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Distribution of Radioactivity in Body Fat and Organs of Rats Treated with Labeled Quinestrol, Ethynylestradiol or 17β-Estradiol. (31678)

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Ethynylestradiol - 3 - cyclopentyl ether (EECPE, quinestrol) differs from its parent compound, ethynylestradiol, (EE) in several respects. Orally administered EECPE is stored unaltered in perirenal fat and brain of rats whereas EE is not(1-3). Unlike EE and most other steroids, EECPE is absorbed in significant amounts through the lymphatic system when administered orally in a lipid vehicle(4). An oil rather than aqueous vehicle also enhances storage of EECPE in both perirenal fat and brain whereas little or no EE is stored in fat or brain regardless of vehicle(3). Absorption of intraduodenally administered EECPE but not EE from an oily solution is prevented by excluding bile and pancreatic juice from the small intestine (3).

The peculiar affinity of EECPE for perirenal fat and brain and the unexplained influence of a lipid vehicle on absorption, transport and storage of this compound prompted further studies. The present communication describes the distribution of radioactivity in different types of body fat, in various areas of the brain and in major organs of rats treated with doubly-labeled (^3H and ^{14}C) EECPE in comparison with that obtained using tritiated EE or 17 β-estradiol (E_2).

Materials and methods. Preparation of radioactive steroid doses. Ethynylestradiol-6,7-^{3}H-3-cyclopentyl ether* (specific activity 0.78 μc/μg) and ethynylestradiol-3-cyclopentyl-1-^{14}C ether* (specific activity 0.0392 μc/μg) were mixed to give specific activities of approximately 0.26 μc ^3H and 0.026 μc ^{14}C per μg EECPE and thus a $^3H/^{14}C$ ratio of 10:1. Ethynylestradiol-6,7-^{3}H* (specific activity 0.93 μc/μg) and 17 β-estradiol-6,7-^{3}H† (specific activity 140 μc/μg) were diluted with the corresponding unlabeled steroids to give similar specific activities (≅ 0.26 μc ^3H/μg).

Radioactive steroids were dissolved in sesame oil or suspended in an aqueous vehicle (0.4% Tween 80, 0.9% benzyl alcohol, 0.9% NaCl, 0.5% carboxymethylcellulose in distilled water). For the distribution studies, doses were approximately 19.1 μg (5 μc ^3H) and in the case of EECPE 5 μc ^3H and 0.5 μc ^{14}C. Actual doses were always determined by liquid scintillation spectrometry and when applicable data were corrected to a constant dose of 5.0 μc ^3H(3). In the studies of distribution of EECPE in brain, a constant dose of 13.8 μc ^3H/20 μg/0.5 ml suspending vehicle (no ^{14}C) was used.

Experimental procedures. (1) *Distribution studies.* Female rats (Hemlock Hollow) weighing 280-300 g were used. One month after bilateral ovariectomy the animals were divided into 3 groups and dosed orally with labeled EECPE or EE or subcutaneously with labeled E_2 in suspending vehicle. The rats were placed in metabolic cages and urine and feces were collected. Twenty-four hours after dosing, the rats were anesthetized with ether and exsanguinated by heart puncture. In addition to plasma and red cells, the following tissues and organs were collected and

* Synthesized by Mr. E. Merrill, radiation officer, Warner-Lambert Research Institute. The steroids were judged radiochemically pure by thin layer chromatography.

† New England Nuclear Corp. Lot. No. 134-193-105. About 90% radiochemically pure by thin layer chromatography.

frozen on dry ice: perirenal, subcutaneous (from the ventral abdominal region), uterine mesenteric, omental and aortic fat (the last is the brown fat just ventral to the spinal cord in which the thoracic aorta is embedded); liver, kidney, heart, thymus, brain, muscle, lung, spleen, thoracic aorta, adrenal, uterus, cervix and vagina.

Fat samples were extracted with methylene chloride as previously described(1-3). After evaporation of the methylene chloride, measured volumes of the liquid fat were added directly to 15 ml scintillation cocktail for determination of radioactivity. Aortic fat samples were small (5-50 μl) and had to be measured with microsyringes. Because of the high viscosity of this fat, the measurements are only approximate ($\pm$ 20%).

