SOME ASPECTS OF THE METABOLISM OF 16-C^{14}-ESTRONE IN NORMAL INDIVIDUALS *

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Until recently, the studies of the physiological disposition of estrogens have been somewhat unsatisfactory because of low recoveries of the administered steroids in urine and feces (1–6). Although recoveries were somewhat increased in investigations in which the steroids were tagged with a radioactive halogen (7, 8) or with deuterium (9), the availability of estrogens tagged with carbon 14 has made possible more precise studies of the physiological disposition of these compounds (10–13).

Beer and Gallagher (10) have reported that the excretion of radioactivity in the urine after injection of estrone-16-C^{14} in two patients was 77 and 58 per cent of the administered dose, while Sandberg and Slaunwhite (12) have reported in nine females an average excretion of 80 per cent of the administered dose, with a range of 41 to 90 per cent. In addition, Sandberg and Slaunwhite, investigating the clearance from plasma of free radioactivity and activity released by β-glucuronidase hydrolysis, noted that a significant amount of activity was associated with the red blood cells (12).

The present paper is concerned with some aspects of the metabolism of 16-C^{14}-estrone when administered to three normal adult subjects, two males and one female. Some of our data are only duplication and confirmation of work already reported in the literature (10–12). However, no study has yet been reported in normal males. Furthermore, in this study an attempt has been made to fractionate and determine the nature of the metabolites of estrone.

METHODS

Experimental subjects. The experimental subjects of this study were two normal males, A. D. and A. N., 32 and 30 years of age, respectively, and a normal 28 year old female (A. K.) who had a regular 29 to 30 day menstrual cycle. This latter subject received the radioactive steroid 11 days after onset of the last menses.

Purity of 16-C^{14}-estrone. The 16-C^{14}-estrone was provided by Charles E. Frosst and Co., Montreal, Canada. Its specific activity was 0.729 mc. per mM. The purity of this material was tested in our laboratory by paper chromatography, using the system of solvents isooctane : toluene : methanol : water (25 : 75 : 80 : 20) of De Courcy (14), and a modified Bush system (15) benzene : skellysolve C® : methanol : water (40 : 60 : 70 : 30). An aliquot of the radioactive steroid was chromatographed and the area of the paper strip corresponding to estrone was eluted; 94 to 98 per cent of the radioactivity of the aliquot was found in the eluate.

Mode of administration of 16-C^{14}-estrone. The steroid was dissolved in redistilled ethanol. An aliquot of this solution was assayed for radioactivity and found to contain 654,000 cpm per ml.

For human administration, a 2 ml. aliquot of the solution was pipetted carefully and diluted with 18 ml. of sterile isotonic saline solution. The methods of intravenous injection and calculation of the dose actually administered were similar to those described for 4-C^{14}-cortisol (16).

The injections were given between 9:30 and 10:30 a.m. over a three minute period.

Mode of collection of samples of blood, urine and feces. Samples of 40 to 50 ml. of blood were collected in heparinized syringes 15, 30, 60, 120, 240 and 360 minutes after the injection of the radioactive material. The blood samples were immediately centrifuged and the plasma separated from the red cells. The red cells were washed twice with twice their volume of isotonic saline solution. It must be noted that the white blood cells were not separated from the red cells.

Urine samples were collected 4, 8, 12, 24 hours following the injection and on the six subsequent days. Feces were collected for 5 to 6 days. All samples were stored in the deep freeze until the time of extraction or counting.

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Method of extraction of the various specimens. Each plasma sample was extracted six times with twice its volume of redistilled chloroform [1] in order to obtain the free steroid fraction. One hundred ml. of absolute ethanol was then added to the plasma residue; after shaking and centrifuging, the liquid phase was decanted and the remainder was washed twice with 50 ml. of ethanol; the pooled extracts were then evaporated to dryness. The ethanol residue was dissolved in 15 ml. of distilled water and incubated with β-glucuronidase [2] (approximately 500 units per ml. of plasma) at pH 4.7 ± 0.1 (acetate buffer), at 37° C. for 48 hours. Extraction was performed with chloroform (30 ml. × 6) to obtain the steroids which had been conjugated with glucuronic acid. The aqueous residue was then subjected to a 48 hour continuous extraction with ether at pH 0.8 to extract the easily hydrolyzable conjugate fraction. Following this procedure, the aqueous residue was boiled for 30 minutes with one-tenth its volume of concentrated HCl and extracted with chloroform to obtain steroids present in plasma as conjugates not hydrolyzed by the preceding procedures.

