subject variations were found. The metabolites other than estradiol, estrone, and estriol comprised a large portion of the total estrogens excreted. A significantly lower amount of estradiol was noted in the group of women with mammary carcinoma as compared to the amount found in the group of women with benign mammary dysplasia, suggesting that the former group metabolizes more rapidly the administered estradiol. Although the excretion patterns of patients who failed to respond to estrogen treatment (nonresponders) differed the greatest from the patterns associated with benign disease, distinctive patterns of the urinary excretion of isotopic estrogens which allowed a statistically significant or clinically useful separation between responders and nonresponders to subsequent estrogen therapy were not discovered.
MATERIALS AND METHODS

Clinical.—Forty-three female patients, postmenopausal by more than 3 years, were studied. Five of these had benign disease of the breast (Group I) and 38 had advanced carcinoma (Group II). All diagnoses were established by pathologic examination of representative biopsy specimens. Carcinoma of the breast was primary without prior treatment in 21 women and recurrent in 17 women. Patients with evidence of renal dysfunction, of disease involving an endocrine organ, or of hepatic or cerebral metastases were excluded from the study. None of the patients had received any hormonal therapy or other anticancer therapy for at least 3 months prior to the study. The extent of the neoplastic disease was determined by physical examination and conventional radiologic and laboratory methods. All accessible deposits of tumor were measured and recorded.

In each patient 5 μg of estradiol-4-C¹⁴ (2 μc per mmole, 680 μg) dissolved in 2.0 ml propylene glycol were infused into a peripheral vein over a 2-min. interval. Three sequential 24-hr. urine specimens were obtained by gravity drainage from an indwelling catheter. Medications administered during the period of study were restricted to a maintenance dose of digitalis, 100 mg of Seconal, and not more than 200 mg of Demerol per 24 hr. The patients were semiambulatory and diet and fluids were not controlled.

Following the 72-hr. urine collection from each patient, the 38 women with proven advanced mammary carcinoma (Group II) were placed in a special program of treatment which consisted of a 12-week trial on therapy with estrogenic hormones. Thirty-three patients received estradiol valerate (Delestrogen 4X), 40—60 mg I.M. once a week, P.O. (Delestrogen 4X estradiol valerate 40 mg/ml) was supplied by E. R. Squibb & Sons). The patients were examined at regular intervals and objective measurements of tumor size were recorded. A final clinical evaluation of response to therapy was made at 3 months. At this time the patients were classified as follows: responders (Group II-a), patients in whom there was an objective, measurable evidence of regression of tumor without the appearance of new lesions; nonresponders (Group II-b), patients who failed to demonstrate objective evidence of regression of their tumor deposits or whose disease increased during the 3-month treatment period; or indeterminate (Group II-c), patients who failed to complete the standard course of therapy because of premature death. At the conclusion of the study the original 43 postmenopausal women were thus divided into two major clinical groups: Group I, women with benign mammary dysplasia (5 patients); and Group II, women with advanced mammary carcinoma (38 patients). Group II, as noted above, was then subdivided into 2 determinant subgroups: II-a, 7 patients who responded to estrogens, and II-b, twenty-two women who failed to respond to estrogen therapy; and an indeterminant group, II-c, women whose response was undetermined because of premature death (data are of no significance for this group and are not presented).

Laboratory.—The isolation and quantitation of estradiol-4-C¹⁴ and its radiometabolites in the urine were performed by methods previously reported from this laboratory (11, 12, 14). In summary, the method consists of the following steps: (a) measurement of the total urinary radioactivity; (b) enzymatic hydrolysis with β-glucuronidase; (c) extraction with ethyl acetate; (d) chromatography (column plus paper, or paper alone); (e) radiochromatogram scanning; (f) triangulation of the scans; and (g) calculation of the percentage composition of the extract. This method accurately identifies and quantitates 3 regions on the paper chromatograms (a, b, and c), as estradiol and its 2 major metabolites, estrone and estriol, respectively, as confirmed by reverse isotope dilution studies and the
crystallization of these 3 compounds to constant specific activity from a pooled specimen of urine (11). In addition the method permits the quantitation of four other regions on the paper chromatogram (d, e, f, and g), which have been designated as 2-methoxyestrone, 16-ketoestrone, ketols (a mixture of 16α-hydroxyestrone and 16-ketoestradiol), and epiestriols (a mixture of 16- and 17-epiestriol), respectively, on the basis of the similarity of their paper chromatographic mobilities with those of authentic reference standards (11, 13). The presence of these metabolites in human urine has been confirmed by other investigators as reviewed by Adlercreutz (1). The original radioactivity applied to the chromatogram which is not accounted for in these 7 regions is referred to as the undesignated fractions.

