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Studies in a chemical method for the determination of human urinary estrogens

Howard Alyn Minners
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by

Howard Alyn Minners, B. A.
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CHAPTER I

Introductory Remarks

In the summer of 1955 there was reported a new chemical method for the determination of human urinary estrogens, estradiol-17β, estriol and estrone (Brown, 1955a). It was the initial object of this laboratory investigation to reproduce this new method, to evaluate it, especially with a view to possible improvements, and to apply it to the number of clinical situations which it may serve to clarify further. With regard to this last point, it appears that such a routine laboratory method would be a valuable, additional tool for the investigation of numerous gynecologic pathologies which may in some way be reflected in urinary estrogen levels. Although this method is designed to measure urinary estrogen levels and thereby might not indicate truly the amount of estrogen produced in the body or the resultant level circulating in the blood, there now exists a small quantity of indirect evidence that the measurable estrogen excreted in human urine mirrors, in general, bodily estrogen production (Brown, 1955b, 1956; Bulbrook and Greenwood, 1957a). Briefly, this evidence indicates a close agreement between measured urinary estrogen levels and the clinically observed, physiological sequence of pregnancy and lactation. In addition, there is a significant correlation between this

quantified urinary estrogen and the degree of sensitivity to estrogenic stimulation of the vaginal mucosa, evident by vaginal smear techniques.

The many clinical problems which may lend themselves to elucidation by this method seem, at present, numberless. After accurately determining the normal levels of human urinary estrogen excretion, any abberations thereof could be studied in such conditions as sexual infancy and immaturity, puberty, the menopause, abnormalities of menstruation, threatened or actual abortion, various endocrine and gynecologic neoplasias, etc. Indeed, the recent British literature contains two articles relating a study, using Brown's method for estrogen determination, of the alterations of urinary estrogen excretion resulting both from oophorectomy and adrenalectomy for metastatic breast cancer and also from hypophysectomy for primary breast cancer (Bulbrook and Greenwood, 1957a,b). In short, it already appears that the application of such a laboratory method will be fruitful. However, during a conversation with Dr. Brown, he mentioned that in preliminary investigations using his method, the number of clinical problems that seem to have been awaiting just such a laboratory method for their solution have exhibited "somewhat less than anticipated sensitivity" to such an approach, i.e. the direct relationship between urinary estrogen levels and a given pathological process remains, in many respects, indistinct.

By way of introduction to this method and of human interest, I shall relate briefly my experiences in Edinburgh in Dr. Brown's laboratory at the Laboratory of Clinical Endocrinology, Edinburgh University. This laboratory was then (Christmas, 1956) engaged in an attempt to develop micro methods for estrogen estimations of less than 5 μg. Also, at the nearby seashore they had collected mussels and snails from which were extracted the various enzymes to be tested in a method involving enzymatic hydrolysis of urine. In the preliminary step, presently reported using acid hydrolysis with HCl, it is hoped to decrease, through this enzyme hydrolysis, the variable, but considerable loss of estrogen encountered using current techniques. Several other workers have likewise suggested a method of estrogen estimation involving enzyme hydrolysis, but to date the determination utilizing acid has been most widely accepted; enzymatic methods, however, certainly could bear further, more extensive exploration. Besides gaining a general knowledge of the mechanics and practical aspects of Dr. Brown's method, I was permitted to photograph the special apparatus designed particularly for the evaporation to dryness of estrogen extracts prior to color development. In addition, I received answers to the several specific questions regarding the practical aspects of the procedure wherein I had met with difficulty.

Although it was the original, stated goal of this

investigation to set up Brown's method and subsequently to use it as outlined, a number of difficulties have been encountered in working through the protocol, and so I have come to an end short of my initial intentions. Therefore, I shall deal with the progress to date, concerned primarily with a consideration of the steps requisite to establishing standard curves for the three estrogens under scrutiny. In passing, one should note that the two additional estrogens, 16-epi-estriol and 16-oxo-estradiol-17β, reported by Marrian and Bauld (1955) and by Watson and Marrian (1955) have not been estimated by Brown's method, and consequently their clinical significance may not be approached for evaluation by the presently available, routine laboratory means. Moreover, another "new" estrogen, 16α-hydroxy-estrone, soon to be reported by Marrian, Watson, Loke, and Panattoni has not been determined by Brown's method. Indeed, the entire field of estrogen determination is presently fast moving and open, and these new estrogens are undoubtedly no more than intermediate metabolites which are excreted in varying amounts in human urine.

CHAPTER II

Standard Curves and a Statistical Analysis

There are a number of recent analytical methods which have been tried in an effort to find a single, successful method for the consistently accurate estimation of human estrogens excreted in urine. Categorically, these methods have involved three basic steps or divisions necessary for each determination: a method of extraction followed by organic solvents, a process of purification, and finally a method of actual measurement. The proposed methods of extraction have been those with acid or enzymatic hydrolysis (Marrian and Bauld, 1951; Katzman et al, 1954; and Oneson and Cohen, 1952). Next, the principal types of purification have specified pigment removal by anion exchange resins (Axelrod, 1954), solvent, i.e. counter current, partition (Engel et al, 1950; Katzman et al, 1954; and Bauld, 1956), column chromotography (Stimmel, 1944; and Bitman and Sykes, 1953), and paper chromotography (Axelrod, 1954; and Harris and Cohen, 1951). Finally, the main methods of actual measurement have consisted of ultraviolet absorption (Carol and Rotondaro, 1946), various modifications (Bates, 1954) of the Kober Reaction (Kober, 1931), and fluorescence reactions with slightly varying concentrations of sulfuric (Bates, 1954) and phosphoric acids (Goldzieher et al, 1952).

