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Studies on a Nitrogen Mustard of Estradiol in Dogs and Rats[1] (39752)

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Introduction. Estracyt, a conjugate of 17β-estradiol (E_2) with a carbamate (Fig. 1), [estradiol-3*N*-bis(2-chloroethyl)carbamate-17β-phosphate], has proven to be of value in the therapy of human prostatic cancer (1-3); consequently, its metabolic fate and biochemical effects in the human have been the subject of investigations communicated from this and other laboratories (4, 5). We have reported on the fate of estracyt labeled with ^3H in the E_2 moiety administered as a mixture with [^{14}C]E_2 to humans and baboons (4). These studies showed that about 10-15% of estracyt is hydrolyzed in the human in such a fashion as to yield [^3H]E_2 which is then metabolized and conjugated similarily to the coadministered [^{14}C]E_2, as evidenced by the fact that the [^3H]E_2 had urinary excretory and countercurrent distribution (CCD) patterns identical to those of the [^{14}C]E_2, even though the former was excreted at about one-third the rate of the latter. The remainder (unhydrolyzed) estracyt could not be accounted for. When doubly labeled estracyt (^3H in the steroid moiety and ^{14}C in the carbamate) was administered to human subjects, it was shown that the excretion of the ^{14}C (carbamate moiety) was much slower than that of the ^3H (estrogen moiety), indicating that hydrolysis of the molecule did, in fact, take place, but that the metabolism and excretion of the ^{14}C-labeled moiety and the bulk of the administered doubly labeled estracyt must follow routes other than those of the [^3H]E_2. Studies in the baboon (4) mirrored those in the human and demonstrated substantial excretion of singly and doubled labeled estracyt in the bile, indicating that this may be the major route by which the compound is excreted in this animal and, by analogy, in the human.

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The present study was performed in order to determine the metabolic fate and excretion patterns of estracyt in dogs and rats, using methods previously described (4), i.e., administration of a mixture of [^3H]-estracyt and [^{14}C]E_2.

Materials and methods. [^3H]Estracyt (^3H at positions 6 and 7 of E_2, 3.65 μCi/μmole) was obtained from AB Leo of Helsingborg, Sweden and checked for purity by extraction as reported (4) and by countercurrent distribution (CCD) in the solvent system ethyl acetate:*n*-butanol:0.2% ammonia in water; 1:3:4 (in which all distributions reported below were performed). In this solvent system, estracyt has a $K = 48.5$ ($N = 97$, $n = 99$). [4-^{14}C]E_2 (53 μCi/μmole) and carrier E_2 were purchased from the New England Nuclear Corp. and Organon Corp., respectively; their purity was checked by paper chromatography and scanning for radioactivity or by spraying with phenol reagent (Fisher) and exposing the paper to fumes of concentrated ammonia, respectively.

All injections were done as described previously (4, 5): Dogs received approximately 20 μCi of an accurately determined amount of ^3H, whereas rats received about 5 μCi. Radioactivity was determined as reported previously (4-6).

Six adult female mongrel dogs were used in this study. The animals with and without bile fistulas were prepared as previously described (6): Anesthesia was carried out using sodium nembutal (25 mg/kg). The common bile duct was surgically exposed using sterile technique, catheterized with PE-260 tubing, and ligated distally for bile collection.

A saline iv drip of 50 ml/hr was maintained during bile and/or urine collections. Urine was collected from female Wistar rats with a technique previously described for guinea pigs (13): The bladder was catheterized suprapubically and ligated around the

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catheter to ensure quantitative recovery of urine. Bile was collected using tubing that had been drawn to proper size after slight heating in a flame (Tygon Micro-Bore Formulation, Norton Plastics and Synthetics Division, Akron, Ohio). The animals were hydrated during collections by an iv drip of saline delivered either into the tail vein or into a vein in the inguinal region. The saline solution containing equimolar amounts of the radioactive compounds was injected into the tubing of the iv drip.

