Pathway of Absorption of Orally Administered Ethynylestradiol-3-Cyclopentyl Ether* in the Rat as Influenced by Vehicle of Administration.† (30998)

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It seems well established that, regardless of the vehicle used for their administration, the pathway of absorption of orally administered steroids is the portal system(1-3).

More recently, Kimbel(3) has presented evidence indicating that: a) steroid esters which are not hydrolyzed by intestinal esterases are transported by the lymph to a greater extent than the corresponding free compounds and b) introduction of a lipophilic group into the molecule greatly enhances the lymphatic transport of steroids.

Meli *et al*(4,5) have shown that, unlike ethynylestradiol, oral administration of its 3-cyclopentyl ether is followed by storage of a considerable amount of unaltered estrogen in body fat and to a much lesser extent in the brain of the rat. These findings indicate that a certain amount of this compound crosses the intestinal barrier unaltered. Thereafter, the compound is either transported to the systemic circulation *via* the lymph thus avoiding liver inactivation and/or follows the portal route but is not efficiently metabolized by the liver.

In view of the high lipophilic properties of this compound it seemed interesting to determine the role of the lymphatic system as a possible pathway of transport.

Materials and methods. A) *General.* The chromatographically pure compounds tested were ethynylestradiol-6,7 H^3 with a specific activity of 0.93 $\mu c/\mu g$ and either ethynylestradiol-6,7 H^3-3-cyclopentyl ether with a specific activity of 0.78 $\mu c/\mu g$ or a mixture (H^3/C^{14} ratio as specified in Table II) of ethynylestradiol-6,7 H^3-3-cyclopentyl ether with a specific activity of 0.78 $\mu c/\mu g$ and ethynylestradiol-3-cyclopentyl-1 C^{14}-ether with a specific activity of 0.0392 $\mu c/\mu g$. Doses of EECPE were adjusted to contain approximately 5 μc H^3 and 0.5 μc C^{14}/19.1 μg/0.5 ml. Doses of EE were adjusted by addition of unlabelled compound to contain 5 μc H^3/19.1 μg/0.5 ml. Aliquots of the doses were checked by liquid scintillation counting.

Male albino rats of 300 to 400 g body weight were used. Cannulation of the thoracic duct was performed under ether anesthesia according to the method of Bollman *et al*(6). Following the operation the rats were placed in individual restraining cages which prevented the animals from turning around but allowed some forward and backward movement. Two to three hours after the operation, the animals received by stomach tube either ethynylestradiol (EE) or its 3-cyclopentyl ether (EECPE) at the doses and in the vehicle specified in Tables I and II. The amount of vehicle used was kept constant at 0.5 ml/animal. The lymph was collected for 24 hours and the amount recorded. During the collection period the animals had access only to water.

B) *Lymph analysis.* One ml from each lymph sample was added directly to 14 ml dioxane base liquid scintillation fluid (7 g PPO, 0.3 g dimethyl POPOP and 100 g naphthalene in 1000 ml freshly redistilled 1,4-dioxane). After centrifugation for 5 minutes at 2500 rpm the supernatant was directly transferred to a counting vial. This procedure quantitatively recovered 95% or more of labelled EE or EECPE added to control lymph samples. The radioactivity of each sample was determined on a 3 channel Packard Liquid Scintillation Spectrometer Mod. 3324 equipped with an external standard for quench correction. In order to count tritium and C^{14} simultaneously, the following setting was used: Red channel (H^3 and C^{14}), gain

* The following trivial names and abbreviations are used in this paper: ethynylestradiol (EE) = 19-nor-17 α-pregna-1,3,5(10)-trien-20-yne-3,17 β-diol; ethynylestradiol-3-cyclopentyl ether (EECPE) = 3-cyclopentyloxy-19-nor-17 α-pregna-1,3,5(10)-trien-20-yn-17 β-ol.

† Labelled ethynylestradiol and its 3-cyclopentyl ether (which has the non-proprietary name of Quinestrol) were synthesized by Mr. E. Merrill.

TABLE I. Amounts of Tritiated EE or EECPE Present in 24 Hr Lymph Samples.