RESULTS

Excretion patterns—all patients.—The patterns of excretion of estradiol-4-C\(^{14}\) and its radiometabolites in the urine of the 43 postmenopausal women with benign and malignant disease of the mammary gland are summarized for each of 3 consecutive 24-hr. periods in Table 1. This profile includes the total urinary radioactivity, the extent of hydrolysis of the conjugated estrogens, and the quantitative distribution of estradiol-4-C\(^{14}\) and its radiometabolites in the hydrolyzed fraction.

In reference to the mean values only, it is apparent that: (a) the excretion of isotope was greatest during the first 24-hr. period, and decreased on successive days; (b) the extent of hydrolysis was similar on all 3 days; (c) estradiol and estrone, the 2 most biologically active compounds, were excreted in relatively large amounts on day 1 and in progressively smaller amounts on days 2 and 3; (d) the excretion of estriol and the other metabolites likewise decreased on successive days (see Table 1, D-3) but their concentration in the hydrolyzed fraction (see Table 1, D-1) increased or remained relatively constant on days 2 and 3; (e) the metabolites (designated as 2-methoxyestrone, 16-ketoestrone, ketols, and epiestriols) accounted for approximately 25% of the hydrolyzed estrogens on all 3
days; (f) the undesignated portion increased from 14 to 23% between the 1st and 3rd days.

The ranges for each parameter are large, indicating a wide variation in the over-all pattern of excretion of exogenous estrogens in this group of women. Further analysis of the data failed to demonstrate any correlations between the urine volume (not shown), the excretion of isotope, the extent of hydrolysis, and the quantitative distribution of the metabolites in the hydrolyzed fraction.

Reproducibility of the excretion patterns.—To determine the extent of the variation in the urinary excretion pattern of estradiol-4-C\(^{14}\) within the same subject, 3 patients were studied on 2 separate occasions under similar conditions. The results revealed considerable variation in the excretion of isotope and/or the extent of hydrolysis found on successive studies. However, the pattern of distribution of estradiol, estrone, and estriol in the hydrolyzed fraction was very similar in the 3 individuals (Table 2). It is evident from these data that an individual may excrete a particular dose of estradiol-4-C\(^{14}\) at a different rate on separate occasions due to unknown factors and that the extent of hydrolysis of the conjugated estrogens may likewise change. However, the quantitative distribution of the major metabolites in the hydrolyzed fraction remained essentially uniform, indicating at least some consistency of the individual in the excretion pattern of exogenous estradiol.

Excretion patterns of clinical groups.—An attempt was made to identify urinary excretion patterns of exogenous estrogens which were distinctive for the previously mentioned clinical categories: Groups I, II, II-a, and II-b. Data identifying and comparing patterns of the 4 clinical categories are summarized in Tables 3, 4, and 5. Values for the excretion of estradiol-4-C\(^{14}\) and its metabolites are given only for day 1 since the amount of radioactivity and the differences in excretion patterns between the groups were always the greatest on day 1.

The total excretion of isotope (expressed as percentage of injected dose) and the extent of hydrolysis of the isotopic estrogens found in the urine were similar for all 4 groups (Table 3).

