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COMPARISON OF THE DISTRIBUTION KINETICS AND METABOLISM TO ACID END-PRODUCTS OF CORTICOSTERONE AND 11-DEOXYCORTICOSTERONE IN BALB/c MICE

ALEXANDRU MARANDICI and CARL MONDER[1]
The Population Council, 1230 York Avenue, New York, NY 10021, U.S.A.

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Summary—The conversion of [4-^{14}C]corticosterone([^{14}C]B) and 11-deoxy-[1,2-^{3}H]corticosterone ([^{3}H]DOC) to steroidal carboxylic acids was studied in the BALB/c mouse. There was rapid and preferential excretion of [^{3}H]DOC metabolites into the gastrointestinal tract. Excretion of ^{14}C through the kidney was higher than ^{3}H excretion. Within minutes of intraperitoneal injection, levels of ^{3}H and ^{14}C in most organs reached their maximal levels and subsequently decreased in an exponential pattern. The majority of the organs took up ^{14}C to a greater extent than ^{3}H. Using tissue blood ratio of tracer (T/B) as criterion, it was found that liver, gall bladder, intestine, and kidney concentrated ^{3}H and ^{14}C-labeled steroid from blood. T/B for ^{3}H exceeded that for ^{14}C in the gastrointestinal tract. Abdominal fat preferentially took up [^{3}H]DOC tracer, whereas [^{14}C]B tracer was not taken up by this tissue. T/B was less than 1 for ^{3}H and ^{14}C in heart, thymus, spleen, brain, skeletal muscle and skin. In these organs uptake of B and its metabolites was greater than that of DOC and its metabolites. In liver, [^{14}C]B and [^{3}H]DOC were converted to carboxylic acid metabolites which accumulated in the intestine. The most abundant acid was 11β,20α-dihydroxy-3-oxo-pregn-4-en-21-oic acid from B. The acid metabolites of DOC were not identified. For both steroids, acids were major metabolic end-products.

INTRODUCTION

Steroid 20-hydroxy-21-oic acids are quantitatively important products of corticosteroid metabolism in mammals [1]. *In vitro*, there is selectivity in the ability of liver preparations to oxidize corticosteroids: in mouse liver, the ketol side chain of 11-deoxycorticosterone is metabolized to the 20-hydroxy-21-oic acid side chain five times faster than the corticosterone side chain [2]. *In vivo*, there is also selectivity, but the extent of oxidative metabolism and patterns of products formed differ from those observed *in vitro*. The major metabolite formed in mice from corticosterone is 11β,20α-dihydroxy-3-oxo-pregn-4-en-21-oic acid [3]. In contrast, 11-deoxycorticosterone is oxidized to several acid end products, none of which has yet been identified [4]. An examination of the differences in the organism's treatment of these two steroids may contribute to an understanding of their different physiological properties. In this paper we compare the metabolic behavior of corticosterone and 11-deoxycorticosterone in the BALB/c mouse.

EXPERIMENTAL

Animals

Male mice of the BALB/cJ strain were obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained in the Laboratory Animal Research Center of the Rockefeller University. They were kept in individual cages at 21–22°C, and fed Purina Rodent Laboratory Chow (Ralston Purina Co., St Louis, MO) and water *ad libitum*. A diurnal cycle of 14 h of light and 10 h of darkness was maintained.

Steroids and reagents

[4-^{14}C]Corticosterone (52 mCi/mmol) and 11-deoxy[1,2-^{3}H]corticosterone (47 Ci/mmol) were bought from Amersham Co. (Arlington Heights, IL). Steroid suspending vehicle was donated by the Pharmaceutical Development Services, National Institutes of Health (Bethesda, MD). Its composition is (per ml in water): sodium chloride, 9 mg; sodium carboxymethylcellulose 7LP,5 mg; polysorbate 80, 0.004 ml; benzyl alcohol, 0.009 ml. Dimethyl sulfoxide, pharmaceutical grade, was obtained from the Crown Zellerbach Corporation (Camas, WA).

