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Synthesis and Release of Steroids in Intestines from Cynomolgus Monkeys (*Macaca fascicularis*)

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ABSTRACT—To examine the synthesis and release of steroids in intestinal tissues from cynomolgus monkeys (*Macaca fascicularis*), we performed the following experiments: 1) incubated prepared intestinal tissues with [^{3}H]testosterone to study the conversion to other steroids; 2) used a radioimmunoassay to determine steroid levels in six segments of intestinal tissues and contents (duodenum, jejunum, ileum, cecum, colon, and rectum); 3) localized testosterone in the six intestinal segments by immunofluorescence histochemistry; and 4) determined steroid levels in feces from males and females of various ages by radioimmunoassay to examine a correlation between steroid levels and age or sex. In prepared intestinal tissues, testosterone was converted into androstenedione, 5α-dihydrotestosterone, and an unidentified substance; all of these steroids were detected in all segments of the intestinal tissues and contents by radioimmunoassay. Immunofluorescence showed that testosterone was located in all segments of intestinal epithelia. Androstenedione, testosterone, 5α-dihydrotestosterone, and the unidentified substance were also detected in feces, and their levels were not affected by the age or sex of the animal. The present findings in cynomolgus monkeys led us to conclude that 1) steroids were synthesized in the intestines; 2) intestinal steroids were released from the six intestinal tissues to the intestinal cavities and excreted outside the body with feces; and 3) intestinal steroids were released irrespective of age or sex of the animal. Intestinal steroids seem to be paracrine or exocrine agents and to have different characteristics from classical serum steroids.

Key words: androstenedione, testosterone, 5α-dihydrotestosterone, intestine, cynomolgus monkey

INTRODUCTION

Steroid hormones are generally released from steroidogenic glands, such as the gonad and adrenal (Ojeda and Griffin, 1996). When the steroids reach specific organs in the body, steroid-converting enzymes play a crucial role in converting the steroids to active or inactive derivatives. For instance, 5α-reductase type 1 and/or type 2, which catalyze the synthesis of testosterone (T) to 5α-dihydrotestosterone (5α-DHT), are expressed in the prostate, seminal vesicle, skin, liver, muscle, and other locations (Normington and Russell, 1992; Russell and Wilson, 1994). In addition, 17β-hydroxysteroid dehydrogenase (17β-HSD) type 2, which catalyzes the synthesis of T to androstenedione or estradiol (E2) to estrone (Wu *et al*., 1993), is expressed in the endometrium, placenta, and liver (Andersson, 1995).

On the other hand, current research has established that the brain, which is traditionally considered a target site for steroid hormones, synthesizes steroids *de novo* from cholesterol (Baulieu, 1997; Tsutsui *et al*., 2000). Thus, it is possible that steroids are also synthesized *de novo* from cholesterol and released from organs other than the classical steroidogenic glands.

In the gastrointestinal tract, which is not a classical steroidogenic organ, the synthesis of some steroids, e.g., T from progesterone, E2 from androstenedione, and estrone from E2, has been reported in rats (Dalla Valle *et al*., 1992; Le Goascogne *et al*., 1995; Ueyama *et al*., 2002) and humans (Sano *et al*., 2001). Steroid hormones have also

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been detected in the feces of various primates (Matsumuro *et al.*, 1999; Miyamoto *et al.*, 2001a, b; Yoshida *et al.*, 2001). Identifying the source and understanding the conversion of these intestinal or fecal steroids in nonhuman primates may reveal a new function of intestines. In this study, biochemical and immunofluorescence histochemical methods were used to examine the synthesis and release of steroids in intestinal tissues of cynomolgus monkeys (*Macaca fascicularis*).

MATERIALS AND METHODS

Animals and samples

Twenty-seven cynomolgus monkeys were used in this study. Animals were bred and kept at the Tsukuba Primate Center for Medical Science, National Institute of Infectious Diseases (NIID), Japan (Honjo, 1985). They were fed fruits and commercial monkey diet (type AS, Oriental Yeast, Japan). To identify the characteristics of steroids in intestines, we divided the study into four parts: 1) conversion of steroids in intestinal tissues, 2) steroid levels in intestinal tissues and contents, 3) localization of testosterone in intestinal tissues, and 4) steroid levels in feces of male and female monkeys of various ages. The profiles of the animals used in each experiment are summarized in Table 1.

The intestines of Subjects 1 to 5 and 11 to 15 were removed after they were euthanized under deep anesthesia with ketamine hydrochloride and sodium pentobarbital for other studies. In experiment 1, cecum tissue of Subject 1 was used to detect T-converting-enzyme activity. In experiment 2, cecum tissue of Subject 2 was used to measure androstenedione levels. Intestines obtained from Subjects 3 to 5 were separated into six segments–duodenum, jejunum, ileum, cecum, colon and rectum–and steroid levels were measured in tissues and contents of each segment. For comparison of steroids found in intestine and serum, blood samples were collected from Subjects 6 to 10 under anesthesia with ketamine hydrochloride. In experiment 3, the intestines of Subjects 11 to 15 were separated into six segments, and T was localized by immunofluorescence histochemistry. Testes were also obtained from Subjects 11 to 15 for use as a positive control (Liang *et al.*, 1999). In experiment 4, the feces of Subjects 16 to 27 were collected for measurement of steroid levels.

All experiments were carried out under the guidelines for animal experimentation of the NIID.

