mately 100 times the growth inhibitory concentration of chloramphenicol.

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Influence of Vehicle of Administration on Intestinal Absorption, Fat Storage, and Biological Activity of Ethynylestradiol (EE) and its 3-Cyclopentyl Ether (EECPE) in Rats. (31432)

B. G. STEINETZ, A. MELI, V. L. BEACH, AND T. GIANNINA
Department of Physiology, Warner-Lambert Research Institute, Morris Plains, N. J.

Ethynylestradiol-3-cyclopentyl ether (EECPE)* is more potent orally than its parent compound, ethynylestradiol (EE)† as a uterotrophic agent and as a gonadotrophin inhibitor in laboratory animals(1-5). Unlike EE, EECPE is stored in body fat and brain (6,7) and sustained release from these depots may account for its enhanced potency. The greater affinity for lipids of EECPE as compared with EE may also influence route of absorption from the gut. Thus, when administered in sesame oil rather than aqueous suspension, a significant amount of EECPE but not EE is transported through the lymphatic system(8).

The above findings suggest fundamental differences in the interaction of EECPE and EE with lipids, both as constituents of sesame oil and of body fat. Present experiments show that intestinal absorption of EECPE from sesame oil is qualitatively and quantitatively different from that of EE and that vehicle of administration greatly affects storage of EECPE in body fat and brain.

Materials and methods. (A) *Preparation of radioactive steroid doses.* Ethynylestradiol-6,7-H^3-3-cyclopentyl ether, specific activity (s.a.) 0.78 $\mu c/\mu g$ and ethynylestradiol-3-cyclopentyl-1-C^{14} ether, s.a. 0.392 $\mu c/\mu g$ were mixed to give an H^3 to C^{14} ratio of approximately 10:1 and a s.a. of approximately 0.26 μc H^3 and 0.026 μc $C^{14}/\mu g$ EECPE. Ethynylestradiol-6,7-H^3, s.a. 0.93 $\mu c/\mu g$ was diluted with unlabeled EE to give a s.a. approximately the same as that of EECPE (*i.e.*, 0.26 $\mu c/\mu g$). Doses for administration (approx. 5 μc or 19.1 μg of steroid) were either dissolved in sesame oil or suspended in an aqueous vehicle (0.9% NaCl, 0.4% Tween 80, 0.5% carboxymethylcellulose, 0.9% ben-

* 3-cyclopentyloxy-19-nor-17 α-pregna-1,3,5(10)-trien-20-yn-17 β-ol.

† 19-nor-17 α-pregna-1,3,5(10)-trien-20-yne-3,17 β-diol.

zyl alcohol). Aliquots of the final dosage forms were always taken for liquid scintillation counting to establish the exact amount of radioactivity. Crude data were then corrected in proportion to a constant dose of 5 μc H^3 and 0.5 μc C^{14} where applicable. This correction was considered legitimate because actual doses did not vary by more than $\pm$ 20% of this value. The correction normalizes values in the same sense as calculating percentage of administered dose.

(B) *Intestinal absorption.* (1) *In the presence of bile and pancreatic secretions.* Male rats, Hemlock Hollow, weighing 300-350 g were used. Under ether anesthesia, the small intestine was exposed through a midline incision of the abdomen. Ligatures were placed at the pyloric and iliac ends and the labeled compounds (as oily solutions or aqueous suspensions) were introduced directly into the intestine by means of a soft catheter through a small incision 2 cm below the pyloric ligature. A loose loop of silk was placed around the catheter and tied off upon its withdrawal to prevent any leakage(9). The animals were exsanguinated by heart puncture at varying time intervals after administration. The segment of intestine between the two ligatures was carefully removed and the intestinal lumen washed consecutively with 50 ml absolute ethanol and 50 ml methylene chloride. The washes were combined for analysis. In addition to plasma, perirenal fat was excised, processed and analyzed.