The method of Brown, subject to evaluation in this paper, consists of acid hydrolysis with HCl according to the principles set forth by Marrian and Bauld (1951), purification using a new step, methylation, devised by Brown, followed by column chromotography and ultimate measurement colorimetrically using an improved modification of the Kober Reaction (Brown, 1952; Bauld, 1954).

The first step after the prescribed reagents were prepared, in reduplicating Brown's method, consisted of an attempt to establish standard curves for the three estrogens to be estimated. Consequently, solutions of the methyl derivatives of estradiol-17β, estriol, and estrone were prepared in absolute ethanol and were stored in a refrigerator at 0-2° C., except when taken out and allowed to warm to 20° C. for purposes of pipetting. All glassware was cleaned, as suggested by Brown, in a sulfuric acid-dichromate solution and was thoroughly rinsed with acid bisulfite, tap, and finally distilled water. In this last regard, it was found that the ordinary distilled water running from the so designated taps was acid in pH and reportedly contained certain amounts of iron, the presence of which was implicated in an early failure of our determinations to obey closely Beer's Law. A subsequent change to twice distilled, demineralized water served to decrease the observed readings on the spectrophotometer. Although this decrease was not measured in a quantitative manner, our readings were then more satisfactory. It is of note that

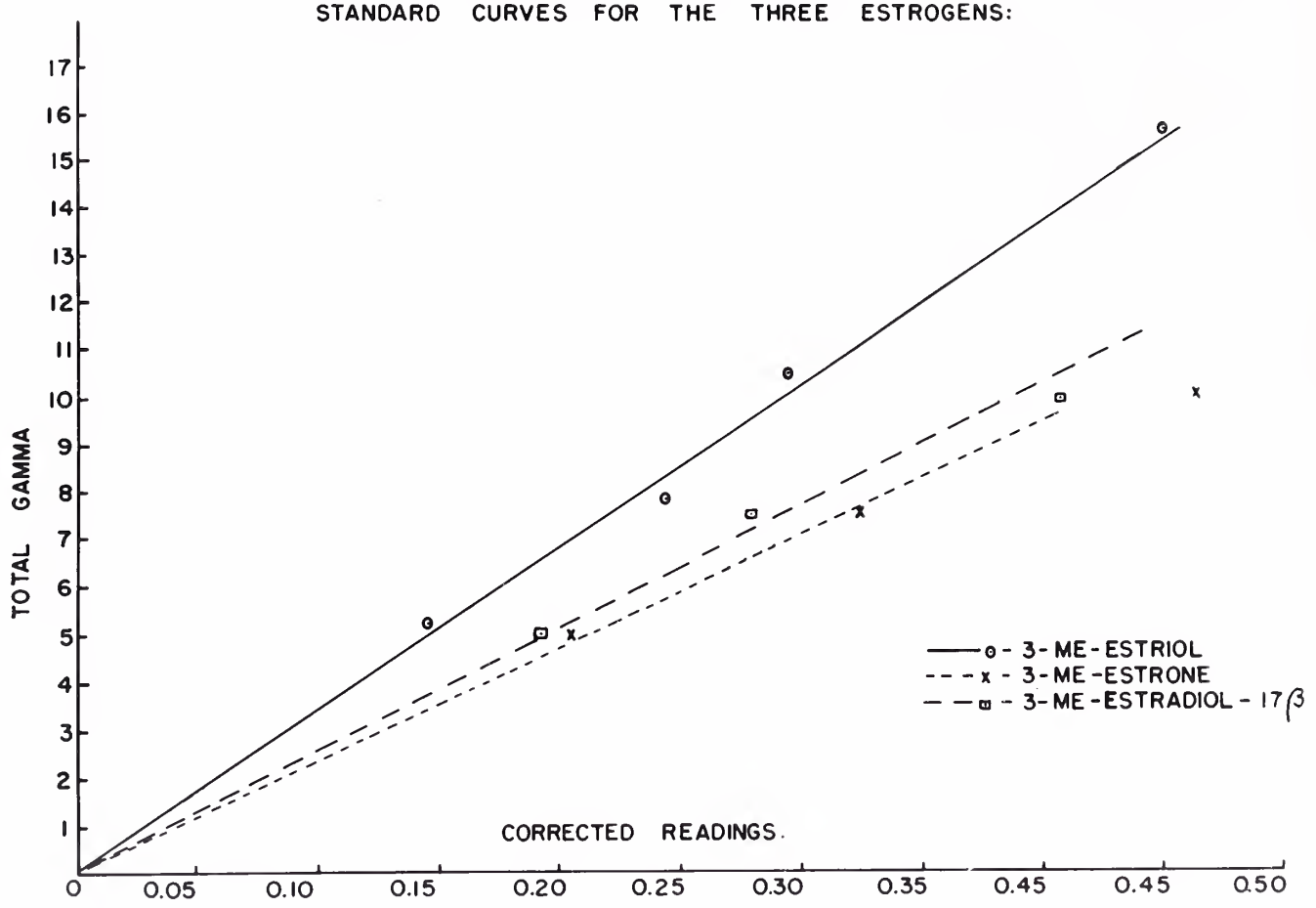
such a change in water would be expected, in general, to effect most markedly the various reagents used in color development, depending upon the percentage of water that each contained. Therefore, one would expect to find the greatest effect upon the estradiol color (60% H_2SO_4), next on estrone (66% H_2SO_4), and, of course, least upon estriol (76% H_2SO_4). Furthermore, this same type of relation would be anticipated regarding the use of the other reagents in preparing the various, prescribed solutions, in particular the brand of sulfuric acid used. Naturally, one is obligated to prove, before such a statement can be put into practice, that any or all of the suspected impurities produce color at the same wavelengths used for measurement of the estrogens. Thereafter, any correction for these impurities could be effected through the use of the Allen Equation (Allen, 1950). In this regard, it is important to realize that with Brown's method and in correcting for additional color using the Allen Equation, the final, corrected value from which the calculated amount of estrogen in urine is measured by reference to standard curves is quite small in relationship to the value read directly from the spectrophotometer. This indicates, logically, that there is considerably more color produced by the Kober Reaction involving substances other than estrogens under investigation than there is produced by these estrogens themselves. Comparing the results published by Brown with our own, we have found that for

