A NEW METHOD FOR THE SEPARATION OF ANDROGENS FROM ESTROGENS AND FOR THE PARTITION OF ESTRIOL FROM THE ESTRONE-ESTRADIOL FRACTION

WITH SPECIAL REFERENCE TO THE IDENTIFICATION AND QUANTITATIVE MICRODETERMINATION OF ESTROGENS BY ULTRAVIOLET ABSORPTION SPECTROPHOTOMETRY*

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It is recognized generally that a qualitative and quantitative knowledge of the excretion pattern of the urinary estrogens is one index to an understanding of the functional activity of the ovary and adrenal cortex. Obviously, such determinations may be useful also in evaluating the normal and abnormal functions of other physiologically related endocrine glands as well as of organs like the liver and kidneys. The clinical applications of these data are self-evident.

Various attempts have been made to circumvent the notoriously inaccurate values which have been obtained for the urinary estrogens by a variety of bioassay methods and colorimetric techniques (1, 2). The acknowledged shortcomings of these methods have led us to investigate the application of ultraviolet absorption spectrophotometry to the quantitative determination of the urinary estrogens in an attempt to develop an objective physical method for their accurate determination. It is known that the infra-red portion of the spectrum yields more differentially characteristic curves, but those of the ultraviolet range are more readily obtainable, and consequently better adapted to clinical use.

This communication is concerned with studies of the following aspects of the problem: (1) spectrophotometric identification and quantitative micro determination of crystalline estrogens; (2) detection by spectrophotometric assay of gross errors in current methods for extraction and partition of estrogens; (3) studies on the ultraviolet absorption of sub-

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stances comprising the background material; (4) separation of the phenolic estrogens from the so called neutral steroid fraction; (5) separation of urinary estrogens from other urinary phenolic substances by steam distillation; (6) micro-Girard separation of estrone from estradiol; (7) an essentially new method for the extraction and partition of crystalline estrone, estradiol, and estriol, and their quantitative assay by ultraviolet spectrophotometry.

Spectrophotometric Identification and Quantitative Microdetermination of Crystalline Estrogens

Ultraviolet absorption spectra of chemically pure crystalline estrone, estradiol, and estriol were determined with the Beckman quartz spectrophotometer. The region from 226 to 300 mμ was investigated, and curves were constructed from points determined at intervals of 2 mμ. The corrected constant melting point of each of these compounds was determined in order to establish the purity of the compounds. These values were found to be 258° for estrone, 273–274° for estriol, and 176.7° for α-estradiol.

Experimental Procedures—4 or 5 ml. of U. S. P. 95 per cent ethyl alcohol were used throughout these experiments as a solvent for the residues of the substances that were subjected to ultraviolet spectrophotometry. This solvent has the advantage of being transparent from the visual range to 200 mμ. The alcohol was not redistilled, because its use as the blank for the setting of the spectrophotometer compensated adequately for the insignificant amount of absorption due to its impurities. All of the experiments currently reported were controlled in this fashion.

25 mg. of each of the three estrogens were dissolved in 95 per cent ethyl alcohol and made up to volume in a 25 ml. volumetric flask. Aliquot portions of each standard were diluted quantitatively subsequently to give concentrations of 125, 100, 75, 50, 25, and 12.5 γ per ml. These dilutions were used in the construction of the individual calibration curves.

Results—The ultraviolet absorption curves of estrone, estriol, and estradiol were found to be very similar. Our observations are in essential agreement with those in the literature (3–8) in so far as the *general* shape of these absorption curves is concerned. The estrone and estriol are characterized by a minimal density at 248 mμ and maximal densities between 280 and 282 mμ with a secondary peak at 288 mμ. Another maximal density is to be found below 230 mμ. Estradiol shows the same peaks as estrone and estriol, but the lowest extinction occurs at 252 mμ. The secondary peak, which was observed at 288 mμ in the curves for all three estrogens, is to be found in the absorption curves for estrone published by Hogness *et al.* (3), Mayneord and Roe (4), and Callow (5), although they do not call attention

to this point specifically in their texts. The constancy of the appearance of this secondary peak in the curves for all three estrogens suggests that it may be of significance in connection with the molecular structure of these substances. Between 280 and 288 mμ, our absorption curve for estrone is in general accord with that of Heard and Hoffman (6), but it differs considerably from the characteristics of their curve below and above these points.

Although the data in the literature indicate that the spectrophotometric curves for estrone, estradiol, and estriol are essentially identical, we have

TABLE I

Differences in Densities and Molecular Extinction Coefficients of Estrogens at 280 mμ

Estriol		Estrone		Estradiol	
	Density		Density		Density
γ per ml.	*100 γ per ml.*	*γ per ml.*	*100 γ per ml.*	*γ per ml.*	*100 γ per ml.*
25	0.716	25	0.760	50	0.754
50	0.664	50	0.760	52	0.769
52	0.664	75	0.774	75	0.717
75	0.713	98	0.793	99	0.707
92	0.714	100	0.758	100	0.689
100	0.682	103	0.779	101	0.724
101	0.710	107	0.769	105	0.734
104	0.734	125	0.740	112	0.734
108	0.708			125	0.736
125	0.690				
Average	0.700		0.767		0.729
ϵ*.....	2000		2073		1970

* $\epsilon = (1/cx) \log_{10} (I_0/I_x)$, in which ϵ = molecular extinction coefficient, c = gm. molecules per liter, x = width of cell in cm., $\log_{10} (I_0/I_x)$ = density.

found a significant difference in the density per unit weight at a given concentration at 280 mμ (Table I). The average density values for 100 γ per ml., as determined by a series of experiments of varying concentrations, are 0.700 for estriol, 0.767 for estrone, and 0.729 for estradiol. These differences are not due solely to differing molecular weights. Apparently, the carbonyl group in Ring D of estrone enhances its density at 280 mμ, because this group exhibits an absorption maximum in that region. On the other hand, the hydroxyl groups in Ring D of estriol and estradiol show a maximal absorption at 186 mμ, and therefore do not influence the density values at 280 mμ. So far as the difference between estriol and estradiol is concerned, one should note that on an absolute weight basis there are a larger number

of estradiol molecules than estriol molecules in a solution of a given concentration. This is in accord with the experimental data, which indicate that the density of the estradiol solution is greater than that of the estriol solution.

We have also determined that the minimal density of the estradiol differs consistently, but not remarkably, from that of the estrone and estriol. Since this difference is relatively small, these three estrogens must be

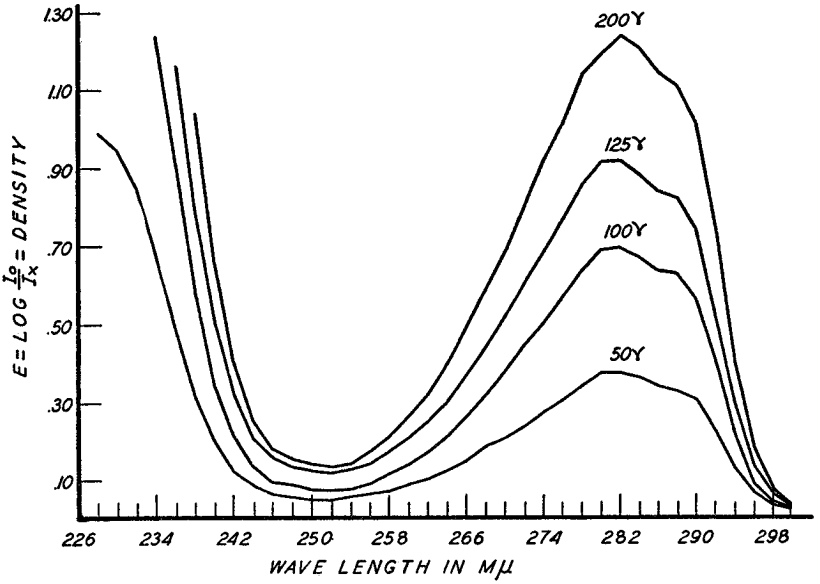


FIG. 1. The effect of concentration upon the ultraviolet absorption of estradiol

separated quantitatively before the spectrophotometric method can be used for their determination.

The effect of concentration of estradiol upon the ultraviolet absorption is shown in Fig. 1. As might be expected from the decreased sensitivity of the instrument above $E = 1.00$ and below $E = 0.10$, the extinction coefficient is proportional to the concentration only between those limits.

The peak at 280 mμ was selected for the construction of a calibration curve for each of the estrogens (Fig. 2). It is clear from these data that the relation between density and concentration follows Beer's law between $E = 0.10$ and $E = 1.00$ at a wave-length of 280 mμ. The reproducibility of results at a wave-length of 280 mμ was tested on 10 successive days with each of the three estrogens in dilutions of 100 γ per ml. The percentage variation from the mean was found to be negligible (Table II). The data

on the concentration-extinction relationship in Fig. 2 are not in agreement with those of Reynolds and Ginsburg (7). Since they omitted reference

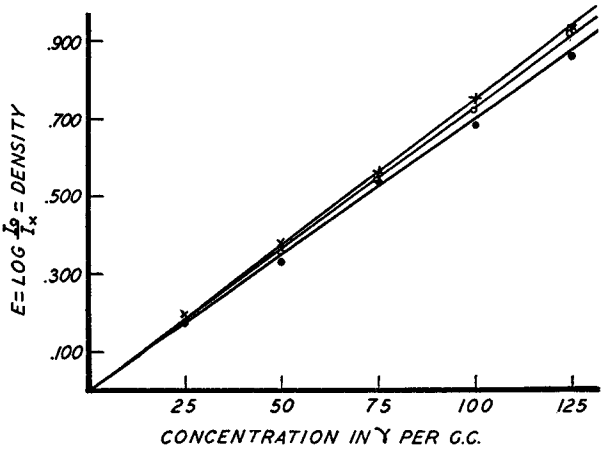


FIG. 2. Calibration curves of estrone, estradiol, and estriol, respectively, in 95 per cent ethyl alcohol at a wave-length of 280 mμ. × = estrone; ○ = estradiol; ● = estriol.