(2) *In the absence of bile and pancreatic secretions.* The aim of these experiments was to prevent emulsification and hydrolysis of the lipid vehicle in order to determine the influences of these two processes on the release and subsequent absorption of the compounds dissolved in such a vehicle. Sex and size of the animals as well as surgical procedures were similar to those described under #1. A ligature was placed at the iliac end of the small intestine and the labeled compounds were injected through a small incision 3-4 cm below the entry of the ductus choledocus. A polyethylene tubing (Intramedic (R) PE 190) was introduced into the upper portion of the intestine through a small incision 2 cm below the entry of the ductus choledocus and secured in place by means of a silk ligature. Upon closure of the abdominal incision, this tubing was exteriorized to prevent accumulation of bile and other extra-intestinal and intestinal secretions. Groups of 3 animals per each compound were killed 1, 3 and 6 hours after administration. The small intestine, with exception of the tract above and immediately below the entry of the ductus choledocus was carefully removed. Thereafter the procedure was similar to that described under #1.

(C) *Fat storage.* Male rats, Hemlock Hollow, 300-350 g body weight, received a single oral dose of 5 μc EECPE-6,7-H^3 either dissolved in sesame oil or suspended in an aqueous vehicle. At various time intervals, groups of 4 animals each were exsanguinated by heart puncture and plasma, perirenal fat and brain were obtained and analyzed.

(D) *Uterotrophic activity.* Oily solutions or aqueous suspensions of scalar doses of EE or EECPE were administered by gavage for 3 consecutive days to immature female rats 30-35 g body weight. On the morning of the 4th day the animals were killed and the wet weight of the uterus (after pressing out the intrauterine fluid) determined to the nearest 0.1 mg.

(E) *Analysis of intestinal washings.* An aliquot (0.5 ml) of the ethanol-methylene chloride wash was added directly to dioxane cocktail‡ and counted. The remainder (approx. 100 ml) was taken to dryness by flash evaporation and then transferred by means of 6 alternate washes with water (total of 10 ml) and ether (total of 20 ml) to a 40 ml glass stoppered centrifuge tube. The washes were shaken together and the ether layer transferred to a clean 50 ml graduated centrifuge tube. The aqueous phase was shaken with another 20 ml ether. The ether extracts were combined, adjusted to 40 ml and dried over Na_2SO_4. One to 10 ml of the ether extract (free fraction) was evaporated in a counting vial and radioactivity determined in 15 ml dioxane cocktail. The remaining

‡ Dioxane cocktail: 7 g PPO (2,5-diphenyloxazole), 0.3 g dimethyl POPOP (1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene) and 100 g naphthalene in 1 L redistilled dioxane.

ether extract was saved for thin layer chromatography (TLC). The aqueous phase (ether non-extractable) was found to contain conjugated metabolites which had been excreted back into the intestine *via* the bile. Hence only the free fraction could be used to measure rate of intestinal absorption. Rate of absorption was calculated at each time interval as:

$$\% \text{ Dose absorbed} = \frac{\text{Dose administered } (\mu c) - \text{Free fraction of intestinal wash } (\mu c)}{\text{Dose administered } (\mu c)} \times 100$$

(F) *Analysis of plasma.* Three ml of plasma were diluted to 5 ml with distilled water and shaken 3 times with 10 ml ether. The radioactivity in 10 ml of the combined ether extracts was determined as above and the remainder saved for TLC. The radioactivity remaining in the aqueous phase was also determined. This fraction was designated "total conjugates" on the basis of other work which demonstrated the presence of glucuronide and sulphate conjugates.

(G) *Analysis of perirenal fat and brain.* Steroids present in these tissues were extracted with methylene chloride as previously described(6,7) and radioactivity was determined by liquid scintillation spectrometry.

(H) *Thin layer chromatography (TLC) of extracts.* The only compound previously found in fat or brain of EECPE-treated animals was EECPE itself(7), while no steroids were detected in fat or brain of rats treated with EE(6,7). Accordingly these tissues were not investigated chromatographically in the present study. The ether soluble ("free") fractions obtained from intestinal washing and plasma were chromatographed on silica gel G using an ascending system of methanol-chloroform-water (485:15:1) as previously described(7). Non-radioactive standards were run on the same chromatograms and visualized by exposure to iodine vapors. The areas corresponding to the standards, as well as 1 cm segments taken consecutively from the origin to the solvent front, were scraped into counting vials, eluted with 0.5 ml benzene-ethanol (1:1) and counted in 14 ml dioxane cocktail.