ethanol solutions of methylayed estrogens, our corrected spectrophotometric readings are lower by approximately 25% than those reported by Brown. This is probably attributable to the relatively less impure solutions that we have employed. Irrespective of the large degree of extraneous color involved in this measurement, but corrected for with Allen's Equation, Brown apparently feels that his method is actually more accurate than was his original supposition (Bulbrook and Greenwood, 1957a). However, there remains little additional evidence that Brown's method is accurate for the determination of a total of less than 5 μg. per aliquot. Such micro methods are currently under investigation, but have not, as yet, been reported.

After some difficulty of a practical nature involving the use of our apparatus, standard curves for 3-methyl-estradiol-17β, 3-methyl-estriol, and 3-methyl-estrone were established (Figure I). An analysis of the figures represented in these curves using accepted statistical methods (in Smillie, 1955) indicates that there is a relatively small standard deviation and standard error for the lower concentrations of the methylated estrogens, whereas this error increases, in general, with increasing concentration. The points used in establishing the curves in Figure I represent mean values for concentrations of 5.2 and 7.8 μg. of 3-methyl-estriol, the points at 10.4 and 15.6 μg. signifying only single determinations and their position not taken into

FIGURE I

STANDARD CURVES FOR THE THREE ESTROGENS:



consideration in defining the slope of the resultant curve. With 3-methyl-estrone the curve is described by mean values of 5 and 7.5 µg.; the point for 10 µg., representing the average of two determinations, is included solely as an interesting finding. Finally, the curve for 3-methyl-estradiol-17β represents mean values for three aliquots of 5, 7.5, and 10 µg. The actually recorded, mean values signified by these curves are as follows:

µg.	3-Me-estradiol-17β	3-Me-estriol	3-Me-estrone
5	0.192(2)	0.146(3)	0.205
7.5	0.283	0.243	0.324(3)
10	0.408(6)	-	-

Since the number of determinations in each case was relatively small, it seems that a more searching statistical evaluation is in order so that the reliability of each value above, and its expected reproducability, may be better grasped. For this purpose, two additional factors, the standard deviation and the standard error, have been calculated as follows:

Standard Deviation -

µg.	3-Me-estradiol-17β	3-Me-estriol	3-Me-estrone
5	±0.003(9)	±0.003(2)	±0.007
7.5	±0.012(6)	±0.004(6)	±0.021(3)
10	±0.023(4)	-	-

Note - figures in parenthesis represent the next decimal point

Standard Error -

µg.	3-Me-estradiol-17β	3-Me-estriol	3-Me-estrone
5	±0.001(2)	±0.001(3)	±0.002(9)
7.5	±0.003(2)	±0.001(9)	±0.007(6)
10	±0.005(9)	-	-

The standard deviation has been calculated using a modified method, applicable to any series wherein the determinations are less than 30 in number, as suggested by Hill (1955). This simply involves multiplying the standard deviation, as calculated by the usual method, by a factor of the square root of "n/n-1," where "n" is the number of determinations.

Since the standard deviation appears to be relatively small, a further analysis of these figures seems in order. Rather than subjecting each of the estrogens to analysis, I shall briefly use 3-methyl-estradiol-17β as an example for this purpose:

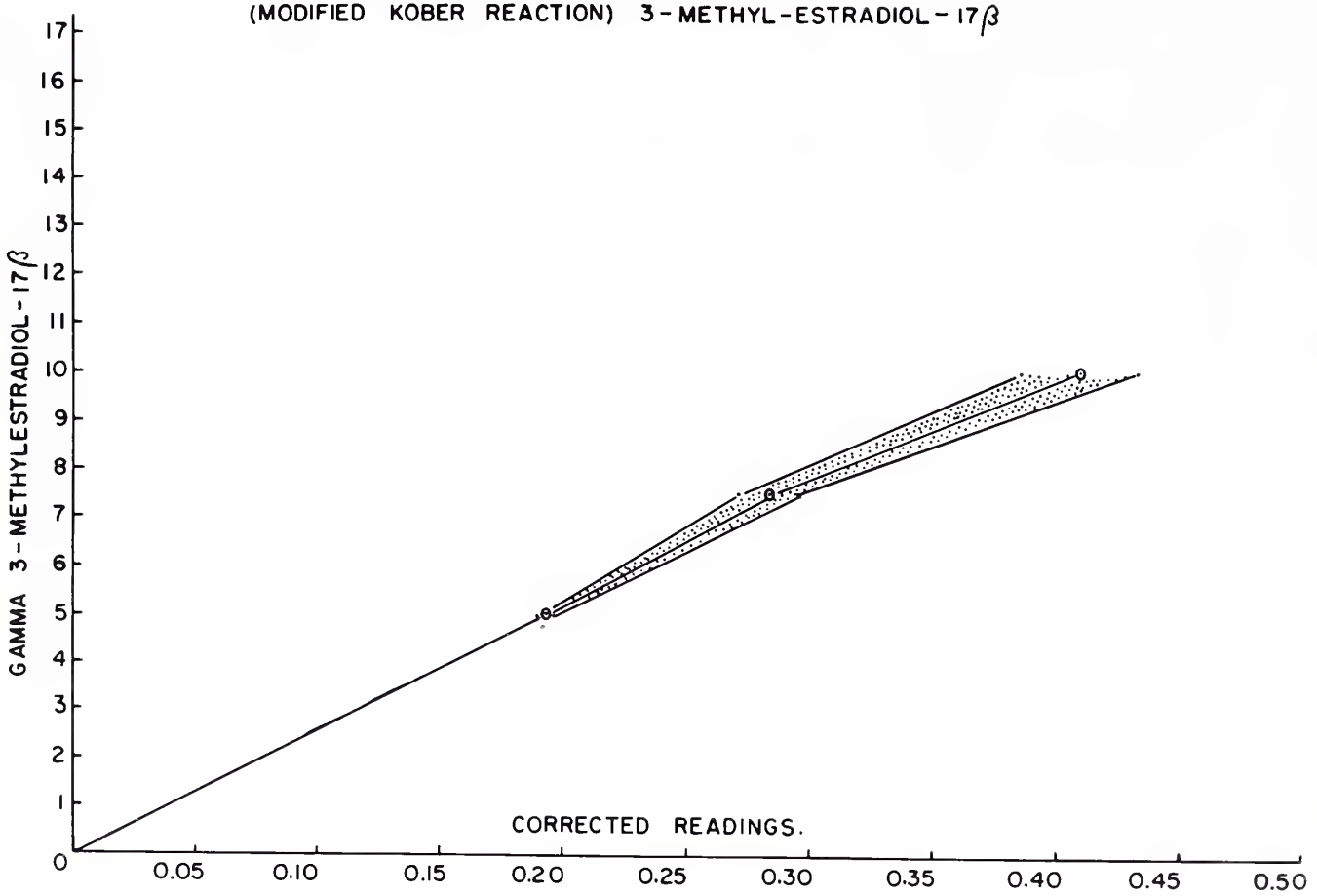
µg.	Mean	Mode	Median	Standard Deviation
5	0.192(2)	0.192	0.192	±0.003(9)
7.5	0.283	0.279	0.279	±0.012(6)
10	0.408(6)	-	0.404	±0.023(4)

Again it can be seen that with increasing concentrations of 3-methyl-estradiol-17β, the scatter of the values increases. These reported values represent, in the case of 5 µg., five determinations; a sixth corrected reading was discarded, after suitable statistical examination, as being an error in reading rather than a chance variation.

For the quantities of 7.5 and 10 μg., a total number of eight determinations for each is recorded, from which a single value was similarly excluded from the corrected readings at 10 μg. Likewise, using the mean corrected readings for 3-methyl-estradiol-17β as central values and the standard deviation as the limit of variation from this mean, a graph may be constructed showing the range wherein 95% of all corrected readings may be expected to fall: the stippled area in Figure II indicates this area. No predictions for values below a concentration of 5 μg. are made since, with the few readings that were made in this range (actually 2.5 μg.), considerable scatter of values was encountered. It therefore is apparent that any attempt to estimate concentrations of less than 5 μg. would be subject to considerable error and that samples of urine should be of such volume as to contain an anticipated quantity in excess of 5 μg. of estrogen. According to the results reported by Brown, this implies that 24 hour urine samples are the optimal quantities for analysis, excepting, of course, the instance of estradiol-17β where the amount that is excreted is quite small. This minimal amount of estradiol-17β can be accounted for by the fact that it is largely metabolized within the body to estrone (Heard and Hoffman, 1941), as is estrone metabolized, to a certain extent, to estriol (Pearlman and Pincus, 1946; Pearlman et al, 1954). Pearlman et al also indicate that some proportion

FIGURE II

TYPICAL STANDARD CURVE
(MODIFIED KOBER REACTION) 3-METHYL-ESTRADIOL-17β



of estrone is metabolized back again to estradiol-17β, the degree depending upon the various tissue levels of the specific enzymes involved. In addition, although estradiol-17β and estrone have both been isolated from species other than man, estriol has been recovered, in the animal kingdom, only from human tissue (Dorfman and Unger, 1954). A schematic representation of estrogen metabolism has been presented by Pincus and Thinmann (1955). The indicative findings from urinary estrogen estimations suggest that these metabolic equilibria ultimately favor the excretion of principally estriol and estrone in urine. Other possible modes of estrogen excretion, e.g. sweat and feces, have not been approached as yet. Regarding a terse reference to the role of these estrogens prior to excretion, Villee and Hagerman (1957) present interesting evidence that estradiol-17β serves to combine with the enzyme, isocitric dehydrogenase of placental origin, and subsequently activates this enzyme. Estriol, estradiol-17α (unknown in human tissues), and a stilbesterol derivative have thereafter been found to be competitive inhibitors for the combination of estradiol-17β with placental isocitric dehydrogenase, although these inihibitors do not seem to be concerned with activation of this enzyme after combination with estradiol-17β has occurred.

