DOI: 10.17311/sciintl.2015.31.36

In vitro Metabolic Changes in the Corticosteroidal Hormones in Faeces of Ruminants

[1]Sabry M. El-Bahr, [2]R. Palme, [2]E. Mostl, [1]N. Taha, [1]M. Korshom and [1]A. Mandour
[1]Department of Biochemistry, Faculty of Veterinary Medicine, Alexandria University, Egypt
[2]Institute of Medicinal Biochemistry, University of Veterinary Medicine, Veterinarplatz 1, A-1210, Vienna, Austria

ABSTRACT

Objective: The present study aimed to investigate the extent of stability of corticosteroid hormones against the bacterial actions and their enzymatic activities that are naturally occurring in faeces. **Materials and Methods:** Faecal samples were freshly collected from each of 10 cattle and 10 sheep. Each sample was immediately incubated in a water bath at 38°C with either 1 μg of cortisol or corticosterone. The incubations were then run at time interval of 4, 8 and 24 h (in cattle) and at 1, 2, 4, 8 and 24 h (in sheep). The samples were extracted with methanol and the aliquots were taken after centrifugation from their supernatants and analyzed for measurement of cortisol and corticosterone by Enzyme Immunoassays (EIAs). **Results:** A dramatically decline in the concentrations of the added cortisol and corticosterone was observed in cattle faeces. Only about 20% of the starting levels of both hormones after 4 h in contrast to almost no cortisol with about 10% of corticosterone after 8 h and about 5% of corticosterone after 24 h was detected. Prolonged incubation of faecal samples of sheep decreased the immunoreactive substances measured by the 3α-hydroxy, 11-oxoandrogens EIA (11-oxo-A-EIA), whereas with the 11, 17 dioxoandrostanes EIA (11, 17 DOA-EIA), the values were increased. **Conclusion:** Cortisol was heavily metabolized by faecal flora being quicker in its metabolism than corticosterone in the faeces of ruminants. In addition, the present study provides a powerful evidence to the absence of native cortisol in the faeces of ruminants.

Key words: Cortisol, corticosterone, faeces, ruminants, metabolism, enzyme immunoassay

Science International 3 (1): 31-36, 2015

INTRODUCTION

The concentration of glucocorticoids (cortisol and corticosterone) in blood is widely used as an indicator of stress, although caution is advised, since an increase does not occur with every type of stressor[1]. Like many other hormones, glucocorticoids have a circadian rhythm in many species but such rhythms may be abolished by prolonged stress[2]. Thus, rhythmicity and episodic secretion demand frequent sampling. One has to consider that sample collection which often involves confinement or handling of animals may by itself be stressful and may confound the results[3]. Therefore, feedback-free sampling methods are preferential. To overcome these problems, several authors have investigated non-invasive sampling procedures such as a corticoid (metabolites) determination in urine[4], saliva[5] or milk[6]. However, there are some major drawbacks related to each: Saliva or urine collection also needs some manipulation of the animal and can be used only to a limited extent in free moving animals and milk is limited to lactating animals. Above all, faecal samples offer the advantages that they can be easily collected without stressing the animals. Methods for measuring faecal metabolites of placental or gonadal origin are well established to evaluate reproductive function[7]. As the measurement of physiological stress also has an importance in wildlife management, conservation biology and behavioural ecology, the measurement of faecal glucocorticoids metabolites is gaining increased importance. For the development of non-invasive techniques to monitor adrenocortical activity, basic information of the metabolism and excretion of glucocorticoids is necessary. The excretion of ^{14}C cortisol was investigated in the sheep[8]. The study stated that two-third of the radioactivity was subsequently

Corresponding Author: Sabry M. El-Bahr, Department of Biochemistry, Faculty of Veterinary Medicine, Alexandria University, Egypt