The quantitative distribution of estradiol, estrone, and estriol and the sum of the other metabolites in the hydrolyzed fraction (Table 4) indicates that the average

### Table 2

**Patterns of Estrogens in Urine of 3 Subjects after Administration of Estradiol-4-C\(^{14}\) on 2 Separate Occasions**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Study Number</th>
<th>Excretion of Isotope (% of Injected Dose)</th>
<th>Hydrolysis of Conjugated Estrogens (% of Urinary C(^{14}))</th>
<th>Metabolites (% of Hydrolyzed Fraction)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td>A</td>
<td>1</td>
<td>31</td>
<td>19</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>58</td>
<td>27</td>
<td>54</td>
</tr>
<tr>
<td>B</td>
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<tr>
<td></td>
<td>2</td>
<td>44</td>
<td>25</td>
<td>90</td>
</tr>
</tbody>
</table>
amount of estradiol and estrone was higher, and reciprocally the amount of estriol lower, in Group I than in Group II. Similarly, slightly higher amounts of estradiol and estrone and lower quantities of estriol were found in the responders (Group II-a) as compared to the nonresponders (Group II-b). However, there is an overlapping of the ranges among all the groups. This overlap is particularly evident between the responders and nonresponders.

The sum of estradiol and estrone (E2 + E1) and the ratios of estradiol/estrone plus estriol (E2/E1 + E3) and estradiol/all metabolites (E2/all metabolites) were also calculated for each clinical group (Table 5). The 2 ratios express numerically the quantitative relationship between estradiol and 2 or more of its metabolites and thus provide a glance a survey (although an imperfect one) of the rate and extent of the metabolism of estradiol in the first 24 hrs. after injection, as reflected by the urinary excretion pattern. The patients of Group I demonstrated significantly larger amounts of estradiol plus estrone (E2 + E1) and also significantly higher ratios of estradiol/estrone plus estriol (E2/E1 + E3) and estradiol/all metabolites than the women in Group II. The responders (Group II-a) showed similar but much less striking differences for these three parameters than the nonresponders (Group II-b), and the overlap of values was extensive. Group II-b differed the greatest from Group I.

A statistical analysis of the differences between the mean

values of the several aspects of the urinary excretion pattern was made comparing (a) the benign group and the group with breast cancer (I versus II), and (b) the responders and nonresponders (II-a versus II-b) (Table 6).

The excretion of estradiol was distinctly higher in the patients with mammary dysplasia (Group I) than in the women with advanced cancer of the breast (Group II)—18.7 % versus 11.9 % (P = <0.001). Similarly, the ratios of the excretion of E2/E1 + E3 and E2/all metabolites were higher in Group I than Group II—0.41 versus 0.25, and 0.27 versus 0.16 respectively (P = <0.001 for both differences). Apparent trends were also noted suggesting that a larger amount of estradiol, and a relatively smaller amount of estriol, in the hydrolyzed fraction tend to distinguish the patients of Group II-a from those of Group II-b. However, statistical analysis revealed significant differences only within 70–80 % confidence limits.

**DISCUSSION**

A prominent feature of the data presented in this report is the magnitude of the variation in the metabolism of the administered dose of estradiol-4-C14 in this group of postmenopausal women with mammary carcinoma. Such factors as methodologic limitations, particular dose of
estradiol employed, and normal or pathologic variations of the \textit{in vivo} metabolism of estradiol may all be related to these observations.

The method for the isolation and quantitation of estradiol and its major metabolites in the urine (11) has proved to be accurate, reliable, and reproducible; it has also been corroborated by the similarity of the excretion patterns in successive studies of 3 patients (see Table 2). The obvious limitation of the method is related to the incomplete enzymatic hydrolysis and the wide variability of the incompleteness of the hydrolysis. Since the chemical nature of the unhydrolyzed fraction and the cause of its wide variation in amount remain unknown, the method provides an incomplete quantitative analysis of the distribution of the sum total of the radiometabolites present in the urine, and allows only a limited interpretation of a complex \textit{in vivo} metabolic process (15).

The quantity of estradiol-4-C$^4$ administered (680 $\mu$g) was conditioned by safety factors related to the labeled estrogen with the highest specific activity available at the initiation of these studies. The amount of 680 $\mu$g greatly exceeds the normal daily secretion of estrogens by the postmenopausal woman since the urinary excretion of natural estrogens in this age group is very low, rarely exceeding 25 $\mu$g in 24 hr. (16, 21). This amount of estradiol, however, approximates the daily dose of estrogens employed in the treatment of women with advanced breast cancer and was a rational dose for this particular study (20). Possibly, normal metabolic events may be altered by the rapid loading of the body with such a large dose (9), or, contrariwise, such rapid loading could possibly magnify and allow easier recognition of the end results of subtle differences in estrogen metabolism.