It is of considerable interest that Brown's method has given values in the 10-20 μg. range for total 24 hour

urinary estrogen excretion, whereas the ranges for other methods have gone as high as 1000 µg./day (Giannettasio, 1953). This large variance, naturally, is why there exists such a need for an accurate and reproducable method for estrogen estimation. A short article, with references to other methods and their reported results, has been published by Sulak and Zimmermann (1955), wherein, incidentally, they obtained mean levels of 600-800 µg./24 hours of total estrogen excreted. In view of the fact that Brown's results are compared from standard curves in which precisely weighed quantities of the estrogens under evaluation are added to the standard solution, it appears that his method is more accurate and consistent than previous procedures, especially those wherein total estrogen, i.e. irrespective of particular constituents, is measured.

CHAPTER III

Experimental Protocol

The experimental protocol is essentially similar to that published by Brown (1955a), and any deviations or innovations are contained within the text. In efforts to establish the necessary standard curves, the enumerated order was followed.

1. Preparation of Reagents - Doubly distilled, demineralized water was used throughout. Solutions (500 μg./100 ml.) of 3-methyl-estradiol-17β, 3-methyl-estrone, and 3-methyl-estriol (520.5 μg./100 ml.) were prepared in absolute ethanol, U. S. P. (U. S. Industries Chemicals Company). Acid sulfuric, Analytical Reagent (Mallinckrodt Chemical Works) was used in the preparation of the following color reagents: for estradiol-17β, 4 gm. hydroquinone in 200 ml. 60% v/v H_2SO_4; for estriol, 4 gm. hydroquinone in 200 ml. 76% v/v H_2SO_4; and for estrone, 4 gm. hydroquinone in 200 ml. 66% v/v H_2SO_4. These color reagents were stored at room temperature in the dark and may be so kept for at least 3 months; they were a very light amber in color. Likewise, a 2% w/v hydroquinone in absolute ethanol solution was prepared, stored in the dark, and kept at 0-2° C. except when allowed to warm to 20° C. for purposes of pipetting.

At first, hydroquinone (Mallinckrodt "Photo Purified")

was used in the preparation of the prescribed color reagents, but it was soon noted that these reagents changed color upon standing and that preliminary results using them were quite irreproducable. Therefore, this same hydroquinone was carefully sublimed and used in the preparation of new color reagents. Whereas the ordinary hydroquinone was a white substance with rather coarse, slightly dusky appearing crystals, the sublimed substance was a "fluffy white," fine crystalline material. Although this sublimed reagent was deemed suitable for our use, coincident with the completion of the sublimation process, I was fortunate enough to receive the same hydroquinone (British Drug Houses Ltd.) that was in use in Dr. Brown's laboratory, and thus the small quantity of hydroquinone which I had sublimed was never put to use. Hydroquinone (British Drug Houses Ltd.) may be obtained commercially from Melville & Hunter, 6 Bristo Street, Edinburgh 8, Scotland. All other reagents used were of Analytical Reagent grade (Mallinckrodt), excepting dimethyl sulfate (Matheson Coleman & Bell) which was carefully distilled. Although dimethyl sulfate toxicity is a theoretical problem to be considered in handling this chemical, only a single death has been reported in the literature, and this was the result of a laboratory technician's excessive exposure received while mopping up a spilled bottle of this agent. Nevertheless, simple precautions should be observed.

2. Evaporation of Solvents - A modified apparatus for evaporation of solvents was designed whereby reflux of vaporized solvent was effectively prevented by bending the exitus tube from the male part of the apparatus to ca. a 45^{o} angle. This modified apparatus, as used, may be seen in Figures III and IV. At first, an attempt was made simply to dry down the methylated estrogen solutions in an ordinary test tube, in a hot water bath, and under a stream of nitrogen. This technique proved to be highly unsatisfactory, since considerable estrogen was lost thereby, and thus recourse to the apparatus used by Dr. Brown, with suitable modifications, was found to be essential. Using this modified apparatus, it was possible to reduce the pressure throughout the entire evaporating system, and so to facilitate a lower temperature, more rapid evaporation process. Moreover, the column of ethanol vapor evaporating from the estrogen solutions served to exclude oxygen from contact with the surface of these solutions while they were being heated. Solvents were treated in specially prepared, 30 ml. pyrex test tubes with a Standard Tapered 19/38 female joint, into which the corresponding male part was fitted. Four sample determinations were run simultaneously. Samples of 0.2-3.2 ml. of standard methylated estrogens (2.5-15 µg.) were added to each tube, with one tube serving as reagent blank, i.e. no estrogen added. Then 0.2 ml. of 2% w/v hydroquinone (4 mg.) was added to each tube and the tubes were evaporated completely