found in the bile. The main metabolites were glucouronides of tetrahydrocortisol, tetrahydrocortisone and cortolone. Also $C_{19}O_3$ steroids (androstanes) were formed. Additionally, enterohepatic circulation of these metabolites occurred and cortisol metabolites were voided via faeces. In farm animals, infused radioactive cortisol is mainly excreted in urine[9]. Excretion via faeces is 28% in sheep. These studies also described a species-specific lag time between the infusion and the peak of radioactivity in the faeces (10-12 h in ruminants). The cortisol metabolite may be further metabolized during this period. For example, the side chain of some C-21 steroids cleaved by the faecal flora of human and rats to C-19 compounds[10]. The elevated level of faecal cortisol can be measured during stress in the rocky mountain bighorn sheep[11]. Three different commercially available radioimmunoassay for cortisol and one assay for corticosterone in a variety of wildlife mammals were investigated[12]. A corticosterone antibody (ICN = Biomedicals, Costa Mesa, CA) gave best results. This antibody may not only be used for radioimmunoassay but also for EIA[13]. The cross-reactions of this antibody are still not fully evaluated. In contrast, radioactive faecal cortisol metabolites using High Performance Liquid Chromatography (HPLC) and EIAs were characterized[14]. They[14] showed that the predominant faecal metabolites were unconjugated steroids but they could not detect immunoreactive cortisol. Especially in ruminants, even after intravenous infusion of 1 g of cortisol, no native cortisol could be detected in the faeces of sheep[15]. Group specific Enzyme Immunoassays (EIAs) for cortisol metabolites using 11-oxoetiocholanolone as immunogen and a biotinylated steroid as label were described[14,16]. The assay system described by Palme and Mostl[14] used antibody against 11-oxoetiocholanolone coupled at position C-3. This EIA (measuring 11,17 dioxoandrostanes-11,17 DOA) proved suited for evaluating adrenocortical activity in sheep, cows and horses[15,17,18] and also for some zoo and wildlife animals (e.g., roe deer, hares ad elephants[16,19-21] raised an antibody against 11-oxoetiocholanolone coupled at C-17 (11-oxo-A). This EIA records higher concentration of cortisol metabolite in cows compared to that described by Palme and Mostl[22], most probably because the new antibody has also cross reactions with $C_{21}O_4$ cortisol metabolites. The aim of this *in vitro* study is to investigate whether the faecal flora of ruminants are capable of converting cortisol or corticosterone clarifying which of these steroids is quickly metabolized. The aim of this study was also extended to investigate the stability of cortisol in faecal samples of sheep clarifying whether or not native cortisol is present in faeces of these species.

MATERIALS AND METHODS

Experiment No. 1 in Cattle: A total of 10 faecal samples were freshly collected from healthy cattle (5 cows and 5 bulls). Forty times 0.5 g faeces from each faecal sample were weighed and incubated in a water bath at 38°C with buffer containing 1 μg cortisol or corticosterone (n = 20 each; Wilton, NH, USA), respectively immediately and then at intervals after 4, 8 and 24 h. Samples were deep frozen (-20°C; n = 5 each) and stored until the time of analysis (10 faecal samples×2 steroids×5 replicates×4 times = 400 incubations). Afterwards all samples were extracted with 4 mL methanol (80%) and centrifuged (2500 g). Then 10 μL aliquot of the supernatant (after further dilution 1:100 for cortisol and 1:1000 for corticosterone) was analyzed with cortisol and corticosterone EIAs, respectively. Cortisol and corticosterone EIAs were performed on microtitre plates using the double antibody technique and biotinylated steroids as labels[14,22]. Antisera were raised in rabbits against cortisol-3-CMO and corticosterone-3-CMO, respectively each coupled with Bovine Serum Albumin (BSA). The labels were cortisol-3-CMO (for both EIAs) linked to DADOO biotin (N-biotinyl-1, 8-diamino-3, 6-dioxaoctane). The standards were 2-80 pg/well for cortisol and 2-500 pg/well for corticosterone. Briefly, standards (50 μL) and samples (50 μL) were incubated in duplicate with label (100 μL) and antibody (100 μL) overnight at 4°C. Following incubation, the plates were four times washed with 0.02% Tween 20 (Merck 822184) washing solution and dry blotted, before 250 μL streptavidin horseradish peroxidase conjugate (4.2 mU, Boehringer, No. 1089153) was added to each well. Plates were then left at 4°C in the dark on stirring tables for another 45 min. The enzymatic reaction was stopped with 50 μL/well of 4 mol L sulfuric acid. Absorbance was measured at a wavelength of 450 nm (reference filter: 620 nm) on an automatic plate reader (Labsystems Multiskan, MCC/340; Szabo).