The red blood cells were placed overnight in the deep-freeze and then extracted with absolute ethanol (100 ml. once and 50 ml. twice). The pooled extracts were then evaporated to dryness. The dry residue was redissolved in 5 to 10 ml. of ethanol and then filtered. This filtrate which represented the total red cell extract was evaporated to dryness. The residue, dissolved in 10 ml. of distilled water, was extracted with chloroform (20 ml. × 6) to obtain the free fraction of the red cell extract. The aqueous residue was then incubated with β-glucuronidase and extracted with chloroform (30 ml. × 6); this constituted the glucuronoside fraction [3] of the red cell extract.

A 25 ml. aliquot of each urine sample was extracted six times with twice its volume of chloroform to obtain the free steroids. The residue was subjected to β-glucuronidase hydrolysis, followed by a 48 hour continuous extraction with ether at pH 0.8, and subsequent hydrolysis with HCl.

Each feces collection was extracted five times with 95 per cent ethanol (3,000 ml.) using a Waring blendor for mixing. The ethanol extracts were separated from feces by centrifugation and filtration. The ethanol was evaporated to dryness and the residue was dissolved in 100 ml. water. This aqueous fraction was extracted five times with twice its volume of chloroform to obtain the free steroids and then β-glucuronidase hydrolysis was carried out.

Radioactivity determinations. All extracts of plasma, red blood cells, urine and feces, as well as whole urine, were assayed for radioactivity in the manner previously described (16).

Benzene-water fractionations. Some of the plasma and urine extracts were evaporated to dryness. The respective residues were dissolved in 15 ml. of redistilled benzene and the benzene was extracted three times with its volume of distilled water.[4]

Paper chromatography. Some of the residues resulting from a benzene: water fractionation were chromatographed on paper using the systems of solvents isooctane: toluene: methanol: water (25: 75: 80: 20) for the benzene fraction, and benzene: methanol: water (100: 55: 45) for the water fraction. The R_f values of various estrogen standards [5] when using the two systems described above and a third system of solvents, benzene: skellysolve C®: methanol: water (40: 60: 70: 30), are shown in Table I.

The estrogens on paper chromatograms were visualized either by coupling with diazotized sulfanilic acid as described by Berry, Sutton, Cain and Berry (17) or by the method of Mitchell (18).

Radioautography. Some of the paper chromatograms were radioautographed using Kodak Medical X-Ray films (tinted, duplitized, no screen). On most occasions the time of exposure was three weeks. Following this procedure the areas of the papergram corresponding to the darkened zones of the radioautogram were excised and the pieces obtained were placed in an Erlenmeyer flask. After the addition of absolute ethanol (10 to 15 ml.) the flask was agitated by means of an automatic shaker for 30 minutes. The procedure was repeated twice more using ethanol and finally once using chloroform. The pooled eluates were filtered, evaporated to dryness and the radioactivity of the residue was determined.

RESULTS

1. *Plasma studies*

The amount of radioactivity readily extractable from plasma by chloroform and that obtained fol-

[1] When using this method of extraction, pure estrone and estradiol-17β added to water were quantitatively recovered while 90 to 94 per cent of the added estriol was recovered.

[2] Ketodase, a beef liver preparation, from Warner-Chilcott Laboratories was used in the present study.

[3] The correct term to designate this type of steroid conjugate is "steroid glucusiduronic acid." However, the colloquial terms "glucuronide" and "glucuronoside" are widely used. In the present paper, "glucuronoside fraction" is used to designate the group of steroids which are liberated by β-glucuronidase hydrolysis.

[4] When pure estrone, estradiol-17β, 16-epiestriol and estriol were submitted to such a fractionation, it was found that an average of 98.5 per cent of the estrone, 95 per cent of the estradiol-17β and 90.4 per cent of the 16-epiestriol could be recovered in the benzene fraction, while 88.5 per cent of the estriol was in the water fraction.

[5] The authors are very grateful to Dr. Max N. Huffman, Professor of Research Biochemistry, Oklahoma Medical Research Institute, Oklahoma City, who kindly provided the following steroid standards: 1,3,5(10),16-estratetraen-3-ol, estrone-16, estradiol-16β, estradiol-16, 16-ketoestrone, 16-ketoestradiol-17β and 16α-epiestriol. Thanks are also due to the Parke, Davis and Company who provided us with estrone and estradiol-17β standards.

TABLE I

R_f values of several estrogens for three different systems of solvents

Estrogens	Systems of solvents		
	Isooctane:toluene: methanol:water (25:75:80:20)	Benzene:Skelly C®: methanol:water (40:60:70:30)	Benzene: methanol:water (100:55:45)
Estriol	0.025	0	0.090
16-Epiestriol	0.156	0.018	0.438
16-Ketoestradiol	0.311	0.084	0.640
16-Ketoestrone	0.488	0.167	0.752
Estradiol-16α	0.494	0.215	0.786
Estradiol-17β	0.536	0.279	0.786
Estradiol-16β	0.567	0.265	0.808
Estrone	0.780	0.648	0.878
Estrone-16	0.802	0.660	0.902
1,3,5(10),16-estratetraene-3-ol	0.890	0.920	0.922

lowing the various hydrolysis procedures are shown in Table II. The results are expressed as percentage of the administered dose per 1,000 ml. of plasma.