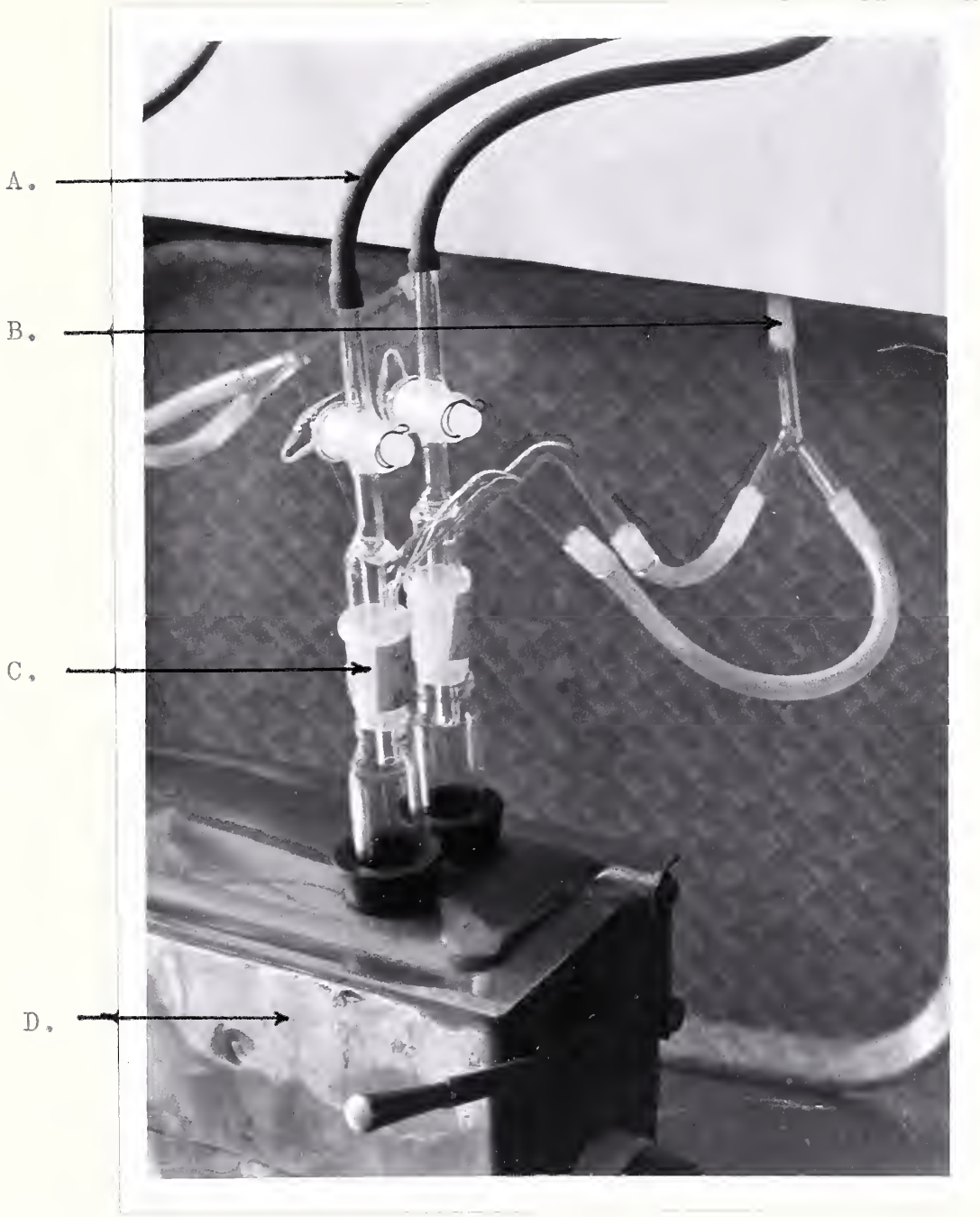


Figure III

A. Nitrogen inlet
B. Suction
C. ST 19/38 joint
D. Hot water bath

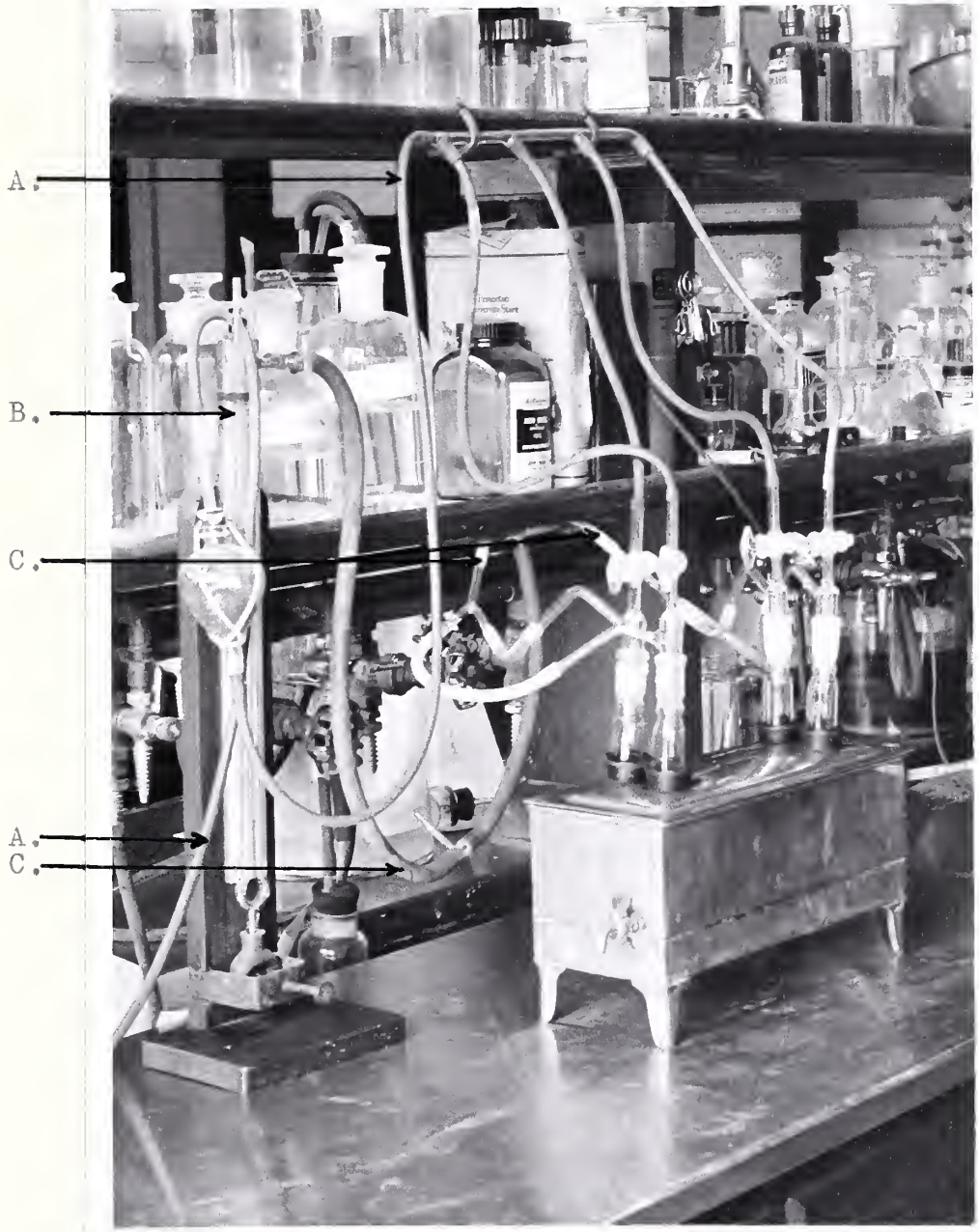


Figure IV

A. Nitrogen inlet

B. Manometer

C. Suction

to dryness in a hot water bath. Pressure in these tubes during evaporation was ca. 0.6 atm., obtained from a water tap suction attachment. A slight flow of anhydrous nitrogen (ca. >1 litre/min.) was allowed into the tubes; the temperature of the water bath was rarely permitted to exceed 90° C., and evaporation took 10-40 minutes, depending upon the quantity of solvent to be evaporated. The tubes were removed from the bath as soon as the solutes were dry and were observed to be white and crystalline in appearance. It is of note that if dried solute was allowed to remain in the water bath, an amber, charred appearance soon replaced the white crystals, and such charred solute was discarded. After all four of the tubes were completely dried, the drying apparatus was such as to allow application of the full force of the water suction (ca. 0.5 atm.), following which the suction was released by filling the tubes with anhydrous nitrogen. This decompression and subsequent filling with nitrogen was accomplished at least twice. More frequent, similar operations had no effect upon the final results.

3. Color Development and Colorimetry - Color was then developed by adding 3.0 ml. of the appropriate hydroquinone-H_2SO_4 reagent to each tube and heating the tubes for 20 minutes in a boiling water bath. During this 20 minute period, each tube was thoroughly shaken twice, at 3 and 6 minutes. After such treatment, the tubes were cooled by placing them in a cold water bath. Water (1.0 ml.

to each estriol tube, 0.5 ml. to each estrone tube, and 0.2 ml. to each estradiol-17β tube) was added, and the tubes were reheated in the boiling water bath for 10 minutes, being shaken at 3 minutes. The tubes were finally cooled again in a water bath for at least 10 minutes, after which their optical densities were read, using the reagent blank as a zero point of reference, on a