

Metabolism of arterial plasma estrogens by the splanchnic organs of the dog *in vivo*

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Research Article

In order to study the splanchnic metabolism of blood-borne estrogens, a constant infusion of estrone-6,7³H was made in a series of dogs, and arteriovenous (A-V) differences at equilibrium were determined for estrone-6,7³H and for its products estradiol-17 β , estrone sulfate, estrone glucosiduronate, and estradiol-17 β glucosiduronate across the splanchnic bed (artery-hepatic vein), the small intestine (artery-superior mesenteric vein), and the spleen (artery-splenic vein). Per cent extractions (100 - [V/A] 100) were calculated. The plasma metabolic clearance rate (MCR) for estrone was measured. Principal findings were as follows: mean MCR was 731 liters/day per m², SEM 50. By comparison with estimated hepatic plasma flow and using the observed splanchnic extraction of estrone, 45-71% of estrone metabolism was calculated to be extrasplanchnic. The significant mean per cent extractions were as follows (SEM in parentheses): splanchnic bedestrone 85.9 (1.92), estradiol-17 β 88.11 (3.36), estrone sulfate 27.9 (5.22), estrone glucosiduronate -48.5 (9.33), estradiol-17 β glucosiduronate -33.3 (80.3); small intestine—estrone 45.3 (2.60), estradiol-17 β 46.1 (12.9), estrone glucosiduronate - 30.8 (7.9); spleen—estrone 35 (3.8), estrone glucosiduronate 12 (3.7). These results lead to the following conclusions. Both estrone and estradiol-17 β are nearly completely extracted in one passage through the splanchnic bed. There is net uptake of estrone sulfate and net production of estrone glucosiduronate and of estradiol-17 β glucosiduronate by the splanchnic bed. There is net uptake of estrone and of estradiol-17 β [...]

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Metabolism of Arterial Plasma Estrogens by the Splanchnic Organs of the Dog In Vivo

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A B S T R A C T In order to study the splanchnic metabolism of blood-borne estrogens, a constant infusion of estrone-6,7-³H was made in a series of dogs, and arteriovenous (A-V) differences at equilibrium were determined for estrone-6,7-³H and for its products estradiol-17 β , estrone sulfate, estrone glucosiduronate, and estradiol-17 β glucosiduronate across the splanchnic bed (artery-hepatic vein), the small intestine (artery-superior mesenteric vein), and the spleen (artery-splenic vein). Per cent extractions (100 - [V/A] 100) were calculated. The plasma metabolic clearance rate (MCR) for estrone was measured. Principal findings were as follows: mean MCR was 731 liters/day per m², SEM 50. By comparison with estimated hepatic plasma flow and using the observed splanchnic extraction of estrone, 45-71% of estrone metabolism was calculated to be extrahepatic. The significant mean per cent extractions were as follows (SEM in parentheses): splanchnic bed—estrone 85.9 (1.92), estradiol-17 β 88.11 (3.36), estrone sulfate 27.9 (5.22), estrone glucosiduronate — 48.5 (9.33), estradiol-17 β glucosiduronate — 33.3 (8.03); small intestine—estrone 45.3 (2.60), estradiol-17 β 46.1 (12.9), estrone glucosiduronate — 30.8 (7.9); spleen—estrone 35 (3.8), estrone glucosiduronate 12 (3.7). These results lead to the following conclusions. Both estrone and estradiol-17 β are nearly completely extracted in one passage through the splanchnic bed. There is net uptake of estrone sulfate and net production of estrone glucosiduronate and of estradiol-17 β glucosiduronate by the splanchnic bed. There is net uptake of estrone and of estradiol-17 β by the intestine, associated with substantial net production of estrone glucosiduronate. There is net uptake of estrone by the spleen and a small but significant net uptake of estrone glucosiduronate.

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INTRODUCTION

The evident importance of the enterohepatic circulation of estrogens in the human has stimulated considerable investigation into the details of its operation. Thus, a number of studies have been devoted to the nature and origin of estrogen secreted into the bile (1-12). Furthermore, it has recently been shown that estrogens are actively metabolized as they pass through the enterohepatic circulation. For instance, estrogens brought to the intestine via the bile undergo transformations in the intestinal lumen and on passage through the intestinal wall (5, 6, 10-12). The transformations have been well documented in the case of estriol conjugates (6, 10-12).

However, these studies relate mainly to estrogens within the human enterohepatic circulation. There is virtually no knowledge in any species regarding the immediate metabolism of estrogens brought to the splanchnic area in arterial blood, and the nature and amounts of estrogens released by the splanchnic area into the systemic venous system. We are also largely ignorant of the separate activities of the major splanchnic organs in the metabolism of arterial estrogens and of the nature and relative concentrations of estrogens in the different parts of the splanchnic venous system.

It is therefore clear that an investigation of over-all splanchnic metabolism of arterial estrogens, together with an analysis of the separate activity of intestines and spleen, would be of a great potential value. Appropriate studies in the human have been impeded by the difficulty in approaching the venous sampling sites. An animal model was therefore necessary, and the male dog was chosen. The metabolism of radioactive estrone by this animal was recently investigated in work from this laboratory and found to be similar (within the limits of the investigation) to that in the human, except for the substitution of the product estriol-16 α , 17 α for the human estriol-16 α , 17 β (13). Furthermore, this animal is

known to have an enterohepatic circulation for estrogens (14).

Accordingly, constant infusions of estrone-6,7-³H were set up in a series of male dogs, and simultaneous arterio-venous concentration differences were determined for radioactive estrone and for its products estradiol-17 β , estrone sulfate, estrone glucosiduronate, and estradiol-17 β glucosiduronate across the splanchnic bed as a whole (artery-hepatic vein), across the intestine (artery-superior mesenteric vein), and across the spleen (artery-splenic vein). In order to relate splanchnic estrone metabolism to over-all estrone metabolism, estrone metabolic clearance rates (MCR) were also estimated.