Experiment 1. Conversion of steroids in intestinal tissues

Materials and solvents. Testosterone was obtained from Nacalai Tesque (Japan). 5β-Dihydrotestosterone (5β-DHT) was purchased from Steraloids (USA). [^{3}H]T (101 Ci/mmol) and [^{3}H]5α-DHT (44 Ci/

Table 1. Characteristics of monkeys studied

Experimental use	Subject no.	Sex (M/F)	Age (yr)	Body weight (kg)
Detection of testosterone-converting enzyme activity	1	M	4.7	3.7
Measurement of androstenedione-like steroid levels in intestinal tissue	2	M	3.4	2.8
Measurement of steroid levels in intestinal tissues and contents	3	M	3.3	3.6
	4	M	3.3	3.8
	5	M	3.5	3.3
Measurement of steroid levels in serum	6	M	6.2	3.9
	7	M	7.2	5.5
	8	M	7.8	4.8
	9	M	15.2	9.8
	10	M	16.8	6.8
Localization of testosterone in intestinal tissues	11	M	3.4	3.0
	12	M	3.5	2.6
	13	M	4.2	3.4
	14	M	15.0	7.2
	15	M	15.1	4.9
Measurement of steroid levels in feces	16	M	1.3	1.6
	17	M	1.4	1.5
	18	M	2.0	2.3
	19	M	10.3	5.6
	20	M	10.8	6.5
	21	M	10.8	5.7
	22	F	1.0	1.3
	23	F	1.6	1.5
	24	F	2.0	2.3
	25	F	10.2	3.8
	26	F	10.6	3.8
	27	F	10.7	2.0

mmol) were purchased from NEN Life Science Products (USA). [^{3}H]Androstenedione (105 Ci/mmol) and [^{14}C]T (56 mCi/mmol) were obtained from Amersham Biosciences (UK).

For the parallelism test, an androstenedione enzyme-linked immunosorbent assay (ELISA) kit (Oxford Biomedical Research, USA) was used.

Homogenization buffer consisted of 10 mM potassium phosphate (pH 7.0), 150 mM potassium chloride, and 1 mM EDTA. Incubation buffer consisted of 3 pmol of [^{3}H]T, 6 μg of T, and 25 mM NADPH. Extraction solvent was a mixture of hexane and ether (3:2, vol/vol).

Procedure. A modified method (for original method, see refs. Andersson and Russell, 1990; Normington and Russell, 1992 and Jakimiuk *et al.*, 1999) was used to examine the activity of T-converting enzymes in the intestinal tissues. The cecum tissue (3.5 g) was homogenized by a Potter-Elvejem homogenizer (B. Braun Biotech International, Germany) in 35 ml of the ice-cold homogenization buffer and centrifuged at 100,000 × g at 4°C for 30 min. The resulting pellet was resuspended in 4 ml of homogenization buffer. The resuspended solution (1.2 ml) was mixed with 300 μl of incubation buffer and incubated at 37°C for 15 or 30 min. As a control, a vehicle alone was used instead of the resuspended tissue. The incubating mixtures (500 μl) were added to 2.5 ml of extraction solvent. After vigorous vortexing for 1 min, upper layers of samples were transferred into glass tubes and then completely dried *in vacuo*. The dried extracts were dissolved in 200 μl of 60% methanol, and 27 pmol of [^{14}C]T was added as a marker. Then 100 μl of each reconstituted sample was fractionated by reverse-phase high-performance liquid chromatography (HPLC; TSKgel, ODS-120T; Tosoh, Japan) using a linear gradient solvent system, from 60 to 100% methanol (1%/min). Flow rate was 500 μl/30 sec/tube, and the temperature of a column was 40°C. The radioactivity (dpm) in each eluate fraction was measured by using a liquid scintillation counter (LSC-3500; Aloka, Japan).

To obtain a chromatogram of standard steroids to be used as a reference, standard steroids and ^{3}H-labeled standard steroids were also fractionated by HPLC, as described above. The elution of nonlabeled steroids was monitored at 204 nm by a combination of UV detector (UV-8020, Tosoh) and HPLC.

Parallelism test. A metabolite of T was examined by the parallelism test. The metabolite and androstenedione were diluted serially with the buffer solution prepared with the androstenedione-ELISA kit and then assayed. Dose-response curves of the metabolite and the standard steroid were compared. The similarity between slopes of both curves was determined by using the parallel line assay method of Sakuma (Sakuma, 1964).

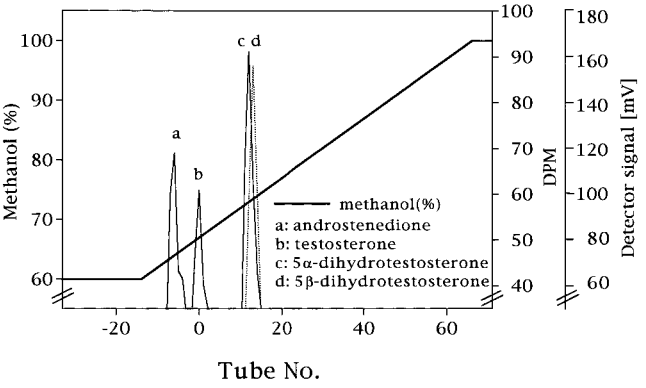


Fig. 1. Typical traces showing a percentage methanol gradient throughout chromatography and a chromatogram of steroids. The x axis shows the tube numbers. The eluting position of [^{3}H]T is standardized as tube 0. Elution of [^{3}H]androstenedione (a), [^{3}H]T (b), and [^{3}H]5α-DHT (c) was determined by measuring the radioactivity (dpm) of each eluate fraction. Elution of 5β-DHT (d) was determined by measuring the absorbance at 204 nm.