Experiment No. 2 in sheep: Ten faecal samples were freshly collected from healthy sheep (5 ewes and 5 rams). Aliquots of 0.5 g faeces in triplicates from each faecal sample were incubated in a water bath at 38°C with 1 mL buffer containing 1 μg cortisol. Bacterial action was stopped immediately by adding 4 mL of 80% methanol and then after 1, 2, 4, 8 and 24 h (10 faecal samples×1 steroid×3 replicates×6 times = 180 incubations). Afterwards all samples were extracted,

centrifuged (2500 g) and a 10 μL aliquot of the supernatant (after further dilution 1:100) was analyzed with 11,17 DOA and 11-oxo-A EIAs which were performed on microtitre plates using the double antibody technique and biotinylated steroids as labels[16,22]. Antisera were raised in rabbits against 11-oxoetiocholanolone-3-HS:BSA and 11-oxoetiocholanolone-17-CMO:BSA, respectively. The labels were 11-oxoetiocholanolone-3-glucuronide (for both) linked to DADOO biotin (N-biotinyl-1, 8-diamino-3, 6-dioxaoctane). The standards were 11-oxoetiocholanolone (range 500-2 pg/well for both). The EIAs were performed as described above.

Statistical analysis: Amounts of measured glucocorticoids (cortisol and corticosterone), present after a given time, were expressed as a percentage of added glucocorticoids (cattle). The concentrations of 11,17-DOA and 11-oxo-A during incubations, measured immediately after collection of the faeces were considered to be 100% and the variations were calculated as percent increase or decrease (sheep). To determine the effect of incubation time on the measured steroid concentrations a repeated measures ANOVA was used. All tests were performed with the software package SIGMASTAT© (SPSS Inc., Chicago, IL, USA).

RESULTS

Experiment No. 1 in cattle: In the cattle faeces (cows and bulls) a dramatical decrease ($p<0.001$) in the concentrations of the added cortisol and corticosterone (1 μg) was observed (Fig. 1a, b) amounts of measured steroids, present after a given time are expressed as a percentage of the starting values. After 4 h about 23% of the starting level of cortisol and 25% corticosterone were reached (Fig. 1a). After 8 h almost no cortisol could be detected but about 10% of corticosterone was detected (Fig. 1a). After 24 h about 5% of the starting levels of corticosterone still present. The same pattern was found in bull (Fig. 1b). There was some individual variation between the five animals (of the same sex) concerning time course and the amount decreased.

Experiment No. 2 in sheep: To demonstrate changes in the 11,17-DOA and 11-oxo-A concentrations during incubation with cortisol (1 μg), the values measured immediately after collection of the faeces were considered to be 100% and the variations were calculated as percent increase or decrease (Table 1). In sheep (both rams and ewes), there was a significant ($p<0.01$) increase in 11,17-DOA concentrations within 1 h (Table 1). After 2, 4, 8 and 24 h of incubation, the concentrations were significantly elevated compared to the starting values (Table 1). The increase in 11,17-DOA concentrations was significantly differed in rams and ewes at all time points of incubations (Table 1). In sheep (both rams and ewes), there was a significant ($p<0.01$) decrease in 11-oxo-A concentrations within 1 h (Table 1). After 2, 4, 8 and 24 h of incubation, the concentrations were also significantly decreased compared to the starting values (Table 1). The decrease in 11-oxo-A concentrations was significantly differed in rams and ewes at 4, 8 and 24 h of incubations (Table 1).

DISCUSSION

The metabolism of glucocorticoids include oxidation at C-11; reduction at C-3 and/or C-20 and/or C21; hydroxylation at C-6; as well as formation of ring A saturated steroids[23]. This metabolism takes place mainly in the liver and the metabolites are subsequently excreted as conjugates (sulphates or glucouronides) via the urine and the bile[8]. In addition, intestinal bacteria in the gut can affect the structure of these steroids. For example, a side-chain cleavage was found for cortisol, resulting in the formation of androstanes which in contrast to androgen metabolites, still bear an oxygen at position C-11[24]. In ruminants at least 21 cortisol metabolites (with $C_{21}O_4$ or $C_{19}O_3$ structure) were detected in faecal samples using HPLC/mass spectroscopy[16]. In the present study, incubation of faecal samples (0.5 g; n = 20) with high dose of cortisol or corticosterone (1 μg), for several hours, revealed a quick metabolism, reflected by a rapid decrease of glucocorticoids concentration to negligible amounts after a few hours (4 h) (Fig. 1). The faecal flora which are capable of metabolizing steroids in the faeces of human and rats[10] may be the cause for such decrease of cortisol and corticosterone in faeces of ruminants. The present study also demonstrated that, cortisol was quickly metabolized in faeces of ruminants than corticosterone (Fig. 1). This might be due to the action of faecal flora which contains a desmolase enzyme which removes the side chain from some C-21 steroids as described in the faces of human and rats[10]. This interpretation can be underlined by the fact that a 17 α-hydroxy group is a prerequisite for a steroid to be undergo desmolase side chain cleavage[10]. In this respect cortisol which contain 17 α-hydroxy group was a preferred substrate for desmolase enzyme and subsequently more quickly