Beckman DU Spectrophotometer at wavelengths of 480, 518, and 556 mμ. for estradiol-17β, and 480, 516, and 552 mμ. for estriol and estrone. The colored solutions were observed to be a light amber-pink and slightly fluorescent; these developed colors were not permitted to stand for more than 12 hours before being read.

4. Optical Density Correction - The observed optical densities were corrected, as suggested by Brown, by a modified application of Allen's formula: for estradiol-17β, $D_{corr.} = 2D_{518} - (D_{480} + D_{556})$, and for estriol and estrone, $D_{corr.} = 2D_{516} - (D_{480} + D_{552})$. The validity of this modification in Allen's Equation has been discussed and substantiated by Diczfalusy (1955). Optical densities of less than zero were considered to be "0" and all other positive readings were used in the calculations. These readings for measured quantities of the methyl derivatives of each of the three estrogens were used to establish standard curves. Amounts of free estrogen could be readily calculated by multiplying those quantities of their 3-methyl derivatives by the respective ratio of their molecular weights.

5. Fluorescence Studies - In addition to colorometric determinations using the Kober Reaction, a preliminary study of a fluorometric method (Goldzieher et al, 1952) was undertaken. Developing fluorescence in 90% H_2SO_4, readings were obtained using an Amico Bowman Spectrophotofluorimeter. Maximum fluorescence was recorded at the following wavelengths:

Substance	Exciting Wavelength	Emitting Wavelength
90% H_2SO_4	350	370
estradiol-17β (5.088 μg./ml.)	400	485
3-Me-estradiol-17β (4.992 μg./ml.)	400	485

Quantitatively, the estradiol-17β maximum was approximately twice in magnitude that of its methylated derivative.

Following the satisfactory establishing of standard curves, recoveries of estrogen added to water and subject to the entire procedure for separation, excepting the initial acid hydrolysis, seemed to be next in order. Preliminarily, this recovery demanded that alumina to be used for chromotography be prepared to the proper activity.