Administration of steroids and preparation of tissues

Ten-week old unfasted male mice weighing 24 ± 2 (SD)g were each given a single intraperitoneal injection consisting of 1 μCi of [4-^{14}C]corticosterone and 5 μCi of 11-deoxy[1,2-^{3}H]corticosterone in 0.2 ml of a 80:20 (v/v) mixture of steroid suspending vehicle and dimethylsulfoxide. Injections were made at about 10 a.m. Groups of these animals were weighed, then killed by cervical dislocation at intervals after injection. Excreted urine and residual urine from the bladder were combined, collected on filter discs, weighed, and transferred to scintillation vials. After opening the abdominal and thoracic cavities, approx 150 mg of blood were collected by heart puncture

[1]To whom correspondence should be addressed.

using a syringe rinsed with heparin. Other organs were dissected out, rinsed in 0.9% aqueous sodium chloride until the wash fluid was clear, blotted, weighed, and transferred to homogenizing tubes. About 150 mg of skeletal muscle (vastus lateralis), 1 cm^2 of skin, and whole brain were homogenized without rinsing. Gall bladder was dissected by holding the cholecystic duct with fine forceps and cutting with scissors carefully to avoid loss of bladder contents. Stomach, segments of the small intestine (corresponding to duodenum, upper and lower jejunum and ileum), large intestine, and gall bladder were homogenized with their contents.

Homogenization of tissues was performed in a glass tube with a Teflon pestle using methanol. Five ml of methanol were used for liver, 1 ml for adrenals and 2 ml for each of the other tissues. Homogenates were centrifuged at 2000 rpm for 5 min. Radioactivity of 200 μl aliquots of the supernatants diluted in 4 ml ACS scintillation mixture (New England Nuclear Corp., Boston, MA) was measured in a Tricarb 460C scintillation counter (Packard Instruments Co. Inc., Dowers Grove, IL).

The remainder of the methanol extracts of liver and the pooled methanol extracts of the intestinal segments were dried under nitrogen at 30°C. To the residues resuspended in 0.1 ml of methanol were added 3 ml of 0.05 M sodium acetate buffer, pH 5.0, containing 0.1% EDTA. The aqueous suspensions were equally distributed into three tubes. Into tubes 2 and 3 were added 2500 I.U. of β-D-glucuronidase (*Helix pomatia*, Sigma Chemical Co., St Louis, MO) in 0.5 ml of acetate buffer. Tube 1 contained 0.5 ml of buffer and no glucuronidase. All tubes were maintained at 37°C for 24 h.

Chromatographic analysis

The incubation mixtures were extracted with ethyl acetate. Solvent was removed under a stream of nitrogen at 30°C. The contents of tube 3 were dissolved in 0.1 ml of methanol and treated with 15–20 drops of diazomethane in ether in order to esterify free carboxylic acids. Solvent was removed under a nitrogen stream. Steroids in the tubes were dissolved in acetone and quantitatively transferred to thin layer plates (Polygram Sil G/UV 254, Brinkmann Instruments, Inc., Westbury, NY). Running channels 1, 2 and 3 corresponded to the tube treatment number. Chromatography was performed in chloroform–methanol–formic acid (90:10:1, by vol). For reference ^{14}C-labeled corticosterone, ^{3}H-labeled 11-deoxycorticosterone, and their corresponding 20-hydroxy-21-oic acids [3,5] were co-chromatographed with the tissue extracts.

The plates were examined under ultraviolet light at 254 nm in order to locate the reference steroids, and the u.v. opaque regions were marked. They were then placed in a Berthold β-camera (Model LB 292, Beta Analytical Inc., Coraopolis, PA) and flushed with P10 gas saturated with methylal. A total of 100,000 scintillations per plate were recorded on Polaroid film (Type 667) in the voltage range of 3.5–3.8 KV. The photographs were used to mark the radioactive positions on the original plates. Profiles of steroids in channel 1 (untreated steroid extract) and 2 (treated with β-glucuronidase) of each thin layer plates were identical, Channel 1 was therefore not analyzed further. Channels 2 and 3 (treated with β-glucuronidase and diazomethane) were cut into 30 segments each of 5 mm length and transferred to scintillation vials for radiotracer analysis.