Results. The excretion data from all dogs and a representative excretion curve from one of the biliary fistula animals (no. 2) are given in Table I and Fig. 2, respectively. After 8 hr of collection, the averages for the urinary excretions in biliary fistula animals were 22.4% (range 19.7–25.5%) for ^{3}H and 24.8% (range 17.4–30.2%) for ^{14}C. Since interruption of bile flow had little effect on the rate of urinary excretion, it is probable that during the 8-hr collection little enterohepatic circulation of the administered estracyt had taken place. Biliary excretions averaged 24.1% (range 17.8–33.3%) for ^{3}H and 24.3% (range 19.6–34.8%) for ^{14}C.

The rates of excretion of the ^{3}H associated with the labeled E_2 moiety of estracyt and of the ^{14}C of the administered E_2 were very similar, both in the bile and urine (Table I and Fig. 2). This indicates that in all probability the administered estracyt is readily hydrolyzed in the dog, leading to the metabolism of its E_2 moiety in the same fashion as that of the coadministered E_2.

The ^{14}C associated with administered E_2 was excreted in the bile and urine of the rats at a much faster rate than ^{3}H associated with estracyt (Table II). Furthermore, excretion of radioactivity (^{3}H and ^{14}C) was much higher in bile than in urine. Thus, the interruption of the biliary route did not affect the urinary excretion of ^{3}H and ^{14}C, and, therefore, in the rat fecal excretion is probably the major route of elimination of the metabolism of E_2 and estracyt. The differences in the excretory rates between ^{3}H and ^{14}C

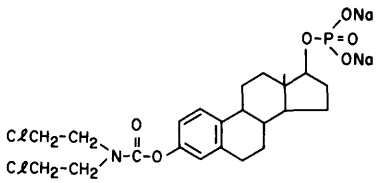

FIG. 1. Chemical formula of the disodium salt of estracyt showing it to be an ester of a nitrogen mustard and E_2, the former being attached to the latter at position 3. A phosphate group is present at position 17.

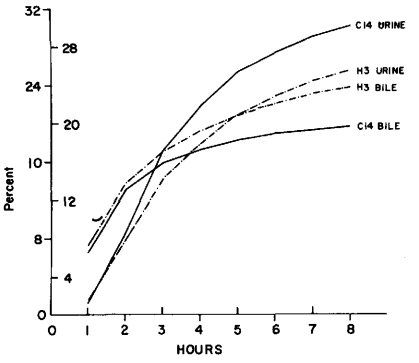

FIG. 2. Cumulative excretion of radioactivity in the urine and bile of dog 2 injected with [^{3}H]estracyt (broken lines) and [^{14}C]E_2 (solid lines). The excretion of the two labels was similar and indicates extensive and ready hydrolysis of estracyt (see text).

TABLE I. EXCRETION OF RADIOACTIVITY IN BILE AND URINE OF DOGS INJECTED WITH A MIXTURE OF [^{3}H]ESTRACYT AND [^{14}C]E_2.

	Percentage of injected dose excreted in			
	Urine		Bile	
Animal	^{3}H	^{14}C	^{3}H	^{14}C
1	23.1	29.2	33.3	34.8
2	25.5	30.2	23.8	19.6
3	21.2	22.5	17.8	20.2
4	19.7	17.4	21.5	22.5
5	28.9	27.8		
6	20.4	17.4		

TABLE II. CUMULATIVE EXCRETION OF RADIOACTIVITY IN BILE AND URINE OF RATS INJECTED WITH A MIXTURE OF [^{3}H]ESTRACYT AND [^{14}C]E_2.

		Percentage of injected dose excreted in			
		Urine		Bile	
Animal	Hours after injection	^{3}H	^{14}C	^{3}H	^{14}C
1	4	0.2	1.3	8.8	22.0
	19	4.4	8.8	16.3	43.6
	24	5.9	10.5	16.9	44.2
2	9	0.7	1.2	36.0	65.6
	28	1.1	6.9	37.6	88.7
3	24	4.5	18.8		
4	17	1.2	3.8		

point to retention within the body of the bulk of the administered estracyt, at least during the period of study (17–28 hr), and when compared to the rate of excretion of E_2.