Fifteen minutes after the injection of 16-C^{14}-estrone, only 2.76 to 3.79 per cent of the dose was present as free steroid in the total plasma volume (estimated as 5 per cent of body weight). The amount of radioactivity in the glucuronoside fraction from plasma was maximal 60 to 120 minutes after the injection and did not significantly change during the next few hours. The fractions obtained from continuous ether extraction at pH 0.8 and strong acid hydrolysis contained a small, but statistically significant, amount of radioactivity. The trend of these values was somewhat similar to that observed for the values of the glucuronoside fraction (Table II). These data differ from those reported by Sandberg and Slaunwhite (12) only in respect to the elevation in the glucuronoside fraction which in the present study appeared more sustained.

The activity in the water extract following benzene: water partition of the free and glucuronoside fractions from plasma of Subjects A. D. and A. K. was found to represent only a small percentage of the total activity (free fraction: 3.1 to 4.2 per cent for Subject A. D., 6.2 to 7.3 per cent for Subject A. K.; glucuronoside fractions: 1.4 to 7 per cent for A. D., 4.8 to 9.3 per cent for A. K.).

TABLE II

Radioactivity in the plasma and washed red blood cells of three normal subjects following 16-C^{14}-estrone administration (expressed as percentage of the administered dose per liter of plasma)

Subjects	Min. after inject.	Per cent dose/1,000 ml. Plasma Free	Plasma Gluc.	Plasma Continuous ether ext. pH 0.8	Plasma Strong acid hydro.	Red cells Total	Red cells Free	Red cells Gluc.
A. D.	15	0.79	0.28	0.02	0.00	0.50	0.12	0.03
	30	0.51	0.71	0.04	0.01			
32 yrs.	60	0.40	0.78	0.03	0.02			
	120	0.19	0.90	0.06	0.12	0.28	0.06	0.00
70 Kg. ♂	240	0.15	0.86	0.06	0.09			
A. K.	15	1.35	0.30	0.07	0.05			
	30	0.81	0.49	0.04	0.04	0.67	0.12	0.08
28 yrs.	60	0.53	0.80	0.06				
	120	0.14	1.25	0.10	0.08			
56 Kg. ♀	240	0.08	0.86	0.11	0.13	0.31	0.03	0.02
	360	0.04	0.77	0.05	0.16			
A. N.	15	0.72	0.50	0.08	0.03	0.75	0.17	0.05
	30	0.52	0.66	0.07	0.04			
30 yrs.	60	0.30	0.72	0.07	0.04	0.46	0.05	0.01
	120	0.12		0.14	0.08			
78 Kg. ♂	240	0.10	0.60	0.24	0.12			
	360	0.06	0.92	0.08	0.09			

TABLE III

Distribution between plasma and red cells of the radioactivity (total, free, glucuronoside) of 1,000 ml. of whole blood

Subject	Min. after injection	Hematocrit (av.)	% Dose in plasma 1,000 ml. blood			% Dose in RBC 1,000 ml. blood		
			Total*	Free	Gluc.	Total	Free	Gluc.
A. D.	15–30	43	0.672	0.370	0.282	0.217 (24.4%)†	0.052 (12.3%)	0.011
	60–120–240	42	0.705	0.143	0.487	0.115 (14.0%)	0.023 (13.9%)	0.000
A. K.	15–30–60	40	0.835	0.537	0.238	0.268 (24.3%)	0.046 (7.9%)	0.030
	120–240–360	39	0.762	0.052	0.586	0.119 (13.5%)	0.012 (18.7%)	0.008
A. N.	15–30	45	0.710	0.342	0.307	0.337 (32.2%)	0.076 (18.2%)	0.024
	60–120	43	0.703	0.121	0.490	0.198 (22.0%)	0.023 (16.0%)	0.006

* Total in plasma is average of sum of various plasma fractions of Table II.
† (RBC activity/plasma activity + RBC activity) × 100.

When the benzene extract of the free fraction was chromatographed on paper, the radioactivity present in the area of the papergram corresponding to estrone constituted a greater percentage of the total activity in the first plasma samples than it did in subsequent ones (85 and 87 per cent of the benzene fraction in the 15 minute samples of Subjects A. D. and A. K., decreasing progressively to 45 and 42 per cent in the 240 minute samples).

2. Red blood cell studies

Substantial amounts of activity were associated with the red cells (Table II), confirming the finding of Sandberg and Slaunwhite (12).