Levels of tracer in the respective ^{14}C and ^{3}H channels of the scintillation counter were corrected for spillover; after application of the appropriate quench corrections using the external standardization method, radioactivity was expressed as dpm. To calculate total content of tracer in various tissues, total weight of blood and muscles were considered to represent 5 and 40% of body wt, respectively. Skin accounted for 15% of body wt by direct measurement ($14.7 \pm 1.3\%$ [SD] for 10 animals).

Statistics

Statistical analyses were performed using the Analysis of Variance [6, 7]. Main effects, strains, and time intervals were considered to be fixed according to Model 1. The preliminary calculations and the analysis of variance were carried out by using a TI-58C Texas Instrument calculator programmed by us. The half time parameters were estimated by running the data under a multiexponential curve fitting program in a DEC-PDP-11 (Digital Equipment Corp) computer.

RESULTS

Accumulation of radioactivity from [^{14}C]-corticosterone ([^{14}C]B) and [^{3}H]11-deoxycorticosterone ([^{3}H]DOC) in mouse organs

Figure 1 shows that there were substantial differences in the accumulation of tracers from [^{14}C]B and [^{3}H]DOC in various organs and tissues. The concentration of ^{3}H exceeded that of ^{14}C in the small intestines. Most of the radioactivity was localized in the intestinal contents with little in the wall, as previously established [4]. The distribution of the small amount of radioactivity in the tissues of the large intestines was consistent with that of most other organs, in which a preferential uptake of ^{14}C occurred. The greater concentration of ^{3}H in the gall bladder and intestinal contents was in accord with the rapid preferential excretion of DOC metabolites through the bowel as we have shown previously [4]. High concentrations of ^{14}C were also found in the small intestine but these were significantly less than ^{3}H, whereas in kidney and urine the ^{14}C concentrations were higher. The relative levels of [^{3}H]DOC were greater than [^{14}C]B in the fat stores and mesentery. The rates of turnover of tracer from [^{3}H]DOC and [^{14}C]B were similar in the liver. The rapid de-