Table 1: Increase (%) in 11,17-DOA and 11-oxo-A concentrations (Mean±SD) after incubation of faecal samples of sheep with cortisol (1 μg) for 1-24 h

	Incubation (h)					
Animal	0	1	2	4	8	24
11,17-DOA						
Rams	100^{Ea}	164 ± 36^{Da}	198 ± 87^{Ca}	235 ± 97^{Ba}	235 ± 75^{Bb}	277 ± 104^{Ab}
Ewes	100^{Fa}	136 ± 14^{Eb}	175 ± 33^{Db}	221 ± 97^{Cb}	274 ± 83^{Ba}	332 ± 105^{Aa}
t-value	1.55^{NS}	40.55^{**}	35.46^{**}	37.97^{**}	42.93^{**}	49.45^{**}
11-oxo-A						
Rams	100^{Aa}	59 ± 13^{Ba}	47 ± 13^{Ca}	35 ± 17^{Db}	25 ± 13^{Ea}	20 ± 12^{Fa}
Ewes	100^{Aa}	59 ± 16^{Ba}	49 ± 15^{Ca}	40 ± 17^{Da}	16 ± 9^{Eb}	12 ± 7^{Eb}
t-value	3.24^{NS}	2.22^{NS}	5.57^{NS}	15.56^{*}	38.49^{**}	37.40^{**}

Capital letter: Indicated that means within the same row carrying different letter are significantly differed at ($p<0.01$), Small letter: Indicated that means within the same column carrying different letter are significantly differed at ($p<0.01$), NS: Non significant, **: Highly significant at ($p<0.01$), *: Significant at ($p<0.05$)

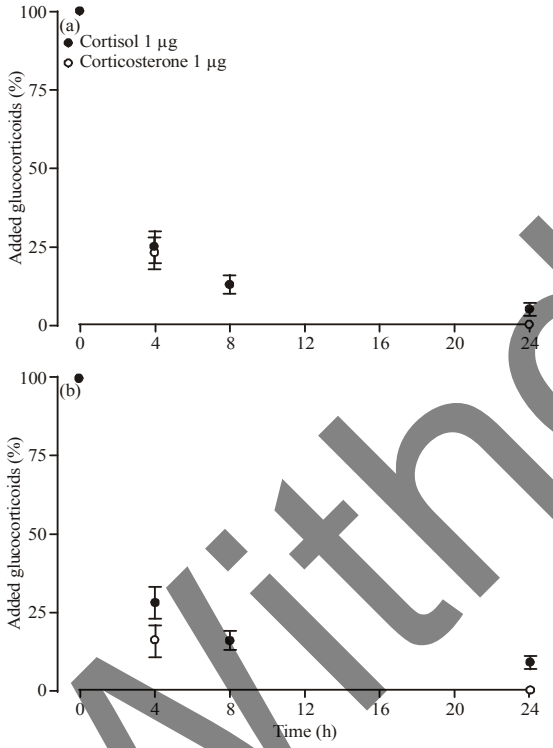

Fig. 1(a-b): Metabolic changes of cortisol and corticosterone in fresh faecal sample of (a) Cows and (b) Bulls (n = 5, Mean±SE)