METHODS

Operative procedure. Mongrel male dogs weighing 14.5-29.9 kg were anaesthetised with i.v. pentobarbital, 25 mg/kg. An intratracheal tube was inserted. A cardiac catheter was passed into the right jugular vein and thence into the hepatic vein under fluoroscopic control. The catheter was passed as far into the liver substance as possible. The position of the catheter tip was checked at intervals by fluoroscopy. The right lobe of the liver was catheterized usually, and the left lobe occasionally. No difference in results relative to the lobe catheterized was observed. Although no studies to determine specifically the effect of pentobarbital anesthesia on splanchnic estrogen metabolism were carried out, the widespread use of this anaesthetic in studies in the dog and its absence of effects on hepatic blood flow (15) and oxygen consumption (16) seemed to justify its use in these experiments without further testing.

A small incision was made in the abdominal wall. A small plastic catheter was inserted into a branch of the superior mesenteric vein draining the small intestine and secured, the tip pointing towards the intestine. A small plastic catheter was also inserted into the splenic vein and secured with its point as near the spleen substance as possible (to avoid reflux from the left gastroepiploic vein). The abdomen was closed leaving the sampling ends of the catheters outside. All three veins (hepatic vein, superior mesenteric vein, and splenic vein) were not necessarily catheterized in the same dog. The femoral artery was catheterized. The left jugular vein was catheterized for introducing the loading dose and infusion. Blood samples (5 ml) were drawn simultaneously from each sampling site into heparinized tubes. Total volume of blood withdrawn was usually 45 ml, or occasionally 60 ml, over a 15-20 min period. No effects due to the withdrawal of this relatively small volume of blood were observed.

Loading dose and infusion of estrone-6,7-³H. Authentic estrone-6,7-³H (see below) was dissolved in 0.1 ml of 95% ethanol, and diluted to 20-30 ml with 0.9% (w/v) saline. The loading dose (radioactivity approximately 20 μ Ci, mass approximately 0.15 ng) was given into the left jugular vein as a single injection. The infusion (radioactivity approximately 100 μ Ci, mass approximately 0.75 ng) was drawn into a calibrated syringe, which was then inserted into a Harvard constant infusion pump (Harvard Apparatus Co., Inc., Millis, Mass.), connected to the left jugular vein catheter, and administered at a constant rate immediately after the loading dose. Aliquots of loading dose and of infusion were taken for subsequent radioactivity measurement. The whole infusion was administered in 35-45 min. After 20

min of infusion, samples were obtained from catheterized sites as described above at approximately 5-min intervals.

Estrogen standards. Estrone-6,7-³H (stated specific activity 30-50 Ci/mmol) was obtained from New England Nuclear Corp. (Boston, Mass.). Each batch, which was used within 2 months of receipt, was tested at intervals for radiochemical purity using column partition chromatography as already described (17). Over 96% of the total radioactivity was contained in a chromatographic peak which corresponded exactly to a peak due to authentic nonradioactive estrone (see below) when both radioactive and nonradioactive substances were chromatographed together. On crystallization of a mixture of the radioactive material and authentic nonradioactive estrone, specific activity of crystals and mother liquor corresponded and did not vary significantly on repeated crystallization. Estradiol-17 β -6,7-³H (stated specific activity 30-50 Ci/mmol) and estrone-6,7-³H sulfate (stated specific activity 10-15 Ci/mmol) were obtained from New England Nuclear Corp. These substances were used only in recovery experiments. Tests for radiochemical purity were carried out as described above for estrone with similar results. Authentic estrone, estradiol-17 β and estrone sulfate standards were obtained from Mann Research Labs. Inc., New York. In each case melting points were in the expected range. On column partition chromatography approximately 95% of the total fluorescence (see below) of each standard was contained in a single peak in the chromatographic position expected for each substance. Nonradioactive estrone glucosiduronate and estradiol-17 β -17-glucosiduronate standards were very kindly given by Dr. A. E. Kellie. The methods of synthesis of these conjugates have been described (18). On testing >95% of total fluorescence was contained in a single peak after column partition chromatography. Estradiol-17 β -3-glucosiduronate was prepared by sodium borohydride reduction of estrone glucosiduronate.

Fluorometry. Fluorescence was obtained by heating the estrogens with sulfuric acid and was measured as described by Preedy and Aitken (19).

Radioactivity measurements. Radioactivity was measured by scintillation counting, using a Packard (Model 3000 or 4000 series) scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.). For estrone, estradiol-17 β , and estriol, the conditions (including scintillation fluid) were those previously described (13). The estrogen conjugates were counted in 0.2 ml of water mixed with 10 ml of dioxane containing 10 g/liter of 2,5-diphenyloxazole, 0.15 g/liter of 1,4-bis-2(4-methyl-5-phenyloxazole)-benzene, 150 g/liter of napthalene, and 20 ml/liter of ethylene glycol.

Determination of estrogens in plasma samples. Plasma was separated promptly from red cells and stored at -15°C until analyzed. Known amounts of estrone, estradiol-17 β , estrone sulfate, estrone glucosiduronate, and estradiol-17 β -17-glucosiduronate were added as internal standards to 8 ml of 95% aqueous ethanol. An aliquot (usually 1 ml) of plasma was then added drop-wise to the aqueous ethanol with stirring to precipitate proteins. The precipitate was separated by filtration or centrifugation, then the filtrate or supernatant was dried. The dried extract was redissolved in 70% aqueous methanol and kept at -15°C for 48 hr to precipitate lipids. The precipitate was separated by centrifugation or filtration at -15°C, and the supernatant or filtrate was dried.

Unconjugated estrone and unconjugated estradiol-17 β . The dried extract was partitioned between ether and water. The aqueous phase was saved. The ether extract was dried,

redissolved in a small amount of stationary phase, and applied to the column partition system of Preedy and Aitken (19). Chromatography was carried out as described (19) except that only the first two sequential mobile phases were used (13, 19), to separate estrone and estradiol-17 β . Fractions were collected and were analyzed for radioactivity and for the mass of the internal standards, estrone and estradiol-17 β .