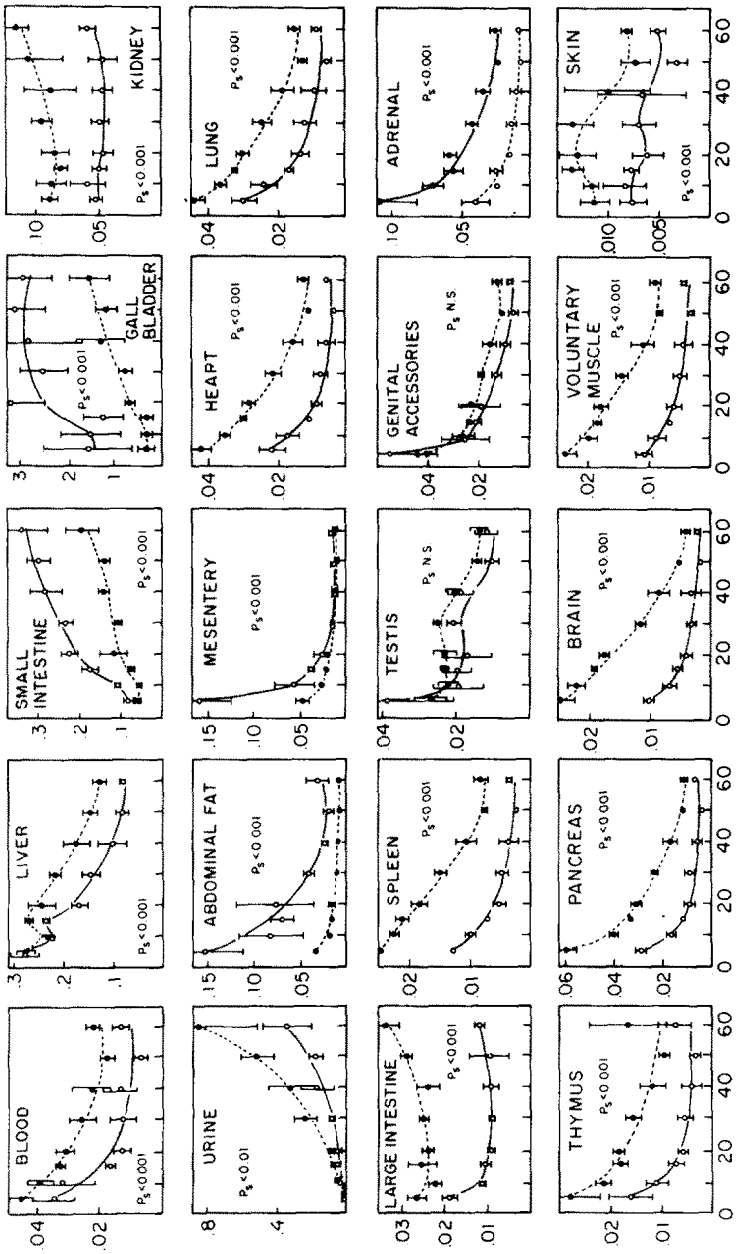

Fig. 1. Patterns of change in the specific radioactivity of various organs and tissues of male BALB/c mice after the i.p. injection of 1 μCi [4-^{14}C]corticosterone (●) and 5 μCi [1,2-^{3}H]deoxycorticosterone (○). Ordinate, microcuries/g of tissue; [^{3}H]DOC values were divided by 5 for comparison with [^{14}C]B data. Each point represents the mean ± SEM for 3 animals. The significance of the overall differences between the corresponding values of the two steroids (P_s) were estimated by analysis of variance.

crease in radioactivity in liver reflects the rapid metabolism, redistribution and excretion of these steroids. In most organs examined, including spleen, reproductive organs, thymus, pancreas, brain, skeletal muscle and skin, the levels of radioactivity associated with either steroid was low, and did not exceed 0.05 μCi/g of tissue. In the testis and genital accessories, there were no differences between ^{3}H and ^{14}C, as was also found for stomach and urinary bladder (data not shown).

Most of the tissues followed a decreasing pattern of radioactivity with time for both steroids. The data satisfactorily fitted exponential decay curves, and the decay rates were calculated. These values, expressed as the time interval in minutes for 50% decrease in radioactivity ($t_{1/2}$), are presented in Table 1. [^{3}H]DOC disappeared faster than [^{14}C]B from all tissues. Residence times for [^{14}C]B were greatest in blood, liver and thymus gland. Decay rates for the other tissues were faster, but did not follow any discernable pattern or order of preference. Decay rates amongst the tissues for [^{3}H]DOC were faster than [^{14}C]B overall and the range of $t_{1/2}$ values was much narrower.

Table 1. Rates of decrease in radioactivity ($t_{1/2}$) derived from [4-^{14}C]corticosterone and 11-deoxy[1,2-^{3}H]corticosterone in various organs and tissues

	^{14}C	^{3}H
Blood	41.43 ± 0.004	21.47 ± 0.001
Liver	40.07 ± 0.003	24.36 ± 0.001
Adrenal	21.30 ± 0.001	16.56 ± 0.001
Spleen	30.16 ± 0.004	18.95 ± 0.001
Pancreas	19.32 ± 0.001	12.10 ± 0.001
Abdominal fat	16.53 ± 0.001	13.79 ± 0.001
Heart	25.90 ± 0.005	16.85 ± 0.001
Lung	28.86 ± 0.001	19.26 ± 0.001
Thymus	44.06 ± 0.037	21.38 ± 0.005
Brain	22.25 ± 0.007	17.56 ± 0.003
Muscles	34.84 ± 0.027	29.21 ± 0.001

*The $t_{1/2}$ for liver was estimated for 15–60 min post injection intervals only since the peak was reached at 15 min after injection. Other values represent the time interval in minutes for a 50% decrease of the initial concentration of radioactivity over the experimental period.

Radioactivity from [^{14}C]B and [^{3}H]DOC in tissues and blood

The concentration of ^{14}C and ^{3}H in tissues and urine relative to blood (tissue–blood ratio, T/B) is shown in Fig. 2. In 15 min, liver reached a steady T/B ratio of 8 and 12 for [^{14}C]corticosterone and [^{3}H]DOC, respectively, which persisted for 50 min. In the gall bladder, the maximum T/B ratios of 70 for [^{14}C]B and 600 for [^{3}H]DOC reflected the preferential excretion of the latter steroid into bile. In the intestine, the ratios of 8 and 50 for ^{14}C and ^{3}H, respectively, are in accord with the preferred biliary excretion of DOC. Concentration of activity in kidney was higher than in blood; both steroids showed similar T/B ratios, 2–5, over the 60 min of observation, reflecting their levels in the residual urine in this tissue. The similarity between the two steroids was further reflected in the accumulation of labeled metabolites in excreted urine with relative concentrations with respect to blood which increased with time to 25- to 30-fold. The patterns for abdominal fat