Treatment	No. of animals	Oral dose in μc/animal	Vehicle of admin	Avg lymph vol (ml/24 hr)	Avg % of admin dose
EE	3	8.6	Sesame oil	16.5	.59 ± .3
EECPE	5	6.4	" "	19.6	7.6 ± 1.5

50%, window 50-1000; Green channel (C^{14} only), gain 11%, window 150-1000. The Blue channel counted the external standard. The instrument had been calibrated with sets of quenched H^3 and C^{14} standards. Details of the procedure and theory of external standardization and of simultaneous counting of H^3 and C^{14} are available from the manufacturer of the instrument.

Several lymph samples from animals treated with either EE or EECPE were subjected to further purification and thin layer chromatography to identify radioactive compounds present. Identity of EECPE was also assessed by determining the H^3/C^{14} ratio. Lymph samples from groups of animals treated with EE or EECPE were pooled. Thirty ml of each pool were added to 150 ml absolute ethanol and filtered. The filter papers were rinsed with 10 ml ethanol and 10 ml methylene chloride. The filtrates (including rinses) were evaporated to dryness on a Buchler flash evaporator. The dry residues were quantitatively transferred to 40 ml glass stoppered centrifuge tubes using a total of 5 ml absolute ethanol, 15 ml distilled water and 20 ml fresh diethyl ether. The mixtures were shaken and the organic phases transferred to clean graduates. The aqueous phases were shaken with 2 additional 20 ml aliquots of diethyl ether. The ether layers were transferred to the graduates and dried over Na_2SO_4. Aliquots of the ether and water fractions were taken for liquid scintillation counting. Ninety-seven percent of the radioactivity was found in the ether fraction of the extract of lymph from EECPE-treated animals whereas only about 50% was found in the ether fraction of the extract of lymph from EE-treated rats. The latter finding suggested alteration of the EE molecule had occurred, inasmuch as 95% of authentic EE could be extracted by this procedure.

The ether extracts were evaporated to dryness and taken up in 0.5 ml benzene-methanol mixture (1:1) for thin layer chromatography. Non-radioactive standards were added for each compound and four 5 lambda aliquots of each extract were chromatographed on silica gel G using the systems chloroform-methanol-water (485:15:1) and benzene-ethanol (90:10) as previously described(5). The EECPE and EE spots were visualized by exposing the chromatograms to iodine vapors and these areas were scraped into liquid scintillation vials, eluted with 0.5 ml benzene-methanol (1:1) and counted in dioxane scintillation cocktail as described above. The remaining silica gel from each chromatogram channel was likewise scraped off and subjected to the counting procedure. Recovery was assessed by comparison with similar amounts of each extract not subjected to chromatography.

Results. a) *Lymphatic transport.* The data are shown in Tables I and II. Small amounts

TABLE II. Amounts of Tritiated EE or Doubly Labelled EECPE in 24 Hr Lymph Samples.

Treatment	Animals	Oral dose in μc/animal based on admin H^3	H^3/C^{14} ratio of admin dose	Vehicle of administration	Average lymph volume (ml/24 hr)	Lymph radioactivity Average % of administered dose based on H^3 recovery	Avg H^3/C^{14} ratio
EE	5	3.90	—	Aqueous	23.4	.59 ± .08	—
EE	6	4.80	—	Sesame oil	20.0	.38 ± .12	—
EECPE	4	4.50	8.8	Aqueous	23.7	1.47 ± .57	9.47
EECPE	6	4.92	9.0	Sesame oil	23.2	7.53 ± 1.30	9.63
EECPE	5	6.15	7.7	" " *	20.6	15.7 ± 1.3	7.92

* Containing 25% w/v of glyceryl mono-oleate. (Myverol®—distilled glyceryl mono-oleate Type 18-71-E-90% pure monoester was kindly supplied by Distillation Products Industries, Rochester, N. Y.).

(averages of 0.4 and 0.6% respectively of the administered dose) were found in the 24-hour lymph samples from animals following oral EE administered as aqueous suspension or as oily solution. Slightly but not significantly greater amounts (average 1.4% of the administered dose) were found in 24-hour lymph samples from animals which had received oral EECPE as aqueous suspension. Significantly greater amounts (average 7.5% of the administered dose) were found in 24-hour lymph samples from animals treated with EECPE dissolved in sesame oil. An even further significant increase (average 15.7% of the administered dose) in the amount of compound transported by the lymph could be achieved by administering EECPE dissolved in sesame oil containing 25% w/v of glyceryl mono-oleate.

b) *Identification of compounds present in the lymph.* Eighty-nine to 91% of the radioactivity in extracts of lymph from animals treated with doubly labelled EECPE either administered as aqueous suspension or dissolved in a lipid vehicle had the same mobility as authentic EECPE in the two TLC systems employed (Table III). Furthermore the ratio H^3/C^{14} was similar to that of the administered dose.

