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PROGESTERONE METABOLISM IN THE BABOON*

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SUMMARY

The metabolism of intravenously administered doubly labeled progesterone [21-^{14}C,7-^{3}H] was studied in three *Papio anubis* baboons: (i) an intact animal, (ii) an intact animal with prior intragastric instillation of Amberlite XAD-2 and (iii) an animal with a biliary fistula. This approach afforded an evaluation of the loss of the side-chain and possible hydroxylation and/or oxidation of ring-B. The radioactivity excreted as per cent of the amount injected was as follows: in the intact animal (i) 39% ^{3}H and 33% ^{14}C in urine after four days, in the intact animal (ii) 22% ^{3}H and 17% ^{14}C in urine after four days and in animal (iii) with a bile fistula, 37% ^{3}H and 31% ^{14}C in the urine and 25% of both ^{3}H and ^{14}C in the bile after 6 h. In both urine and bile the predominant (>99%) conjugates were glucosiduronates. Side-chain cleavage of progesterone was observed and is postulated to occur at sites other than those of the intestinal tract. The major urinary metabolite was androsterone. Biliary metabolites were shown to be mainly C_{21} compounds.

INTRODUCTION

The excretion rate of progesterone, its modes of conjugation and specific metabolites in bile and urine have been extensively studied and found to vary from species to species. Baboons have acquired a particular importance as suitable models for human work because of their phylogenetic relationship to man. Results on progesterone metabolism in subhuman primates have been reported in the pig-tail and Rhesus monkeys [1, 2] chimpanzee [3] and baboon [4, 5]. Goldzieher *et al.*[5] have shown that the major identifiable metabolites in baboon urine were pregnanediols (41%) and androsterone (36%). Plant[6] and Reddy[7] studied progesterone metabolism in the Rhesus monkey and reported conflicting results. The major metabolite in urine was androsterone which accounted for 6% of the progesterone administered. Pregnanediol was not an important urinary metabolite in this species. In the pig-tail monkey, Jeffery[1] has shown a considerable (5–19%) conversion of radioactive progesterone to androsterone and has obtained preliminary evidence suggesting a 2–6% conversion to 6-hydroxypregnanolone.

We present results of a study on the patterns of urinary and biliary excretion of labeled progesterone. In the results presented below, it is demonstrated that the normally lengthy procedure of isolation and identification of metabolites can be circumvented under the conditions of this experiment by the use of the properly doubly labeled compound. The enterohepatic circulation in one animal was modified, without recourse to surgery or biliary fistulation, by the intragastric instillation of an Amberlite XAD-2 resin, the rationale being that the resin adsorbed conjugates.

Three baboons were injected intravenously with [21-^{14}C,7-^{3}H]-progesterone: (i) an intact animal, (ii) an intact animal with prior intragastric instillation of Amberlite XAD-2 resin and (iii) an animal with a biliary fistula. Upon fractionation of urine and bile from these animals, determination of the ^{3}H to ^{14}C ratio of each fraction, and comparison of the conjugation pattern in bile with that in urine, information regarding the integrity of the side-chain and a specific site (position 7) in the steroid nucleus was obtained without the necessity of investigating the detailed chemical structure.

Figure 1 represents the four possible products obtained through the metabolism of the injected [21-^{14}C,7-^{3}H]-progesterone. Group I are compounds labeled with ^{14}C and in which ^{3}H has been removed through metabolic oxidation at position 6 and/or 7. Group II are compounds having only ^{3}H; these compounds have lost the side-chain. Group III are compounds with both ^{3}H and ^{14}C and with a ^{3}H/^{14}C ratio identical to that of the injected material and are, therefore, C^{21} compounds. Group IV are compounds which are not labeled and, therefore, cannot be detected.