Estrone sulfate. The aqueous phase previously saved was adjusted to pH 6.0 using 0.1 M acetate buffer and 2 mg of the phenolsulphatase preparation Mylase P (Wallerstein Laboratories) per ml of solution added. The solution was incubated at 37°C for 18 hr. The aqueous phase was then extracted with ether as described above. The aqueous phase was saved. The ether fraction was evaporated and the residue chromatographed as already described for unconjugated estrone and estradiol-17 β . Fractions were collected and analyzed for radioactivity and for the mass of estrone (derived from added estrone sulfate) as described above. (No estradiol-17 β sulfate was found in any sample.)

Estrone glucosiduronate and estradiol-17 β glucosiduronate. The aqueous phase previously saved was adjusted to pH 5.0 using 0.1 M acetate buffer. Beef liver β -glucuronidase (Ketodase, Warner-Chilcott Laboratories, Morris Plains, N. J.) was added (100,000 β -glucuronidase U/100 ml of solution) and incubated for 16 hr at 37°C. The solution was then extracted with ether, and the ether extract was subjected to column partition chromatography as described above. Fractions were collected, and radioactivity and mass of the added standards were measured (as the aglycones), as described above. This procedure measures estrone glucosiduronate and estradiol-17 β glucosiduronate (the term estradiol-17 β glucosiduronate is here used to mean any glucosiduronate or mixture of glucosiduronates of estradiol-17 β).

Correction for methodological losses. Values obtained for the concentration of radioactive estrogens were corrected for losses by means of the internal standards in the normal way. Recoveries of the internal standards were in the following range: estrone and estradiol-17 β , 75-85%; estrone sulfate, 60-75%; and estrone and estradiol-17 β -17-glucosiduronate, 50-70%.

In later experiments estradiol-17 β -17-glucosiduronate was used as the only glucosiduronate internal standard, since the amounts of estrone glucosiduronate available were limited. However, it had previously been shown that recovery of estrone glucosiduronate and of estradiol-17 β -17-glucosiduronate standards were similar, so that this imposed restriction did not alter the analytical conditions significantly.

In the last few experiments (dogs 19, 20, and 21) no standards were added. In the previous experiments the rate of recovery of each of the standards used has been established. Consequently, it was considered justifiable to use the observed mean recovery rate to correct for losses in those dogs where this was necessary (as in the MCR determinations, Table I). For the A-V differences this correction was not applied, since recovery rates from arterial and venous samples would be similar.

Test of the extraction procedure. Known amounts of the internal standards, estrone (about 10 μ g), estradiol-17 β (about 10 μ g), estrone sulfate (about 10 μ g), estrone glucosiduronate (about 30 μ g), and estradiol-17 β -17-glucosiduronate (about 30 μ g), were added separately and in turn to the plasma ethanol mixture described above using 1 ml of plasma from untreated dogs. The analytic procedure was then continued. When either estrone or estradiol-17 β were added, virtually all added material was recovered in the first

ether extract, as demonstrated after partition chromatography and sulfuric acid fluorescence. None of the added estrone or estradiol-17 β could subsequently be detected after Mylase P and Ketodase treatments with ether extraction, partition chromatography, and fluorometry after each treatment. On estrone sulfate addition, no estrone or estradiol-17 β was detected in the first ether extract or third ether extract after Ketodase. After Mylase P most of the estrone sulfate was recovered as estrone in the second ether extract. When estrone glucosiduronate or estradiol-17 β -17-glucosiduronate were added, no estrone or estradiol-17 β was detected either in the first ether extract or in the second ether extract after Mylase P treatment. Most of the estrone glucosiduronate and estradiol-17 β -17-glucosiduronate was recovered after the Ketodase treatment and the third ether extraction.

Identification of radioactive unconjugated estrogens. Further identification of the radioactivity peaks presumed due to estrone and estradiol-17 β both in the unhydrolyzed and the hydrolyzed plasma samples was obtained in the following two ways: (a) demonstration that the specific activity of a mixture of the material forming a radioactive peak with the corresponding nonradioactive estrogen standard did not change significantly after acetylation and rechromatography carried out as already described (13), and (b) demonstration that on crystallization of a mixture of the material forming a radioactive peak and the corresponding nonradioactive estrogen standard the specific activity of crystals and mother liquor were similar and did not change significantly after repeated crystallization (13).

Identification of radioactive conjugated estrogens. More direct identification of the radioactive conjugates (estrone sulfate, estrone glucosiduronate, and estradiol-17 β glucosiduronate) were carried out as follows. Plasma from artery and from hepatic, superior mesenteric and splenic veins (one sample from each site) was extracted with ether, and the ether fraction discarded. The aqueous phase was made 80% in respect of ethanol and was centrifuged. The following nonradioactive standards were added to the supernatant: estrone sulfate, estrone glucosiduronate, estradiol-17 β -3-glucosiduronate, and estradiol-17 β -17-glucosiduronate. The dried extract was applied to a Florisil column and eluted successively with 50 ml each of ethyl acetate, ethyl acetate-methanol (1:1), methanol, and methanol-water (1:1). In this system estrogen sulfates are eluted in the ethyl acetate-methanol mixture and glucosiduronates in the methanol and methanol-water mixtures.

The ethyl acetate-methanol fraction (sulfates) was dried down, a further estrone sulfate standard was added, and the material was rechromatographed on a celite column using chloroform: n-heptane: *t*-butanol: 1 M NH₄OH (150:75:25:300). Aliquots of the fractions containing estrone sulfate were analyzed for radioactivity and mass as already described. The remainder of the material was chromatographed again on Silica Gel G plates with benzene: methyl ethyl ketone: ethanol: water (3:3:3:1) (20) as eluent, with standard estrone sulfate as marker. Radioactivity and mass were again determined. Specific activity of the material did not change significantly after the two chromatographic procedures. An aliquot was treated with Mylase P under conditions already described, and the hydrolysis product identified as estrone, using chromatography, derivative formation, and repeated crystallization as already described.