6. Alumina Standardization - Non-alkaline alumina (Woelm) activity grade I was deactivated with doubly distilled, demineralized water by shaking for two hours to insure homogeniety. Considerable care was required in the standardization of this alumina, and various activities were tested before the proper result could be secured. After trials wherein 9.5, 10, 12, and 14 ml. of water was added to the

alumina, a value of 11.3 ml./100 gm. alumina seemed optimal. A 2 gm. column of this alumina was prepared in light petroleum and a solution containing 10 µg. of 3-methyl-estrone in 25 ml. of light petroleum, which had been washed with water, was applied to the column. The column was eluted with a 25% benzene in light petroleum solution and the 3-methyl-estrone first appeared in the 23rd ml. of eluate. Following this procedure, another 2 gm. column was prepared in light petroleum, and water washed solutions of 3-methyl-estrone and 3-methyl-estradiol-17β (10µg. of each in a total of 25 ml. of light petroleum) were applied to the column which was then eluted, first with 12 ml. of 25% benzene in light petroleum, and then fractionally with 40% benzene in light petroleum. The estrone methyl ether was eluted in the initial 12 ml. of 40% benzene in light petroleum, and the estradiol-17β methyl ether was first identified in the 30th ml. of total eluate, being completely eluted between 30 and 40 ml. of eluate. Another 2 gm. column was similarly prepared in light petroleum, and a solution of methylated estradiol-17β was applied and eluted with 12 ml. of 25% benzene in light petroleum, and then fractionally with benzene alone. The 3-methyl-estradiol-17β was found to come out in the first 5 ml. of benzene. Following this, a solution of 3-methyl-estriol in 25 ml. of benzene was washed with water and applied to a column prepared in benzene. This column was eluted with 1.4% ethanol in benzene, and the estriol methyl ether

appeared after 33 ml. of elution. Lastly, a similar column was prepared and first eluted with 12 ml. of 1.4% ethanol in benzene, followed by 2.5% ethanol in benzene, wherein the methylated estriol appeared in the initial 10 ml. of eluate. The appearance of each of the methylated estrogens was identified qualitatively by the addition of 1 ml. of the appropriate hydroquinone-H_2SO_4 color reagent and the subsequent development of a characteristic pink color.

After this qualitative standardization of our alumina, it was decided to determine quantitatively exactly how much methylated estrogen was eluted. Therefore, a 2 gm. column of alumina was prepared in light petroleum, and a solution containing 10 μg. each of 3-methyl-estrone and 3-methyl-estradiol-17β, taken up in 25 ml. light petroleum and washed with 3 ml. water, was applied to this column. The column was then eluted with 12 ml. of a 25% benzene in light petroleum solution, which was discarded, and with 18 ml. of 40% benzene in light petroleum, upon which eluate specific estrone color was later to be developed. The column was then further eluted with this same 40% benzene in light petroleum and the 30th-45th ml. were kept for estradiol-17β color development. Recoveries from this experiment yielded 83% for 3-methyl-estrone and 57% for 3-methyl-estradiol-17β. In view of these relatively poor results, another attempt at estradiol-17β recovery was made. Another 2 gm. column was

prepared in light petroleum and a solution of 10 μg. of 3-methyl-estradiol-17β, taken up in 25 ml. light petroleum and washed with 3 ml. water, was applied to this column. The column was first eluted with 12 ml. of 25% benzene in light petroleum followed by 27 ml. of benzene (40%) in light petroleum, which eluate was discarded. Then four fractional samples, totalling 32 ml., of benzene alone were collected from the column. The first 12 ml. were found to contain all of the 3-methyl-estradiol-17β, but with a discouraging yield of only 50%. A similar operation of chromotography was carried out, except that the original 3-methyl-estradiol-17β solution was not washed with water in hopes that this is where the loss had occurred, but a yield of a scant 33% (the corrected reading fell within the range of statistical insignificance) led to no solution to this problem. It therefore appears that the estrogen has remained within the column, possibly adsorbed to the alumina. This difficulty was not mentioned by Dr. Brown, and the possibilty exists that Woelm alumina is sufficiently different from the alumina used by Brown (Savory and Moore Ltd., London) to explain our losses. Finally, a single run through the entire method proposed by Brown, excepting the initial acid hydrolysis with concentrated HCl, beginning with known added amounts of the non-methylated estrogens, gave yields of 43%, 68%, and 26% respectively

for estradiol-17β, estrone, and estriol. The curves in Figure I were used as the standard reference. Obviously, other sources for the loss of estrogen must and shall continue to be sought in this laboratory. And indeed, the purity of our beginning, so-called standard estrogens might be questioned, especially in light of the fact that Stern and Braunsberg (1955) have identified other substances in "crystalline" estriol.

CHAPTER IV

Summary and Conclusions

Studies were made in the method proposed by Brown (1955a) for the chemical determination of human urinary estrogens, estradiol-17β, estriol and estrone.

1. The usefulness and possible significance of such a routine laboratory method has been briefly presented.

2. A terse outline of the principal current means of approach to the problem of estrogen determination has likewise been presented, and the resultant variability of measurements is indicated.

3. Certain pitfalls regarding the use of relatively impure reagents were discovered and are reported.

4. Standard curves for the 3-methyl derivatives of the three estrogens under surveillance have been established and their statistical significance and accuracy is assayed.

5. Certain pertinent metabolic considerations are reported.

6. All experimental procedures are delineated and their results are tabulated.

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