Plasma samples (2-3 ml) were diluted to 5 ml with distilled water and shaken 3 times with 10 ml volumes of ether. The ether extracts ("free fraction") were assayed for radioactivity and also chromatographed. One ml of the remaining aqueous phase, shown in a previous study(3) to contain glucuronidase hydrolyzable and solvolyzable conjugates, was added to 14 ml scintillation fluid in a centrifuge tube and the resulting precipitate sedimented. The ("water soluble") clear supernatant (which contained more than 95% of the radioactivity) was used for counting purposes.

Organs were homogenized in 50 ml ethanol on a high speed homogenizer and filtered on a Buchner filter. The alcoholic extracts were taken to dryness on a flash evaporator and transferred to clean 50 ml stoppered tubes by means of 6 alternate rinses with ether (total 20 ml) and water (total 10 ml). After shaking, the ether layer was transferred to a clean tube and the aqueous phase shaken twice more with 20 ml volumes of ether. The ether extracts ("free fraction") were combined and used for determining radioactivity and for chromatography. One ml of the aqueous phase ("water soluble fraction") was added directly to 14 ml scintillation cocktail for counting.

"Free fractions" of plasma and organ extracts were chromatographed with appropriate standards on silica gel G using an ascending system of chloroform, methanol and water (485:15:1) as previously described(2,3). One cm segments of each chromatogram taken from origin to solvent front were scraped into counting vials, eluted with 1 ml benzene-methanol (1:1) and counted in scintillation cocktail.

Urine (0.2-0.5 ml) was added directly to the scintillation cocktail for counting. Feces were homogenized and extracted with methanol according to Arai *et al*(5). An aliquot of the extract was counted in scintillation fluid.

(2) *Concentration of EECPE in rat brain.* Six rats were dosed by gavage with 13.8 μc (20 μg) EECPE-6,7-^{3}H in 0.5 ml sesame oil. Half were killed at 24 and the remainder 72 hours post treatment. Brains were dissected into the following areas: Cerebellum, cortex, thalamus plus hypothalamus, pons plus medulla, and hypophysis. Brain areas were extracted with methylene chloride as previously described(2) and after evaporation of solvent, extracts were dissolved in scintillation fluid.

Counting procedures. The ^{3}H and ^{14}C were determined simultaneously on a 3 channel liquid scintillation spectrometer (Packard Model 3324) equipped with external standard for quench correction. All samples were counted in 15 ml of a scintillation cocktail consisting of 7 g PPO, 0.3 g dimethyl POPOP and 100 g naphthalene in 1 l redistilled dioxane.

Results. Distribution of radioactivity in body fat and organs. Twenty-four hours after oral administration of doubly labeled EECPE, high ^{3}H and ^{14}C counts were found in subcutaneous, perirenal, uterine mesenteric, omental and aortic fat (Table I). The ^{3}H/^{14}C ratios in each type of fat did not differ significantly from that of the administered EECPE (*i.e.*, 10:1). In rats treated orally with tritiated EE or subcutaneously with tritiated E_2 only 1/10-1/30 as much radioactivity was found in the various fat samples (Table I). Although there was marked within-group variation in the amount of radioactivity present in each type of fat, the data showed great consistency in the relationships between the various fat com-

TABLE I. Radioactivity in Different Types of Fat of Rats Treated 24 Hours Previously with EECPE, EE or E_2.*

	EECPE			EE	E_2
Type of fat	^3H, dpm/g	^{14}C, dpm/g	$^3H/^{14}C$	^3H, dpm/g	^3H, dpm/g
Subcutaneous	21600 ± 4450	2180 ± 473	10.1 ± .25	1230 ± 91	632 ± 69
Perirenal	18100 ± 2160	1800 ± 368	10.1 ± .18	1410 ± 113	854 ± 45
Uterine mesenteric	18000 ± 3960	1790 ± 410	10.2 ± .20	1570 ± 122	1710 ±351
Omental	20200 ± 4210	2050 ± 450	10.1 ± .28	1925 ± 122	—
Aortic	60800 ± 7670	6620 ± 950	9.8 ± .45	1205 ± 241†	1200 ± 81†

* Doses were as follows: EECPE: 5 μc ^3H, .5 μc ^{14}C/.5 ml p.o. ($^3H/^{14}C$ = 10.0); EE: 5 μc ^3H/.5 ml p.o.; E_2: 5 μc ^3H/.2 ml s.c. Each treatment group had 5 rats.