MATERIALS AND METHODS

Doubly labeled progesterone; mixing, purification and administration

[21-^{14}C]-Progesterone (55·6 mCi/mmol) and [7-^{3}H]-progesterone (10 μCi/mmol) were purchased from New England Nuclear Corporation and were checked for purity by chromatography on paper in the Bush A system (benzene–hexane–methanol–water; 33:66:80:20 by vol.). The total amount of [21-^{14}C]-progesterone available to us was only 25 μCi. Approximately 85–90% of the ^{3}H label resides at C-7, the remainder being at C-4; the ratio of 7α- to 7β-^{3}H

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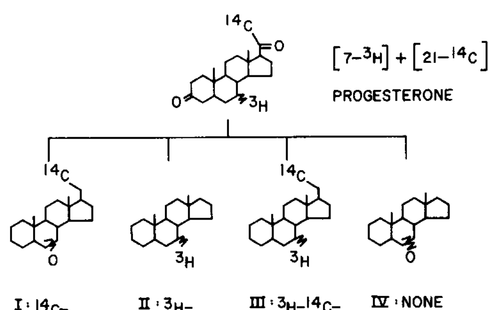

Fig. 1. Possible fate of doubly labeled metabolites used in the present study.

is variable, dependent on the batch. The ^{3}H and ^{14}C labeled material was mixed and the mixture rechromatographed in the same system. A final determination of the isotope ratio showed it to be 22·5. For injection, the radioactive steroid was taken up in 2 ml 95% ethanol, mixed thoroughly with 8 ml of isotonic saline, and injected into the antecubital vein of a previously prepared baboon. The syringe needle and vial which had contained the radioactive steroid were washed thoroughly with aqueous methanol and the amount of residual radioactivity was determined (see Table 1 for the actual administered radioactivity and the $^{3}H/^{14}C$ ratio). Sulfate conjugates of [1,2-^{3}H]-testosterone and [7-^{3}H]-pregnenolone were purchased from Amersham–Searle. [7-^{3}H]-Dehydroepiandrosterone sulfate was synthesized by us.

Animals; preparation and collection of excreta

Three adult (20 kg) male *Papio anubis* baboons were used in this study: N-7, N-8 and S-2. The animals were sedated and anesthetized with Valium (7-chloro-1,3-dihydro-1-methyl-5-2H-1,4-benzodiazepin-3-one, Roche Lab.) and Sernylan (phencyclidine hydrochloride, Parke–Davis), respectively. Both N-7 and N-8 (i and ii) were left intact; urine was collected for 4 days. During the daytime the animals were anesthetized and catheterized into the urinary bladder (Nelaton catheter), urine thus being collected continuously. During the night time, the animals were returned to their metabolic cages and urine collected there. S-2 (iii) was surgically cannulated with a polyethylene tube in the common bile duct, concomitant with urinary bladder catheterization as above. Urine and bile were collected from S-2 for 6 h. N-8 (ii) received 100 ml of a suspension of 33 g Amberlite XAD-2 (Rohm & Haas, Co., Philadelphia) intragastrically through a Fr. No. 26 Nelaton catheter 10 min before the administration of the labeled steroid, this time interval being judged as adequate for the resin to pass into the duodenum at the same time that radioactivity appeared in the bile.

Determination of radioactivity

A premixed scintillation fluid, Aquasol (New England Nuclear Corp.), diluted to one-third of its volume with toluene was used. For the determination of radioactivity in urine, 0·5 ml was added to 10 ml of diluted scintillation fluid. The collected bile was diluted with distilled water and 25λ aliquots absorbed on filter paper which was oxidized in a Packard Iricarb sample oxidizer (model 305 or 306). Radioactivity was measured in a Packard Tricarb spectrometer model 3375 to a relative standard error of 2% and corrected for background and quenching. Efficiencies for counting ^{3}H and ^{14}C varied with the type of sample, but for determination of radioactivity in column fractions, the ^{3}H efficiency was generally 38%, while that for ^{14}C was 56% with a crossover of 14%.

Column chromatography

After the volume and pH of urine and bile were determined they were adjusted to pH 8 and the urine or bile was passed through Amberlite XAD-2 resin columns. These glass columns had an inside diameter of 15 mm and were equipped with a valve and fitted with glass-wool at the bottom and top of the resin, as described by Osawa and Slaunwhite [10]. The amount of resin was approximately 100 ml/l of urine. The urine or bile was passed through at the rate of approximately 5 ml/min. The columns were then washed with distilled water totalling 3-bed vol. of resin. The washes were collected in three fractions. The metabolites were eluted with methanol with a volume of four times that of the resin and collected in four fractions. All fractions were counted and the percentage calculated for pooling. Methanolic eluates so obtained were evaporated under vacuum. The residues were dissolved in 15 to 20 ml of 50% aqueous ethanol in preparation for application to the DEAE-Sephadex A-25 columns.