Material eluted in the methanol and methanol-water fractions (glucosiduronates) from the Florisil column was combined with additional estrone glucosiduronate, estradiol-17 β -3-glucosiduronate, and estradiol-17 β -17-glucosiduronate

standards and chromatographed with DEAE-Sephadex, using as eluent increasing concentrations of NaCl (0.1-0.2 mole/liter) in water in the form of a linear gradient (21). This system separates estrone and estradiol-17 β glucosiduronates. The specific activity of material forming the estrone glucosiduronate peak was estimated. The material was then subjected to thin-layer chromatography on Silica Gel G plates with benzene: methanol: acetic acid (70:30:10) and instant thin-layer chromatography (ITLC) (silica gel impregnated glass fiber sheets, Gelman Instrument Company, Ann Arbor, Mich.) with chloroform: acetone: acetic acid mixtures (75:25:5 or 75:50:5) as eluent. Specific activities of the material corresponding to estrone glucosiduronate were again determined and did not change significantly after chromatography. The material was then hydrolyzed using Ketodase (as already described), and the hydrolysis product identified as estrone by derivative formation and by repeated crystallization as already described.

The estradiol-17 β glucosiduronate zone from the DEAE-Sephadex column contained two adjacent radioactivity peaks. The smaller peak corresponded with the added estradiol-17 β -17-glucosiduronate standard. The specific activity of this peak was estimated, and the material rechromatographed in the thin-layer systems described above. Specific activity of the material corresponding to estradiol-17 β -17-glucosiduronate was determined and was found not to change significantly after chromatography. The larger estradiol-17 β glucosiduronate peak corresponded with the estradiol-17 β -3-glucosiduronate standard. The material contained in each estradiol-17 β glucosiduronate peak was hydrolyzed separately using Ketodase. The product of hydrolysis in each case was identified as estradiol-17 β by derivative formation and repeated crystallization as already described. Details of the above procedures for identifying estrogen conjugates will be described elsewhere.

Calculation of metabolic clearance rate (MCR) and per cent extraction (% extr.). The MCR of estrone was calculated in the usual way (22) using the formula $MCR = r/x_i$, where r is the rate of radioactive estrone infusion (cpm/min), and x_i the concentration of arterial radioactive estrone at equilibrium (cpm/ml). This gives the MCR in ml/min, which can then be converted to ml/min per kg, or liter/day per m^2 (Table I). The surface area of the dog (m^2) was calculated from the weight of the dog by the Meeh-Rubner formula (23). The per cent extraction = $100 - [V/A]$ 100, where V is the concentration (cpm/ml) of a radioactive estrogen in a vein and A the concentration (cpm/ml) of the same radioactive estrogen in an artery, when samples are obtained simultaneously.

Statistics. The statistical design consisted of the following two complementary analyses: (a) analysis for regression and (b) analysis for random variation or scatter. To determine the significance of trends related to time, linear regression analysis was carried out on the serial arterial and venous concentrations in each dog, where a series contained more than two observations. The series of per cent extractions in each dog were similarly analyzed. For the analysis for random variation, means (m), standard error of the mean (SEM), and coefficients of variation (C of $V = SEM \times 100/m$) were calculated by standard procedures. For the A-V differences the mean difference (d) and standard error of the mean difference (SEM_d) were calculated by the usual methods, and the paired t test used to test the significance of a mean difference. The significance of a mean per cent extraction was estimated by testing the significance of the difference between the mean per cent extraction and zero. A

value for P of < 0.03 is taken to indicate "significance." The importance of each form of statistical analysis in the interpretation of the data is brought out in the Discussion section.

RESULTS

Significant regression was found in a small percentage of series only, as follows: 2 (artery, dog 18; superior mesenteric vein, dog 5) of 38 series of estrone values, 2 (hepatic vein, dog 19; superior mesenteric vein, dog 10) in 23 series of estrone sulfate values, and 8 in 35 series of estrone glucosiduronate values (artery, dogs 12, 18, 21, 25; hepatic vein, dogs 6, 12; superior mesenteric vein, dogs 6, 18). There was no significant regression in any of the series of estradiol-17 β or estradiol-17 β glucosiduronate values. There were therefore only 12 instances of significant regression in a total of 138 arterial and venous series. Analysis of the per cent extractions showed that significant regression was present only for estrone glucosiduronate, 3 of 24 series (artery-hepatic vein, dogs 8, 18; artery-superior mesenteric vein, dog 22). There were therefore only three instances of significant regression in a total of 81 series of per cent extractions. Where regression was observed the values for the regression coefficient were positive (indicating rising values with time), except in two of the three per cent extraction series for estrone glucosiduronate, where the values were negative (dogs 18, 22).

The plasma metabolic clearance rate (MCR) for estrone in 11 dogs is given in Table I. MCR's are expressed as ml/min, ml/min per kg, and liter/day per m^2 for internal comparison and for comparison with published results from the human. Mean MCR as ml/min was 931 (SEM 76), as ml/min per kg was 42.2 (SEM 2.9) and as liter/day per m^2 was 731 (SEM 50). Coefficients of variation were 8.1, 6.9, and 6.8, respectively, indicating a decrease in scatter when correction for weight was applied. There was little further decrease when the figures were corrected for estimated surface area. The series from which the average arterial estrone values (Table I, column 3) were calculated are given in Table IV. There was no significant regression in any of these series.

The values for estrone, estradiol-17 β , estrone sulfate, estrone glucosiduronate, and estradiol-17 β glucosiduronate in artery (A) and hepatic vein (HV) plasma sampled simultaneously are given in Table II, together with the per cent extractions. There was a significant mean A-HV difference (12 dogs, 39 samples) for estrone ($P < 0.001$) and a mean per cent extraction of 85.9 (SEM 1.92), which was significantly different from zero ($P < 0.001$). The results for estradiol-17 β (11 dogs, 26 samples) were similar. There was a significant mean A-HV difference ($P < 0.001$) and a mean per cent extraction of 88.1 (SEM 3.36), which was significantly different from zero ($P < 0.001$).

TABLE I
Plasma Metabolic Clearance Rate of Estrone in 11 Dogs

Dog	Estrone-6, 7- ³ H		Plasma metabolic clearance rate		
	Infusion rate cpm/min $\times 10^3$	Average* arterial plasma concentration cpm/ml	ml/min	ml/min per kg	liter/day per m ²
6	2190	1920	1140	57.0	959
7	1920	2190	877	51.0	817
8	1930	2380	810	32.8	592
9	2310	1730	1330	44.6	857
10	2270	3070	738	29.1	530
12	2400	5000	481	33.2	502
15	2080	1730	1200	55.2	953
17	1860	2330	800	33.3	596
19	3020	2660	1140	44.0	801
20	1350	1810 [†]	744	38.2	636
21	1310	1320 [†]	991	46.5	799
	Mean	931	42.2	731	
	SEM	76	2.9	50	
	Coefficient of variation	8.1	6.9	6.8	

* Average of three or four consecutive samples obtained at approximately 5-min intervals.