No concentration of H^3 was found in the eluate of EE spots on chromatograms of extracts of lymph from animals treated with tritiated EE whether administered as aqueous suspension or dissolved in sesame oil. The low level of radioactivity in extracts of lymph from EE-treated animals discouraged further attempts to identify the steroid(s) present.

TABLE III. Thin Layer Chromatograms of Pooled Extract of Lymph Obtained from Rats Treated with EECPE.

Material	Radioactivity in 20λ of extract: H^3 dpm	H^3 %	C^{14} dpm	C^{14} %	H^3/C^{14} ratio
A. Unchromatographed extract	5550	100	620	100	9.0
B. Chromatographed extract (chloroform-methanol-water, 485:15:1)					
1. EECPE spot (Rf 0.83)	5050	91	537	87	9.4
2. Remainder of channel	308	6	34	6	9.1
C. Chromatographed extract (benzene-ethanol, 90:10)					
1. EECPE spot (Rf 0.81)	4900	89	514	83	9.6
2. Remainder of channel	370	7	66	11	5.6

Discussion. The present data on lymphatic transport of ethynylestradiol (EE) whether administered as aqueous suspension or as oily solution agree well with previous findings on other steroidal compounds(1-3) which similarly indicate that: a) the portal system is the major, if not the exclusive, pathway of transport of orally administered steroids and b) the pathway of transport is independent of the vehicle of administration.

Ethynylestradiol-3-cyclopentyl ether (EECPE) behaved quite differently. When administered orally as aqueous suspension, the amount of EECPE present in the lymph was slightly greater than that following oral EE but not of such a magnitude as to indicate that the lymphatic system was playing any important role. On the other hand, when EECPE was administered orally dissolved in sesame oil, a significant amount (average 7.6% of the administered dose) could be recovered from the lymph. A further and highly significant increase (average 15.7% of the administered dose) in the amount of EECPE transported by the lymphatic system could be achieved by administering the compound dissolved in sesame oil containing 25% w/v of a monoester of a long chain fatty acid (glyceryl mono-oleate). Identity of unaltered EECPE as the major component present in the lymph was assessed by both H^3/C^{14} ratio and TLC determinations. On the other hand, in spite of numerous attempts, we were unable to characterize by TLC the compound(s) present in extracts of lymph from EE-treated animals. In view of the small amounts of compound transported by the lymph, no further consideration was therefore given to this problem.

That absorption of either compound from the gut took place, was assessed by determining the rate of disappearance of labelled EE or EECPE injected directly into the duodenum of the rat according to a method pre-

viously described(7). When administered as aqueous suspensions, about 90% of the administered dose of EE or EECPE had disappeared from the intestine at 30 minutes after administration. On the other hand, when the two compounds were dissolved in sesame oil only 61% of the administered dose of EECPE and 85% of that of EE had disappeared from the intestine at 8 hours after administration. A similar amount (56% of the administered dose) had disappeared from the intestine at 8 hours after administration of EECPE dissolved in sesame oil containing 25% w/v of glyceryl mono-oleate (Steinetz and Meli, unpublished data). These findings confirm Kimbel's observation(3) that the use of a lipid vehicle considerably slows down the rate of intestinal absorption of steroids.

There are two possible explanations for the peculiar behavior exhibited by EECPE: a) When administered as aqueous suspension, the lymphatic drainage could not keep pace with the extremely fast rate at which the compound was absorbed, b) when administered dissolved in a lipid vehicle, an aliquot of EECPE could have been bound (chemical or physical binding) to some long chain fatty acids and/or monoglycerides of long chain fatty acids (fatty acids having a chain length of 16 or more carbon atoms) which are known to be exclusively transported from the intestine *via* the lymphatic route(8-13).

The fact that a similar phenomenon did not occur in the case of EE which, like EECPE, was absorbed from the intestine at a significantly slower rate when administered dissolved in sesame oil than as aqueous suspension suggests that the first explanation is less likely to be true.