TABLE 1

SCHEDULES OF COLLECTIONS AND DOSES OF LABELLED PROGESTERONE ADMINISTERED TO BABOONS

Baboon Ident. #	Treatment	Doses	Specimen	Interval
1) N-7	intact	39.2 μg ^{3}H:141.3 μCi ^{14}C:6.3 μCi ratio:22.4	urine	30 min.1,2,3, ...,6,24,48, 72 hr.
2) N-8	Amberlite XAD-2 resin in digestive tract	48.8 μg ^{3}H:174.5 μCi ^{14}C: 7.7 μCi ratio:22.2	urine	30 min.1,2,3, ...,6,24,48, 72 hr.
3) S-2	bile fistula	51.8 μg ^{3}H:182.2 μCi ^{14}C:8.2 μCi	urine bile	30 min.1,2,3, ...,6 hr.

DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Inc.) was added as a slurry in water and allowed to settle in K9/60 Sephadex columns (Pharmacia, Ltd.) to yield gel columns 60 cm. in length and 0·9 cm. in dia. The mixing vessel initially contained water. After application of the sample, the column was washed with water and then eluted with a linear NaCl gradient of 0 to 0·4 M (400 ml). The column was then washed with 2·0 M NaCl solution (200 ml). A flow rate of about 80 ml/h was maintained and 10 ml fractions were collected. Either ^{3}H labeled standard testosterone sulfate, pregnenolone sulfate or dehydroepiandrosterone sulfate was added to the material to be chromatographed prior to its application to the DEAE-Sephadex A-25 columns to help in the localization of the fractions in which sulfate was eluted.

Enzymatic hydrolysis

More than 90% of the fractions eluted were in the "glucosiduronate" area of the column elution pattern. Enzymatic hydrolysis (500 units of β-glucuronidase per ml of eluate) with Ketodase (Warner–Chilcott) at 37°C for 48 h followed by three ethyl acetate extractions were performed on aliquots of each fraction to obtain "Ketodase-released metabolites". In all hydrolyses a control was simultaneously incubated to ascertain that the radioactive compound did not decompose spontaneously. To ascertain that glucosiduronidate was being hydrolyzed, a third incubation was simultaneously carried out using enzyme plus inhibitor (saccharo-D-lactone, 10^{-3} M). An example of resulting Ketodase hydrolysis is given below:

Ident. No.	Specimen	Yield of hydrolysis % ^{3}H	% ^{14}C
N-7	Urine	94 ± 0·5	92 ± 4·1
N-8	Urine	93 ± 3·8	85 ± 7·0
S-2	Urine	94 ± 4·7	88 ± 2·0
S-2	Bile	92 ± 4·2	79 ± 15·5
Inhibition by saccharolactone		38	30
control (Labeled material with buffer only)		8	8

RESULTS

Excretion of radioactivity

The excretion of radioactivity in urine and bile was studied as a function of time. Figure 2 shows the cumulative excretion of ^{3}H and ^{14}C as per cent of the doubly labeled progesterone injected in intact baboons N-7 and N-8. Also shown are the $^{3}H/^{14}C$ ratios in the urine collections. In four days, 39% of the ^{3}H and 33% of the ^{14}C were excreted in the urine of baboon N-7 and 22% of the ^{3}H and 17% of the ^{14}C from baboon N-8. The $^{3}H/^{14}C$ ratios were higher than that of the injected material in urine collections from baboon N-7 and markedly higher from N-8.

Excretion of radioactivity in the bile and urine collected from baboon S-2 with a biliary fistula is shown in Fig. 3. In 6 h, 37% of the ^{3}H and 31% of the ^{14}C were recovered in the urine. During that time, 25% of each label was recovered in bile. The ratio was slightly higher in urine than that of the injected compound, but almost identical to the injected ratio in the bile.