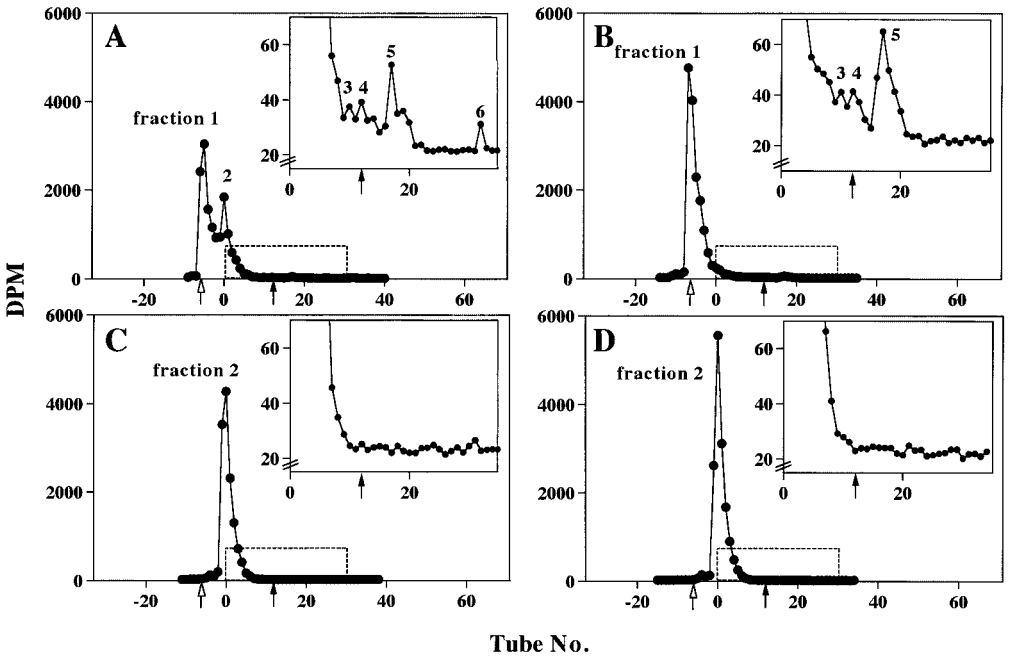


Fig. 2. Chromatograms of T metabolites. T was incubated with prepared intestinal tissue for 15 min (A) or 30 min (B) or without tissue for 15 min (C) or 30 min (D). The x axis shows the tube numbers. The eluting position of [^{14}C]T is standardized as tube 0. Insets are enlargements of the areas enclosed by dashed lines. Eluting peaks are labeled as fractions 1 to 6 in order of eluting time. The white arrow indicates the expected eluting position of androstenedione, and the black arrow indicates the expected eluting position of 5α-DHT.

Experiment 2. Steroid levels in intestinal tissues and contents

Materials and solvents. For measurement of steroid levels, two commercial kits were used: a T radioimmunoassay (RIA) kit (Testosterone 'Eiken', Eiken Chemical, Japan) and an androstenedione-ELISA kit. The manufacturers of each kit reported that the antibody used in the T-RIA was cross-reactive to 5α-DHT in 36.6%; to 5α-androstanediol, androstenedione, androsterone, and progesterone in less than 3%; and to E2, 17α-hydroxy-progesterone, deoxycortisol, dehydroepiandrosterone, estrone, estriol, and cortisol in less than 0.01% and that the antibody used in the androstenedione-ELISA was cross-reactive to estrone in 1.5% and to pregnenolone, deoxycorticosterone, estrone-3-sulfate, E2, hydrocortisone, prednisolone, and estriol in less than 0.2%. In addition, we confirmed that the latter antibody was also cross-reactive to 5α-DHT. LH-20 solvent was a mixture of benzene and methanol (85:15, vol/vol).

Extraction and fractionation of steroids in intestinal tissues. Extraction solvent (20 ml) was added to 2.0 g of minced intestinal tissues. Samples were gently shaken and kept at room temperature overnight. The upper layers of samples were transferred into glass tubes and then completely dried *in vacuo*.

The dried extracts were dissolved in 300 μl of LH-20 solvent, and 300 fmol of [^{3}H]T was added. These reconstituted samples were gel filtrated on a 0.7 × 20-cm column of Sephadex LH-20 (Amersham Biosciences) at a flow rate of 500 μl/90 sec/tube. Then the radioactivity in each eluate fraction was measured with a liquid scintillation counter. Fractions containing radioactivity were mixed together and dried completely *in vacuo*. After gel filtration, the dried extracts were redissolved in 200 μl of 60% methanol, and 700 fmol of [^{3}H]5α-DHT was applied. Then 100 μl of the reconstituted samples were fractionated by HPLC, as described above. The radioactivity in each eluate fraction was measured with a liquid scintillation counter, and 250 μl of eluate was collected from each tube and completely dried *in vacuo*.

Measurement of T-like steroid levels. After fractionation by HPLC, the dried extracts were redissolved in 300 μl of the buffer solution prepared with the T-RIA kit and then assayed.

Measurement of androstenedione-like steroid levels. After fractionation by HPLC, the dried extracts were redissolved in 500 μl of the buffer solution prepared with the androstenedione-ELISA kit and then assayed.

Examination of steroids in intestinal contents. Intestinal contents from six segments were freeze-dried, and extraction solvent was added to 50 to 500 mg of dried intestinal contents at a concentration of 4–10 ml/100 mg. Samples were gently shaken and then kept at room temperature for 1 hr. Upper layers were transferred into glass tubes and then completely dried *in vacuo*. HPLC fractionation and steroid assays were the same as those for intestinal tissues.

Examination of steroids in serum. The serum samples from Subjects 6 to 10 were mixed together, and 5 ml of extraction solvent was added to 1 ml of the serum mixture. After vigorous vortexing for 1 min, an upper layer of sample was transferred into a glass tube and then completely dried *in vacuo*. HPLC fractionation and steroid assays were the same as those used for intestinal tissues.

Recovery test. [^{3}H]T (1 pmol) was applied to 1 to 2 g of minced intestinal tissues, and [^{3}H]T (300 fmol) was applied to 200 to 600 mg of dried intestinal contents. After 1 hr of incubation, 20 ml of extraction solvent was added, and samples were gently shaken and kept overnight or for 1 hr, as described above. The radioactivity in the upper layer of each sample was measured with a liquid scintillation counter.

Parallelism test. Substances detected by T-RIA or androstenedione-ELISA were examined by the parallelism test. Serially diluted substances and standard steroids of androstenedione, T, 5α-DHT, and 5β-DHT were assayed with each kit. The similarity between slopes of both dose-response curves was determined as described above.