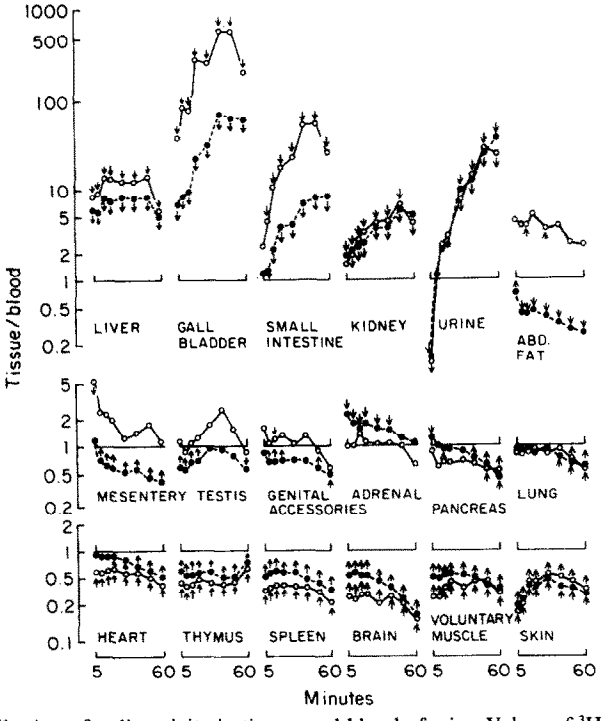

Fig. 2. Relative distribution of radioactivity in tissues and blood of mice. Values of ^{3}H and ^{14}C in tissues are represented relative to their respective concentrations of the two isotopes in blood, taken as unity. Ordinate is tissue–blood ratio plotted on a logarithmic scale. Arrows mark the values significantly different from blood at $P > 0.05$, estimated on the basis of log ratio transforms. ○, [^{3}H]DOC; ●, [^{14}C]B.

and mesentery showed a preferential uptake from blood of ^{3}H derived from DOC. [^{14}C]B, in contrast, was not taken up efficiently, since the T/B ratio was always below 1. Adrenals selectively took up B, while DOC was maintained at about the same concentration as in blood.

None of the other organs studied showed a selective uptake of either steroid. In pancreas and lung, the radiotracer levels were only occasionally different from those in blood. Heart, thymus, spleen, brain, skeletal muscle, and skin all appeared to have a barrier to the uptake of radioactivity from either steroid. The barrier appeared to be selective, since in general, the T/B ratios for these tissues were less for [^{3}H]DOC than for [^{14}C]B.

Formation of acidic metabolites from B and DOC

The data presented above, supplementing those of our earlier publications [3, 4], show that the liver is the most active organ for the uptake of corticosterone and DOC. We tested for the presence of metabolites containing the carboxylic acid moiety by reacting the combined steroid metabolites isolated from liver with diazomethane. On the basis of the differences in the radioactive profiles of two adjacent channels on thin layer plates before and after treatment with diazomethane, we concluded that both [^{14}C]B and [^{3}H]DOC are metabolized to acidic products.

The profile of [^{14}C]B metabolites was similar in liver and small intestine. The most abundant acid was, as reported earlier [3], 11β,20α-dihydroxy-3-oxo-pregn-4-ene-21-oic acid.

The profile of [^{3}H]DOC metabolites was more complex. In liver, there appeared to be at least three acid metabolites 5 min after injection of steroid. One, corresponding to 20-hydroxy-pregn-4-en-21-oic acid (pregnolic acid), disappeared within 15 min. The results are shown in Fig. 3. In the Figure, free acids are represented as areas below the reference line, and neutral esters derived from the acids are above the reference line. Pregnolic acid is DHA; its methyl ester is DHE. Within 15 min after injection multiple metabolites of unknown structure, but clearly different from the pregnolic acid initially present at 5 min, emerged. In the small intestine, pregnolic acid was never a significant acidic metabolite of DOC. Other acids more polar than pregnolic acid were observed between 15 and 60 min after injecting [^{14}C]DOC. These are seen in the difference profiles of Fig. 3 as elevations corresponding to their ester derivatives.