† Values corrected for average recovery rate of 80% (see text).

There was a significant mean A-HV difference (10 dogs, 28 samples) for estrone sulfate ($P < 0.001$) and a mean per cent extraction of 27.9 (SEM 5.22), which was significantly different from zero ($P < 0.001$).

There was a significant mean A-HV difference (13 dogs, 41 samples) for estrone glucosiduronate ($P < 0.001$), but in this case the mean difference was negative. The mean per cent extraction was -48.5 (SEM 9.33) which was significantly different from zero ($P < 0.001$). Exclusion of the two cases in which there was significant regression in the series of per cent extractions (dogs 8, 18) had no significant effect on these statistics.

The findings for estradiol-17 β glucosiduronate were similar to those for estrone glucosiduronate. There was a significant mean A-HV difference (11 dogs, 27 samples) ($P < 0.01$), and this was negative. The mean per cent extraction was -33.3 (SEM 8.03) and was significantly different from zero ($P < 0.001$).

Corresponding values for artery (A) and superior mesenteric vein (SMV) plasma are given in Table III. There was a significant mean A-SMV difference (9 dogs, 29 samples) for estrone ($P < 0.005$) and a mean per cent extraction of 45.3 (SEM 2.60), significantly different from zero ($P < 0.001$). Similar results were obtained for estradiol-17 β . The mean A-SMV difference (5 dogs, 12 samples) was significant ($P < 0.01$) as was the mean per cent extraction of 46.1 (SEM 12.9) ($P < 0.003$).

There was no significant A-SMV difference for estrone sulfate (7 dogs, 17 samples) or for estradiol-17 β glucosiduronate (5 dogs, 13 samples) and no significant mean extraction. However, the comparatively high values for SEM_A and SEM should be noted.

There was a significant mean A-SMV difference (8 dogs, 25 samples) for estrone glucosiduronate ($P < 0.02$), and this was negative. The mean per cent extraction was likewise significant and was negative, -30.8 (SEM 7.9) ($P < 0.003$). Exclusion of the single case in which significant regression in the per cent extraction series occurred (dog 22) did not significantly affect the statistical results.

The mean A-SMV difference and mean per cent extraction for estradiol-17 β was not significant (5 dogs, 13 samples). Here again the relatively high values for SEM_A and SEM should be noted.

Values for arterial (A) and splenic vein (SPV) concentrations of estrone and estrone glucosiduronate are given in Table IV. (Values for estradiol-17 β , estrone sulfate, and estradiol-17 β glucosiduronate are omitted since many concentrations were too low to be estimated accurately, and the remaining figures were too few to allow meaningful statistical analysis.) There was a significant mean A-SPV difference (5 dogs, 15 samples) for estrone ($P < 0.01$) and a significant mean per cent extraction of 35 (SEM 3.8) ($P < 0.01$). There was a

TABLE II

Simultaneous Arterial (A) and Hepatic Venous (HV) Plasma Concentrations and Per Cent Extraction (% extr.) of Radioactive Estrone, Estradiol-17 β , Estrone Sulfate, Estrone Glucosiduronate, and Estradiol-17 β Glucosiduronate after Infusion of Estrone-6,7- 3 H in 14 Dogs

Dog No.	Sample No.	Estrone			Estradiol-17 β			Estrone sulfate			Estrone glucosiduronate			Estradiol-17 β glucosiduronate			
		A	HV	% extr.*	A	HV	% extr.*	A	HV	% extr.*	A	HV	% extr.*	A	HV	% extr.*	
<i>cpm/ml</i>			<i>cpm/ml</i>			<i>cpm/ml</i>			<i>cpm/ml</i>			<i>cpm/ml</i>			<i>cpm/ml</i>		
5	1	8680	630	93	544	73	87	296	215	27	10800	16000	— 54	1400	1950	— 39	
	2	10500	443	96													
	3	12900	517	96													
6	1	1970	245	88	219	0	100	296	215	27	713	1110	— 56	713	1110	— 56	
	2	1630	100	94													
	3	2060	77	96													
	4	2020	125	94													
7	1	2100	190	91	299	0	100	239	147	38	845	1200	— 42	406	348	14	
	2	1872	122	94	316	0	100	208	144	31	501	1361	— 171	440	449	— 2	
	3	2600	144	95	384	0	100	306	261	15	1390	2040	— 47	392	391	0	
8	1	2410	375	84	253	0	100	149	179	— 20	2410	3450	— 43	580	960	— 65	
	2	1740	385	78	190	0	100	316	223	29	1820	2820	— 55	499	550	— 10	
	3	2990	425	86	178	0	100	233	325	— 39	3180	5310	— 67	983	1400	— 42	
9	1	1699	568	67	462	105	77	192	118	39	657	1110	— 69	236	385	— 63	
	2	1796	512	72	440	86	81	374	303	19	617	1100	— 78	328	708	— 116	
	3	1702	273	84	459	80	83	444	121	73	668	1150	— 72	331	463	— 331	
10	1	3250			713	0	100	403	288	29	2100	1900	10	455	610	— 34	
	2	2950			866	160	82	617	431	30	1804	3100	— 72	717	927	— 29	
	3	3000			1200	0	100	626	423	30	2230	3180	— 43	801	1070	— 34	
12	1	4190	231	95	615	65	89	337	75	78	450	754	— 68	274	200	27	
	2	3450	1180	66	254	167	34	474	259	45	932	3150	— 238	570	731	— 28	
	3	5540	455	92				439	204	54	2030	3250	— 60				
	4	6803	1822	73				581	250	57	2580	5270	— 104	805	1760	— 118	
15	1	1900			175			205			948			331			
	2	1880			186			160			1210			463			
	3	1410			224			206			1620						
17	1	2170	79	96	296	0	100	181	222	— 22	1370	1550	— 13	229	292	— 28	
	2	2070	153	93	305	77	75	284	118	58	741	1190	— 60	292	473	— 62	
	3	2740	111	96	433	0	100	248	215	13	956	1370	— 43	176	325	— 85	
18	1	816	36	96							212	384	— 81				
	2	974	78	92							484	798	— 65				
	3	1370	97	93							856	1333	— 56				
	4	1488	98	93							954	1323	— 39				
19	1	2328	772	67				168	103	39	369	1020	— 176				
	2	2829	752	73				208	189	10	861	631	58				
	3	2816	1120	60				478	290	39	1124	884	21				
20	1	1091	68	94				144	140	3	230	165	28				
	2	1612	625	61				184	189	— 3	202	536	— 165				
	3	1645	723	56							1198	1460	— 22	188	262	— 39	
21	1	989	65	93							1386	1509	— 9	132	279	— 111	
	2	1037	41	96	148	0	100				1513	2085	— 38	233	370	— 59	
	3	1144	65	94	223	0	100										
22	1	1639	276	83	376	92	76	114	92	19	485	418	14				
	2	2238	208	91	415	31	93	140	100	29	613	643	— 5	162	116	28	
	3	3028	239	92	715	112	84	194	75	62	984	760	13	318	280	12	
Mean difference		2512			388			99			— 896			— 212			
SEM _A		399			49.3			20.6			213			58			
P		<0.001			<0.001			<0.001			<0.001			<0.01			
Mean % extr.		85.9			88.1			27.9			— 48.5			— 33.3			
SEM		1.92			3.36			5.22			9.33			8.03			
P _†		<0.001			<0.001			<0.001			<0.001			<0.001			