Experiment 3. Localization of testosterone in intestinal tissues

Materials and solvents. Rabbit anti-T antiserum (anti-T) was obtained from Biogenesis (UK). Biotinylated goat anti-rabbit immunoglobulins and fluorescein isothiocyanate-conjugated streptavidin

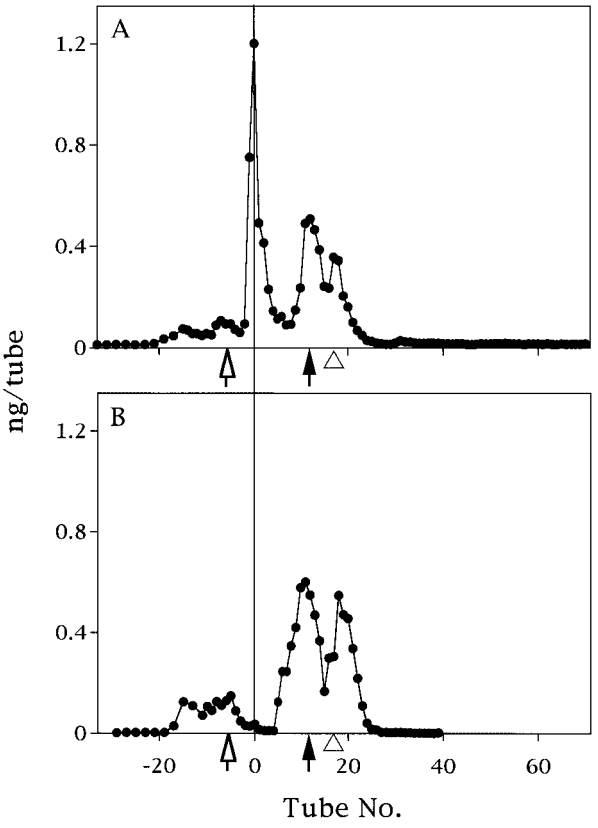


Fig. 3. Chromatograms of steroids detected by T-RIA (A) or androstenedione-ELISA (B) in the intestinal tissue (the cecum) from Subject 3 (A) and Subject 2 (B). Amounts of steroids were converted to relative amounts derived from 1 g of tissue. The x axis shows the tube numbers. The eluting position of [^{3}H]T is standardized as tube 0. The black arrow shows the eluting position of 5α-DHT, and the white arrow shows the expected eluting position of androstenedione; the triangle shows the expected eluting position of the substance in fraction 5.

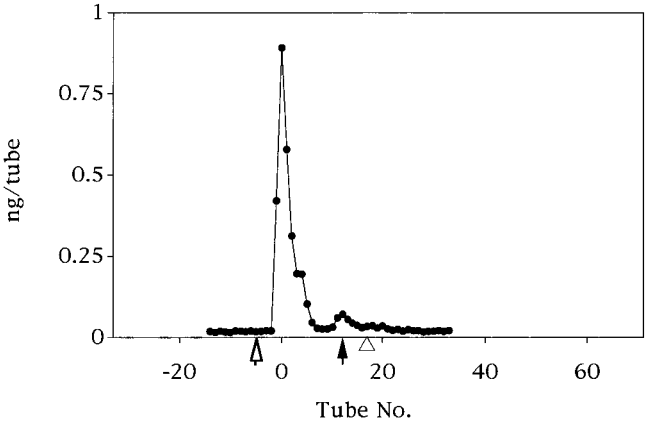


Fig. 4. Chromatogram of steroids detected by T-RIA in the serum mixture collected from five male monkeys. The x axis and symbols are the same as those described in the legend to Fig. 3.

were from Dako Cytomation (Denmark).

Dilution buffer consisted of 10 mM phosphate-buffered saline (PBS; pH 7.2) with 15 μg/ml of collagen type I (Wako Pure Chemical Industries, Japan), 15 μg/ml of collagen type III (Wako Pure Chemical Industries), and 1.5% bovine serum albumin (albumin, bovine fraction V powder; Sigma, USA). Ten millimolar citrate buffer (pH 6.0) was used in pretreatment.

Procedure. Six segments of intestinal tissues and testes were fixed in Bouin's solution for 4 to 5 hr and embedded in paraffin wax, according to the method of Liang *et al*. (2000). Anti-T was diluted at 1:2 in the dilution buffer and then kept at 4°C overnight.

Deparaffinized 2-μm-thick sections were pretreated by microwave for 15 min in citrate buffer before the first antiserum incubation. The sections were incubated with anti-T at 4°C overnight and then incubated with the anti-rabbit immunoglobulins (diluted 1:500) at room temperature for 30 min. Sections were further incubated with fluorescein isothiocyanate-conjugated streptavidin (diluted 1:200) at room temperature for 30 min. A negative control was prepared in adjacent sections by incubation with normal rabbit serum (diluted 1:500) instead of anti-T.

Experiment 4. Steroid levels in feces of male and female monkeys of various ages

Procedure. Twenty milliliters of extraction solvent was added to 500 mg of freeze-dried feces. Extraction, fractionation, and steroid assays were the same as those used for intestinal contents.

Recovery test. The recovery of [^{3}H]T from dried feces was investigated by the same method as that for dried intestinal contents.

RESULTS

Recovery

Recovery of [^{3}H]T in samples incubated with extraction solvent was 74.1±7.3% (mean±SD) for intestinal tissues, 90.6±11.2% for intestinal contents, and 64.3±17.8% for feces.

Fractionation by HPLC

Figure 1 shows typical traces of a percentage methanol gradient throughout HPLC and a chromatogram of the steroids. The eluting position of T was standardized as tube 0. The eluting positions of androstenedione, 5α-DHT, and 5β-DHT were tubes –6, 12, and 13, respectively.