The total activity in 1,000 ml. of whole blood was calculated on the basis of the hematocrit and of the sum of the activity in the various plasma fractions and red cell extracts. From this, it was determined that 13.5 to 32.2 per cent of the total activity in a liter of blood was associated with the red cells (Table III).

A similar calculation was made for the radioactivity readily extractable by chloroform. As seen in Table III, 7.9 to 18.7 per cent of the free steroids in a liter of blood was associated with the red cells.

The glucuronoside fraction from red cells was very small so that a large amount of the total radioactivity in red cells was not accounted for. In Subject A. D., the residues obtained after removal of the free and glucuronoside fractions from the total red cell extracts contained 0.341 per cent of the dose per 1,000 ml. of red cells for the 15 to 30 minute samples and 0.200 per cent for the 60 to 240 minute samples. These data would tend to show that an important proportion of the compounds found in red cell extracts is in a state other than as free steroids or their glucuronosides.

3. Urine studies

The data on the urinary excretion of radioactivity are shown in Table IV. From 79.9 per cent (Subject A. D.) to 89.5 per cent (Subject A. K.) of the administered dose was recovered during the first six days following the injection of 16-C^{14}-estrone. No significant amount of radioactivity could be detected after this time. These data are in accordance with the results published by other investigators (10, 12).

The unaccounted-for fraction of urinary radioactivity. A rather large amount of urinary radioactivity was unaccounted for in all cases, and some attempts were made to determine whether this was due to technical error. The following experiment was carried out:

At the end of the first 24 hours, 51.10 per cent of the administered dose was excreted in the urine of Subject A. K.; of this, 40.82 per cent was recovered in the free fraction plus the fractions obtained after β-glucuronidase hydrolysis and con-

tinuous extraction at pH 0.8 (see Table IV). Following these procedures the radioactivity in the urine residue was 6.50 per cent of the administered dose, and therefore there was a net loss of 3.78 per cent, representing the sum of the experimental errors (handling and counting of each individual sample) to that point.

Benzene:water partition of the various urinary fractions. The benzene:water partition of the various urinary fractions is shown in Figures 1 and 2. The percentage of activity found in the benzene phase was relatively constant for the free fraction of the various urine collections and represented 40 to 60 per cent of the total activity (Figure 1). In the glucuronoside fraction, the percentage of activity in the benzene phase was 75 to 90 per cent of the total in the 0 to 4 hour urine specimens, but this percentage had a definite tendency to decrease with time and was only 42 to 60 per cent of the total in the 48 to 72 hour urine collection. A similar but less marked variation was observed for the pH 0.8 hydrolysis fraction as well as the strong acid hydrolysis fraction (Figure 2).

Paper chromatography of the urinary glucuronoside fractions. The urinary metabolites freed by β-glucuronidase hydrolysis were partitioned between benzene and water. Each fraction was chromatographed on paper and a radioautogram of the papergram was prepared.

The results obtained with the water extract of the various samples was difficult to interpret because of unsatisfactory separation of the compounds present in this fraction. However, it was observed that: *1*) the radioautograms showed a very faint spot corresponding to 16-epiestriol but there were no spots corresponding to compounds less polar than 16-epiestriol; *2*) a spot corresponding to estriol was detected in every instance; but, *3*) there was also a large amount of material which had not moved from the origin, and therefore was more polar than estriol.

The radioautograms of the benzene extracts showed a series of spots: *1*) a spot representing

TABLE IV

Cumulative urinary excretion of radioactivity following injection of 16-C^{14}-estrone (expressed as percentage of the administered dose)

Subject	Time after inject.	Per cent dose excreted in urine					
		Total	Free	β-Gluc.	Cont. extract.	Strong acid	Unaccounted
A. D.	4 hrs.	5.56	0.10	4.25	0.58	0.28	0.35
	8 hrs.	11.55	0.15	7.51	1.36	0.59	1.94
	12 hrs.	17.07	0.20	11.39	1.84	0.84	2.80
	1 day	37.81	0.45	22.52	3.57	1.40	9.87
	2 days	63.30	0.83	38.57	6.02	2.18	15.70
	3 days	71.10	1.00	43.50	6.81	2.53	17.26
	4 days	74.40					
	5 days	78.30					
	6 days	79.90					
	7 days	79.90					
A. K.	4 hrs.	8.28	0.19	5.88	0.82	0.44	0.95
	8 hrs.	17.41	0.35	11.92	1.49	0.90	2.75
	12 hrs.	26.03	0.49	18.50	2.02	1.40	3.62
	1 day	51.10	0.74	36.35	3.73	2.63	7.65
	2 days	71.95	0.93	52.60	5.30	3.75	9.37
	3 days	80.70	0.99	57.00	5.59	4.09	13.03
	4 days	86.20					
	5 days	88.90					
	6 days	89.50					
	7 days	89.50					
A. N.	4 hrs.	6.58	0.17	3.25	0.94	0.56	1.66
	8 hrs.	15.04	0.34	7.66	1.92	1.45	3.67
	12 hrs.	22.22	0.49	11.18	2.61	1.98	5.96
	1 day	37.40	0.72	20.30	3.48	2.75	10.15
	2 days	59.40	1.06	33.35	4.87	3.51	16.61
	3 days	71.00	1.28	40.30	5.59	3.89	19.94
	4 days	78.40					
	5 days	79.80					
	6 days	80.60					
	7 days	80.60					