† Due to small sample sizes, actual counts were too low to be considered more than approximations.

TABLE II. Ratios of 24-Hour ^3H Concentrations in Various Types of Fat of Rats Treated with EECPE, EE or E_2 (Subcutaneous Fat = 1.00).*

Treatment	Perirenal Subcutaneous	Uterine mesenteric Subcutaneous	Omental Subcutaneous	Aortic Subcutaneous
EECPE	.83 ± .04	.82 ± .03	.92 ± .05	2.61 ± .49
EE	1.14 ± .03	1.28 ± .05	1.58†	.90 ± .16
E_2	1.32 ± .08	2.59 ± .29	—	1.76 ± .22

* Ratios shown are the average of individual ratios calculated for each type of fat in each rat. Each treatment group had 5 rats. Dose schedule as in Table I.

† Different rats were used for omental fat samples, thus ratio is calculated on mean values and included only for completeness. However, the mean concentration of tritium in omental fat was significantly higher than that in subcutaneous fat ($p < .01$) of EE-treated rats.

partments of each individual rat. Accordingly, the ratios of the tritium concentrations in perirenal, uterine mesenteric, omental and aortic fat to the tritium concentration in subcutaneous fat were calculated for each individual and the mean ratios for each group were determined. In the case of EECPE, perirenal and uterine mesenteric fat contained significantly less and aortic fat significantly more tritium than did subcutaneous fat ($p<0.01$, Table II). Omental and subcutaneous fat contained similar amounts of radioactivity. When EE was administered orally or E_2 subcutaneously the tritium ratios in perirenal/subcutaneous and uterine mesenteric/subcutaneous fat were significantly greater than unity (Table II).

Tritium counts were higher in the free fraction of plasma of EECPE than of EE or E_2-treated rats while there were no significant differences between the water soluble fractions of the 3 groups (Table III). Both the free and water soluble fractions of red cells of EECPE-treated animals exhibited higher ^3H counts than similar blood fractions of EE-treated rats (Table III). Radioactivity in the free fractions of the various organs were higher than that of plasma. After EECPE treatment, the highest concentrations of free radioactivity were found in adrenal and liver, followed by kidney, heart, brain, uterus, aorta, lung, thymus and cervix. Lesser amounts were found in spleen and vagina (Table III). In the case of the EE-treated rats, the highest accumulation of free radioactivity appeared in liver and adrenal, with much lower amounts in the other organs (Table III). Following injection of E_2, the highest concentrations of free radioactivity were found in adrenal, heart and liver (Table III).

The ratios of free (F) to water soluble (WS) radioactivity were unique for each test compound as well as for each organ, and did not reflect the ratios found in plasma where water soluble metabolites predominated (Table IV). In case of EECPE, F/WS ratios indicated a significantly greater accumulation of free radioactivity in liver, kidney, heart and thymus ($p<0.01$, Table IV). Many organs did not have significant radioactivity in the WS fraction (Tables III and IV).

The $^3H/^{14}C$ ratios were also calculated for the fractions obtained from tissues

TABLE III. Concentration of Radioactivity in Tissues and Organs of Rats Treated 24 Hours Previously with EECPE, EE or E_2 (5 Rats per Group).

Tissue or organ	Fraction	Treatment: EECPE ^{3}H, dpm/g	EE ^{3}H, dpm/g	E_2 ^{3}H, dpm/g
Plasma	Free	557 ± 185	119 ± 20	87 ± 11
	Water sol.	2020 ± 251	1690 ±108	1520 ± 153
Erythrocytes	Free	340 ± 50	78 ± 10	—
	Water sol.	1240 ± 77	710 ±110	—
Liver	Free	12900 ± 4300	2360 ±240	1440 ± 181
	Water sol.	3500 ± 83	1110 ± 95	1560 ± 175
Kidney	Free	5450 ± 1740	440 ± 52	383 ± 36
	Water sol.	890 ± 238	354 ± 37	770 ± 34
Heart	Free	5240 ± 1500	375 ± 32	1780 ± 930
	Water sol.	930 ± 550	0	0
Thymus	Free	3910 ± 740	0	—
	Water sol.	860 ± 160	0	—
Brain	Free*	5220 ± 570	205 ± 38	—
Muscle	Free*	2310 ± 360	268 ± 38	172 ± 28
Lung	Free*	3920 ± 810	0	0
Spleen	Free*	1690 ± 244	0	—
Aorta	Free*	4240 ± 1250	545 (pool)	314 (pool)
Adrenal	Free*	15050 ± 3280	1700 (pool)	2670 (pool)
Uterus	Free*	4700 ± 985	747 (pool)	644 (pool)
Cervix	Free*	3510 (pool)	0	0
Vagina	Free*	1400 (pool)	0	0