TABLE II

Reproducibility of Density Values for Estrogens at 280 mμ

Estrone		Estradiol		Estriol	
Density	Per cent variation from mean	Density	Per cent variation from mean	Density	Per cent variation from mean
0.824	0.2	0.708	0.4	0.700	0.4
0.830	0.5	0.718	1.0	0.695	0.3
0.815	1.1	0.709	0.3	0.690	1.0
0.824	0.2	0.705	0.8	0.696	0.1
0.828	0.2	0.708	0.4	0.700	0.4
0.825	0.1	0.712	0.1	0.700	0.4
0.824	0.2	0.720	1.3	0.700	0.4
0.838	1.4	0.712	0.1	0.700	0.4
0.830	0.5	0.712	0.1	0.695	0.3
0.820	0.7	0.710	0.1		
Mean....0.826		0.711		0.697	

to the melting points of their preparations, there is no way of determining the purity of the hormones employed in their studies.

One may conclude from the calibration curves of Fig. 2 that each of the estrogens may be determined with accuracy only in concentrations above 12 to 15 γ per ml. of alcoholic solution. Various studies indicate that the total estrogen excretion in certain phases of the menstrual cycle may be such that the concentration of estrogens in the final alcoholic extracts is well below this amount. This would make it necessary in certain circumstances to extract pooled urine specimens of several days excretion in order to determine their estrogen content. We have applied the technique of lyophilization to urine in order to make it possible to collect and concentrate it in relatively large quantities without running the risk of chemical alteration of its steroid content (9).

Detection by Spectrophotometric Assay of Gross Errors in Current Methods for Extraction and Partition of Estrogens

Critique of Currently Accepted Methods for Partition of Estrogens—A detailed analysis of the literature on data obtained by methods in general use for the partition and assay of urinary estrogens discloses that the quantitative accuracy claimed for them is not in accord with the results on which this contention is based. The foregoing statement may seem startling in view of the wide-spread and relatively unquestioned acceptance of these methods. The facts speak for themselves, however.

Working with aqueous solutions of crystalline estrogens, Cohen and Marrian (10) observed that their method of partition yielded a recovery of 78 to 93 per cent of estriol and 96 to 106 per cent of estrone. Furthermore, from the urines to which crystalline estriol and estrone were added, respectively, they recovered 97 per cent of the estriol and 93 per cent of the estrone. In the light of what we now know of the expected 20 per cent loss of estriol in the Na_2CO_3 washings, added to the inevitable large loss of estriol during a 2 to 4 hour hydrolysis at pH 1 to 2, these high recoveries are open to question. As a consequence, one is inclined also to wonder about the quantitative accuracy of their values for the recovery of estrone, particularly since they themselves have acknowledged that in the estrone-estriol partition 20 per cent of the estrone is carried over into the estriol fraction.

Using the Cohen and Marrian technique, except for a recognized improvement in the conditions for hydrolysis, Smith, Smith, and Schiller (11) found that the results obtained in *exploratory partition experiments*, which they singled out for special study, could not be duplicated when estrogens were submitted to the *entire procedure of extraction* and *partition*. They reported that the three estrogens could be separated quantitatively when submitted to partition alone. However, when carried through the entire procedure, they observed that the recovery of estrone was between 60 and 105 per

cent, that the recovery for estriol varied between 33 and 103 per cent, and that estradiol was recovered in amounts varying from 40 to 99 per cent of the added material.

To explain this discrepancy, Smith, Smith, and Schiller attributed the inconsistent results to an inadequate recovery from NaOH of the separated estrogens, rather than to the Cohen and Marrian technique of partition. Although they claimed uniformly higher recoveries of added estrogens when they modified the technique in accordance with their theory, an analysis of the data suggests that their conclusions were based on insufficient evidence.

In view of the foregoing, it would seem that the data in the literature have not been examined critically enough. The fact that nearly perfect recoveries were obtained under experimental conditions in which substantial losses are known to occur leads us to question the validity either of the methods of assay or the extraction and partition procedures, or both. In this connection, one may consider the data published by three reliable groups of investigators, who checked the Kober colorimetric assay for urinary estrogens against the bioassay method. It is well recognized that the Kober reaction is affected by non-estrogenic urinary substances, and consequently gives overestimates of the actual total estrogen content. The error thus introduced may be one of the factors contributing to the vast discrepancies disclosed by the data in Table III. For example, two samples analyzed by the bioassay method showed 1.100 and 1.150 mg. of estrone, respectively, as compared with 0.672 and 4.140 mg. of estrone determined colorimetrically. Reference to the ratios for the Kober colorimetric-bioassay in Table III shows that these difficulties are the rule rather than the exception, and that they are of a significant order of magnitude. In spite of the fact that perennial attempts have been made to establish the accuracy of one of these methods by checking it against the other, the results in Table III indicate that this cannot be done.

Because of the doubt which has been expressed concerning the quantitative accuracy of the bioassay and the Kober colorimetric techniques, and because we have demonstrated already that the spectrophotometric method can be used for the quantitative assay of crystalline estrogens, it seemed advisable to evaluate the efficiency of the currently accepted partition methods by this physical means.

There are in common use today two types of methods for the separation of estriol from the estrone-estradiol fraction. This partition is accomplished generally by the equilibration of either an alkaline salt or a hydroxide with one of several organic solvents.

Ultraviolet absorption spectrophotometry, as a method of assay in the partition of estrogens, could not be applied satisfactorily to the Cohen and Marrian technique because of the toluene used in the procedure. Toluene

TABLE III

Illustrations of Inconstant Relations Between Kober Colorimetric and Bioassay Methods

Period of gestation	Estrone			Estriol		
	Kober	Bioassay	K:B*	Kober	Bioassay	K:B*
Cohen and Marrian (10), mg. per 100 ml. urine						
Sample 3†				0.205	0.243	0.84
" 3†				0.240	0.231	1.04
" 4†	0.033	0.024	1.38	0.182	0.171	1.06
" 4†	0.042	0.031	1.36	0.178	0.178	1.00
" 5†	0.071	0.070	1.01	0.316	0.265	1.19
" 5†				0.306	0.218	1.40
" 5†				0.275	0.224	1.23
" 5†				0.280	0.231	1.21
" 6a†	0.100	0.107	0.94	0.691	0.624	1.11
" 6a†				0.700	0.607	1.15
" 6b†	0.127	0.126	1.01			
" 6b†	0.100	0.115	0.87			
" 7a†				0.680	0.593	1.15
" 7a†				0.705	0.558	1.26
" 7b†	0.100	0.092	1.09			
" 8†	0.127	0.125	1.02	1.070	1.070	1.00
" 8†	0.123	0.121	1.02			
Smith, Smith, and Schiller (11), mg. per 24 hr. sample						
3 mos.	0.840	0.060	14.00	3.960	0.146	27.12
5½ "	0.900	1.300	0.69			
6 "	3.020	2.500	1.21	10.800	10.700	1.01
7 "	2.254	1.350	1.68	13.600	10.800	1.26
8 "	2.368	2.450	0.97	30.710	11.800	2.60
8 "	4.270	5.100	0.84	39.979	24.500	1.63
8 "	0.672	1.100	0.61	38.000	10.600	3.59
8 "	4.140	1.150	3.60	36.400	7.350	4.95
41 days before delivery	1.800	0.800	2.25	14.000	10.700	1.31
22 " " "	3.040	1.500	2.03	11.600	8.000	1.45
15 " " "	5.400	6.600	0.83	46.880	64.000	0.74
9 " " "	3.600	2.500	1.44	42.080	21.300	1.98
6 " " "	2.520	1.335	1.89	34.080	21.200	1.61
Pincus, Wheeler, Young, and Zahl (12), rat units						
Approximately 5th day	606	148	4.09	647	148	4.37
" 5th "	432	252	1.71	1812	840	2.16
4 mos.	1140	675	1.69			
4½ "	1318	450	2.93	1241	685	1.83
5 "	663	330	2.01	998	600	1.66
5½ "				1463	1780	0.82
6 "	1044	550	1.90	1788	2200	0.81
7 "				1502	1800	0.83
7–9 "	891	670	1.33	5800	8900	0.65

* (Colorimetric data)/(bioassay data). † Period of gestation not stated.

exhibits a significant optical density between 230 and 290 mμ (13), a range which includes the absorption peak of the estrogens. The present experiments were limited, therefore, to a consideration of those methods in which estriol was separated from the estrone-estradiol fraction by the use of Na_2CO_3 and benzene. Consequently, the investigation centered around the Mather principle for the partition of estrogens; namely, the use of Na_2CO_3 to remove estriol from the benzene solution. This principle is incorporated in the methods recommended by Pincus (1) and by Bachman and Pettit (14). In view of these considerations, one of the methods selected for study was the Pincus modification of the Mather technique (1). The other method studied spectrophotometrically was that originally recommended by Mather (15). Since neither Mather nor Pincus attempted any purification of the so called "strong" and "weak" phenolic fractions, it seemed feasible to determine whether such a procedure would facilitate the application of the spectrophotometric technique to the assay of estrogens. The Bachman and Pettit method (14) was used for the process of purification after the Mather partition. It was hoped that these steps would prepare extracts relatively free from background material, which is anathema to the spectroscopist.