Activity of T-converting enzymes

Figure 2A and B shows chromatograms of metabolites derived from T in prepared intestinal tissue at different incubation times. After 15 min of incubation, six eluting peaks were detected; they are labeled as fractions 1 to 6 in order of eluting time (Fig. 2A). After 30 min of incubation, the amount of substances in fractions 1, 3, 4, and 5 increased, whereas no substance was detected in fractions 2 and 6

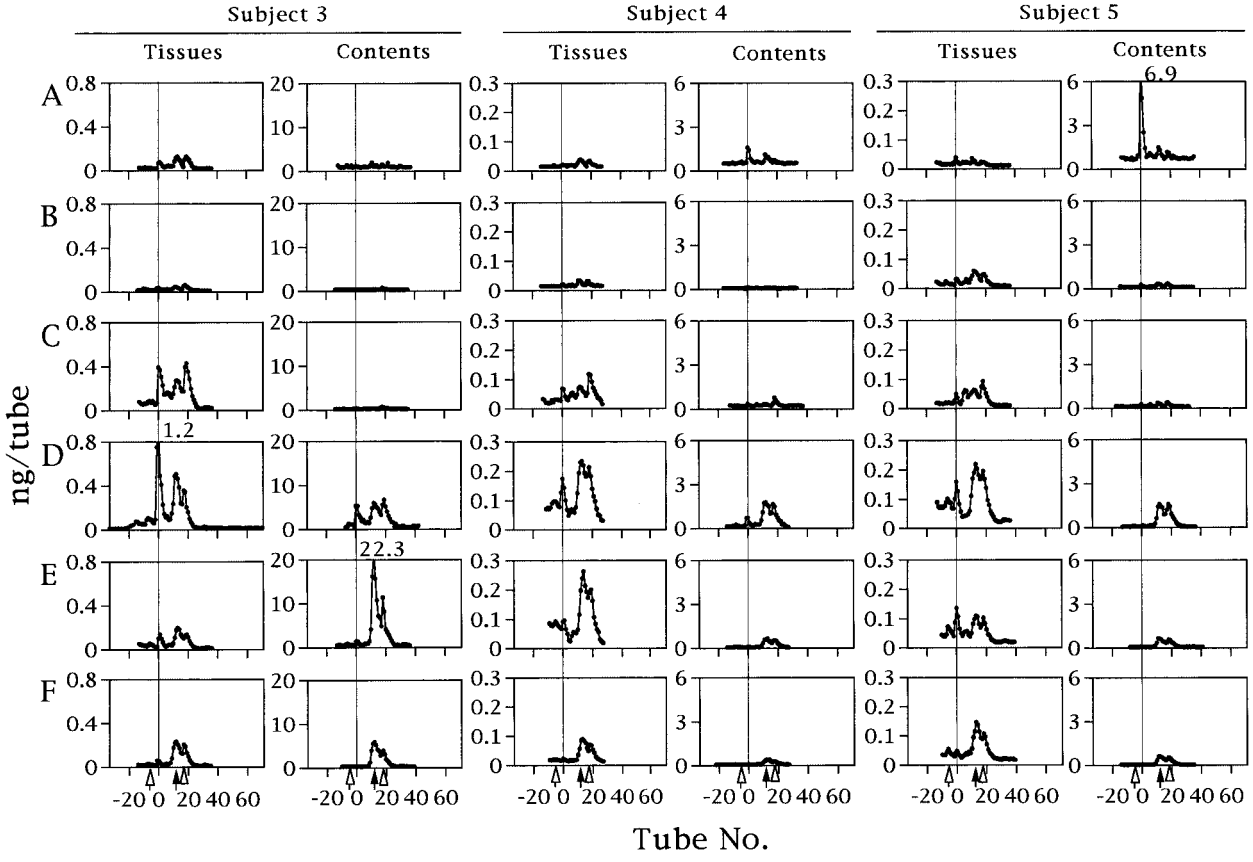
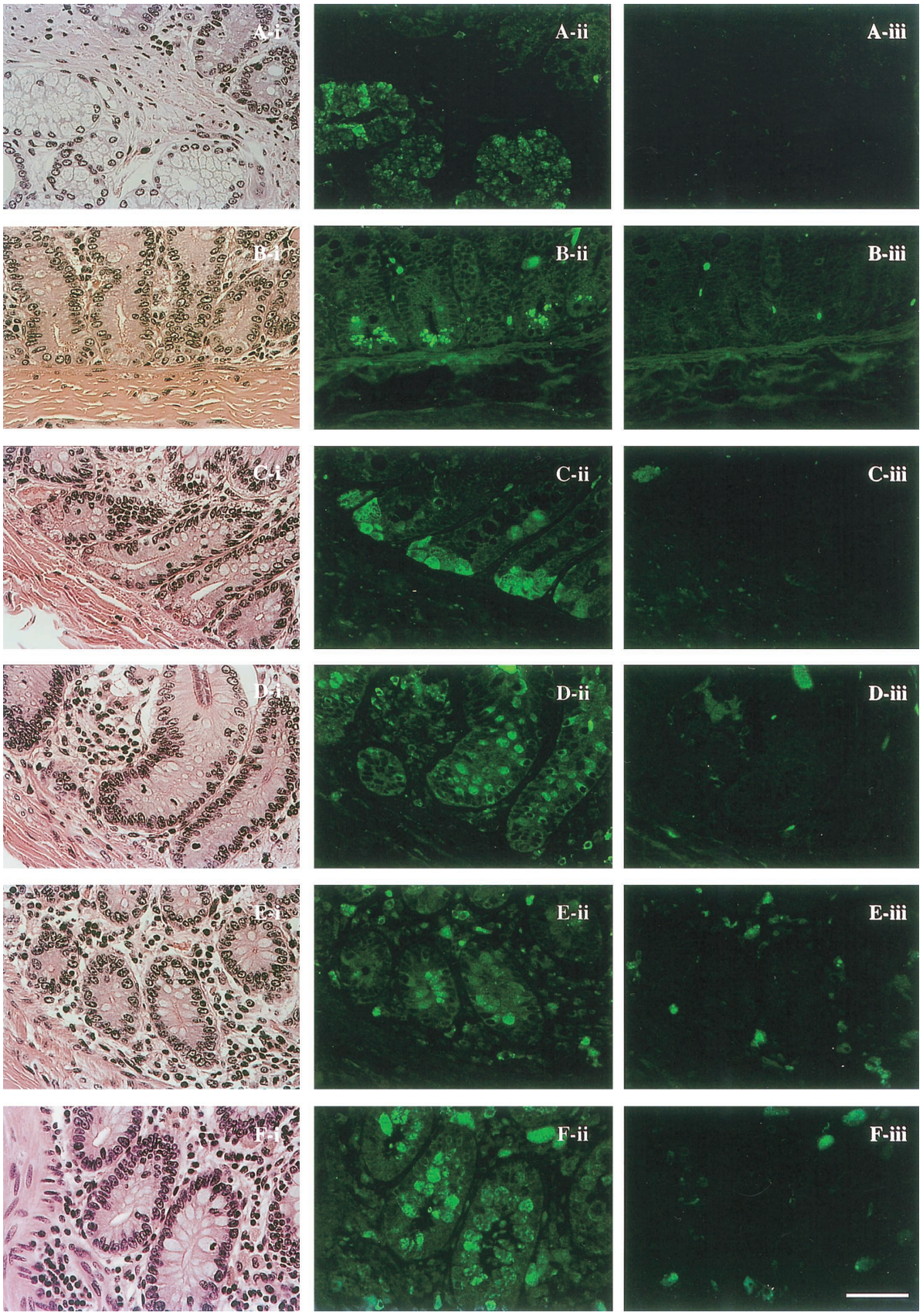