In order to ascertain whether, in fact, the E_2 released from estracyt was metabolized very similarly to the administered E_2, the patterns of the conjugate metabolites excreted in the urine and bile were analyzed by CCD. Even though there were a few minor peaks in the CCD of the biles and urines associated with the ^3H which were not seen in the ^{14}C curves (Figs. 3–5), the bulk of the radioactivity of both administered compounds was distributed in peaks which were very similar. The fact that some of the peaks did not match in altitude may be indicative that the hydrolysis of the labeled estracyt occurred at sites which ultimately resulted in higher excretion of one metabolite over another, when compared to the distribution of the [^{14}C]E_2. However, qualitatively the CCD pattern for the E_2 in the estracyt was very similar to that of the [^{14}C]E_2 administered to dogs and rats. The small ^3H peaks for which ^{14}C analogs were not present may be indicative of the excretion of intact estracyt, either metabolized and/or conjugated. This was true for both the bile and the urine.

Discussion. The approaches utilized in the present study, i.e., the injection of labeled estracyt (in the E_2 moiety) and differently labeled unconjugated E_2 (4), allow a comparison of the fate of the administered substances in at least two areas. One is a comparison of the excretion rate which, if it is equal for both isotopes, indicates that the estracyt molecule had been almost quantitatively hydrolyzed in the body, thus resulting in the metabolism of the E_2 moiety very similar to that of unconjugated E_2. This is predicated on the assumption that the excretion of the intact estracyt molecule probably cannot occur as rapidly and readily as that of E_2. To further substantiate that the E_2 released upon hydrolysis of estracyt does, in fact, take a metabolic route very similar to that of unconjugated E_2, a comparison of the CCD patterns is a second crucial step. If the CCD patterns are very similar, they indicate that the released E_2 had been metab-

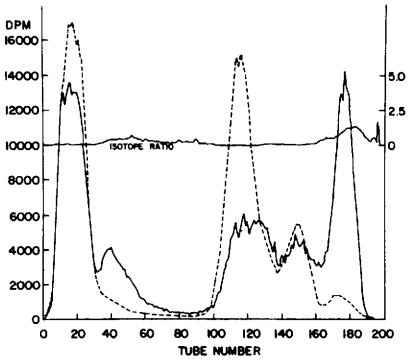

FIG. 3. CCD pattern of the bile of dog 4 following the administration of [^3H]estracyt (solid line) and [^{14}C]E_2 (broken line). Qualitatively the patterns are similar, with only a minor peak of ^3H at tube 40 not being reflected in the ^{14}C curve (see text).

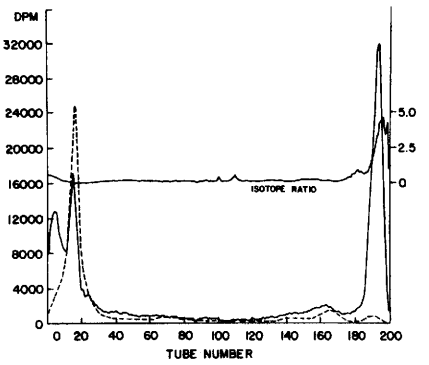

FIG. 4. CCD patterns of the urine of dog 4 (see legend to Fig. 2) showing qualitatively similar patterns for ^3H and ^{14}C. A ^3H peak at the beginning of the CCD is not seen in the ^{14}C curve and may indicate excretion of some intact estracyt (see text).

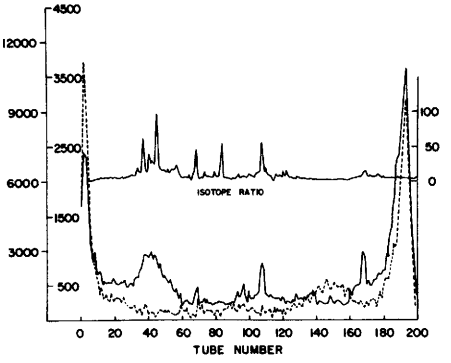

FIG. 5. CCD patterns of the urine of rat 4 injected with [^3H]estracyt and [^{14}C]E_2. Qualitatively the patterns are similar, except for a ^3H peak at the beginning of the distribution (see text).