Normal or pathologic alterations of the \textit{in vivo} metabolism of estradiol causing a variation in the individual patterns of urinary excretion may be related to the woman's age, genetic background, extent of replacement of normal tissue by the neoplasm, and general nutritional and endocrine state.

This study confirms the findings of Engel (7) and Fishman (8) that a measurement of the excretion of estradiol and its 2 major metabolites, estrone and estriol, does not constitute a complete picture of the urinary excretion of an administered amount of estradiol. The metabolites other than estradiol, estrone, and estriol constitute a sizable portion (averaging 25%) of the total excretion of estrogens in a vast majority of subjects.

Of some interest and possible importance is the observation that women in the postmenopausal state with advanced breast cancer excrete a dose of exogenous estradiol in a fashion characterized by a significantly smaller amount of estradiol in the hydrolyzed fraction on day 1 and by a significantly lower ratio of estradiol to all its metabolites than seen in women with mammary dysplasia. This is particularly true of women whose neoplastic process continues to progress while on estrogen therapy (Group II-b). This suggests that a common and possibly distinctive feature of the postmenopausal woman with advanced breast cancer is her more rapid conversion of estradiol to estrone and on to estriol and other less biologically active metabolites. These findings, suggesting an increased rate of conversion of estradiol, supplement and tend to confirm the work of Brown (2) who demonstrated, following I.M. injection of estradiol-17$\beta$, an increased excretion of estriol and a higher estradiol/estrone plus estrone (E$_2$/E$_1$ + E$_3$) ratio in postmenopausal women with mammary carcinoma as compared to normal women. Bulbrook et al. (9) found that women of all ages with advanced carcinoma of the breast exhibited patterns of urinary excretion of steroid hormones characterized by a higher 17-hydroxycorticoid/etiocholanolone ratio than found in normal controls. Our finding of a lowered estradiol/all metabolites ratio in the urinary excretion pattern of Group II suggests the possibility of multiple changes in the internal hormonal milieu of women with breast cancer.

Differences between the responders (Group II-a) and the nonresponders (Group II-b) to estrogen therapy are not statistically significant. However, the patterns of the former group tended to resemble the benign tumor group whereas the nonresponders differed the greatest from the nonmalignant group. The nonresponders showed the greatest conversion of estradiol in the first 24 hr., as reflected by a very low estradiol/all metabolites ratio (0.16). This characteristic of the patient who is not responsive to estrogen therapy is in agreement with and may complement the observation by Loraine et al. (17) that postmenopausal women with advanced mammary carcinoma failing to respond to treatment with diethylstilbestrol had elevated levels of urinary gonadotrophins. Diminished levels of estradiol and elevated levels of estriol in the urine may be related homeostatically (10, 22, 25) to the elevated levels of urinary gonadotrophins found by Loraine in nonresponders. It may be postulated that a rapid conversion of the biologically active estradiol by the liver or other tissues may prevent a potentially endocrine-sensitive tumor or intermediary endocrine gland (such as the pituitary) from having sufficiently prolonged contact with the hormonal agent initiating the antineoplastic action to allow therapeutic benefit.

From the studies reported it is clear that none of the variables in the urinary excretion patterns of administered estradiol-C$^4$ and its metabolites can be employed effectively to distinguish in advance between women with breast cancer who will respond to estrogen therapy and those who will fail to respond. This parallels the results of other investigators who have failed to discover characteristic patterns of the urinary excretion of endogenous estrogens by women with advanced mammary carcinoma which could distinguish responders from nonresponders prior to or after various endocrine ablative procedures (3, 4, 5, 23, 24). However, the finding that a more rapid conversion of estradiol is characteristic of women with breast cancer (particularly nonresponders to estrogens) suggests that further investigations of the relationships between estrogen utilization and metabolism in patients with mammary carcinoma are indicated.

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Excretion Patterns of Urinary Metabolites of Estradiol-4-C\(^{14}\) in Postmenopausal Women with Benign and Malignant Disease of the Breast

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