Fig. 5. Chromatograms of steroids in six segments (A, duodenum; B, jejunum; C, ileum; D, cecum; E, colon; and F, rectum) of the intestinal tissues and contents obtained from three monkeys. Amounts of steroids measured by T-RIA were converted to relative amounts derived from 1 g of tissues or dried contents. The x axis and symbols are the same as those described in the legend to Fig. 3. Numbers above certain plots show the value of data points that extend beyond the y-axis scale.



(Fig. 2B). The substance in fraction 1 had the highest peak, and its eluting position coincided with that of androstenedione. In the parallelism test, the dose-response curve prepared from the substance in fraction 1 was parallel to that from androstenedione ($P>0.05$). The eluting position of the substance in fraction 2 coincided with that of T, and the eluting position of the substance in fraction 4 coincided with that of 5α-DHT. The eluting positions of the substances in fractions 3, 5, and 6 were in tubes 10, 17, and 32, respectively. No metabolite was detected in the control incubation (Fig. 2C and D).

Steroids in intestinal tissue detected by T-RIA

A number of T-like substances were detected in the cecum (Fig. 3A). According to chromatograms, the largest amount of substance was found at the eluting position that coincided with fraction 2 (see Fig. 2). A parallelism test verified that the dose-response curve prepared from the substance in fraction 2 was parallel to that from T ($P>0.05$). The second largest amount of substance was detected at the eluting position that coincided with fraction 4 (see Fig. 2). A parallelism test verified that the dose-response curve prepared from this substance was parallel to that from 5α-DHT ($P>0.05$) but not to that from 5β-DHT ($P < 0.05$). Peaks were also detected in the eluting positions of fractions 1 and 5 but not in those of fractions 3 and 6 (see Fig. 2).

Steroids in intestinal tissue, detected by androstenedione-ELISA

Some androstenedione-like substances were detected in the cecum (Fig. 3B). A low peak was detected in the eluting position of fraction 1 (see Fig. 2). A parallelism test verified that the dose-response curve prepared from the substance in fraction 1 was parallel to that from androstenedione ($P>0.05$). A high peak was detected in the eluting position of fraction 4 (see Fig. 2). A parallelism test verified that the dose-response curve prepared from this substance was parallel to that from 5α-DHT ($P>0.05$). A peak was also detected in the eluting position of fraction 5 but not fractions 2, 3, and 6 (see Fig. 2).

Steroids in serum, detected by T-RIA

The eluting position of the most abundant T-like substance (Fig. 4) coincided with that of fraction 2 (see Fig. 2). A small amount of substance was detected in the position of fraction 4 (see Fig. 2), but no substance was found in the positions of fractions 1, 3, 5, or 6 (see Fig. 2).

Distribution of steroids in six segments of intestinal tissues and their contents

T-like substances were detected in all six segments of intestinal tissues and contents. The eluting positions of T-like substances (Fig. 5) coincided with those of fractions 1, 2, 4, and 5 (see Fig. 2). Of the three monkeys used in this experiment (Subjects 3, 4, and 5), the largest amount of substance was found in the tissue and contents of Subject 3. In the six separate intestinal segments from three monkeys, there was a similarity in the distribution of a substance whose eluting position coincided with that of fraction 2 (see Fig. 2). The largest amount of substance was found in the intestinal tissues and contents of the ceca, and the next largest amount of substance was detected in adjoining areas of the cecum, such as the ileum and the colon, except