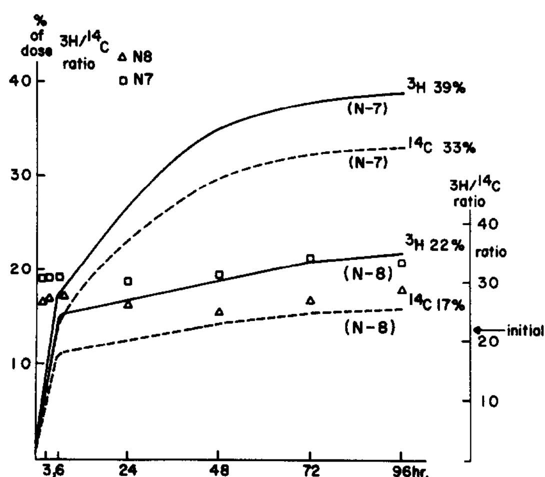

Fig. 2. Excretion of radioactivity in urine of baboons N-7 and N-8 following administration of doubly labeled progesterone. The curves consisting of unjoined squares or triangles represent the isotope ratios in the urines of these two baboons.

The nature of conjugates in bile and urine

Typically, in order of elution from DEAE-Sephadex A-25 columns, four types of "areas" of compounds appeared: (1) "non-charged", (2) "glucosiduronates", (3) "sulfates" and (4) "diconjugates". Figures 4–7 are examples of elution patterns obtained upon chromatography of representative samples on DEAE-Sephadex A-25. These samples are: Figure 4, urine of N-7; Fig. 5, urine of N-8; Fig. 6, bile of S-2 and Fig. 7, urine of S-2. The small peak at the beginning of these curves (tubes 0–5) represents uncharged steroid. The large peak represents glucosiduronates. This was ascertained in a preliminary manner by (a) the elution volume of the peak, and (b) the fact that a co-chromatographed sulfate standard was eluted later. For the final identification of the conjugated moiety hydrolysis and hydrolysis inhibition were

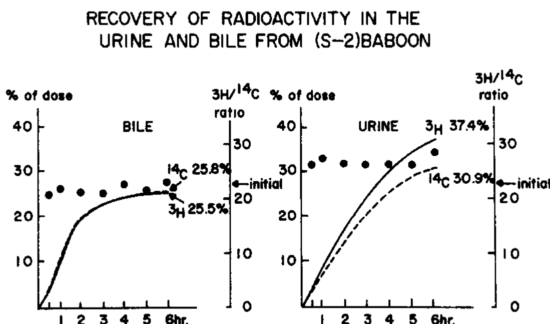

Fig. 3. Excretion of radioactivity in bile and urine of a baboon injected with doubly labeled progesterone.

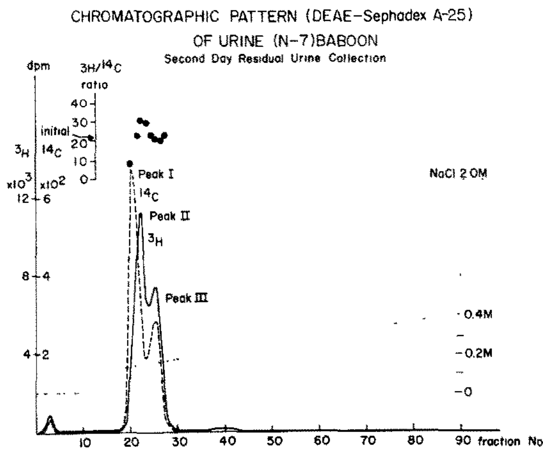

Fig. 4. Pattern of elution of radioactivity from DEAE-Sephadex A-25 column following chromatography of urine of baboon N-7.

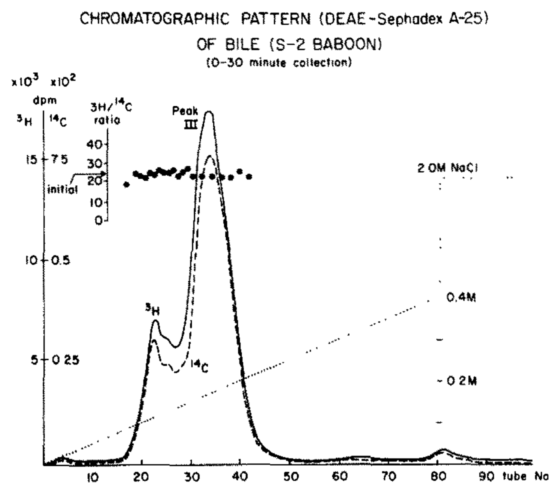

Fig. 6. Pattern of elution of radioactivity from DEAE-Sephadex A-25 column following chromatography of bile of baboon S-2.

used. In the case of bile of S-2, the glucosiduronate peak was quite broad and encompassed the fractions where sulfate conjugates are normally eluted. However, the fact that this peak was composed only of glucosiduronates was ascertained by almost quantitative hydrolysis with Ketodase and inhibition of this hydrolysis with saccharo-D-lactone.