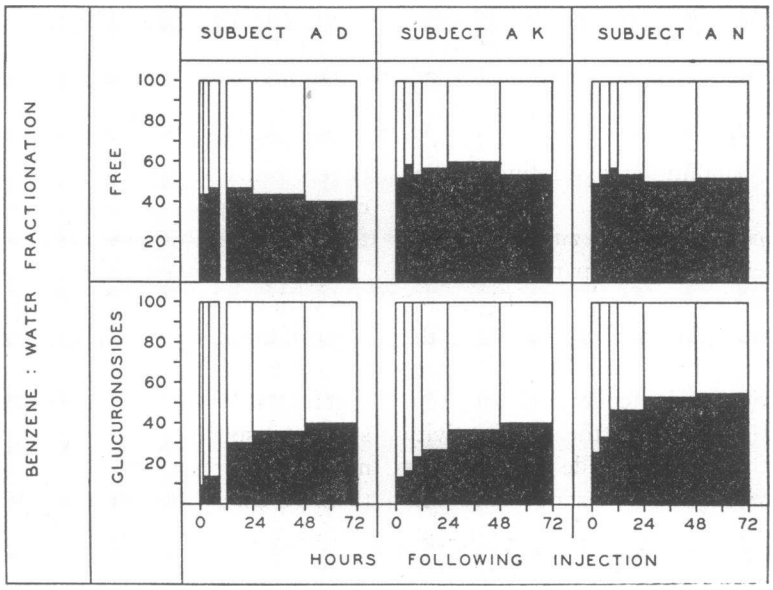

FIG. 1. PARTITION BETWEEN BENZENE AND WATER OF THE FREE AND GLUCURONOSIDE FRACTIONS OF THE VARIOUS URINE COLLECTIONS

The activity in benzene and in water are expressed as per cent of the sum of the activity of both phases. The black columns represent the water fraction.

the least polar compound with an R_f value of approximately 0.850, *2*) a large spot of R_f value 0.775 to 0.790, corresponding to estrone, *3*) a spot of R_f value 0.545 to 0.530, corresponding to estradiol-17β, *4*) a spot of R_f value 0.495 to 0.480 corresponding to either estradiol-16α or more probably 16-ketoestrone, *5*) a spot of R_f value 0.305 to 0.315, corresponding to 16-ketoestradiol or pos-

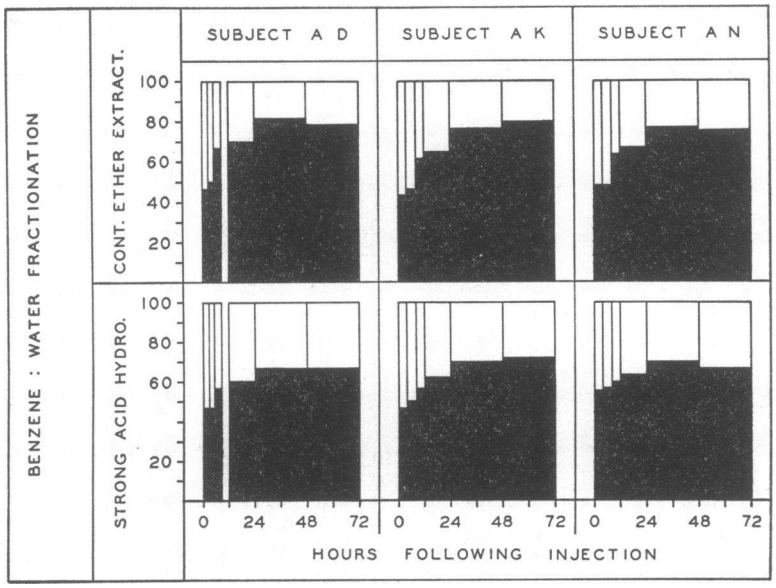

FIG. 2. PARTITION BETWEEN BENZENE AND WATER OF THE PH 0.8 HYDROLYSIS FRACTION AND OF THE STRONG ACID HYDROLYSIS FRACTION OF THE VARIOUS URINE COLLECTIONS