Although this possibility cannot be completely ruled out, in favor of the second explanation are the observations that: a) unlike EE, the lymphatic transport of EECPE could be significantly increased by use of a lipid vehicle (sesame oil). b) Addition of 25% w/v of a monoglyceride to the sesame oil could further and significantly increase the lymphatic transport of EECPE without any concomitant change in the rate of intestinal absorption.

Our results with EECPE indicate that not only the chemical nature of the compound but also the nature of the vehicle plays an important role in determining pathway of transport.

Although when administered dissolved in a lipid vehicle, a significant amount of EECPE is transported directly by the lymphatic system, the portal route still remains the major pathway of transport of this compound.

Experiments are under way to determine whether or not the lymphatic transport of EECPE can be further influenced by the use of different monoglycerides and/or various concentrations of monoglycerides in sesame or other oils.

Summary. The pathway of transport of labelled ethynylestradiol (EE) or its 3-cyclopentyl ether (EECPE) has been studied following oral administration to rats.

The data on lymphatic transport of EE, whether administered as aqueous suspension or dissolved in a lipid vehicle, agree well with previous findings on other steroidal compounds which similarly indicate that: a) the portal system is the major, if not the exclusive pathway of transport of many steroids and b) the pathway of transport is independent of the vehicle of administration. By contrast, the lymphatic transport of EECPE could be significantly increased by use of a lipid vehicle indicating that not only the chemical nature of the compound but also the nature of the vehicle plays an important role in determining pathway of transport.

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Effects of Acetylcholine and Calcium Ions on the Spontaneous Release of Epinephrine from Catecholamine Granules.* (30999)

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The recent work of Douglas and Rubin(1,2) on perfused adrenal glands has led to the hypothesis that calcium ions are involved in "stimulus-secretion coupling." They suggest that acetylcholine and/or potassium evoke adrenal medullary secretion through an action on the chromaffin cell membrane leading to an increased influx of calcium ions(3) which are the immediate stimulus for the release of the catecholamines. The stimulant effect of acetylcholine and potassium required the presence of calcium ions; the stimulant effect of calcium ions, under appropriate conditions, did not require the presence of acetylcholine or potassium.

70-80% of the catecholamine content of the adrenal medullary cell is contained within the chromaffin granules which are easily isolated from the tissue homogenates(4,5). The physiologic role of these granules is uncertain. Is their catecholamine content held primarily as a reserve store or is their catecholamine content continuously available? The morphologic basis of the catecholamine extrusion is uncertain. A number of authors have described an intracellular leakage of electron dense material, presumably the catecholamine, from the chromaffin granules(6,7,8). On the other hand, De Robertis and Vaz Ferreira(9) have described a complex chain of events during which the chromaffin granules migrate towards the cell membrane and attach themselves to it before extruding their content into the extracellular space. Thus, an agent or agents (*e.g.* acetylcholine, calcium) interacting with these granules need not penetrate the cell membrane if they are available when the granule membrane becomes continuous with cell membrane.

The effect of Ca^{++} on the release of catecholamines from bovine chromaffin granules in suspension in sucrose has been investigated with varying results. Phillipu and Schumann (10) obtained a dose related release of catecholamine from 50-150% above control with increasing concentrations of Ca^{++} from 2.5-12.5 mM. Other workers(11,12,13,14) observed a negligible release; however, they used lower concentrations of Ca^{++} in the range from 1-5 mM. Therefore, as discussed by Douglas and Rubin(2) it seemed appropriate to determine whether Ca^{++} might have an enhanced or different effect on chromaffin granules prepared in electrolyte media rather than in sucrose.

We had studied the spontaneous release of epinephrine from chromaffin granules in suspension in homogenates prepared from the adrenal gland of the rat(5). The spontaneous release was accelerated in electrolyte media and at increased temperature as had been noted with bovine(12,14) and rabbit granules (15); and acetylcholine, which depletes the adrenal gland *in vivo*, was ineffective as had been noted with bovine(10,12,16) and rabbit granules(15). However, reserpine was ineffective either as a releaser or as an inhibitor of release(5) in contrast with data obtained by others with bovine(13,17,18,19,20) and rabbit chromaffin granules(15).

In this report(21), we have examined for the effect of calcium ions, over a wide range of concentrations (.0025-25 mM), on the

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