(I) *Counting procedures.* Tritium and

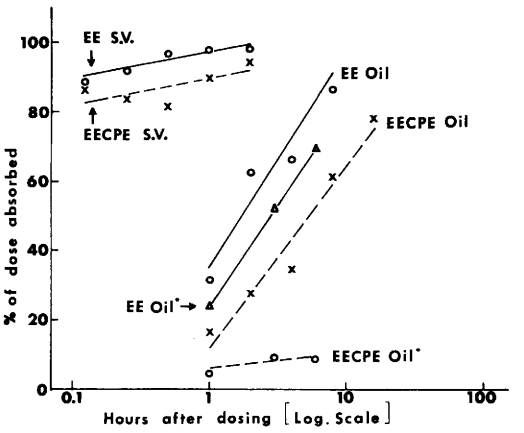


FIG. 1. Intestinal absorption of EE and EECPE from suspending vehicle (S.V.) or sesame oil. Doses were 5 μc H^3/19.1 μg steroid introduced directly into small intestine. Each point represents mean value for 2-4 rats. Slopes were: EE oil (in presence or absence of bile and pancreatic secretions) 45.44, 52.66; EECPE in oil 58.72; EECPE in oil in absence of bile and pancreatic secretions 6.11 (not significantly greater than a slope of zero). The other slopes did not differ from each other significantly.

C^{14} were determined simultaneously on a 3 channel liquid scintillation spectrometer (Packard Model 3324) equipped with external standard for quench correction.§

Results. (A) *Intestinal absorption.* (1) *In the presence of bile and pancreatic secretions.* When 5 μc aqueous suspensions of EE or EECPE were introduced into the intestine more than 80% of the administered dose had disappeared after 7½ minutes. Thereafter, the data obtained fall on the upper asymptote of the absorption curve and do not reflect true rates (Fig. 1). When administered as oily solutions EE or EECPE were absorbed at a much slower rate. However, during the first hour 3 times as much EE as EECPE had disappeared from the intestine. Thereafter, the 2 compounds were absorbed at a rate equivalent to 15.2% of the dose for each doubling of time (Fig. 1). When aliquots of free steroid fractions of intestinal washings of EECPE-treated rats were subjected to TLC about 50% of the radioactivity corresponded to unaltered EECPE and 30-40% was more polar than either

§ See Packard Instrument Bulletin on quench correction by automatic external standarization.

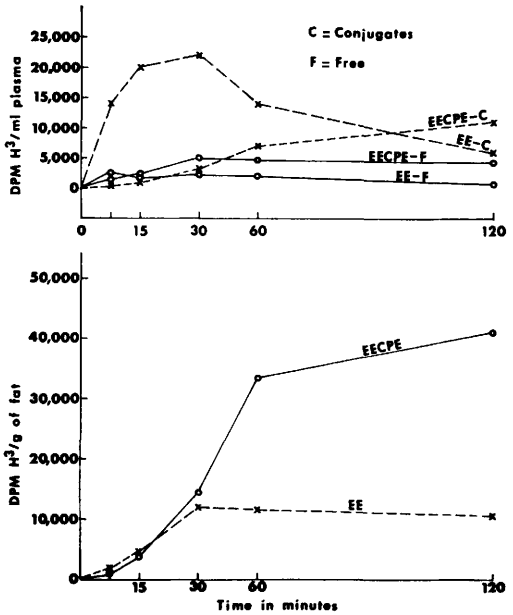


FIG. 2. Fat and plasma levels of radioactivity following intraduodenal administration of EE or EECPE in suspending vehicle. Doses were 5 μc H^3/19.1 μg steroid. Each point represents mean value for 2-4 rats. "Free" refers to ether extractable H^3 "conjugates" refer to ether insoluble radioactivity.

EECPE or EE. The H^3/C^{14} ratio was 10:1 in both areas. Similarly, chromatograms of intestinal washings of EE-treated animals showed only about 40% of the radioactivity at the EE spot with 30-40% more polar materials. Whether these polar substances in each case are products of intestinal metabolism or represent recycled biliary free metabolites is unknown at present. If the latter possibility is correct, then the amounts absorbed, but not the absorption rates themselves, have been underestimated.