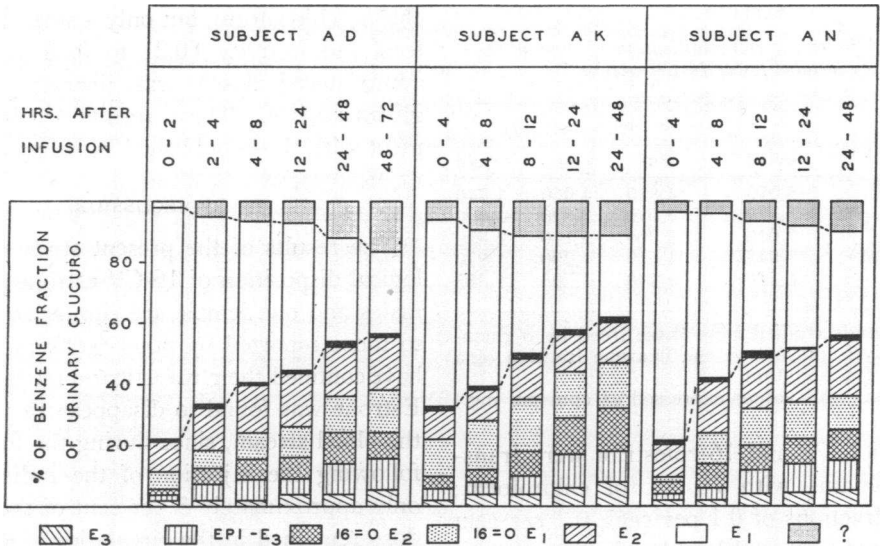

FIG. 3. BENZENE FRACTION OF A BENZENE: WATER PARTITION OF THE URINARY GLUCURONOSIDES

This fraction was chromatographed on paper and a radioautogram was prepared. The activity of each spot on the papergram is expressed as per cent of the sum of the activity obtained for the various spots. Each column represents the results obtained for the various urine samples collected from the experimental subjects. The black section of the columns represent the percentage of activity found in the area of the papergrams situated between the spots of estrone and estradiol-17β. The closely stippled section at the top of each bar represents the material with an R_f value of 0.850.

sibly 16-hydroxyestrone, *6*) a spot of R_f value 0.150 to 0.161 corresponding to 16-epiestriol, *7*) and finally, a small amount of material very close to the origin corresponding to estriol.

A radioautogram of the benzene extract of each of the urine collections was prepared. The corresponding spots of the chromatograms were excised carefully and the material present on each of these pieces of paper was eluted using 100 per cent ethanol as eluant. The radioactivity of each eluate was determined. Figure 3 presents the values obtained for each eluate expressed as percentage of the sum of the values obtained for the various spots. The recoveries were 91 to 106 per cent of the material applied on paper for chromatography.

Partial identification of some of the spots observed on the radioautograms of the benzene extract of the urinary glucuronoside fractions. The compound of R_f 0.850 and that corresponding to estriol were not studied. A known quantity of the urine collected between four and 48 hours after the administration of 16-C^{14}-estrone to Subject A. K. was extracted with $CHCl_3$ to remove the free steroids. The urine residue was treated with β-glucuronidase and the steroids liberated by this procedure were extracted with $CHCl_3$. This extract was partitioned between benzene and water and the benzene extract was chromatographed on several paper strips. Radioautograms of these papergrams were prepared, and the areas of the papergrams corresponding to estrone, estradiol-17β, 16-ketoestrone, 16-ketoestradiol-17β and 16-epiestriol were extracted with ethanol. Paper chromatography of the pooled extracts of each individual compound was repeated until the respective radioautograms showed only one well defined spot suggesting the presence of a single compound in each case.

The eluates of each spot were then chromatographed individually on an 11 × 70 mm. column of florisil. The florisil was activated as described for the determination of plasma 17-hydroxycorticosteroids (19). The dry residues from the eluates of estrone and estradiol-17β were transferred on the column with 20 ml. of chloroform, and the steroids were eluted with 60 ml. of 0.4 per cent methanol in chloroform. 16-Ketoestrone,

TABLE V

*Specific activity following recrystallization of some of the urinary metabolites of 16-C^{14}-estrone**

Compounds	Initial	Recrystallization		
		First	Second	Third
Estrone	5,070	4,850	4,960	4,740
Estradiol-17β	3,120	3,070	2,890	3,090
16-Keto-estrone	3,010	2,900	3,110	2,820
16-Keto-estradiol	1,945	1,630	1,290	1,070
16-Epiestriol	1,540	1,490	1,505	1,385

* The compounds studied were those freed by β-glucuronidase hydrolysis present in the benzene fraction of a benzene : water fractionation.
(Fractionation. All values expressed as cpm per mg.)