The quantities of acids in liver and intestine formed from B or DOC were calculated from chromatographic profiles similar to those shown in Fig. 3. The values, shown in Fig. 4, confirm that acid metabolites were major products of corticosterone metabolism in these organs. Acids represented 23% of the total metabolites of corticosterone in liver 5 min after injection. At 60 min, the proportion increased to 49%. In the intestinal tract, the appearance of acid metabolites from injected B was slower than in liver. The values in liver and intestine converged at 60 min. The acidic metabolites of [^{3}H]DOC in liver represented 35% of the total DOC metabolites in liver 5 min after injection of steroid. This value remained unchanged over the 60 min interval. In the intestine, the proportion of acid metabolites of DOC relative to total DOC metabolites was 18% at 5 min, increased to 33% at 30 min, with no further change at 60 min.

The quantities of acids found in liver and intestine, expressed as a percentage of the dose injected are also shown in Fig. 4. In liver, the percent of acids derived from B changed little, rising from 8 to 12% during the initial 30 min, returning to 8% at 60 min. In the intestine, the proportion of B derived acids progressively increased to 15% of the dose in 1 h; acid

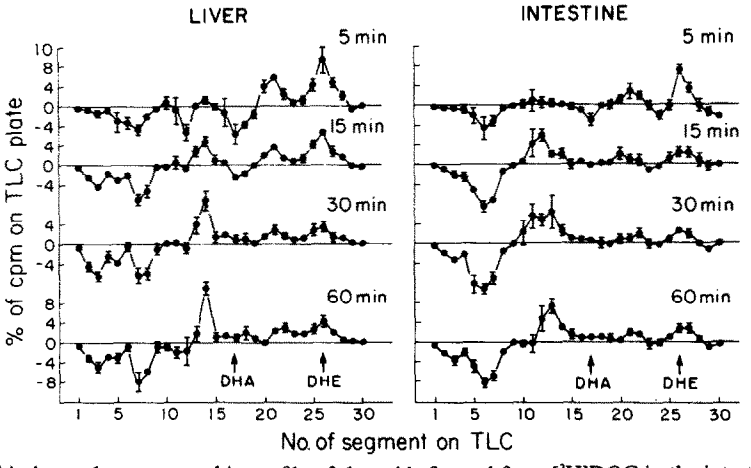

Fig. 3. Thin layer chromatographic profile of the acids formed from [^{3}H]DOC in the intestine and liver. Abscissa, sequence of 0.5 cm segments on TLC plate; ordinate, percentage of the total radioactivity in segment. The profiles represent the differences between the corresponding fractions of the same sample before and after esterification with diazomethane as described in the text. Negative values represent the free acids and positive values, their esters. DHA, position of pregnolic acid standard; DHE, position of 21-methyl ester of pregnolic acid.

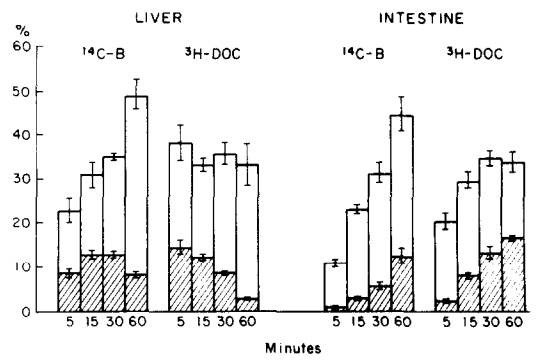

Fig. 4. Content of acids found in the liver and intestines of BALB/c mice at various intervals after injection with [^{14}C]B and [^{3}H]DOC. Total bar height represents the percentage of acids relative to total metabolites in the organ. Hatched regions represent percentage of acids relative to dose. Values are the mean for 3 individuals ± SEM.

metabolites of DOC increased in a parallel manner to 16% of the dose. Thus, in mice, a significant fraction of the intestinal metabolites of DOC and B were acids.