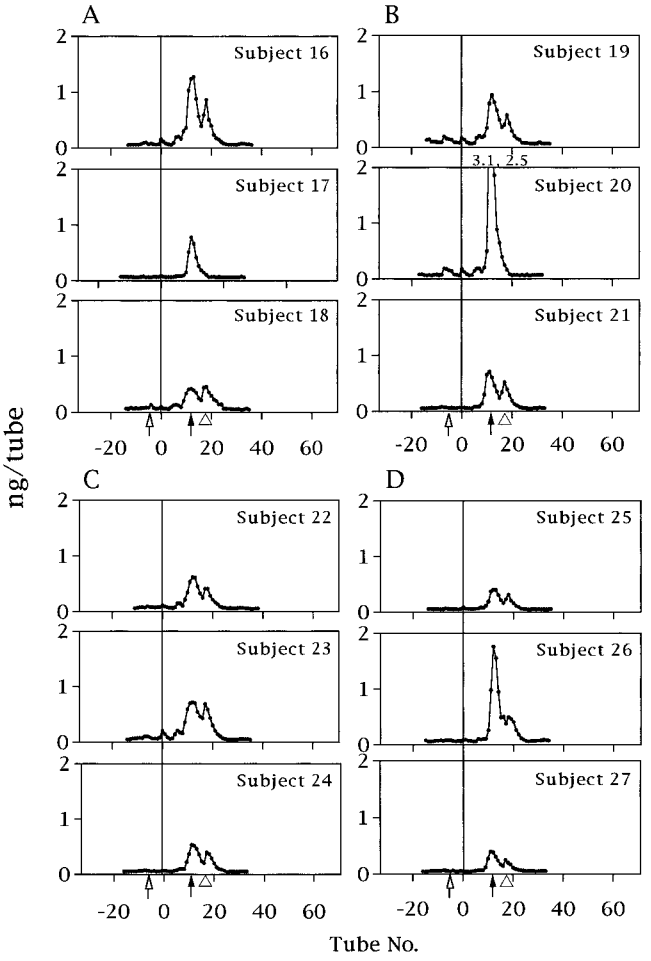


Fig. 7. Chromatograms of steroids in the feces of immature males (1 to 2 years of age) (A), mature males (10 years of age) (B), immature females (1 to 2 years of age) (C), and mature females (10 years of age) (D). Amounts of steroids measured by T-RIA were converted to relative amounts derived from 1 g of dried feces. The x axis and symbols are the same as those described in the legend to Fig. 3. Numbers above certain plots show the value of data points that extend beyond the y-axis scale.

Fig. 6. Immunofluorescence histochemical stains of T in six segments of the intestinal tissues (A, duodenum; B, jejunum; C, ileum; D, cecum; E, colon; and F, rectum). Typical results, obtained from Subject 13, are shown (magnification, × 400). The left column shows sections stained with hematoxylin and eosin (A-i to F-i); the middle column shows sections stained with anti-T antiserum (A-ii to F-ii); and the right column shows sections of the negative control with normal rabbit serum (A-iii to F-iii). Bar, 200 μm.

in the intestinal contents from Subject 5. A similar tendency was seen in the distribution of other substances.

Localization of testosterone, identified by immunofluorescence histochemistry

Six segments of intestinal tissues were collected from precise regions that showed no histopathologic changes (Fig. 6A-i to F-i). In all six segments, epithelia were stained with anti-T. In the duodenum, the glandular cells of duodenal glands were stained (Fig. 6A-ii). In the jejunum and ileum, the glandular cells at the basement of intestinal crypts were stained (Fig. 6B-ii and C-ii). In the cecum, colon, and rectum, the glandular cells at the intestinal crypts and the goblet cells distributed over the mucous membrane were stained (Fig. 6D-ii to F-ii). These positive cells, found in all six segments, showed granular cytoplasm. Distribution of anti-T staining in the intestines did not differ among animals of different ages, although Liang *et al.* (1999) have shown that the reactivity against T in the testes changes during development.

Steroid levels in feces, measured by T-RIA

T-like substances were detected in the feces of all 12 monkeys used in this experiment. According to chromatograms, the eluting positions of T-like substances (Fig. 7) coincided with those of fractions 1, 2, 4, and 5 (see Fig. 2). The amount of these substances was different in each animal. No relation was found between the amount of T-like substances and the age or sex of the animal.

DISCUSSION

This is the first study to document the synthesis and release of steroids in the intestinal tissues of immature and mature cynomolgus monkeys. Since biochemical assays of steroids in intestinal tissues have not been well established, we identified intestinal steroids based on the eluting positions revealed by HPLC and the affinity of steroids to antibodies estimated by the parallelism between dose-response curves. We concluded that the substance detected in fraction 1 was androstenedione, and the substance detected in fraction 2 was T. DHT contains two kinds of isomers–5α-DHT, which is a more potent androgen than T (Dorfman and Kincl, 1963), and 5β-DHT, which is an inactive androgen (Segaloff and Gabbard, 1962). Although the eluting positions of these two DHTs were close, the affinity to antibodies revealed that the substance detected in fraction 4 was 5α-DHT. The antibodies used in the T-RIA and androstenedione-ELISA were also cross-reactive to the substance detected in fraction 5, however, this substance was not identified yet.

To determine whether the steroids were synthesized in the intestinal tissues, we incubated the prepared intestinal tissues with [^{3}H]T *in vitro*. We used cecum tissue because the cecum has fewer digestive enzymes that might interfere with the reactions catalyzed by T-converting enzymes, and because it has a thinner muscle layer that is easy to homogenize. The results suggested that the steroids in intestinal tissues of cynomolgus monkeys were metabolized with certain steroidogenic enzymes, such as 17β-HSD type 2 and 5α-reductase. Sano *et al.* (2001) reported the expression and activity of 17β-HSD type 2 in the human gastrointestinal tract, and Normington and Russell (1992) reported the expression of transcripts of 5α-reductase in the rat intestine. Furthermore, in the rat gastrointestinal tract, the enzyme activities of cytochrome P450 17α-hydroxylase/17, 20-lyase, 17β-hydroxysteroid oxidoreductase, cytochrome P450 aromatase and 3β-hydroxysteroid dehydrogenase were reported (Dalla Valle *et al*., 1992; Le Goascogne *et al.,* 1995; Ueyama *et al*., 2002). Thus, intestinal tissues of cynomolgus monkeys may also have more enzymes as well as 17β-HSD type 2 and 5α-reductase and use steroid which is different from T as the first precursor for successive steps.