Under these experimental conditions, plasma and fat levels of EECPE were also found to be influenced by the vehicle. When EECPE had been administered as aqueous suspension, radioactivity in body fat was still increasing at 2 hours although more than 80% of the administered dose had been absorbed in 7½ minutes (Fig. 2). When administered as oily solution, deposition of EECPE in body fat was slower and appeared to parallel roughly the absorption curve (Fig. 3). Plasma free and conjugated radioactivity rose much more slowly than in the case of aqueous vehicle (Fig. 3). Following EE administration, very little radioactivity accumulated in body fat, plasma free steroid H^3 remained low and plasma conjugated H^3 rose abruptly regardless of vehicle of administration (Fig. 2 and 3). The free steroid fraction of plasma did not have sufficient radioactivity for adequate characterization by TLC. However, radioactivity with an Rf value corresponding to that of EECPE or EE was observed in plasma of rats treated with EECPE or EE respectively. More polar substances were also detected in the plasma free fraction of EECPE- or EE-treated rats.

(2) *In the absence of bile and pancreatic secretions.* When EE in oil was introduced into a segment of small intestine from which bile and pancreatic juice were excluded, its initial absorption was delayed. However, after the first hour, EE was absorbed from the oil at a rate similar to that observed in the presence of bile and pancreatic secretions (Fig. 1). In contrast, absorption of EECPE from oil was severely hindered in the absence of bile and pancreatic juice (Fig. 1). Thus, 4 hours after administration in oil, the following percentages of the doses had been absorbed: EE (no interference) 73%; EE (exclusion of bile and pancreatic juice) 60%; EECPE (no interference) 40%; EECPE (exclusion of bile and pancreatic juice) 10%.

(B) *Influence of vehicle on amount and duration of storage of EECPE in body fat and brain.* Because vehicle appeared to influence the amount of EECPE stored in body fat (Section A), further experiments were performed to investigate this phenomenon. These experiments differed from the absorption study in that EECPE was administered by stomach tube to intact animals. Rats were killed at various time intervals up to 96 hours and perirenal fat, brain and plasma samples were obtained. Under these conditions, storage of EECPE in body fat reached a maximum at 8 hours and appeared to plateau for 48 hours (Fig. 4). However, twice as much compound was stored following administration in oil as was stored after administration in aqueous suspension (Fig. 4). Despite this difference, plasma free and conjugated steroids followed a remarkably similar pattern regardless of vehicle (Fig. 4). When the

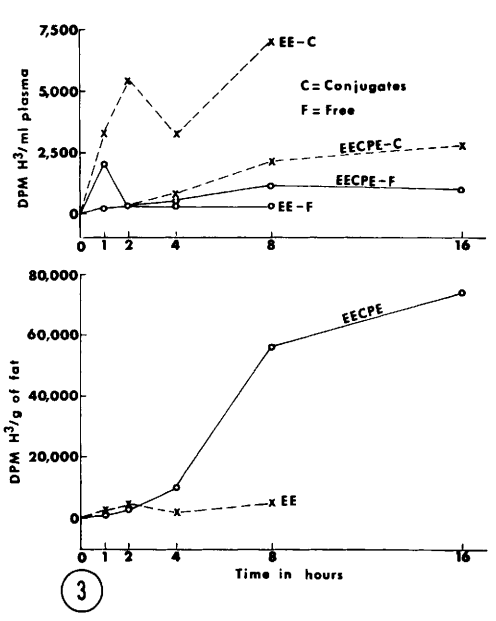
loss of radioactivity from brain fat was compared with that from perirenal fat, it was found that the slopes of the 2 lines differed significantly, $p < 0.01$ (Fig. 5). Choice of vehicle did not significantly influence this phenomenon (Fig. 5). These results confirm the previous report of Meli *et al*(7) that (non-radioactive) EECPE was cleared from brain more rapidly than from body fat.