DISCUSSION

The distribution and metabolism of [^{3}H]DOC and [^{14}C]B in the tissues of the mouse showed some similarities and many differences. Although the qualitative patterns of uptake and loss in most tissues were similar for both steroids, there were significant quantitative differences between them.

The relative hydrophobic properties of steroids and their metabolites influences their relative pathways of excretion. A significant fraction of the metabolites of the more polar steroid, corticosterone, was excreted in the urine, while the less polar DOC metabolites were preferentially excreted into the intestine in bile. This pattern of preference was also seen in the accessory organs. More radioactivity from B than DOC was found in the kidney and more DOC associated radioactivity was found in the gall bladder. The behavior of DOC, B, and their metabolites is consistent with the observations of previous workers on the relationship between excretion route and lipophilicity [8].

The majority of the organs took up [^{14}C]-corticosterone or its metabolites to a greater extent than [^{3}H]DOC or its metabolites. The exceptions were the reproductive organs, in which differences in accumulation were not observed, and the lipid stores, where the hydrophobic properties of DOC favored its uptake. Subsequent rates of loss of ^{3}H and ^{14}C from tissues differed. For purposes of comparison, rates of decay in steroid content of the organs were expressed as the time interval for 50% decrease in radioactivity. In every case, B associated radioactivity decreased more slowly than that for DOC. For most organs, ^{3}H was lost twice as fast as ^{14}C. Therefore, corticosterone was taken up by the organs more efficiently than DOC, and was retained more effectively. We have not determined the reasons for the selective retention of [^{14}C]B over extended periods of time in many tissues. It may be the consequence of specific binding to cellular sites [9].

Additional differences in the kinetic behavior of B and DOC were found when their uptake into tissues was analyzed with respect to their concentrations in blood. The liver removed the steroids efficiently from circulation in proportion to their levels in blood. In kidney, concentrations of ^{3}H and ^{14}C representing DOC and B, respectively, increased relative to blood, due to the retention of steroid in the face of a declining concentration of steroid in blood. We interpret this to mean that a portion of radioactivity from either steroid was bound in the kidney in a form that exchanged slowly with unlabeled endogenous steroid, while the remaining tracer rapidly passed into the urine. Abdominal fat and mesentery took up [^{3}H]DOC and excluded [^{14}C]B. Both steroids were excluded from heart, thymus, spleen, brain, skeletal muscle, and skin. Many of the tissues from which corticosterone was excluded are responsive to glucocorticoids. Therefore, selective uptake or exclusion of a steroid from a tissue relative to blood cannot be related to the sensitivity of the tissue as a target for steroid action.

In addition to differences in the kinetics of distribution of DOC and B, there are differences in their specific metabolism. We have shown that mice convert corticosterone to 11β,20α-dihydroxy-3-oxo-pregn-4-en-21-oic acid and several minor acids, while DOC is converted to pregnolic acid and two or three additional metabolites [3, 4]. In this paper, we have provided an estimate of the relative quantitative importance of these metabolic modifications. The acid metabolites were a major fraction of the total steroid metabolites. They represented one third of the total DOC metabolites and half the total B metabolites in the liver and intestine. Acids become a constant proportion of the products of DOC metabolism in liver within 5 min of administration of DOC, but the proportion of corticosterone derived acids increased over the entire period studied.

We have analyzed the fate of two chemically similar steroids that differ in composition only at C-11. This small structural distinction is sufficient to affect the behavior of the steroids in many ways. Absolute rates of accumulation and net accumulation in individual organs, rates of loss from organs, relative uptake of steroids by tissues from the blood, the nature of the metabolites, and the rates of appearance of these metabolites are different, as are their routes of excretion.

Qualitatively, there are similarities between the steroids. Soon after injection, both [^{3}H]DOC and [^{14}C]B become widely distributed among the organs, undergo similar changes in levels with time, and are excreted mainly through the gastrointestinal tract. Both are oxidized to acids of similar structure, and

for both steroids the acids are major metabolic products.

The contribution of oxidative metabolism to the biological effectiveness of the corticosteroids remains unknown. The differences in the rates of appearance, the number, proportion, and chemical composition of acid end products of corticosterone and 11-deoxycorticosterone are great. The biological meaning of these characteristic differences remains to be determined.

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