* Insignificant counts in water soluble fraction.

and organs of EECPE-treated rats. The ratios were significantly higher than the administered ratio of 10:1 in the free fractions of plasma, liver, kidney and adrenal and in the water soluble fraction of plasma and liver (Table V). The water soluble fraction of kidney had the remarkably low ratio of 2.6:1 (Table V).

Thin layer chromatograms of free fractions of plasma showed only a small percentage of radioactivity with Rf values similar to those of the administered compounds. The values were: EECPE, 22%; EE, 16%; E_2, 25%. The $^3H/^{14}C$ ratio in the peak corresponding to EECPE was 10.5:1.

Chromatograms of the free fractions of organ extracts are summarized in Table VI. In extracts of liver and kidney of EECPE-treated rats the major radioactive peak had

TABLE IV. Ratios of Radioactivity in Free (F) and Water Soluble (WS) Fractions Obtained from Various Tissues and Organs of Rats Treated with Tritiated EECPE, EE or E_2.

Tissue or organ	EECPE F/WS	EE F/WS	E_2 F/WS
Plasma	.25 ± .05	.07 ± .01	.06 ± .01
Erythrocytes	.28 ± .04	.13 ± .04	—
Liver	3.41 ± .32	2.05 ± .13	.94 ± .01
Kidney	5.90 ± .38	1.24 ± .10	.52 ± .07
Heart	9.76 ±2.3	†	†
Thymus	4.80 ±1.03	*	—

* Insufficient counts in either fraction.

† Insufficient counts in water soluble fraction.

Note: No radioactivity was found in the water soluble fraction of brain, muscle, lung, spleen, aorta, adrenal, uterus, cervix or vagina.

TABLE V. $^3H/^{14}C$ Ratios in Tissues and Organs of EECPE-Treated Rats.

Tissue or organ	Free fraction $^3H/^{14}C$	Water soluble fraction $^3H/^{14}C$
Plasma	13.8 ± .61*	22.7 ± 3.47*
Liver	13.0 ± .43*	13.4 ± .61*
Kidney	13.0 ± .84*	2.6 ± .32†
Spleen	9.0 ± .23†	No counts
Thymus	8.6 ± .10†	No ^{14}C detected
Brain	9.4 ± .36	No counts
Lung	10.0 ± .24	No counts
Heart	11.1 ± .18*	No ^{14}C detected
Muscle	10.3 ± .25	No counts
Adrenal	13.2 ± .24*	No counts

* Significantly higher than administered ratio of 10.0 ($p < .5$-$.01$).

† Significantly lower than administered ratio of 10.0 ($p < .05$-$.01$).

Note: No ^{14}C detected in free fraction of red cells, aorta or reproductive structures.

TABLE VI. Summary of Thin Layer Chromatography Data on Free Fractions of Liver, Kidney and Heart from Rats Treated with EECPE, EE or E_2.*

Compound	Rf_{std}	Liver R_s of ^3H peaks	Kidney R_s of ^3H peaks	Heart R_s of ^3H peaks
EECPE	.73	.18	.54	*.83*
		1.00	.76	1.00
			1.00	
EE	.52	.57	None	None
		.72		
		1.00		
E_2	.40	.67	None	2.03
		1.00		
		1.35		

* The table presents average Rf values for authentic EECPE, EE and E_2 and mobilities of unknown radioactive peaks eluted from the chromatograms of free fractions of the 3 organs.

$$R_s = \frac{\text{Rf unknown}}{\text{Rf standard}}.$$

System used was chloroform-methanol-water (485:15:1) on silica gel G.
Italicized values represent major peak.