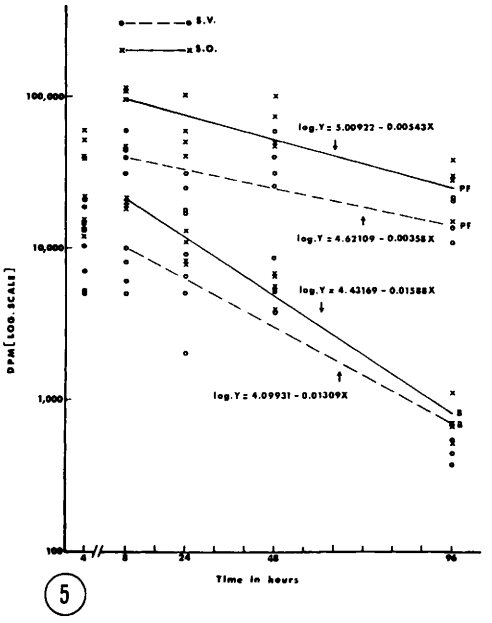
(C) *Influence of vehicle on biological activity of EE and EECPE.* The results of uterotrophic assays of the 2 estrogens in oil or suspending vehicle are shown in Fig. 6. Whereas vehicle did not influence the slope of the EE dose-response curve ($b = 33.1$ for oil or water), the slope of the curve for EECPE in oil was significantly greater than that for EECPE in aqueous suspension (56.2 *vs* 44.3; $p < 0.01$). Each of the EECPE slopes was significantly greater than that of EE ($p < 0.01$). Because of this, it was impossible to assign a fixed relative potency for the 2 steroids. However, at an arbitrary mid-response level (52 mg uterus) the relative potencies were: EECPE in aqueous suspension, 100; EECPE in oil, 71; EE in oil or aqueous vehicle, 53. The difference between EECPE in oil *vs* water is due entirely to the difference in the responses elicited by the low dose (0.05 μg) and vanishes at the higher response levels. This phenomenon was observed in 3 separate assays and does not appear to be an artifact. More likely, it is related to the combined effects of slower absorption and greater fat storage observed with EECPE in an oil vehicle. One might anticipate that as dose is lowered to near the threshold necessary for biological response, these factors might have an increasingly greater effect to reduce the amount of active compound available to the target organs.

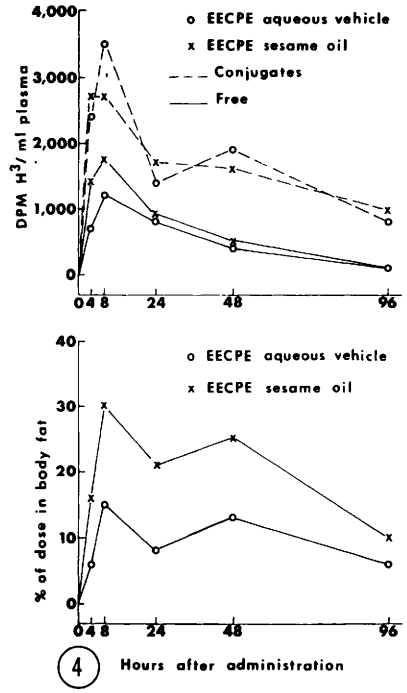
Discussion. When introduced directly into the small intestine, oily solutions of EE or EECPE were absorbed much more slowly than when administered as aqueous suspensions. Although oily solutions of EE or EECPE were absorbed at the same rate between the 1st and the 8th hour, there was an initial lag in absorption of EECPE so that 3 times as much EE as EECPE had disappeared from the intestinal lumen during the 1st hour following administration. Steroids are normally absorbed from the intestine by simple diffusion in both rats(10) and humans(11). Since absorption (diffusion) of EE or EECPE from aqueous suspensions was extremely rapid, the slower absorption from oily solutions could be attributed to the rate at which the steroid was released from the solvent and therefore free to diffuse through the intestinal wall.