Experimental Procedure—2.569 mg. of estrone, 2.793 mg. of estradiol, and 2.532 mg. of estriol were each dissolved in 25 ml. of 95 per cent ethyl alcohol. 20 ml. of each of these solutions were pooled. Two 25 ml. aliquots of this pool were evaporated to dryness with suction. The residues of these aliquots were subjected to the methods of partition selected for study (Tables IV and V).

Results—The results of these experiments, which are to be found in Figs. 3 and 4, are clear cut. The following conclusions concerning the estrone-estradiol fraction may be drawn from these data: (1) The experimental curves (P and M-BP) in Fig. 3 show that the shapes of the curves are characteristic of estrogens, and that the minimal and maximal densities occur at 248 and 280 mμ. (2) Judging by the relatively high level of the minimal densities of Curves P and M-BP at 248 and 296 mμ, there is an appreciable amount of background material present. Since it is probable that the maxima at 280 mμ have been affected similarly, the data at this point cannot be taken at face value. (3) Because of essentially equal amounts of background material, a rough comparison can be made between Curves P and M-BP at 280 mμ. Judging by the differences between the minimal densities of the experimental (P and M-BP) and the standard (S) curves, it would appear that about three-quarters of the estrone-estradiol mixture was recovered after the Pincus modification, and that approximately one-half of these estrogens was recovered after the Mather-Bachman and Pettit procedure. On the basis of this observation, it occurs to us that

whatever one stands to gain by the manifold attempts to remove urinary impurities by the Bachman and Pettit procedure may be more than overbalanced by the losses sustained in these extensive chemical manipulations.

A similar study was made of the estriol fraction treated by the two methods which have been described. Analysis of the curves in Fig. 4 discloses a marked discrepancy between the density of the absorption peaks of recovered estriol (Curves P and M-BP) and that of the standard solution (Curve S), which represents the quantity of estriol with which each experiment was started. It should be noted in connection with Curves

TABLE IV

Pincus Modification of Mather Partition

(1) Residue of 25 ml. aliquot of pooled estrogens

(2) Dissolve in 50 ml. benzene; extract with 3 × 50 ml. 0.3 M Na_2CO_3

(3) Benzene phase

(4) Distil to dryness; perform Girard separation on residue

(5) Estrone fraction

(6) Estradiol fraction

(7) Carbonate phase

(8) Acidify with concentrated HCl to Congo red; extract with 4 × 50 ml. Et_2O

Aqueous phase discarded

(9) Ethereal phase

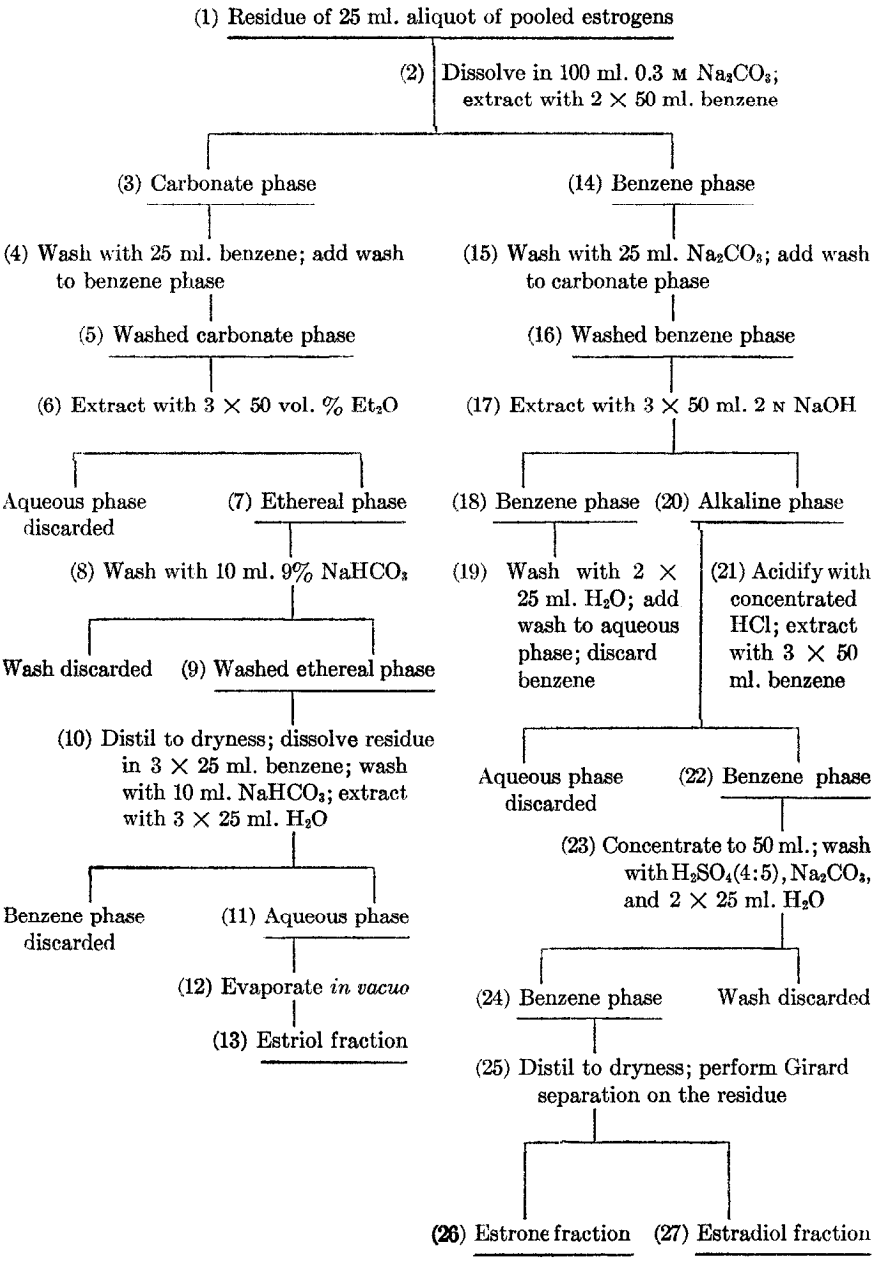
(10) Distil to dryness

(11) Estriol fraction

P and M-BP that their configuration is distorted greatly by the background material, inasmuch as the characteristic outline of the estrogen curve is no longer recognizable. Judging from the amount of background material indicated by the readings at 248 and 296 mμ, the recovery of estriol is practically nil. The latter is not surprising in view of the relatively low solubility of estriol in benzene, a point which was not taken into account either by Mather or Pincus (15, 1). In confirmation of Bachman and Pettit's (14) observation, we have found that the preliminary solution of estriol in alcohol is absolutely essential if estriol is to be taken up by

TABLE V

Mather Partition with Bachman and Pettit Purification of "Weak" and "Strong" Phenolic Fractions



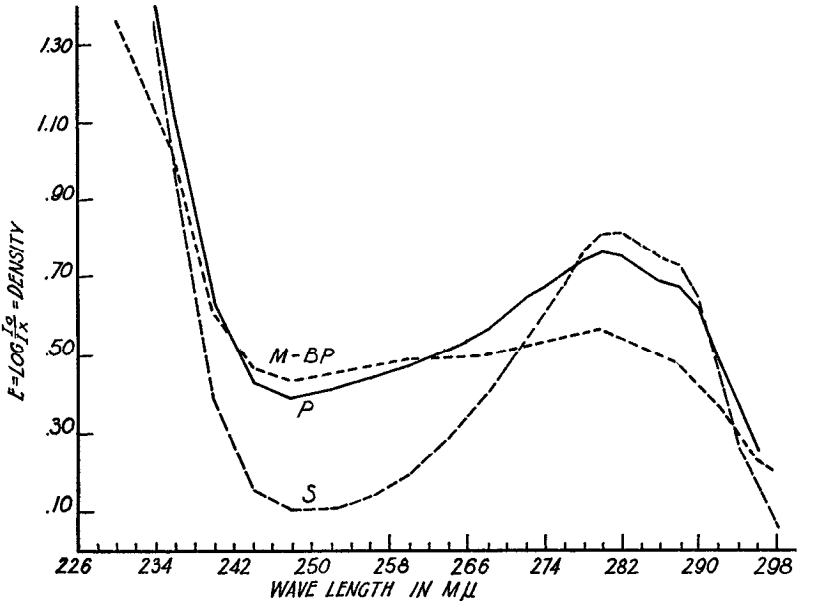


FIG. 3. Comparison of the ultraviolet absorption curve of a standard estrone-estradiol solution (Curve S) with the curves representing the amounts recovered from identical aliquots by the Pincus modification of the Mather partition (Curve P) and by the Mather-Bachman and Pettit partition (Curve M-BP).