Serial samples were taken at approximately 5-min intervals.

* % extr. = 100 — [HV/A]100.

† Significance of difference between mean per cent extraction and zero.

TABLE III

Simultaneous Arterial (A) and Superior Mesenteric Venous (SMV) Plasma Concentrations and Per Cent Extraction (% extr.) of Radioactive Estrone, Estradiol-17 β , Estrone Sulfate, Estrone Glucosiduronate and Estradiol-17 β Glucosiduronate after Infusion of Estrone-6,7- 3 H in Nine Dogs

Dog No.	Sample No.	Estrone			Estradiol-17 β			Estrone sulfate			Estrone glucosiduronate			Estradiol-17 β glucosiduronate		
		A	SMV	% extr.*	A	SMV	% extr.*	A	SMV	% extr.*	A	SMV	% extr.*	A	SMV	% extr.*
		<i>cpm/ml</i>			<i>cpm/ml</i>			<i>cpm/ml</i>			<i>cpm/ml</i>			<i>cpm/ml</i>		
5	1	8680	2570	71												
	2	10500	3880	63	544	312	43									
	3	12900	4890	62	915	219	76									
6	1	1970	822	58												
	2	1630	1330	18												
	3	2060	1290	37	219	0	100	150	236	— 57	2320	2390	— 3	282	287	— 2
	4	2020	1490	26	120	163	— 36	296	363	— 23	4940	3820	23	1080	611	— 43
8	1	2410	1880	22	253	0	100	149	603	— 304	2410	3220	— 34	580	805	— 39
	2	1740	1100	37	190	216	— 14	316	291	8	1820	1970	— 8	499	394	21
	3	2990	1530	49	178	0	100	233	658	— 182	3180	4150	— 30	983	926	6
10	1	3250	1910	41	713	680	26	403	478	— 19	2100	2720	— 30	455	739	— 62
	2	2950	1960	34	866	590	32	617	604	2	1804	1790	1	717	550	23
	3	3000	1790	40	1200	578	52	626	811	— 30	2230	2270	— 2	801	885	— 11
18	1	816	350	57												
	2	974	644	34												
	3	1370	805	41												
	4	1488	700	53												
19	1	2328	1522	35												
	2	2829	1718	39												
	3	2816	1598	43												
20	1	1091	531	51												
	2	1621	646	60												
	3	1645	715	57												
21	1	989	376	62												
	2	1037	382	63	148	127	14									
	3	1144	511	55	223	89	60									
22	1	1639	1250	14												
	2	2238	1462	35												
	3	3028	1616	47												
Mean difference		1444			216				— 61.3			— 205			5.0	
SEM _d		359			67.7				41.5			78.8			52.3	
P		<0.005			<0.01				NS			<0.02			NS	
Mean % extr.		45.3			46.1				— 34.8			— 30.8			— 10.5	
SEM		2.60			12.9				20.8			7.9			10.5	
P _f		<0.001			<0.003				NS			<0.003			NS	

Serial samples were taken at approximately 5-min intervals.

* % extr. = 100 — [SMV/A]100.

† Significance of difference between mean per cent extraction and zero.

small but significant mean A-SPV difference (5 dogs, 15 samples) for estrone glucosiduronate ($P < 0.01$) and a mean per cent extraction of 12.0 (SEM 3.7) which was also significant ($P < 0.01$). It is to be noted that the per cent extraction in this instance was positive.

DISCUSSION

The necessity for establishing a steady state in the determination of the metabolic clearance rate (MCR) is apparent. In particular, there can be no trend with time in the serial arterial concentrations of the infused sub-

stance. On regression analysis any value for the regression coefficient significantly different from zero will invalidate the MCR calculation since the concentration at which the system may eventually stabilize is not known. Difficulty has been experienced by some workers in obtaining a steady state both of precursor and of products during the infusion of radioactive estrone and of estradiol-17 β in the human (24). In the present work there was no significant linear relationship to time in any of the series of arterial concentrations from which the MCR was calculated (Tables I and II).