No attempt was made to identify the "sulfate" peaks other than by the position in the elution pattern, since they represented a minor fraction of the radioactivity eluted. The $^{3}H/^{14}C$ ratios of the glucosiduronate peaks are also plotted in these graphs. According to these ratios, the glucosiduronate peaks were divided into three parts designated as: I (^{14}C dominant), II (^{3}H dominant) and III (had an isotope ratio compatible with the injected material). Shortly after the administration of the steroid, and lasting at least until the second day of collection, the heights of peaks I and II were different in baboons N-7 as compared to N-8 (see Figs. 4 and 5, for examples).

Since the three parts of the glucosiduronate peak (I, II and III) were difficult to separate, the following method was applied to these peaks from each individual urine and bile: the fractions comprising the peaks were combined and desalted by passage through an XAD-2 column. The aglycones extracted upon β-glucuronidase hydrolysis were reacted with Girard-T reagent and, thus, the ketonic and non-ketonic fractions could be determined. With the exception of the material from the first urine collections of N-7 and N-8 (65% ketonic), the ketonic fraction resulting from Peak II in the glucosiduronate peaks of all processed urine represented 80–90% of the radioactivity.

The ketonic fraction from Peak II resulting from application of the above method to the 2–3 h urine collection from baboon N-8 was chromatographed on neutral alumina column (deactivated by

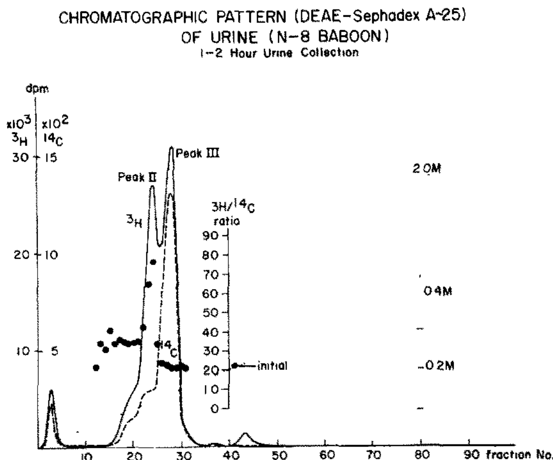

Fig. 5. Pattern of elution of radioactivity from DEAE-Sephadex A-25 column following chromatography of urine of baboon N-8. XAD-2 resin was pre-instilled intragastrically prior to injection of the doubly labeled progesterone. See Methods.

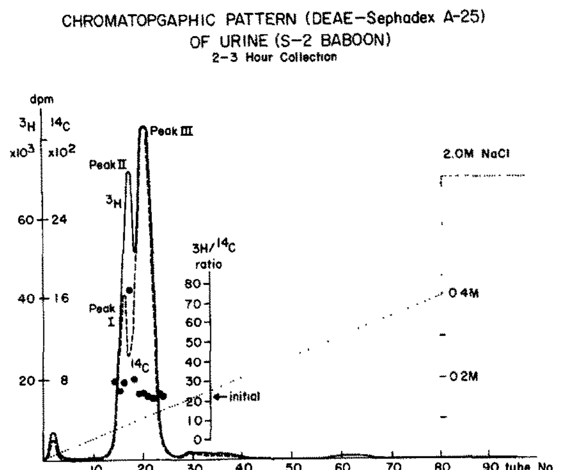

Fig. 7. Pattern of elution of radioactivity from DEAE-Sephadex A-25 column following chromatography of urine of baboon S-2.

addition of 8% water, Woelm) using a gradient of 0 to 1·6% methanol in benzene followed by methanol. Three peaks resulted, the least polar of which was the largest. The first peak was found to contain only ^3H and upon the thin-layer chromatography (silica gel F) in the system 5% acetone–benzene, gave a single peak of radioactivity with an R_F value identical to the co-chromatographed androsterone standard. The latter standard was visualized on the plate by spraying with phosphomolybdic acid. The ^3H-labeled material was eluted from the plate, mixed with carrier androsterone and crystallized to constant specific activity (1·87 × 10^4 d.p.m./mg, 1·91 × 10^4 d.p.m./mg).