metabolized than corticosterone which has no 17 α-hydroxy group. The profound and quick changes of cortisol by faecal flora of cattle (almost no cortisol could be detected after 8 h of incubation, Fig. 1) was also confirmed by the second experiment in faeces of sheep as prolonged incubation of faecal samples of sheep (ewes and rams) decreased the immunoreactive substances measured by the 11-oxo-A-EIA, whereas with the 11,17-DOA enzyme immunoassay, the values were increased (Table 1). This may be explained by the effect of bacterial side-chain cleavage, forming an increased amount of $C_{19}O_3$ steroids. The 11-oxo-A EIA cross-reacts with some $C_{21}O_4$ steroids, whereas the 11,17- DOA EIA does not. Hence, an explanation for the decrease in immunoreactive substances using 11-oxo-A EIA may be that the assay has more cross-reactions with some $C_{21}O_4$ steroids than with the $C_{19}O_3$ metabolites formed from the precursors during incubation. An explanation for the increase in immunoreactive substances using 11, 17-DOA EIA may be that the assay has more cross-reactions with some $C_{19}O_3$ steroids than with the $C_{21}O_4$ metabolites formed from the precursors during incubation. The increase and the decrease in the concentration of 11,17-DOA and 11-oxo-A, respectively indicated that cortisol was heavily metabolized by bacterial side chain cleavage caused most probably by desmolase activity[10]. No explanation for the sex variation in the increase in 11, 17-DOA concentrations (Table 1) and the decrease in 11-oxo-A concentrations (Table 1). This finding was found to be in agreement with previous study[22] showed that, the predominant faecal metabolites were unconjugated steroid and they could not detect immunoreactive cortisol. The same study concluded that even after intravenous infusion of 1 g cortisol, no native cortisol could be detected in the faeces of sheep. The present results was also closely related with the results of previous study[17] which found an increase in immunoreactive 11,17-DOA after incubating faecal samples at room temperature which was probably caused by bacteria. However, this findings disagreed with Miller *et al*.[11] who described that elevated level of faecal cortisol can be measured during stress in the Rocky mountain bighorn sheep. The findings also disagreed with other study[12] which investigated three different commercially available radioimmunoassay for

cortisol and one assay for corticosterone in a variety of wildlife mammals. A corticosterone antibody (ICN Biomedicals, Costa Mesa, CA) gave best results. This antibody may not only be used for radioimmunoassay but also for EIA[13]. The cross-reactions of this antibody are still not fully evaluated. The present study concluded that cortisol is heavily metabolized by faecal flora and seems quicker in its metabolism than corticosterone in the faeces of ruminants. In addition, the present study provide a powerful evidence that no native cortisol could be detected in the faeces of ruminants.

ACKNOWLEDGMENTS

The author thanks Institute of Biochemistry (University of Veterinary Medicine, Vienna, Austria) for providing all antibodies and enzyme labels for different EIAs used in this research. The whole experiment was exhibited in Institute of Biochemistry, University of Veterinary Medicine, Vienna, Austria.