16-ketoestradiol-17β and 16-epiestriol were transferred on columns with 70 ml. of chloroform; then, 20 ml. fractions of 0.1 per cent, 0.2 per cent and 0.5 per cent methanol in chloroform were successively added to the column and discarded, the steroids being eluted with 50 ml. of 2 per cent methanol in chloroform.

The radioactivity present in the final residues was determined. A carefully weighed amount of pure crystalline steroid (19.5 mg.) was added to the presumably corresponding compound. Repeated recrystallization from aqueous methanol showed the specific activity to be constant for estrone, estradiol-17β, 16-ketoestrone and 16-epiestriol (Table V). However, the specific activity for the 16-ketoestradiol-17β fraction had a tendency to decrease, indicating that we were not dealing with 16-ketoestradiol-17β or, at least, not in pure form.

In addition, a 25 transfer countercurrent distribution with the system of solvents methanol: water: carbon tetrachloride (50: 50: 100) for estrone and estradiol and the system methanol: water: carbon tetrachloride (20: 80: 100) for 16-ketoestrone and 16-epiestriol was carried out on the various samples; the radioactivity was found to distribute as the corresponding standard steroids.

An identical study was carried out on the urine of Subject A. N. with similar results.

4. Feces studies

The three experimental subjects excreted 8 to 14 per cent of the injected dose within five days following the injection of 16-C^{14}-estrone. One-half or more of this activity was directly extractable by chloroform, but only a small fraction of the fecal radioactivity (0.21 to 0.58 per cent of the administered dose) was liberated following β-glucuronidase. These results are in good agreement with those published by others (12).

DISCUSSION

The results of the present study of the physiological disposition of 16-C^{14}-estrone in two normal males and one female are similar in most respects to those reported by other workers (10, 12) who have confined their investigation to female subjects. Estrone was found to disappear very rapidly from the blood stream, since during the first 15 minutes following the injection of the radioactive steroid only approximately 5 per cent of the administered dose remained in the circulation. After this time, however, the free activity disappeared from plasma at a progressively slower rate while the high levels of the glucuronoside fraction were sustained during the entire period.

As previously noted by Sandberg and Slaunwhite (12), significant amounts of radioactivity appeared to be associated with the red blood cells. *In vitro* studies of the distribution between plasma and red cells of 16-C^{14}-estrone have shown that 11.6 to 25 per cent of the total blood activity was recovered in the red cell extracts (20). Similar results have been reported by other investigators (21, 22). These values are similar to those calculated for the free red cell activity in this work. Since the distribution *in vitro* was found to be very rapid and not influenced by temperature (20) it is difficult to decide whether the distribution takes place before or after the withdrawal of the blood.

Only 10 to 20 per cent of the total activity detected in red cells was readily extractable by chloroform, suggesting that a large fraction of this total activity was in a conjugated form. This finding is in contrast with *in vivo* studies carried out with cortisol where it was observed that none of the conjugated metabolites of cortisol were associated with the red cells (23). Only very small amounts of activity were liberated by β-glucuronidase hydrolysis and since no attempt was made to further hydrolyze the conjugated steroids associated with the red cells, it is impossible to determine in what form the remaining radioactivity occurred.

Several metabolites of estrone were detected in the benzene extract of the urinary glucuronoside fractions. Our identification of 16-ketoestrone confirms the results of Slaunwhite and Sandberg (24). Levitz, Spitzer and Twombly (13) have demonstrated the conversion of estradiol-17β-16-C^{14} into 16-ketoestradiol-17β in man. Marrian, Loke, Watson and Panattoni (25), who have isolated 16α-hydroxyestrone from pregnant women's urine, pointed out that this latter compound readily undergoes rearrangement in alkaline solution to 16-keto-estradiol-17β. One of the compounds detected in the present work appeared to be 16-keto-estradiol-17β; however, it did not have a constant specific activity and possibly represented a mixture of the isomers of 16α-hydroxyestrone and 16-ketoestradiol-17β. It must be noted that our glucuronoside fraction was studied directly without phenolic fractionation. The 16-epi-isomer of estriol has been isolated from the urine of pregnant women (26) and has been found also in the urine of the three subjects of the present study as one of the metabolites of estrone. It is of interest that these various metabolites of estrone had previously been detected on the paper chromatograms of a nonhomogenous "fraction E_4" prepared from urinary extracts of pregnant women (27). Finally, the spot of R_f value 0.850 which appeared on our radioautograms could possibly be 2-methoxyestrone recently isolated in man as a new metabolite of estradiol-17β by Kraychy and Gallagher (28). A spot of R_f value slightly greater than that of estrone had previously been detected in the urine extracts of pregnant women (27) which possibly could be the same compound, 2-methoxyestrone.