DISCUSSION

In the studies described above, doubly labeled progesterone was injected into baboons. Normally in tracer methodology, biochemically "stable" labeled atoms are used for steroid work, e.g., ^{14}C at position 4 or ^3H at positions 6 and 7. In the present work, biochemically "stable" atoms were intentionally not used with the hope of gleaning insight into the metabolism of progesterone. The normal course of metabolism of this steroid includes desmolation at position-17 which would lead to the loss of the ^{14}C atom of position-21. Another reaction is oxidation at position-6 or 7 which, if it occurs, would give a molecule devoid of ^3H.

In the present studies, we have used three baboons, two intact (N-7 and N-8) and one with a biliary fistula (S-2). The objective of using N-8 was an attempt to create the conditions associated with an interruption of the enterohepatic circulation, without recourse to surgery, as is used in cannulation of the common bile duct. To this end, XAD-2 resin was intragastrically instilled into the animal shortly before, and not simultaneously with, the injection of labeled progesterone. It was hoped that as the resin travelled down the duodenum, it would adsorb the radioactive conjugates excreted in the bile.

Examination of the excretion data from baboons N-7 and N-8 (Fig. 2) reveals that baboon N-8 excreted about half the amount of both labels excreted by baboon N-7 (the control animal) and that the isotope ratio in the urine from N-8 was higher than that found in the urine of N-7. While the factor of individual variation was taken into account, it must be concluded that the presence of the resin in the intestine of N-8 did, indeed, affect the reabsorption and urinary excretion of the radioactivity.

The per cent excretion in the control N-7 not only agrees with that published by Goldzieher and Axelrod (37% excretion in the urine after three days) [8], but is also close to the figure for human excretion (51%), a fact helpful to those seeking a model for the human [9]. Baboon S-2 presented a different picture; it excreted a total of 63·2% of the ^3H and 56·4% of the ^{14}C in urine and bile in 6 h (N-7 excreted 39% ^3H and 33% ^{14}C in 4 days). It appears from the above data that progesterone is involved in a minimal enterohepatic circulation in the baboon. This conclusion is tentative until more data are obtained in a larger group of animals.

Upon fractionation, all urine and bile collections gave a predominant peak (95%) which upon hydrolysis proved to be glucosiduronates. Very little radioactivity was detected in the "sulfate" and "uncharged" fractions, respectively. Comparison of the total radioactivity in a column with the amount applied indicated good recovery and little or no methodological losses. Even though primary alcohol sulfates may have been found and are known to be quite stable, the possibility of their breakdown cannot be ruled out with certainty.

Isotope ratios higher than those of the injected material were encountered in some fractions comprising the "glucosiduronate" peaks, and point to desmolation of the side-chain of the C_{19} compounds; indeed, androsterone was isolated and identified. The presence of fractions in the "glucosiduronate" peaks with isotope ratios identical with or very close to that of the injected material indicated the presence of the C_{21} compounds in which both the 21-^{14}C and the 7α-^3H atoms were intact. Fractions with isotope ratios lower than that of the injected compound were detected. We, therefore, conclude that:

1. The urinary and biliary excretion of [21-^{14}C,7-^3H]-progesterone administered to male baboons was determined.
2. The predominant conjugate in both urine and bile was found to be glucosiduronates. Less than 2–3% of the radioactivity in the urine was in the sulfate fraction. In bile, slightly larger amounts of "sulfates" (4%) were detected. "Diconjugates" were observed in trace amounts in the bile, but not in the urine.
3. The major biliary metabolites were shown to be C_{21} compounds because the isotope ratio in the peaks was identical to the ratio of the injected compound.
4. The administration of Amberlite XAD-2 resin into the intestinal tract of one animal appeared to have an effect on progesterone metabolism as manifested by changes in elution patterns from DEAE-Sephadex columns upon column chromatography of urine; the ^{14}C-dominant peak I was not detected.
5. Side-chain cleavage of progesterone was evident from metabolites (e.g., androsterone) in urine which were only labeled with ^3H (peak II). Since peak II occurred in urines of all animals (including XAD treated), desmolation probably occurred in an extraenteric system.
6. The double isotope technique and *in vivo* use of Amberlite XAD-2 resin were very helpful in the study of the metabolism and the enterohepatic circulation of progesterone.

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