To establish whether or not this process was in turn dependent upon the rate at which the oil vehicle was emulsified, hydrolyzed and absorbed, absorption of EE and EECPE was studied under conditions where bile and pancreatic secretions were excluded from the intestine. Because sesame oil is a mixture of long chain triglycerides(12), extensive hydrolysis (dependent upon the presence of bile and pancreatic lipase) to free fatty acids and monoglycerides is necessary for its absorption(13-15). The rate of disappearance of EE from the intestinal lumen after the first hour was similar whether or not bile and pancreatic secretions were present, whereas absorption of EECPE was severely hindered in the absence of these factors. Thus the initial lag phase in EECPE absorption, (which could be extended by excluding bile and pancreatic secretions from the intestine) likely represents the time required for hydrolysis of long chain triglycerides which in turn appears to be necessary for release of EECPE from the solvent. The factors responsible for the release of EE or EECPE from an oily solution under these experimental conditions may be similar to those which in general govern the release of steroids from subcutaneous injection sites(16). Among these factors are: a) the extension of the absorbing area which is proportional to the amount of solvent, b) the compound-binding ability of the solvent which determines the rate at which the compound is released and c) the rate at which the solvent itself is absorbed. All these factors are obviously interdependent and their interplay will determine the rate at which a particular compound is absorbed.

Although plasma levels of radioactivity were in harmony with intestinal absorption rates of labeled EECPE from oily solutions







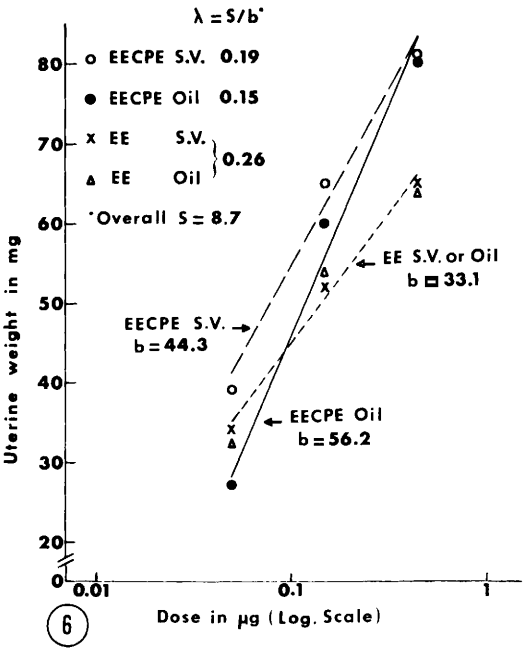


FIG. 3. Fat and plasma levels of radioactivity following intraduodenal administration of EE or EECPE in sesame oil. Conditions as in Fig. 2.

FIG. 4. Clearance of H^3 from perirenal fat (PF) and brains (B) of rats treated with single oral doses of 5 μc H^3 (19.1 μg) EECPE in suspending vehicle (S.V.) or sesame oil (S.O.). Each point represents a single rat. Storage in brain and fat was significantly greater ($p < 0.01$) when steroid was administered in S.O. rather than S.V. Clearance from brain was faster ($p < 0.01$) than from fat regardless of vehicle.

FIG. 5. Comparison of body fat stores and plasma levels of radioactivity following oral administration of 5 μc H^3 (19.1 μg) EECPE in sesame oil or suspending vehicle. Each point repre-

sents mean value of 4 rats. "Free" refers to ether extractable H^3 while "conjugates" refer to ether insoluble H^3. The fraction of the dose in body fat was approximated by multiplying concentration of H^3 found by estimated total weight of body fat (20% of the body weight) and dividing by dose administered.

FIG. 6. Uterotrophic assays of EE and EECPE in suspending vehicle (S.V.) or sesame oil. Each point represents mean value for 30 rats. S = standard error of assay, b = slope, λ= index of precision. The 3 slopes shown differ significantly from one another ($p<0.01$).

or aqueous suspensions, deposition in body fat appeared to be favored by the lipid vehicle. EE did not accumulate in fat regardless of vehicle of administration so that vehicle *per se* is not responsible for fat storage.

Enhancement of fat storage of EECPE by the lipid vehicle was confirmed in rats dosed by gavage. Plasma free and conjugated radioactive steroids were in general similar over the entire period studied (4-96 hours) regardless of vehicle employed. There was, however, a significantly lower concentration of free radioactive steroids at the 4th hour in plasma of rats treated with the aqueous suspension. This was not accompanied by an increased level of conjugated metabolites and therefore does not necessarily indicate a faster rate of metabolism. It might be argued that EECPE is absorbed so rapidly from aqueous suspensions that fat storage sites are overwhelmed. This seems unlikely since following an oral dose of 12.5 mg of EECPE in sesame oil, as much as 200 μg were stored per gram of fat (unpublished results). The potential storage capacity of each gram of fat is therefore more than 10-fold greater than the total dose (19.1 μg) given in our present experiments. Since lymphatic transport of EECPE in oily solutions only amounts to 7% of the administered dose(8) this additional route could hardly explain a doubling of the fat storage. The reason for the influence of the vehicle on fat storage remains unknown.