REFERENCES

1. Broom, D.M. and K.G. Johnson, 1993. Stress and Animal Welfare. Champan and Hall, London, pp: 87-144.
2. Przekop, F., E. Stupnicka, E. Wolinska-Witort, K. Mateusiak, B. Sadowski and E. Domanski, 1985. Changes in circadian rhythm and suppression of the plasma cortisol level after prolonged stress in the sheep. Acta Endocrinologica, 110: 540-545.
3. Morton, D.J., E. Anderson, C.M. Foggin, M.D. Kock and E.P. Tiran, 1995. Plasma cortisol as an indicator of stress due to capture and translocation in wildlife species. Vet. Record, 136: 60-63.
4. Hay, M. and P. Mormede, 1998. Urinary excretion of catecholamines, cortisol and their metabolites in Meishan and Large White sows: Validation as a non-invasive and integrative assessment of adrenocortical and sympathoadrenal axis activity. Vet. Res., 29: 119-128.
5. Cooper, T.R., H.R. Trunkfield, A.J. Zanella and W.D. Booth, 1989. An enzyme-linked immunosorbent assay for cortisol in the saliva of man and domestic farm animals. J. Endocrinol., 123: R13-R16.
6. Verkerk, G.A., A.M. Phipps, J.F. Carragher, L.R. Matthews and K. Stelwagen, 1998. Characterization of milk cortisol concentrations as a measure of short-term stress responses in lactating dairy cows. Anim. Welfare, 7: 77-86.
7. Schwarzenberger, F., E. Mostl, R. Palme and E. Bamberg, 1996. Faecal steroid analysis for non-invasive monitoring of reproductive status in farm, wild and zoo animals. Anim. Reprod. Sci., 42: 515-526.
8. Lindner, H.R., 1972. Enterohepatic circulation and the pattern of urinary excretion of cortisol metabolites in the ewe. J. Endocrinol., 52: 19-20.
9. Palme, R., P. Fischer, H. Schildorfer and M.N. Ismail, 1996. Excretion of infused ^{14}C-steroid hormones via faeces and urine in domestic livestock. Anim. Reprod. Sci., 43: 43-63.
10. Cerone-Mclernon, A., J., Winter, E., Mosbach and V.D. Bokkenheuser, 1981. Side-chain cleavage of cortisol by fecal flora. Biochimica Biophysica Acta (BBA)-Lipids Lipid Metab., 666: 341-347.
11. Miller, M.W., N.T., Hobbs and M.C. Sousa, 1991. Detecting stress responses in Rocky Mountain Bighorn sheep (*Ovis canadensis Canadensis*): Reliability of cortisol concentrations in urine and feces. Can. J. Zool., 69: 15-24.
12. Wasser, S.K., K.E. Hunt, J.L. Brown, C. Cooper and C.M. Crockett *et al*., 2000. A generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species. Gen. Comp. Endocrinol., 120: 260-275.
13. Goymann, W., E. Mostl, T. Van't Hof, M.L. East and H. Hofer, 1999. Noninvasive fecal monitoring of glucocorticoids in spotted hyenas, *Crocuta crocuta*. Gen. Comp. Endocrinol., 114: 340-348.
14. Palme, R. and E. Mostl, 1994. Biotin-Streptavidin Enzyme Immunoassay for the Determination of Oestrogens and Androgens in Boar Faeces. In: Advances of Steroid Analysis, Gorog, S. (Ed.). Akademiai Kiado, Budapest, pp: 111-117.
15. Palme, R., C.H. Robia, S. Messmann, J. Hofer and E. Mostl, 1999. Measurement of faecal cortisol metabolites in ruminants: A non-invasive parameter of adrenocortical function. Wiener Tierarztliche Monatsschrift, 86: 237-241.
16. Mostl, E., J.L. Maggs, G. Schrotter, U. Besenfelder and R. Palme, 2002. Measurement of cortisol metabolites in faeces of ruminants. Vet. Res. Commun., 26: 127-139.
17. Mostl, E., S. Messmann, E. Bagu, C. Robia and R. Palme, 1999. Measurement of glucocorticoid metabolite concentrations in faeces of domestic livestock. J. Vet. Med. Ser. A, 46: 621-631.
18. Palme, R., C. Robia, W. Baumgartner and E. Mostl, 2000. Transport stress in cattle as reflected by an increase in faecal cortisol metabolite concentrations. Vet. Rec., 146: 108-109.

19. Stead, S.K., D.G. Meltzer and R. Palme, 2000. The measurement of glucocorticoid concentrations in the serum and faeces of captive African elephants (*Loxodonta africana*) after ACTH stimulation. J. South Afr. Vet. Assoc., 71: 192-196.
20. Teskey-Gerstl, A., E. Bamberg, T. Steineck and R. Palme, 2000. Excretion of corticosteroids in urine and faeces of hares (*Lepus europaeus*). J. Comp. Physiol. B, 170: 163-168.
21. Dehnhard, M., M. Clauss, M. Lechner-Doll, H.H.D. Meyer and R. Palme, 2001. Noninvasive monitoring of adrenocortical activity in roe deer (*Capreolus capreolus*) by measurement of fecal cortisol metabolites. Gen. Comp. Endocrinol., 123: 111-120.
22. Palme, R. and E. Mostl, 1997. Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. Int. J. Mamm. Biol., 62: 192-197.
23. Brownie, A.C., 1992. The Metabolism of Adrenal Cortical Steroids. In: The Adrenal Gland, James, V.H. (Ed.). 2nd Edn., Raven Press, New York, USA., pp: 209-224.
24. MacDonald, I.A., V.D. Bokkenheuser, J. Winter, A.M. Mclernon and E.H. Mosbach, 1983. Degradation of steroids in the human gut. J. Lipid Res., 24: 675-700.