TABLE IV
Simultaneous Arterial (A) and Splenic Venous (SVP) Plasma Concentrations and Per Cent Extraction (% extr.) of Radioactive Estrone and Estrone Glucosiduronate after an Infusion of Estrone-6, 7-³H in Five Dogs

Dog No.	Sample No.	Estrone			Estrone glucosiduronate		
		A	SVP	% extr.*	A	SVP	% extr.*
<i>cpm/ml</i>							
21	1	989	771	22	1198	1074	11
	2	1037	735	29	1386	1248	10
	3	1144	686	40	1513	1238	19
22	1	1639	1480	10	485	462	5
	2	2238	1498	33	613	646	-5
	3	3028	1755	42	984	626	36
23	1	995	780	21	372	390	-5
	2	1220	776	36	379	414	-9
	3	1289	976	24	590	540	8
24	1	214	121	43	216	160	26
	2	222	111	50	210	150	29
	3	251	122	51	121	110	9
25	1	203	63	69	130	106	18
	2	184	125	32	151	156	-3
	3	200	155	23	179	123	31
Mean difference		313		72.3			
SEM _d		84		29.0			
P		<0.01		<0.03			
Mean % extr.		35.0		12.0			
SEM		3.8		3.7			
P _‡		<0.01		<0.01			

Serial samples were taken at approximately 5-min intervals.

* % extr. = 100 - [SVP/A]100.

† Significance of the difference between the mean per cent extraction and zero.

Inspection of the serial arterial estrone concentrations for those dogs in which the MCR was calculated (Table II) indicates considerable variation in some instances. In the absence of significant regression such variation can be considered random and will include possible variation in the experimental system and variation due to the collection and analysis of samples. The coefficient of variation for all MCR values (expressed as liter/day per m²) was found to be 6.8 (Table I). This compares with coefficients of variation for estrone MCR of 6.1 (males) and 5.3 (females) found in humans by Longcope, Layne, and Tait (22), 8.1 (males) and 3.6 (females) found by Hembree, Bardin, and Lipsett (24), and 7.4 and 8.0 for testosterone and Δ_4 -androstenedione MCR's in dogs found by Chapdelaine (25). Thus, the variation observed in the present study

is in line with that found by other workers, and our MCR determinations appear valid insofar as they can be tested.

The mean plasma MCR for estrone in our series of male dogs can be compared with those reported for the male human. Mean plasma estrone MCR in nine males reported by Longcope et al. (22) was 1310 liters/day per m² (SEM 80) and in five males by Hembree et al. (24) was 1168 liters/day per m² (SEM 95). These mean values are substantially greater than those found in dogs in the present study (mean 731 liters/day per m²) presumably indicating a species difference. The mean plasma estrone MCR in the present series may also be compared with the estimated hepatic plasma flow (EHPF). The estimated hepatic blood flow (EHBF) in dogs lies in the range 23-44 ml/min per kg (26). Using an assumed mean hematocrit of 40%, we get an EHPF of 14-26

ml/min per kg. (Adjusting the EHBF figures for hematocrit is appropriate since the hematocrit has already been used for calculating the EHBF.) It will be seen that the values for MCR (mean 42.2 ml/min per kg, SEM 2.9) are substantially greater than the values for EHPF. Since the mean splanchnic extraction of estrone has been shown in the present study (Table II) to be 85.9% (SEM 1.92), 45-71% of the total metabolism of estrone in dogs must therefore take place outside the splanchnic area. This in general agreement with the finding in human males where a minimum of 35% of the metabolism of estrone was calculated to be extrasplanchnic (22). (A minimal figure only can be given for the human since the splanchnic extraction of estrone in the human is as yet unknown.) In our dogs, red cells contained < 10% of total blood estrone-6, ^7H , and this was true of both arterial and hepatic venous blood.

A steady state is also required in general if a comparison of simultaneous arterial and venous concentrations is to yield valid information of uptake or release of substances by tissues. This subject has been fully discussed by Zierler (27). Ideally tissue metabolism, blood flow, arterial, and venous concentrations should all remain constant during the period of observation. However, these parameters can seldom be measured independently, particularly in the case of tissue metabolism. The monitoring of blood flow is obviously desirable but was not considered practicable in the present study. Splanchnic blood flow estimations usually depend on hepatic extraction of dyes such as sulfobromophthalein (BSP). There is good evidence that estrogens depress the hepatic $T_{1/2}$ for BSP (28-30). Conversely it seemed likely that BSP administration would depress the hepatic extraction of estrogens. In the present study the volume of blood drawn was considered too small to have any significant effect; and since nothing else was done specifically to alter blood flow during arterial and venous sampling, any alterations in the splanchnic flow that may have occurred during the course of the experiments were assumed to be small and random.

As already stated the results of regression analysis of serial arterial and venous concentrations and of per cent extractions in Tables II-IV indicated no relationship to time in the majority of instances. In the few instances where significant regression in the arterial series did occur, it was possible to apply a correction. An arterial sample can be corrected for one transit time through the system before comparison with the simultaneously obtained venous sample (27). Although transit times through the splanchnic bed of the dog specifically for the various estrogens is not known, the transit time for blood has been measured and is in the range of 30-50 sec (31). When this correction was applied, only trivial alterations in the observed A-V differences resulted indicating

that the transit time of the blood through the splanchnic bed is not a significant factor in interpretation of the A-V differences obtained in the present study.

The variation to be observed in some of the arterial series has been mentioned above. This variation also extends to some of the venous and per cent extraction series (Tables II-IV). The sources of this variation have already been referred to and in the absence of regression in the vast majority of cases may be considered random. Such variation for all dogs in the appropriate categories is measured as SEM_A or SEM (see Statistics). Where "significance" is obtained, the total variation must necessarily be small in relation to the effect (i.e., mean A-V difference, or difference between a mean per cent extraction and zero). Even though the variation in some individual dogs may be considerable, this clearly does not affect the validity of the analysis. Such variation is included in the estimate of total variation. Conclusions are based on the analysis of the results as a whole rather than on individual cases.