The rate of disappearance of fat or brain stored EECPE is independent of vehicle of administration. However, the steroid is cleared significantly faster from the brain than from body fat. Since EECPE is likely stored in the fat compartment of the brain, the accelerated disappearance from it may reflect either a lesser affinity for this type of fat and/or a different turnover of brain fat constituents.

Once released from fat, EECPE appears to lose its lipophilic properties. If this were not true, the amount of steroid stored in brain would be expected to decrease at a much slower rate due to a feedback to the brain from the much greater pool of steroid in body fat. Plasma free steroid levels declined more rapidly than did body fat stores, whereas the pattern of water soluble metabolites roughly paralleled loss from body fat. These data, as well as chromatographic evidence of free metabolites with greater polarity than EECPE further suggest that the compound is released from fat as one or more polar relatively lipophobic derivatives which are rapidly conjugated. A point is reached between 48 and 96 hours where, despite the existence of a significant concentration of EECPE in body fat, plasma free steroid levels become negligible.

Despite the marked effects of the vehicle on rate of absorption of EE and EECPE and on the amount of EECPE stored in body fat, biological activity was independent of vehicle of administration. Although lack of correlation between rate of absorption and biological activity has already been pointed out by others(10,11), the failure of the oil vehicle to increase the biological activity of at least EE is surprising. Enhancement of oral activity by a lipid vehicle has been reported in the case of several androgens and some glucocorticoids(17). This apparent difference between phenolic steroids and Δ^4-3-ketosteroids might be due either to the intrinsic nature of the molecule or to a different sensitivity and/or reactivity of the target organs.

Summary. When introduced directly into the small intestine of the rat, as oily solutions, ethynylestradiol (EE) or its 3-cyclopentyl ether (EECPE) were absorbed much more slowly than when administered as aqueous suspensions. These findings are in agreement with data in the literature on other steroidal compounds. Hydrolysis of the components of sesame oil (dependent upon the

presence of bile and pancreatic secretions) seems to be necessary for release and subsequent absorption of EECPE but not EE from the lipid vehicle. Storage in body fat and brain of the unaltered EECPE is increased by its administration as an oily solution instead of an aqueous suspension. The nature of the vehicle of administration, however, had no influence on the degree of biological activity of EECPE as well as EE.

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The Excretion of Taurine During Deoxypyridoxine Administration in Downs' Syndrome Patients and Controls.* (31433)

ERNEST E. MCCOY AND HARRY WEHRLE (Introduced by D. K. Meyer)
Department of Pediatrics, University of Missouri School of Medicine, Columbia

The metabolism of the sulfur-containing amino acid cysteine involves, as shown in Fig. 1, a number of anabolic and catabolic reactions. Among the principal catabolic reactions is the oxidation of cysteine to form cysteine sulfinic acid. Cysteine sulfinic acid or its oxidation product cysteic acid are in turn decarboxylated by a vitamin B_6 dependent reaction to hypotaurine or taurine. Urinary taurine is the most frequently measured metabolite that is used as an indicator of sulfur amino acid metabolism. The excretion of taurine in mammals is influenced in varying degrees by protein intake(1), cysteine loading(2), by irradiation(3,4), by trauma(5) and by vit B_6 intake(2). Decreased taurine excretion has been proposed as an indicator of inadequate vit B_6 levels(6). It has been shown by Goodman *et al*(7) that Downs' syndrome patients have a low base line excretion of taurine. Administration of the vit B_6 antagonist deoxypyridoxine was studied to determine if the effect on taurine excretion was similar to that of actual vit B_6 deficiency and if these effects were similar in a group of Downs' syndrome patients and matched controls. Of further interest was to determine if the previously demonstrated greater effect of deoxypyridoxine on the excretion of certain tryptophan metabolites in Downs' syndrome also resulted in greater changes in taurine excretion compared to control subjects. The results indicated that, after a significant ini-

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