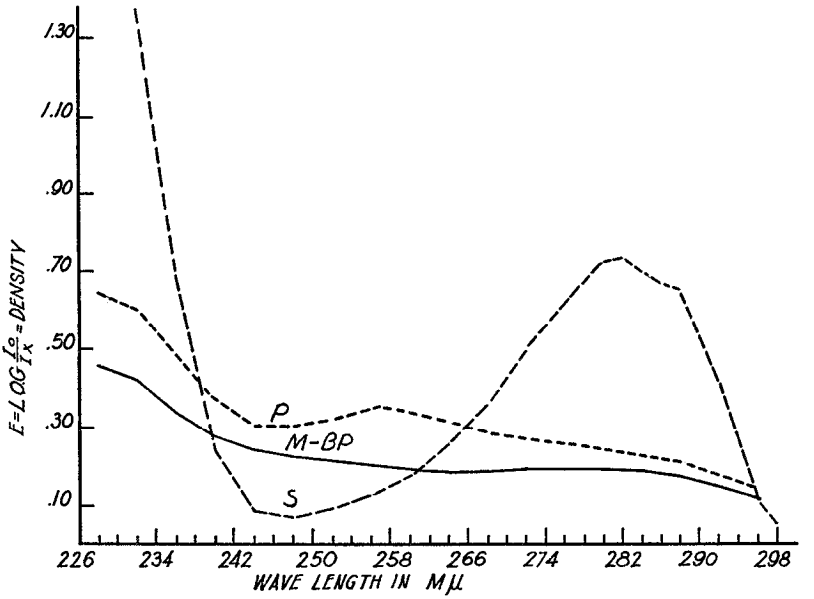


FIG. 4. Comparison of the ultraviolet absorption curve of a standard estriol solution (Curve S) with the curves representing the amounts recovered from identical aliquots by the Pincus modification of the Mather partition (Curve P) and by the Mather-Bachman and Pettit partition (Curve M-BP).

benzene quantitatively. These findings also raise a question with regard to the efficacy of sodium carbonate for the quantitative separation of estriol from estrone and estradiol.

It is clear from the studies which have been described that one must submit to reevaluation the methods currently used for the partition and assay of estrone, estradiol, and estriol. The fact that ultraviolet spectrophotometry provides a sensitive method for the quantitative determination of chemically pure estrogens makes it a feasible means for checking the accuracy of the partition methods, if one eliminates or reduces to an acceptable minimum the optical density of the background material.

Studies on Ultraviolet Absorption of Substances Comprising Background Material

Experimental Procedure—In view of the foregoing findings, and on the basis of preliminary exploratory experiments, it seems likely that the background material, which interferes with a quantitative interpretation of the estrogen absorption curves, consists entirely of substances used in the extraction and partition procedure; *i.e.*, reagents and their impurities. Consequently, a study was made of the absorption characteristics of these reagents and their values for E at 280 mμ. The experimental conditions under which the reagents were used originally were duplicated carefully. Included in these observations were Na_2HPO_4 and CCl_4 (*cf.* below) for reasons which will appear later on in this communication.

It is well known that petrolatum, rubber, and lubricating greases exhibit absorption in the ultraviolet range. It was suspected early in these experiments that stop-cock grease was carried along into the extracts by the organic and aqueous alkaline solvents used in the partition and extraction of estrogens. An effort was made, therefore, to determine whether or not these lubricants interfere with the quantitative determination of the estrogens. Aqueous alkaline solutions were passed through stop-cocks lubricated with Lubriseal, Trutest, and bentonite-glycerol mixture, respectively. The alkaline solutions were then acidified and extracted with ether. The residues after ether distillation were dissolved in 95 per cent alcohol and subjected to ultraviolet spectrophotometry. Benzene and ether were put through similarly lubricated stop-cocks, and evaporated to dryness subsequently. The residues were taken up in alcohol and then studied spectrophotometrically. The aqueous and organic solvents were used also in testing other substances which belong to classes of compounds that do not show significant absorption in the ultraviolet, but which do exhibit lubricating properties; *e.g.*, powdered agar, granular gelatin, and flaked graphite.

Results—Of the reagents commonly used in the extraction and partition of estrogens the following were found to be relatively free of interfering

optical density in the range covered by the ultraviolet absorption curve of the estrogens: alcoholic solutions of the residues of U. S. P. ether, freshly distilled from ferrous sulfate, Merck's redistilled thiophene-free benzene, carbon tetrachloride freed from carbon disulfide, U. S. P. ethyl alcohol, disodium acid phosphate, U. S. P. powdered agar, granular gelatin, flaked graphite, C.P. sulfuric acid.

The successful application of ultraviolet spectrophotometry to the assay of urinary estrogens necessitated substitutions for other reagents and substances exhibiting an optical density of more than 0.1 at 280 mμ. Agar was adopted instead of the commercial stop-cock lubricants ordinarily used, and H_2SO_4 was substituted for HCl. The use of filter paper was omitted entirely in view of its contribution to the optical density. Because the absorption curve of the carbonate radical was found to interfere with the measurement of estrogens at 280 mμ, carbonates were omitted from the procedure for the extraction and partition of estrogens whenever possible. It was for this reason that calcium hydroxide was not used in the removal of peroxides from ether, and that No. 10 mesh glass chips washed with sulfuric acid, water, and ether were substituted for porcelain boiling chips in the distillation of ether from extracts. Moreover, traces of moisture were removed from the residues of ether extracts exclusively by suction in order to avoid the use of anhydrous sodium sulfate, which may contain detectable amounts of carbonate.

Substitution of Dibasic Sodium Acid Phosphate for Sodium Carbonate in Partition of Estriol from Estrone-Estradiol Fraction—The foregoing data, considered in conjunction with other findings which suggested that sodium carbonate may be inefficient in the separation of estriol from estrone and estradiol, posed the possibility of substituting sodium acid phosphate for sodium carbonate.

Samples of crystalline estrone, estradiol, and estriol were dissolved in 95 per cent ethyl alcohol to a concentration of 100 γ of estradiol per ml., 98 γ of estriol per ml., and 103.6 γ of estrone per ml. Since the immediate purpose of this experiment was to determine the benzene-sodium carbonate distribution of each estrogen, 5 ml. aliquots of each solution were dried and carried independently through the entire partition procedure. The residues were dissolved in 1.0 ml. of 95 per cent ethyl alcohol, and 100 ml. of thiophene-free benzene were added to each. The benzene solutions were extracted three times with 50 ml. volumes of 0.3 M Na_2CO_3. The benzene phase, containing the estrone-estradiol fraction, was taken to dryness with suction on a warm water bath, and the residue thereof was dissolved in 10 ml. of 95 per cent ethyl alcohol. The aqueous alkaline phase, containing the estriol, was acidified with 6 N H_2SO_4 to Congo red and extracted with four 50 ml. volumes of ethyl ether, which was distilled off subsequently.

The residue of the ether extract was dissolved in 5 ml. of 95 per cent ethyl alcohol. Spectrophotometric curves were prepared of the alcoholic solutions of these organic and inorganic phases.

The foregoing experiment was repeated with 0.075 and 0.2 M Na_2HPO_4 in place of 0.3 M Na_2CO_3.

TABLE VI

Distribution of Estrone, Estradiol, and Estriol between Benzene and Alkali

Experiment No.	Estrone recovery		Estradiol recovery		Estriol recovery	
	Benzene phase	Alkaline phase	Benzene phase	Alkaline phase	Benzene phase	Alkaline phase
	0.3 M Na_2CO_3					
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	90.8		66.7	31.7	2.1	
2	92.1		68.6	33.9	0	105.6*
3	91.4	2.4	68.5	33.6	0.6	98.9
4	94.9	5.1	70.2	35.7	0	101.7
5	94.7	3.4	64.5	33.3	0	96.0
6	91.5	3.0				
	0.075 M and 0.2 M Na_2HPO_4†					
1	102.4	0	100.0	0	8.2	90.3
2	98.7	0	102.0	0	4.0	94.8
3	100.4	0	102.0	0	8.6	90.8
4					6.1	91.9
5	101.2	0.6	102.1	0.8	2.7	100.4

* The range of these values is not an indication of the accuracy of this method of assay. At this point in the partition the reagent control shows a relatively low density reading at 280 mμ, which interferes with the exact quantitative estimation of the estrogen content of these samples. This is of no consequence, inasmuch as the estrogen content of the sample is not tested for until the end of the procedure, when the density of the reagent control is at an optimum level for exact quantitative work. The data in this table are of value, nevertheless, since they disclose large enough deviations to merit attention.

† The difference in pH between 0.075 M and 0.2 M Na_2HPO_4 is negligible. The values lie between pH 8.7 and 8.8.

Results—The results of the experiments with 0.3 M Na_2CO_3 are recorded in Table VI. These data indicate that 0.3 M Na_2CO_3 is not a satisfactory substance for the partition of estrogens, previous statements in the literature to the contrary. Approximately one-third of the estradiol is carried over into the alkaline phase with the estriol. This results in a large error in the determination of the estradiol as well as of the estriol.

Reference to Table VI discloses, on the other hand, that 0.075 and 0.2 M

Na_2HPO_4 achieve an acceptable quantitative separation of estriol from the estrone-estradiol fraction. Since estradiol is not carried into the aqueous alkaline phase, its quantitative recovery is not interfered with. Furthermore, estradiol no longer masks the extent to which estriol is soluble in the alkaline solution. The small fraction of estriol, which remains in the benzene phase, does not affect the final recovery values for either estrone or estradiol, because it is removed later by the discarded Na_2CO_3 washings during the Girard separation.

It appears, therefore, that the method in common use for the separation of estriol from the estrone-estradiol fraction is grossly inaccurate. This situation may have been overlooked, because the bioassay method used currently for the measurement of these substances is not quantitative as compared with the micro precision of ultraviolet spectrophotometry.

In view of the foregoing, it seemed essential to check up on the accuracy of the remainder of the method employed in the separation of the estrogens from other urinary materials and from one another.