The values for the mean per cent extraction of estrone (85.9) and estradiol-17 β (88.1) (Table II) indicate nearly complete extraction of these two estrogens in one passage through the splanchnic bed. A comparable per cent extraction has been found for aldosterone (32) in the human. The nearly complete extraction of estradiol-17 β derived from estrone by the splanchnic bed is of interest, since it indicates no *net* formation of estradiol-17 β by the splanchnic bed. This means either that the splanchnic bed forms no estradiol-17 β from estrone for release into the systemic circulation or that estradiol-17 β is formed to a very limited extent, in which case the extraction of that estradiol-17 β which enters the splanchnic bed in arterial plasma would be even higher than the observed *net* extraction. This suggests in turn that the estrone \rightleftharpoons estradiol-17 β interconversion may occur principally outside the splanchnic area. This is consistent with the previous calculation that a large proportion (45-71%) of estrone metabolism is extra-splanchnic. Substantial extrasplanchnic metabolism of the similarly interconverting steroids testosterone and Δ_4 -androstenedione was observed in dogs by Chadelaine (25).

The smaller but significant extraction of estrone sulfate (Table II) means that this conjugate is either excreted from the splanchnic bed, presumably into the bile (7), or further metabolized within the splanchnic bed.

In contrast, the negative A-HV per cent extraction of estrone glucosiduronate (Table II) indicates net production of this conjugate by the splanchnic bed. The size of the per cent extraction (-48.5%) suggests that the splanchnic bed may be the major site of production of this metabolite. The negative per cent extraction of estradiol-17 β glucosiduronate (-33.3%, Table II) like-

wise indicates substantial production of this group of conjugates by the splanchnic bed.

The relationship (Table II) between the mean A-V differences (ignoring sign) for estrone (2512 cpm, SEM 399) and for estrone glucosiduronate (896 cpm, SEM 213) on the one hand, and for estradiol-17 β (388 cpm, SEM 49.3) and for estradiol-17 β glucosiduronate (212 cpm, SEM 58.0) on the other, shows that the estrone glucosiduronate released from the splanchnic system into the systemic circulation is a significantly smaller proportion of estrone extracted by the splanchnic system than that in the case of estradiol-17 β glucosiduronate and estradiol-17 β , respectively. This presumably indicates that estrone and estradiol-17 β are metabolized somewhat differently by the splanchnic bed.

The separate activity of two major splanchnic organs, namely the small intestines and the spleen, can be estimated from the data in Tables III and IV. The data in Table III show that the intestine is highly active in the metabolism of blood-borne estrogens, as judged by the uptake of estrone and estradiol-17 β and the release of estrone glucosiduronate. The mean per cent extraction of estrone (45.3, SEM 2.60) and of estradiol-17 β (46.1, SEM 12.9) were similar. Both were considerably lower than the corresponding extraction of the splanchnic bed as a whole. This indicates that further extraction of both substances takes place at some other site within the splanchnic bed. Since the per cent extraction due to the spleen (Table IV) is approximately the same as for the small intestine and since the large intestine is thought to be inactive in estrogen metabolism (35), the probable site of further estrone and estradiol-17 β extraction is the liver.

The mean per cent extraction (-30.8 , SEM 7.9) for estrone glucosiduronate indicates substantial net production of estrone glucosiduronate by the intestines. The intestines must therefore be a principal site for the formation of estrone glucosiduronate from blood-borne estrone within the splanchnic system. Failure to show significant A-SMV differences for estrone sulfate and estradiol-17 β glucosiduronate suggests that large quantities of these substances are not taken up or released by the intestine.

In the interpretation of the A-SMV differences, the excretory and absorptive properties of the intestines have to be taken into account, together with the possible effects of the enterohepatic circulation of estrogens previously referred to. The significant uptake of blood-borne estrone and estradiol-17 β could represent excretion into the intestinal lumen rather than metabolism in the intestinal wall. Equally the significant release of estrone glucosiduronate into the superior mesenteric vein could be due to absorption of this substance (or of a precursor) brought to the intestinal lumen via the

bile. However, it is unlikely that these two separate processes are involved. The experiments were designed to be short-term specifically in order to exclude effects due to the enterohepatic circulation. The duration of the experiments (30-45 min) would hardly allow enough time for the formation of conjugates, transfer into the bile, transport to the intestinal lumen, further metabolism, and transfer into the superior mesenteric vein. This process is thought to take about 6 hr in the human (1, 2). A single explanation is both simpler and more obvious, namely, that arterial plasma estrone and estradiol-17 β are taken up by the intestine and metabolized, resulting in the formation of estrone glucosiduronate by intestinal tissue. The intestinal wall is known to have the capacity to conjugate estrogens with glucuronic acid, but this has so far been shown only for estrogens introduced into the intestinal lumen (6, 11, 33-38).

A different pattern in estrogen metabolism is seen in the spleen (Table IV). There was again uptake of estrone (mean per cent extraction 35.0, SEM 38) which was of the same order as that by the intestine, but less than that of the splanchnic bed as whole. However, in contrast to the intestines and the splanchnic bed as a whole, there was a small but significant uptake of estrone glucosiduronate (mean extraction 12%, SEM 3.7). Since excretion is not possible, the most likely explanation is that the spleen metabolizes small quantities of estrone glucosiduronate by hydrolysis. This organ is known to contain substantial amounts of β -glucuronidase activity in various animal species (39).

The splanchnic metabolism of blood-borne estrogens in the dog is complex in terms of the number of the major splanchnic organs involved and in the different patterns of activity exhibited. The contribution of each organ to over-all splanchnic metabolism must depend upon relative blood flow through that organ, as well as on the A-V difference. Venous drainage from the intestine accounts for about 60% of the blood flow through the portal vein in the dog and is approximately 12% of the cardiac output (40). These figures taken with the values for uptake of arterial estrone and estradiol-17 β and release of estrone glucosiduronate gives some indication of the quantitative potential of the intestine in estrogen metabolism. Venous drainage from the spleen accounts for only about 10% of the portal venous flow and is approximately 2% of the cardiac output (40). Nevertheless, the demonstration of uptake of estrone and of estrone glucosiduronate indicates that the spleen plays some role in splanchnic estrogen metabolism, which could assume importance in certain circumstances. It seems possible that in those forms of human liver disease where liver function is insufficient and the enterohepatic circulation of estrogens is impaired, but

where collateral venous circulation allows adequate venous drainage from the intestine and spleen (e.g., in hepatic cirrhosis), the intestine, with the spleen, could take over the greater part of splanchnic estrogen metabolism.

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