TABLE VII. Radioactivity in 24-Hour Urine and Fecal Specimens of Rats Treated Orally with EECPE or EE (4-5 Rats/Group).

Treatment	Urinary radioactivity, % of dose		Fecal radioactivity, % of dose	
	^3H	^{14}C	^3H	^{14}C
EECPE	5.9 ± .51	36.4 ± .87	7.3 ± 1.3	3.6 ± .81
EE	3.9 ± .74	—	5.5 ± 1.2	—

an Rf like that of EECPE. More polar minor peaks were also present. Although heart extracts exhibited a peak with an Rf like that of EECPE, the major peak was more polar. The major peaks in extracts of liver of EE or E_2-treated rats corresponded to their appropriate standards. No distinct peaks were found in extracts of kidneys of EE or E_2-treated rats or in hearts of EE-treated animals. A single peak with twice the Rf of E_2 was found in heart extracts of the E_2 group.

Excretion of tritium in urine and feces was similar in rats treated with EECPE or EE and accounted for 10-13% of the administered doses. In the EECPE group, 36% of the administered ^{14}C was eliminated in the urine and only 3.6% in the feces (Table VII).

Concentration of radioactivity in brains of rats treated with EECPE. The distribution of tritium in the various areas of the rat brain at 24 and 72 hours is shown in Table VIII. Concentration of radioactivity at 24 hours was highest in pons plus medulla and in hypophysis, followed by cerebellum and thalamic areas, and least in cortex. However, due to the differences in mass of the various areas, the total amount of tritium present was greatest in cortex followed by pons plus medulla, thalamic areas, cerebellum and least in hypophysis. Approximately 80% of the radioactivity had disappeared from the various areas 72 hours post treatment.

Discussion. Ten to 30 times as much ^3H was found in the various body fat depots of EECPE-treated rats as in those of rats treated with equivalent doses of radiolabeled EE or E_2. The distribution of radioactivity among the various fat compartments was remarkably consistent although the concentration found in any given type of fat varied markedly from rat to rat. Furthermore, the affinity for each type of fat varied with the steroid administered. Thus when EECPE was administered, the accumulation of radioactivity was greatest in aortic, next in subcutaneous and omental and least in perirenal and uterine mesenteric fat. The differences, though not large, were highly significant. When EE was administered, accumulation of

TABLE VIII.

Area	^{3}H concentration at 24 hours: Avg wt, mg	dpm/area	dpm/g	^{3}H concentration at 72 hours: Avg wt, mg	dpm/area	dpm/g	% of ^{3}H released between 24th & 72nd hr on the basis of dpm/g
TH*	249	3830 ± 440¶	15300 ± 1870¶	258	710 ± 390¶	2570 ± 1050¶	83.2
PM†	207	4800 ± 220	23700 ± 3300	272	1270 ± 550	4530 ± 1680	80.9
C‡	823	6500 ±1060	7670 ± 970	739	1150 ± 680	1330 ± 580	82.7
CB§	209	2870 ± 280	15700 ± 3200	235	693 ± 390	2800 ± 1300	82.3
PIT‖	6.7	193 ± 114	26700 ±12600	6.3	—	—	—

* Thalamus plus hypothalamus. † Pons plus medulla. ‡ Cortex. § Cerebellum. ‖ Pituitary.
¶ Average ± standard error.

radioactivity was greatest in omental, followed by uterine mesenteric and perirenal and least in subcutaneous and aortic fat. On the other hand, after injection of E_2, the highest concentration of radioactivity appeared in uterine mesenteric, followed by aortic and perirenal, with the smallest concentration in subcutaneous fat. The reason for the differences in distribution of radioactivity between the various fat compartments is not apparent. However the degree to which a steroid is taken up and retained by a fat depot is probably determined by two major factors: the lipophilic properties of the steroid and the constituents of the fat depot itself. This type of interaction may also have a bearing upon the accumulation of steroids in target organs. Different organs have differing uptakes and retention times for estrogens(6). In the anterior pituitary, but not in the liver 17 β-estradiol associates with the nuclear membrane(7,8). These phenomena might be due to the affinity of a steroid for a particular constituent or arrangement of the lipid present in a given tissue, cell or organelle.