Separation of Phenolic Estrogens from So Called Neutral Steroid Fraction

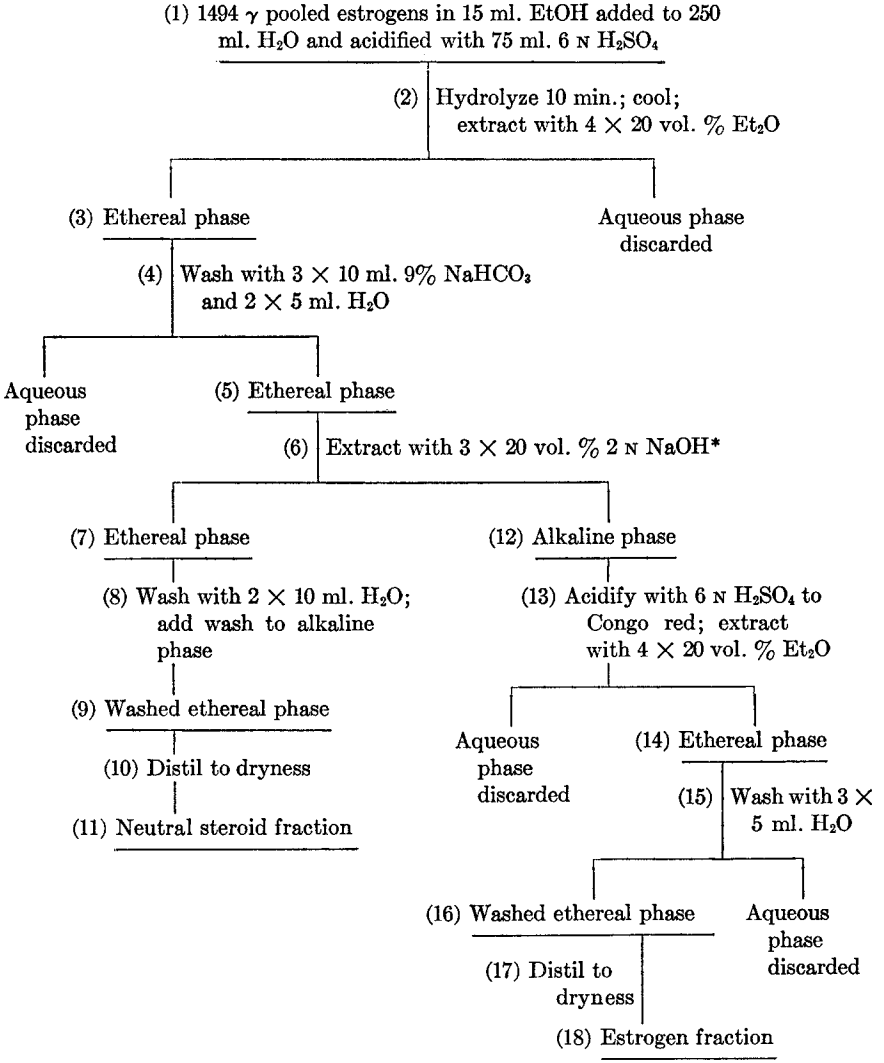
In general, estrogens have been separated from androgens by one of two types of method; *viz.*, simultaneous extraction of the three urinary estrogens from an ether or toluene extract of hydrolyzed urine by a given concentration of KOH or NaOH (1, 16–22), or differential extraction of the estrogens by two concentrations of alkali (10, 11, 16, 23). The latter method, or its modification, involves the extraction of estriol by 0.1 N NaOH from ether, and the removal from toluene of the estrone-estradiol fraction by N NaOH. The latter leaves the neutral steroid fraction in the toluene.

Since toluene is known to exhibit interfering absorption over that part of the ultraviolet spectrum which is occupied by the estrogen curves (13), it was inadvisable to use the ether-toluene method for the separation of estrogens from androgens. Consequently, we studied the application of ultraviolet spectrophotometry to the other type of method, which involved the removal of estrogens by NaOH from an ether extract of hydrolyzed urine.

Experimental Procedure—In order to duplicate as closely as possible the steps (Table VII) that are involved in the extraction of estrogens from urine samples, an aqueous alcoholic solution of estrone, estradiol, and estriol was prepared and subjected to hydrolysis for 10 minutes with 30 volumes per cent of 6 N H_2SO_4 (equivalent to 15 volumes per cent of concentrated HCl). The hydrolysate was cooled rapidly and extracted four times with 20 volumes per cent of ethyl ether. The ethereal extract was washed with three 10 ml. volumes of 9 per cent $NaHCO_3$ solution to remove acids, and the washings were discarded. The ether phase was then extracted three

TABLE VII

Procedure for Separation of Estrogens from So Called Neutral Steroid Fraction



* Nineteen experiments were done in all. Three extractions with alkali were used in the first two experiments, six extractions with alkali were carried out in the next twelve experiments, and four extractions were done in the last five experiments. Concentrations of NaOH or KOH varied from 1.5 N to 6 N (see Table VIII).

times with 20 volumes per cent of 2 or 2.5 N NaOH solution. The ether phase was washed twice with 10 ml. volumes of distilled water, and the washings were added to the alkaline phase. The ethereal solution was distilled to dryness, taken up in 4 ml. of 95 per cent ethyl alcohol, and the ultraviolet absorption curve of this solution was determined. The alkaline phase was acidified with 6 N H_2SO_4 to Congo red and extracted four times with 20 volumes per cent of ethyl ether. This ethereal extract was washed three times with 5 ml. volumes of distilled water, and the ether removed by distillation. The residue thereof was dissolved in 4 ml. of 95 per cent ethyl alcohol and the ultraviolet absorption curve of this solution was prepared.

Results—The results recorded in Table VIII indicate that a significant proportion of the estrogenic hormones, amounting approximately to 26 per cent of the total estrogen content of the initial sample, is left behind in the so called neutral steroid fraction by 2 N or 2.5 N NaOH.

In order to determine which of the three estrogens, or what combination of the three, remained in the organic phase, each of them was subjected to extraction from ether by a variety of concentrations of NaOH ranging from 1.5 to 6 N.

The results of these studies showed that estriol is extracted from ether quantitatively by 1.5 to 2.5 N NaOH. It was apparent, therefore, that the 26 per cent of estrogen lost in the androgen fraction must have consisted either of estrone or estradiol, or both. Because estrone is relatively less soluble in alkali than estriol, other experiments were done in which estrone was extracted from an ethereal solution by 3 and 6 N NaOH. Estradiol, which has an intermediate solubility in alkali, was extracted from an ethereal solution by 3 N NaOH.

The results of this investigation showed that 46 per cent of the estrone and 46 per cent of the estradiol were left in the androgen fraction by the 3 N NaOH, and 81 per cent of the estrone was unextracted by the 6 N NaOH.

One must conclude, therefore, that estrone and estradiol cannot be removed quantitatively from an ethereal extract by four to six extractions with NaOH solutions ranging in concentration from 2 to 6 N. Although it is possible to effect a quantitative separation under these experimental conditions by a considerably greater number of extractions, the latter would render the procedure impractical. Confronted with this impasse, we sought other means to achieve a quantitative removal of estrone and estradiol from an ethereal extract.

Further Experimental Procedures—The quantitative removal of estrone and estradiol from an ethereal extract was accomplished through a reduction in the solubility of these estrogens in the organic phase by the addition to the ether of carbon tetrachloride, in which estrogens are considerably less

soluble (24). Thus the distribution of estrogens between the organic and inorganic phases was altered in favor of the latter.

By trial and error it was determined that a 1:18 ratio of ether to carbon tetrachloride results in a quantitative removal of estriol, estrone, and estradiol by N KOH. KOH was substituted for NaOH because KOH exhibits a slightly greater alkalinity for the same normality. This is de-

TABLE VIII

Separation of Estrogen Fraction from So Called Neutral Steroid Fraction

Experiment No.	Estrogen	Organic solvent	Alkali	No. of extractions	Alkali	Estrogen in total neutral steroid fraction	Estrogen in estrogen fraction
					vol. per cent	*per cent*	*per cent*
1	Pool*	Et_2O	NaOH, 2 N	3	20	37	
2	"	"	" 2 "	3	20	26	67†
3	"	"	" 2.5 "	6	15	27	76†
4	Estriol	"	" 1.5 "	6	15	0	94‡
5	"	"	" 2.5 "	6	15	0	99‡
6	Estrone	"	" 3 "	6	15	46	51‡
7	"	"	" 6 "	6	15	81	16‡
8	Estradiol	"	" 3 "	6	15	46	55‡
9	Estrone	Et_2O-CCl_4, 1:1	" 2.5 "	6	15	41	65‡
10	"	" " 1:1	" 2 "	6	15	28	
11	"	" " 1:1	KOH, 2 "	6	15	22	
12	"	" " 1:1	" 1 "	6	15	17	
13	"	" " 1:1	" 0.5 "	6	15	28	
14	"	" " 1:9	" 1 "	6	15	0	
15	"	" " 1:9	" 1 "	4	15	3.8	96‡
16	Estradiol	" 1:9	" 1 "	4	15	0	96‡
17	Estriol	" 1:9	" 1 "	4	15	0	91‡
18	Pool*	" 1:18	" 1 "	4	50	0.8	
19–25	"	" 1:18	" 1 "	4	50	0	

* Estrone, estradiol, and estriol in approximately equal amounts.

† Aliquot of ether extract of acidified alkaline phase.

‡ Total estrogen fraction.

sirable, inasmuch as the salt content of the final residues should be kept as low as possible in spectrophotometric work.

It remained to find out whether or not the androgens are carried over into the estrogen fraction, and vice versa, under the foregoing experimental conditions. Accordingly, three groups of experiments were set up in which single specimens of androgens, pools of androgens, and pools of androgens plus estrogens were submitted to the separation procedure.