To determine if these steroids were also synthesized *in vivo*, we examined steroids in the cecum tissues by T-RIA and androstenedione-ELISA and compared them with the steroids in serum detected by T-RIA. In this experiment, we used a mixture of serum samples (i.e., samples from five monkeys combined into a single mixture) to determine the average relative concentrations of serum steroids. Previous studies (Bercu *et al*., 1983; Plant and Dubey, 1984) reported that the secretion of T shows a pulsatile pattern and that serum T concentrations fluctuate for several hours. On the other hand, temporal changes in serum 5α-DHT concentrations have not been reported. Steroids detected in the cecum tissues were different from those detected in serum, and therefore our results suggest that steroids were also synthesized *in vivo*. In the incubation with the intestinal tissue preparation, a great portion of T was converted into androstenedione, and T hardly remained after 30 minutes. Neverthless only a small amount of androstenedione and a large amount of T were detected in intestinal tissues. Thus, we supposed that androstenedione synthesized excessively was an artifact produced under *in vitro* experiment conditions. Sano *et al*. (2001) suggested that 17β-HSD type 2 in the gastrointestinal tract might take part in the inactivation of excessive endogenous and exogenous active sex steroids. We observed both the conversion from T to androstenedione that was involved in biological inactivation and the conversion from T to 5α-DHT that was involved in biological activation. Therefore, we propose that the enzymes expressed in the intestine might act cooperatively to regulate biological activity of the steroids in the intestine itself. Between T-RIA and androstenedione-ELISA, we judged T-RIA to be the more useful assay for this study, because it detected more intestinal steroids (androstenedione, T, 5α-DHT, and the unidentified substance detected in fraction 5) by cross-reactivity of the antibody used.

To determine if these steroids were also synthesized in other segments and released into the intestinal cavities, we examined steroids in the tissues and contents of six intestinal segments by T-RIA. The results suggest that steroids

detected in the intestinal contents were released from the intestinal tissues of the six segments. We then localized T in the six segments by using immunofluorescence. Immunohistochemical demonstration of the steroids still left plenty of room for dispute. For example, fixatives such as formaldehyde solution and Bouin's solution are not able to fix with certainty the steroids themselves in the cells or tissues for immunochemical reaction. However, according to Kawaoi *et al.* (1978), if the enzymes that localize in the microsomal or mitochondrial fraction and catalyze the biosynthesis of the steroids are properly fixed, the steroids bound to these enzymes could be indirectly stabilized in the cells. Indeed, many researchers have localized steroids in paraffin-embedded fixed tissues by using immunohistochemistry (Dornhorst and Gann, 1978; Kurman *et al*., 1979; Wong *et al*., 1984; Dobashi *et al*., 1985; Peute *et al*., 1989; Regadera *et al*., 1991; Liang *et al*., 1999, 2000). The present observations of steroids localized in the epithelia of all six intestinal segments are compatible with the results from T-RIA. This result is also consistent with the reports demonstrating the existence of steroidogenic enzymes in the gastrointestinal tract in rats (Dalla Valle *et al*., 1992; Le Goascogne *et al*., 1995; Ueyama *et al*., 2002) and humans (Sano *et al*., 2001) by biochemical and immunohistochemical means. Steroids have been generally considered to be secreted as endocrine agents from steroidogenic cells to blood (Ojeda and Griffin, 1996). However, our demonstration of steroids in the intestinal cavities suggests the possibility that intestinal steroids can also function as exocrine agents.

T and 5α-DHT are major androgens. The T concentrations in serum correlate with age and sex (Steiner and Bremner, 1981; Westfahl *et al*., 1984; Meusy-Dessolle and Dang, 1985; Yoshida, 1990). Thus, to determine if concentrations of these steroids in the intestine also correlate with age and sex, we measured concentrations of T and 5α-DHT in the feces of 12 immature and mature monkeys of both sexes. Cynomolgus monkeys are immature at 1 to 2 years of age and mature at 10 years of age (Steiner and Bremner, 1981; Meusy-Dessolle and Dang, 1985; Yoshida, 1990). In our study, steroids were detected in the feces of both immature and mature monkeys of both sexes, and their levels were not affected by the age or sex of animal. Furthermore, immunofluorescence showed that the distribution of T in the intestines did not differ with age in male monkeys. Thus, the characteristics of steroids released in the intestines seem to be different from those found in serum.

Although the specific function of intestinal steroids is still not known, some effects of T in the intestines have been reported, such as the following: 1) T treatment induces increased uptake and concentrations of 1,25-dihydroxy vitamin D_3 in intestines (Otremski *et al*., 1997); 2) T enhances cell proliferation in the epithelia of small intestines (Carriere, 1966; Wright and Morley, 1971; Wright *et al*., 1972; Tutton and Barkla, 1982); and 3) T is an important factor for determining susceptibility of small intestines to *Toxoplasma gondii* infection (Liesenfeld *et al*., 2001). For these effects, the source of the steroid in intestines is considered to be steroidogenic organs, such as gonads and adrenals, whereas our study demonstrates the synthesis of steroids in the intestinal tissues themselves. Most of the released steroids were excreted outside the body as feces without being converted, although various conversions are accomplished in the intestine by enzymes produced by the digestive system of the host animal or microbes (Stevens, 1988). Thus, the intestinal steroids seem to have different aspect from classical serum steroids, e.g., in their effects as pheromones in humans (Cowley and Brooksbank, 1991; Grosser *et al*., 2000).

In conclusion, we have shown that intestinal steroids in cynomolgus monkeys are synthesized in the intestinal tissues themselves. Released steroids were detected not only in intestinal cavities but also in feces. These results suggest the possibility that intestinal steroids are paracrine or exocrine agents. Therefore, further study should focus on whether there is a steroid receptor in the intestinal tissues. Some studies (Labrie, 1991; Baulieu, 1997; Tsutsui *et al*., 2000) have revealed new functions of steroids, such as neurosteroids, from organs other than classical steroidogenic organs. Our findings contribute to these new research directions.

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