olized in a fashion very similar to that of E_2. Major deviations from the CCD pattern of unconjugated E_2 would indicate that the route taken by the E_2 in estracyt is quite different and indicative that the estracyt moiety had remained intact. In the dog the rate of excretion of the E_2 in estracyt was very similar to that of the unconjugated E_2 and points very strongly to rapid and almost quantitative hydrolysis of the molecule following its iv injection into that animal. That this does, in fact, occur is pointed to by the finding of hemopoietic toxicity following estracyt injection as one of the major manifestations in the dog (unpublished observations from our laboratory and AB Leo, Helsingborg, Sweden). Such toxicity has not been observed in other animals or the human (1–4). The essential similarity between the CCD patterns observed in the bile and urine of the dog is further evidence that hydrolysis of the estracyt molecule had occurred in this animal. On the other hand, the excretion in the rat of the radioactivity associated with estracyt was very slow and at a much lower rate than that of the unconjugated E_2. Retention by rat tissues of intact estracyt has been reported (7, 8) and the results obtained in the present study substantiate these findings. It should be pointed out, however, that the E_2 released upon hydrolysis of the estracyt molecule in the rat behaved metabolically in a manner very similar to that of unconjugated E_2. However, the total amount of E_2 in estracyt which was released by hydrolysis during the time of the study was relatively small.

Due to the unavailability, at present, of doubly labeled estracyt, the approach utilized in the present studies appears to offer a direct comparison between the metabolic fate of labeled estracyt, particularly of its estradiol moiety, with that of labeled unconjugated E_2. A comparison of the metabolism of unconjugated E_2 and estracyt in the human, baboon, dog, and rat (4, 5) indicates that each species metabolized estracyt differently and, hence, in evaluating the clinical use of drugs of a nature similar to that of estracyt it may be imperative to determine its metabolic fate in the human, rather than decide upon its behavior in man from extrapolation of results obtained in animals.

In developing drugs in which a cytotoxic agent is chemically bonded to a steroid, e.g., in the case of estracyt a mustard bonded to E_2, advantage is taken of the possibility that the drug may be preferentially localized in certain tissues due to their content of receptors for E_2. The presence of such receptors for E_2 in the prostates of the rat, human, and baboon has been reported (9–11). In the human localization of E_2 has been described not only in normal prostatic tissue, but also in cancerous prostates (10, 12). However, quantitative hydrolysis of the steroid conjugate prior to its exposure to the target cells would nullify the advantages of such a conjugate. The persistence of some intact steroid conjugate may lead to its preferential localization within certain target tissues; this may be the case with estracyt in some animal tissues, e.g., the prostate in the rat and man. Thus, it has been demonstrated that following administration of estracyt (100 mg/kg) to rats intact estracyt accounted for the preponderant amount of the metabolites present in the prostate (ventral), though a significant portion of it had been oxidized to the estrone mustard (8). The value of estracyt in the treatment of cancer of the prostate in man points to the necessity of applying similar approaches in developing conjugate drugs with predilection for other tissues and, thus, possibly for cancers at these sites.

Summary. Estracyt, a conjugate of 17β-estradiol (E_2) and a cytotoxic agent (carbamate), labeled with ^3H in its E_2 moiety was administered to dogs and rats and the fate of the compound was compared to that of coadministered [^{14}C]E_2. Excretion of radioactivity was determined in bile and urine and the excretory patterns were established by CCD. This approach allows a determination of the hydrolysis of the estracyt molecule by comparing the behavior of the two labels. In the dog, the radioactivities appeared in the bile and urine at the same rate, indicating ready hydrolysis of the estracyt. In the rat, however, the ^3H was excreted at a much slower rate with very small amounts appearing in the urine within 24 hr and relatively large amounts appearing in the bile; however, most of the estracyt was retained in the body of the rat. When hydrolysis takes place in the dog and rat, the released E_2 behaves

similarly to that of administered E_2.

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