In Experiment 1, an alcoholic solution containing 4.98 mg. of androsterone was dried by suction in a separatory funnel. The residue thereof was dissolved in ether and carbon tetrachloride, and extracted four times with N KOH. The latter pooled aqueous extract was washed two times with carbon tetrachloride. This wash was treated as the original organic phase and kept separate from it. (Subsequent trials have shown that such a wash does not carry estrogens along with it.) The aqueous fraction, ordinarily containing the estrogens, was then acidified and extracted with ether. The ethereal extract was washed with sodium bicarbonate solution and with water and distilled to dryness by steam. The final residue was dissolved in alcohol, and the solution examined spectrophotometrically. The organic fraction containing the androgens was washed with water (the wash being added to the original aqueous phase) and dried by suction. The residue was dissolved in alcohol in preparation for spectrophotometry. A 5.15 mg. sample of isoandrosterone was treated in an identical manner.

In Experiment 2, a pooled sample of 19.92 mg. of androsterone and 2.2 mg. of dehydroisoandrosterone was subjected to the procedure outlined for Experiment 1. An identical sample was treated likewise, except that it was hydrolyzed for 30 minutes instead of 10 minutes.

In Experiment 3, two identical aliquots of a pooled sample of estrogens and androgens were subjected to the procedure employed in Experiment 1, except for the wash of the aqueous phase which was omitted. Each ml. of this pool contained 10.04 mg. of androsterone, 1.32 mg. of dehydroisoandrosterone, 29.4 γ of estrone, 30.0 γ of estradiol, and 29.7 γ of estriol.

Results—The results in Table IX indicate that the foregoing procedure separates androgens from estrogens quantitatively.

Separation of Urinary Estrogens from Other Urinary Phenolic Substances by Steam Distillation

The foregoing studies with crystalline estrogens have had as their eventual goal the application of the spectrophotometric method to the assay of the estrogens in urine. In this connection, one should recall that the ultraviolet absorption curves characteristic of the estrogens are simulated by phenolic substances, including the urinary phenols, in that they show pronounced absorption over the same range of the spectrum with maximal density in or about the region of 280 mμ (3, 4, 20). It follows, therefore, that the estrogens must be separated quantitatively from the other urinary phenolic substances before they can be determined quantitatively by the ultraviolet spectrophotometric method.

In accordance with equations formulated by Naumann (25) and by Virtanen and Pulkki (25), it can be predicted that steam distillation can be used to effect this separation.

Because of the relative insolubility in water and the low vapor pressure of the estrogenic phenols, they are practically non-steam-volatile. This may be contrasted with the ready solubility and relatively higher vapor pressure of the other urinary non-estrogenic phenols, such as phenol and *p*-cresol, which consequently are steam-volatile. In accord with these theoretical considerations, Dobriner, Lavin, and Rhoads (20) found that estriol cannot be detected spectroscopically in the *steam-volatile* fraction after steam distil-

TABLE IX

Separation of Crystalline Androgens from Crystalline Estrogens

Experiment No.	Hydrolysis	Steroids recovered	
		Estrogen fraction	Androgen fraction
		per cent	*per cent*
1. Androsterone, 4.98 mg.	None	0	
Isoandrosterone, 5.15 mg.	"	0	112*
2. Each ml. of pooled sample contained androsterone 0.905 mg., dehydroisoandrosterone 0.100 mg.			
Aliquot 1	10 min.	0	106*
" 2	30 "	0	113*
3. Each ml. of pooled sample contained androsterone 10.04 mg., dehydroisoandrosterone 1.32 mg., estrone 29.4 γ, estradiol 30.0 γ, estriol 29.7 γ			
Aliquot 1	None	107*	109*
" 2	"	104*	112*

* These spectrophotometric curves disclose small traces of a residue of carbon tetrachloride, which is difficult to get rid of at this point, but which is apparently removed later on in the procedure. This may account for the experimental error represented by the difference between 100 per cent and these values. The latter cannot be attributed to the inefficient separation of estrogens and androgens, inasmuch as there is no indication of the presence of a steroid in the contour of the curve representing the difference between the reagent control and the recovered androgens or estrogens.

lation, whereas the absorption bands of phenols, such as phenol and the cresols, are absent from the *non-steam-volatile* fraction after steam distillation. They did not attempt a quantitative recovery of the estriol, which may be a necessary step, inasmuch as the steam distillation took place in an acid medium in which destruction of estrogens might have occurred.

The present report deals with two aspects of this problem: (1) to determine the effect of steam distillation on the quantitative recovery of crystalline estrone, estradiol, and estriol; (2) to ascertain whether or not

the steam-volatile phenols of hydrolyzed male urine can be separated completely from the non-steam-volatile fraction by steam distillation.

Experiment 1—Approximately equal amounts of chemically pure crystalline estrone, estradiol, and estriol were dissolved in 95 per cent ethyl alcohol to make a solution containing 99.6 γ of pooled estrogens per ml. 15 ml. of this solution were placed in a small boiling flask and dried by suction. The residue was dissolved in 0.5 ml. of 95 per cent ethyl alcohol, diluted with 5 ml. of distilled water, and steam-distilled in an all-glass still to 500 ml. of distillate. The steam distillation having been completed, the aqueous solution remaining in the flask was dried by suction, and the residues dissolved in 15 ml. of 95 per cent ethyl alcohol. An absorption curve was determined on this solution.

Results—Steam distillation permitted the quantitative recovery of the three estrogens with which the experiment was started. Comparison of the absorption characteristics of the pooled sample, which had been subjected to steam distillation, with that of an identical untreated aliquot disclosed a difference of only 0.6 per cent when measured at 280 mμ.

Experiment 2—1 liter of male urine was hydrolyzed with 15 volumes per cent of concentrated hydrochloric acid for 10 minutes and extracted with 20 volumes per cent of ethyl ether (24). The ether extract was washed with 9 per cent sodium bicarbonate solution, and the androgenic fraction separated from the estrogenic fraction by N KOH. The alkaline estrogenic fraction was acidified and extracted with ethyl ether. The ether was distilled off, and the residue dissolved in 50 ml. of 95 per cent ethyl alcohol. The equal aliquots of this solution were diluted 10-fold in order to reduce the optical density of the latter to a readable level. This dilution factor served also to eliminate from spectrophotometric consideration an already relatively low content of estrogen, so that this extract contained essentially only non-estrogenic urinary phenols. One aliquot was steam-distilled by the method described for Experiment 1. Both aliquots were tested spectrophotometrically.

Results—In accordance with the observations of other investigators (3 4, 20), we have found that urinary phenols, other than the estrogens, are characterized by ultraviolet absorption curves, which resemble closely those of estrone, estriol, and estradiol. Curve A, Fig. 5, represents the ultraviolet absorption of the total phenolic fraction of a 1000 ml. sample of male urine. The shape of this curve, and the fact that it exhibits minimal density at 250 mμ and maximal density at 280 mμ, endow it with the general and specific absorption characteristics of the urinary phenols, which include the estrogens. Curve B represents the absorption of this urinary extract after steam distillation. It is clear from the shape of Curve B that the substances which produced the typical phenolic absorption are steam-vola-

tile. This was verified by calculating the difference between Curves A and B, the results of which are plotted in Curve C. Curve C is typically phenolic. Although Curve B, which represents the non-steam-volatile fraction, does not disclose the presence of phenolic substances, this does not constitute proof of their absence, since the ratio of the density of the urinary background material to that of the estrogen content determines whether the typical estrogen absorption curve is detectable. It should be noted that the foregoing experiment was not meant to be quantitative. It

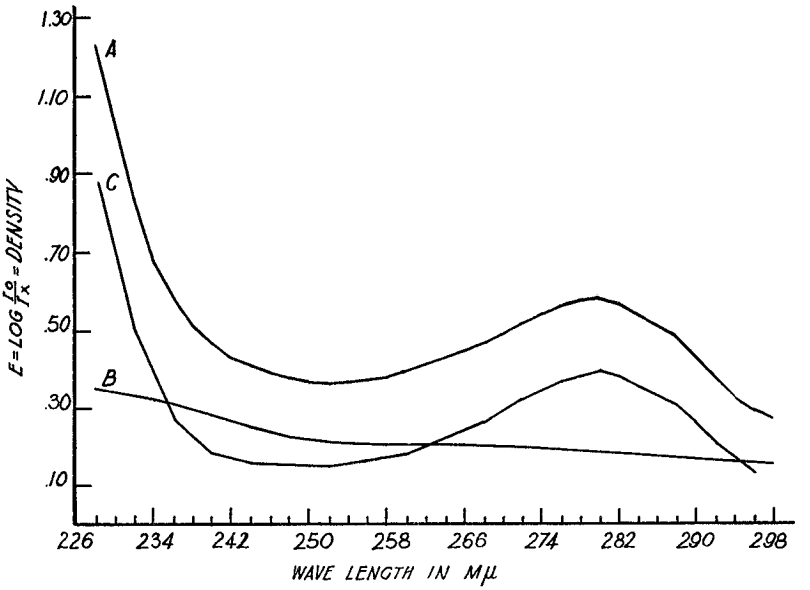


FIG. 5. Steam distillation of extract of male urine. Curve A, total phenolic fraction; Curve B, non-steam-volatile fraction; Curve C, steam-volatile fraction (Curve A minus Curve B).