Beer and Gallagher (11) have noted that following administration of estradiol-17β the amount of estriol recovered from urine increased with time. Comparable results were obtained in the present study; furthermore, it was also true for the other metabolites of estrone. It is possible that this dynamic change in the relative proportion of the various metabolites is due to the fact that estrone undergoes extensive entero-hepatic circulation (12) and, therefore, has a better chance to be metabolized by either repeated perfusions through the liver or in the gut.

In recent years, chemical assay methods for estrogens naturally occurring in human urine have been developed (29, 30). These methods were demonstrated to be quite sensitive and specific. Using such techniques, it was observed that one could recover in the urine of the experimental subjects 22 per cent of the injected estrone, 9.5 per cent of the dose being identified as estrone, 2.4 per cent as estradiol-17β and 10.1 per cent estriol (31). Our work and other isotopic studies (10, 12) show that two-thirds to three-fourths of the administered dose of estrone can be recovered in the urine. Nevertheless, as can be seen in Table VI, the percentages of the dose recovered in the urinary glucuronoside fraction as estrone (8.36 to 14.75 per cent) and estradiol-17β (2.95 to 4.94 per cent) were not very different from those reported by Brown (31). It must be noted that our figures do not include the amounts of estrone and estradiol freed by continuous ether extraction at pH 1.0 and by strong acid hydrolysis. On the other hand, the new chemical methods of estrogen determination do not measure certain compounds present in the urine in significant amounts (Table VI) since some of these compounds such as 16-ketoestrone do not give any color with the Kober reagents. Furthermore, the percentage of estriol in Brown's work is only one-half of the sum of our water fractions, but as mentioned in the Results section the water fraction is not homogenous and probably contains, in addition to estriol, the various estriol isomers and perhaps compounds more oxygenated than estriol.

We believe that isotopic estrogen studies of the type presented in this paper will help in improving present chemical methods of estrogen determination by indicating the ideal recoveries that might be expected and the nature of the metabolites to be measured.

SUMMARY

The physiological disposition of 16-C^{14}-estrone administered intravenously has been studied in three normal subjects (two males and one female). The results of blood, urine and feces studied were in accordance with those reported by Beer and Gallagher (10) and Sandberg and Slaunwhite (12).

Investigation on the nature of the urinary metabolites of radioestrone were carried out: 59.40 to 71.95 per cent of the administered dose was excreted in 48 hours with only 42.79 to 62.58 per cent of the administered dose accounted for after the various techniques of hydrolysis. Ap-

TABLE VI

Calculated amounts of some of the urinary metabolites of 16-C^{14}-estrone

Urinary activity excreted during the first two days following 16-C^{14}-estrone injection			Experimental subjects		
			A. D.*	A. K.	A. N.
Total activity			71.10	71.95	59.40
Activity accounted for†			53.84	62.58	42.79
Sum of water fractions‡			37.51	19.31	20.22
	Total		43.50	52.60	37.35
	Water fraction		12.91	13.61	14.73
Gluc. fraction	Benzene fraction	E_3§	1.15	2.11	0.75
		16-Epi-E_3	2.18	3.19	1.40
		16-Keto-E_2 (?)	2.18	3.94	1.53
		16-Keto-E_1	3.17	5.85	1.82
		E_2	4.94	4.86	2.95
		E_1	13.67	14.75	8.36
		2-Methoxy-E_1 (?)	2.78	3.97	1.37

* Urinary activity excreted during the first three days following 16-C^{14}-estrone injection.
† Sum of radioactivity in free, glucuronoside, pH 1.0 hydrolysis and strong acid fraction.
‡ Sum of the water fractions of the free, glucuronoside, pH 1.0 hydrolysis and strong acid fractions.
§ E_3, estriol and compounds more polar than estriol; 16-epi-E_3, 16-epiestriol; 16-keto-E_2, compounds which had R_f value similar to that of 16-ketoestradiol; 16-keto-E_1, 16-ketoestrone; E_2, estradiol-17β; E_1, estrone; 2-methoxy-E_1, compound which is possibly 2-methoxyestrone.

proximately one-half of the latter values represented metabolites as polar as, or more polar than, estriol. The urinary glucuronoside fraction consisted of: estrone (8.36 to 14.75 per cent of the administered dose), estradiol (2.95 to 4.94 per cent), estriol and more polar compounds (14.06 to 15.72 per cent), 16-ketoestrone (1.82 to 5.85 per cent), 16-epiestriol (1.40 to 3.19 per cent), a mixture of the isomers of 16-ketoestradiol and 16α-hydroxyestrone (1.53 to 3.94 per cent) and a compound which could be 2-methoxyestrone representing 1.37 to 3.97 per cent of the administered dose.

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