Previous work established that EECPE is stored unaltered in rat brain(2). Present data show that the steroid is not uniformly distributed throughout the brain but rather its concentration varies in specific areas being highest in pons, medulla and hypophysis and lowest in cortex. However, a similar percentage (80-82) of the stored estrogen was released from each area between the 24th and 72nd hour after administration regardless of initial concentration. On an absolute basis a greater amount of steroid was therefore released during this period from areas which had a greater initial concentration. Since, as in the case of the other body fat depots, the amount of steroid stored is probably related to the binding ability of the components of each area, it would appear that rate of release is likely to be dependent upon a different metabolic turnover.

The dissimilarity of the patterns of free and water soluble radioactivity in the various tissues and organs as compared with those in plasma suggests that the substances are contained in extravascular compartments and that contamination of tissues by plasma was low. The amount of free radioactivity present in tissues and organs may be correlated with lipid content. Thus adrenal and liver showed relatively high tritium levels following administration of any of the estrogens while skeletal muscle was low. With the exception of liver, water soluble metabolites were low in or absent from the various organs. This suggests that water soluble metabolites have a lesser affinity for organs than do nonpolar ("free") metabolites. It is generally believed that conjugation of estrogens occurs in the liver, and the relatively high levels of water soluble radioactivity may reflect this process.

Certain inferences can be drawn from $^3H/^{14}C$ ratios for possible metabolic pathways for degradation of EECPE. Thus a ratio greater than that of administered compound (10:1) suggests cleavage and loss of the ^{14}C-labeled cyclopentyl group, while a ratio lower than 10:1 suggests either accumulation of the cyclopentyl group derivatives or, less likely, removal of significant amounts of ^3H by hydroxylation of C_6 or C_7. Whereas all types of fat and the free fractions of brain,

muscle and lung showed the expected ratio of 10:1, significantly higher ratios were observed in free fractions of plasma, liver, kidney and adrenal and in the water soluble fractions of plasma and liver. The water soluble fraction of kidney had a very low ratio (2.6:1). We interpret these data to suggest that cleavage of the ether linkage may occur prior to conjugation of metabolites and that ^{14}C-labeled cyclopentyl fragments are removed from the circulation more rapidly than derivatives of the steroid moiety. A likely site of removal of cyclopentyl group derivatives is the kidney because (a) the low $^{3}H/^{14}C$ ratio in the water soluble fraction of this organ indicates accumulation of ^{14}C fragments and (b) 24-hour urine collections contained 36% of the administered ^{14}C but only 6% of the tritium dose.

The nature of the metabolites of the 3 steroids investigated is unknown, but the chromatographic evidence suggests that the free fractions of plasma and heart contain primarily metabolic derivatives rather than the original estrogens. On the other hand, chromatograms of the free fractions of liver showed major peaks with Rf values identical to those of the administered compounds. The low levels of radioactivity present in the various extracts did not permit further study.

Summary. The distribution of radioactivity in body fat and organs, 24 hours after administration of radiolabeled ethynylestradiol-3-cyclopentyl ether (EECPE), ethynylestradiol (EE) and 17 β-estradiol (E_2) has been studied in castrated female rats. Radioactivity was highest in various types of fat of EECPE-treated animals. Only 1/10-1/30 as much tritium was detected in fat of rats treated with EE or E_2. Each steroid exhibited an individual preference for each particular type of fat. Concentration of steroid in the various organs did in general correspond to the amount of fat present. Free (ether soluble) metabolites were in general more concentrated in organs than in plasma whereas water soluble metabolities (presumably conjugates) were generally higher in plasma than in the various organs. The data on the $^{3}H/^{14}C$ ratio suggests that the ^{14}C labeled cyclopentyl group is cleaved from EECPE and eliminated *via* the kidney at a faster rate than the steroid moiety. The distribution of radioactivity in different brain areas following administration of radiolabeled EECPE was studied in male albino rats. EECPE is not uniformly distributed throughout the brain, but rather localized to specific areas. Rate of release of stored EECPE seems to be dependent upon metabolic turnover rather than binding capacity of the components of the different areas.

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