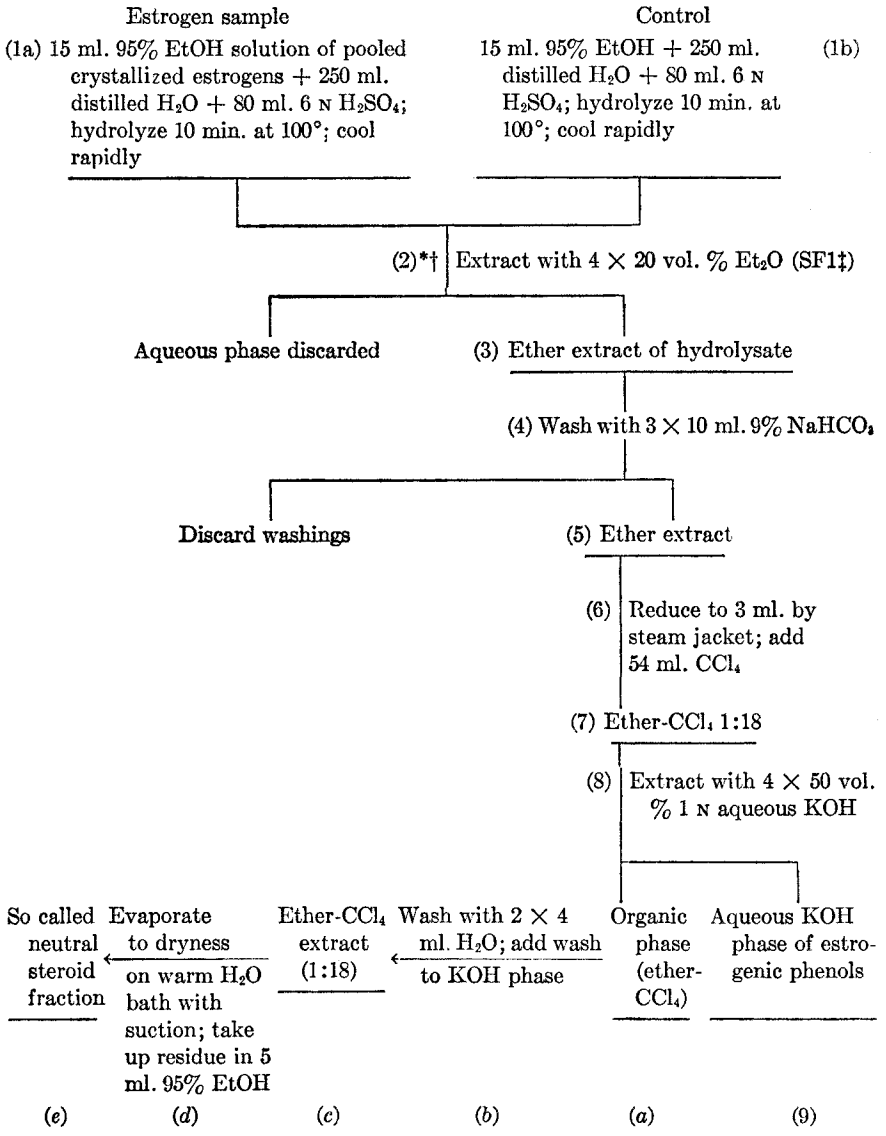
was devised only for the purpose of demonstrating what type of absorption curve is characteristic of the steam-volatile urinary phenols.

Micro-Girard Separation of Estrone from Estradiol

Having developed a satisfactory quantitative method for the separation of estrogens from androgens, and estriol from the estrone-estradiol fraction, it remained for us to check the accuracy of the micro-Girard method, which has to do with the separation of estrone from estradiol. Sulfuric acid was not substituted for hydrochloric acid, as recommended in other parts of the extraction and partition procedure, because its use in the Girard reaction is disadvantageous. During this reaction sulfuric acid causes the formation

TABLE X

Extraction and Separation of Estrogens from Androgens



* Quantitative check by ultraviolet curve on aliquot of ether extract.

† Quantitative check by ultraviolet curve of fifth ether extract (for completeness of extraction).

‡ SF1 represents extraction in a 500 ml. separatory funnel.

of more interfering background material than is encountered with the use of hydrochloric acid.

TABLE XI

Separation of Non-Steam-Volatile Estrogenic Phenols from Steam-Volatile Substances

(9) Aqueous KOH extract of estrogenic phenols

(10)* † Acidify with 6 N H_2SO_4 to Congo red; extract with 4 × 50 vol. % Et_2O (SF1‡)

(11) Ether phase — Discard aqueous phase

(12) Wash with 1 × 5 ml. distilled H_2O

Wash discarded — (13) Total phenols in Et_2O

(14) Distilled to dryness by steam jacket; taken up in 5 ml. 95% EtOH; add 20 ml. distilled H_2O

(15) Total phenols in aqueous EtOH

(16) Steam distil to 500 ml. of distillate, maintaining volume in funnel at 20 ml.; dry by suction with aid of warm bath

(17) Residue of non-steam-volatile phenolic estrogens estrone, estradiol, estriol

(18) Steam-volatile substances

(19) Dissolve residue in 1 ml. 95% EtOH; add 100 ml. benzene

(20) Benzene solution of estrogens (SF1‡)

* Quantitative check by ultraviolet curve on aliquot of ether extract.

† Quantitative check by ultraviolet curve of fifth ether extract (for completeness of extraction).

‡ SF1 represents extraction in a 500 ml. separatory funnel.

Experimental Procedure—Exploratory experiments to determine the accuracy of the Pincus-Pearlman modification (26) of the micro-Girard

method were carried out on three samples of estrogens. A pooled sample of 500 γ each of estrone, estradiol, and estriol and a single sample of 500 γ of estradiol were subjected to separation at the point of the benzene-phosphate partition; the benzene fraction was carried through the micro-Girard pro-

TABLE XII

Separation of Estriol from Estrone-Estradiol Fraction

(20) Benzene solution of estrogens (SF1*)

(21) Extract with 3 × 50 ml. 0.075 M Na_2HPO_4

(22) Aqueous phase, "strong" phenol (estriol) (SF2*)

(23)† Acidify with 6 N H_2SO_4 to Congo red; extract with 4 × 75 ml. Et_2O

(24) Ether extract — Discard aqueous phase

(25) Wash with 2 × 5 ml. distilled H_2O

(26) Washed ether extract (SF2) — Discard washings

(27) Distil off Et_2O; dissolve residue in 5 ml. 95% EtOH

(28) Estriol fraction; read ultraviolet curve

(29) Benzene phase, "weak" phenols (estrone, estradiol)

(30) Reduce volume in SF1 to 25 ml. by suction with aid of warm H_2O bath; transfer directly to 250 ml. funnel (SF3) and dry benzene; rinse SF1 into SF3 by 3 × 5 ml. 95% EtOH; dry EtOH

(31) Estrogen residue (SF3‡)

(32) SF3 with residue over P_2O_5 in vacuum desiccator overnight

(33) Dry residue of estrone-estradiol fraction for micro-Girard (SF3)

* SF1 and SF2 represent two 500 ml. separatory funnels.

† Quantitative check by ultraviolet curve of fifth ether extract (for completeness of extraction).

‡ SF3 represents extraction in a 250 ml. separatory funnel.

cedure subsequently. A third pooled sample containing 500 γ each of estrone and estradiol was subjected to the micro-Girard procedure only. The concentration of estrogen in each instance was about 100 γ per ml.

Results—The results of these experiments were as follows:

92 per cent of the estriol from the pooled sample of three estrogens was recovered from the phosphate phase. Of the estrogens in the benzene

TABLE XIII

Modified Micro-Girard Separation of Estrone from Estradiol

(33) Dry residue of estrone-estradiol fraction (SF3*)

(34) Add 2 ml. glacial acetic acid, then 400 mg. Girard's Reagent T. Stopper funnel with adapter fitted with $CaCl_2$ tube; heat on H_2O or glycerol bath at 90–100° for 20 min.; cool in desiccator; add 60 ml. ice-cold distilled H_2O, then 14 ml. chilled 10% NaOH; mix; extract with 4 × 50 ml. Et_2O†

(35) Ether phase, estradiol fraction (SF3)

(36) Wash with 1 × 20 ml. ice-cold distilled H_2O; add wash to aqueous phase

(37) Washed ether extract

(38) Wash with 1 × 20 ml. 2.5% Na_2CO_3 and 3 × 10 ml. H_2O

(39) Ether extract (SF3)

Aqueous phase discarded

(40) Distil to dryness; dissolve residue in 5 ml. 95% EtOH

(41) Alcoholic solution of estradiol

(Read ultraviolet curve)

(42) Aqueous alkaline phase, estrone fraction

(43) Acidify with 12 ml. concentrated HCl; keep at room temperature for 2 hrs.; extract with 3 × 50 ml. Et_2O in SF4*†

Aqueous phase discarded

(44) Ether extract

(45) Wash with 1 × 20 ml. 2.5% Na_2CO_3 and 3 × 10 ml. distilled H_2O

(46) Ether extract (SF4)

Aqueous phase discarded

(47) Distil to dryness; dissolve residue in 5 ml. 95% EtOH

(48) Alcoholic solution of estrone

(Read ultraviolet curve)

* SF3 and SF4 represent extraction in a 250 ml. separatory funnel.

† Quantitative check by ultraviolet curve of fifth ether extract (for completeness of extraction).

phase, 100.4 per cent of the estrone and 103 per cent of the estradiol were recovered after the Girard separation.

97 per cent of the estradiol and 102 per cent of the estrone were recovered

from the pool of these two estrogens after they had been subjected to the Girard procedure only.

TABLE XIV

Recovery of Crystalline Estrogens from Aqueous Alcoholic Solution by Proposed New Procedure

Experiment No.		Amount added	Remainder after removal of aliquots	Amount recovered	
		γ	γ	γ	*per cent*
Estrone	1	504	325	312	96.0
	2	539	464	381	82.0*
	3	539	477	444	93.0
	4	539	477	441	92.0
	5	535		490	91.5
	6	535		498	93.0
	7	535		535	99.9
	8	535		506	94.6
Estradiol	1	500	323	296	91.6
	2	513	441	358	81.0*
	3	513	454	436	96.0
	4	513	466	392	84.0*
	5	495		470	95.0
	6	495		479	96.7
	7	495		495	100.0
	8	495		490	99.0
Estriol†	1	540		491	91.0
	2	540		502	93.0
	3	540		509	98.0
	4	540		506	93.7
	5	540		517	95.8

* Although not a part of the methodology, it was deemed essential to check at strategic points the accuracy of the extraction and partition procedure by removing and assaying quantitatively aliquot portions of the estrogen pool. The sum total of the estrogens removed during these periodic checks was subtracted from the amount originally added, and the remainder was used as a basis for comparison. This added experimental maneuver probably accounts for the occasional low recovery values for estrone and estradiol.

† Prior to these experiments, six determinations were done in which the recovery of estriol varied from 82.9 to 89 per cent. Since higher recovery values were obtained consistently by increasing the number of extractions in the benzene-phosphate partition from two to three, and lower values were found consistently when the number of extractions was limited to two, it is assumed that the low values were due to incomplete extraction of estriol from the estrone-estradiol fraction.

Of the estradiol that was carried through the benzene-phosphate partition, none was found in the phosphate phase, in which only estriol is

extracted under these conditions. After the Girard treatment, 93 per cent of the estradiol was found in the non-ketonic fraction, in which it is to be expected, and 5 per cent was lost in the ketonic fraction where it would have interfered with the determination of the estrone ordinarily. This separation was carried out with three 50 ml. volumes of ether. Further investigation disclosed that a fourth ether extraction obviated this 5 per cent loss of estradiol. Several attempts to detect estradiol in a fifth ether extraction failed to disclose its presence, even with the sensitive spectrophotometric technique.

In accordance with our general policy of reducing the number of transfers of material from one piece of apparatus to another, all of the steps up to the actual separation of estrone from estradiol were conducted in a single 250 ml. separatory funnel, which was fitted with a straight adapter closed with a $CaCl_2$ tube. The funnel was heated in a horizontal position on a water bath at 90–100° for 20 minutes, during which time it was rotated through 180° every 5 minutes. The neck of the funnel was protected against condensation of steam by a cardboard guard. A glycerol bath was found to be more satisfactory than the water bath. Because of the size of the funnel, it was necessary to increase the volume of glacial acetic acid from 0.5 to 2 ml., with proportionate increases in the amounts of the other reagents in the Girard procedure.

Essentially New Method for Extraction and Partition of Crystalline Estrone, Estradiol, and Estriol, and Their Quantitative Assay by Ultraviolet Spectrophotometry

A quantitative ultraviolet spectrophotometric method for the assay of estrogens was developed on the basis of the data which have been recounted in detail throughout this communication. The new features of this method included the following: (*a*) separation of the androgens from the estrogens by equilibration between ether-carbon tetrachloride (1:18) and N KOH; (*b*) partition of the estrogens into strongly and weakly phenolic fractions by the use of benzene and dibasic sodium acid phosphate; (*c*) the use of specially designed apparatus which makes it possible to reduce to a minimum the number of transfers of extracts and residues;[1] (*d*) application of

[1] We are indebted to Dr. W. Dean Fraser for the design of a special adapter which was used to connect an all-glass steam distillation apparatus with a 500 ml. separatory funnel, thus obviating the necessity of transferring the residue to a boiling flask. Furthermore, a steam jacket, designed to be used on a 500 ml. separatory funnel, enabled us to reduce the volume of ether extracts directly from the funnel in which the extraction was made. Particular attention should be directed to the fact that the extraction and partition procedure for each estrogen was performed in a limited amount of glassware, which consisted of one boiling flask in which the initial hydrolysis was done, two 500 ml. separatory funnels, one 250 ml. separatory funnel, and four Erlenmeyer flasks.

the sensitive ultraviolet spectrophotometric method of assay for the quantitative determination of crystalline estrone, estradiol, and estriol; (*e*) adoption of agar as the lubricant for stop-cocks of the separatory funnels, because it does not act as an interfering substance from a spectrophotometric view-point.[2]

Integrated Experimental Evidence Bearing on Accuracy of New Method—Each of eight pooled samples of an aqueous alcoholic solution of crystalline estrone, estradiol, and estriol and three single samples of estriol were subjected to the entire procedure outlined in the flow sheets of Tables X to XIII. The pooled samples contained approximately 500 γ of each estrogen. The exact amounts may be ascertained by reference to the detailed data

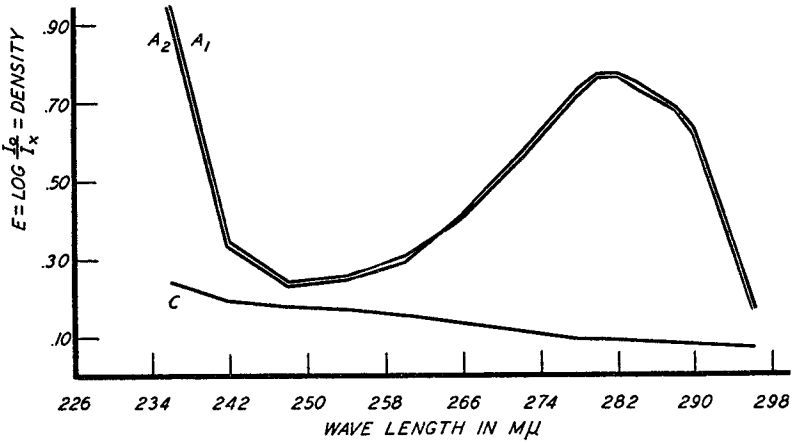


FIG. 6. Recovery of crystalline estradiol from pool of estrone, estradiol, and estriol. Curves A_1 and A_2, estradiol fractions from duplicate pooled samples; Curve C, reagent control subjected to the identical procedure.

(Table XIV). Reagent controls were set up for each of these experiments in order to correct for the background material. The density value of the curve of the reagent control was subtracted from that of the corresponding estrogen curve at 280 mμ.

Results—Analysis of the ultraviolet absorption curves of the final residues of each of the estrogens indicates that the results of these experiments

[2] The agar was used in the form of a thin, soft jelly made by adding 75 ml. of distilled water to 3 gm. of powdered agar. The mixture is heated in an oven at 110° for 30 minutes and then allowed to stand covered at room temperature for several hours. This substance remains soft and ready for use if it is packed into collapsible tubes. Agar has the additional advantage of being easily removed from stop-cocks with warm water. If it dries on the stop-cock when the funnel is in use, it may be softened by introducing a drop or two of water to the surface by capillary action at each end of the stop-cock.

are reproducible. Reference to Fig. 6 discloses the quantitative accuracy with which estradiol may be recovered from equal aliquots of a pooled sample of estrone, estriol, and estradiol. Identical experiments aimed at the quantitative recovery of estrone and estriol, respectively, yielded equally satisfactory results. The same high degree of accuracy, *i.e.* reproducibility, was achieved in experiments designed to study the optical density of the reagent controls for each of these estrogens. Fig. 7 illustrates this point for estradiol. Although the absorption curves of the reagent controls are of the same order of magnitude, it is advisable to run a reagent control on each determination.

The recovery of estrogens subjected to the foregoing procedure is quantitative, and the results are consistent. Estrone and estradiol were each

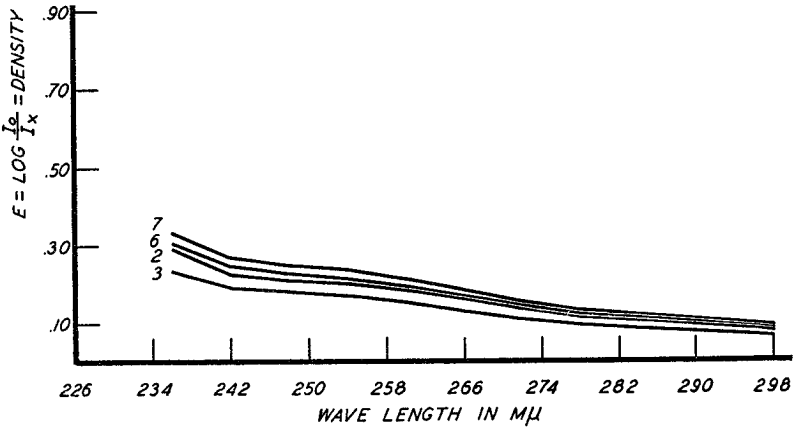


FIG. 7. Reagent controls subjected to the procedure for extraction and partition of estradiol (see Tables X to XIII); four different experiments.

recovered to the extent of 92 to 100 per cent, and estriol to the extent of 91 to 98 per cent.

SUMMARY

1. Critical analysis of the literature on data obtained by methods in current use for the partition and assay of urinary estrogens discloses that the quantitative accuracy claimed for them is not in accord with the results on which this contention is based.

2. Restudy of the crucial steps contributing to the inaccuracies of presently accepted methods led to the adoption of the following new features: (*a*) separation of the androgens from the estrogens by equilibration between ether-carbon tetrachloride (1:18) and N KOH; (*b*) partition of the estrogens into strongly and weakly phenolic fractions by the use of benzene

and dibasic sodium acid phosphate (0.3 M Na_2CO_3 is not an efficient solvent for this separation, because one-third of the estradiol is carried over into the estriol fraction); (*c*) the use of specially designed apparatus which makes it possible to reduce to a minimum the number of transfers of extracts and residues; (*d*) application of the sensitive ultraviolet spectrophotometric method of assay for the quantitative determination of crystalline estrone, estriol, and estradiol.

3. When subjected to the new procedure, crystalline estrogens were recovered quantitatively and the results were consistent.

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