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Original article

Incorporation of oxyphytosterols in tissues of hamster

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Abstract – Oxyphytosterols (OPS) were fed to hamsters, at different concentrations, in order to observe their eventual incorporation into plasma, aorta, liver, kidneys and heart. The animals receiving the very high level (2500 ppm) presented 7β-hydroxycampesterol, β-epoxycampesterol, campestanetriol, 7-ketocampesterol, 7β-hydroxysitosterol, β-epoxysitosterol, sitostanetriol and 7-ketositosterol in all tissues. The same compounds were observed in the tissues of animals receiving 500 ppm of OPS in their diet, but with much lower levels. In hamsters fed 100 ppm of OPS, as well as in control animals, in most cases, the only observed OPS was sitostanetriol, which seems to be difficult to eliminate from the animal.

oxyphytosterol / sitostanetriol / phytosterol / plasma / aorta / liver / kidneys / heart

1. INTRODUCTION

Phytosterols are minor compounds present in plants. Their structure is close to that of cholesterol. The main phytosterols (Fig. 1) have a methyl (campesterol) or an ethyl (sitosterol) group on carbon 24 and another ethylenic bond on 22–23 (stigmasterol) or on 24–28 (Δ5-avenasterol). The intestinal absorption of these compounds is low (3 to 5% for sitosterol, 10 to 12% for campesterol). They are now considered as very important in the diet. They indeed allow a noticeable decrease of cholesterol absorption, inducing a lowering of LDL plasma cholesterol [1]. Some food enriched in phytosterols (spreads, yogurts, …) are now proposed to consumers. However, these phytosterols can be oxidized as well as cholesterol [2, 3]. These oxyphytosterols (Fig. 2) have been identified in some foods [4–8]. Very little is known on the physiological effects of these oxyphytosterols. However, it has been shown that small levels of these compounds could pass the intestinal barrier in rats [9]. Moreover, some oxyphytosterols have been recently identified in the plasma of healthy human subjects [10]. The present experiment was effected in order to see if these oxyphytosterols could be observed in tissues of animals fed with these compounds.

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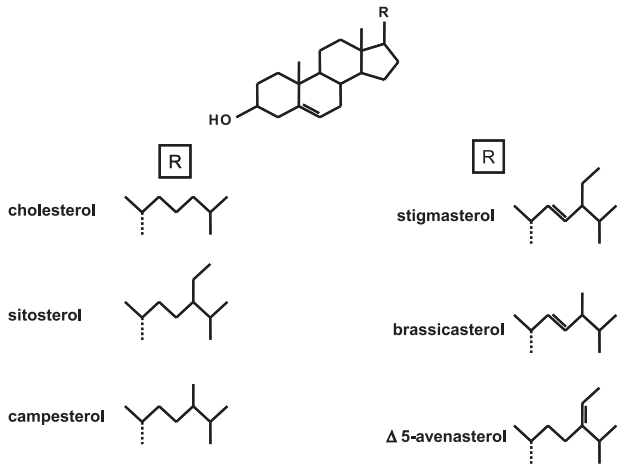

Figure 1. Structure of the main phytosterols and of cholesterol.

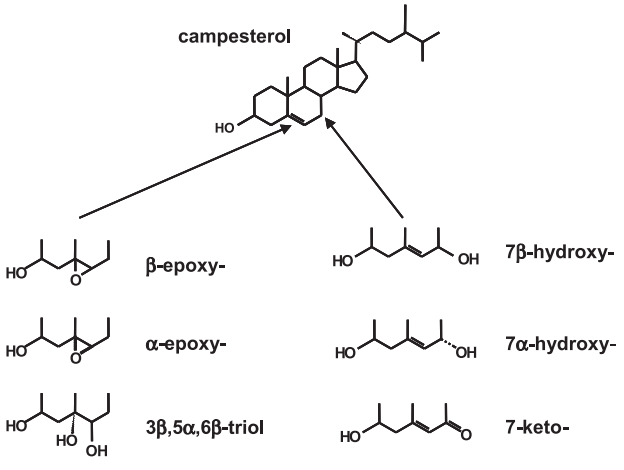

Figure 2. Structure of the main oxyphytosterols. (The compounds presented are the oxidized compounds of campesterol. However, analogue compounds are issued from the other phytosterols.)

2. MATERIALS AND METHODS

2.1. Preparation of oxyphytosterols

A blend of phytosterols (ICN, Irvine, Ca, USA), containing 34.3% campesterol, 4.6% stigmasterol, 56.7% sitosterol, 0.8% Δ5-avenasterol and some other minor phytosterols, was heated at 135 °C, during 24 h. The oxyphytosterols (OPS) formed were purified by silica column chromatography. The unoxidized phytosterols were first eluted using increasing levels of *tert*-butylmethyl ether (TBME) in hexane: (1/9; V/V), (2/8; V/V), (1/2; V/V), (1/1; V/V) and (2/1; V/V). The oxyphytosterols were then

Table I. Composition of the oxyphytosterol fraction (%).

7α-hydroxycampesterol	0.9
6β-hydroxycampesterol	0.9
7β-hydroxycampesterol	1.8
β-epoxycampesterol	2.4
α-epoxycampesterol	2.8
6-ketocampesterol	0.7
7-ketocampesterol	2.4
Total oxidized campesterol derivatives	11.9
7α-hydroxystigmasterol	0.1
7-ketostigmasterol	0.8
Total oxidized stigmasterol derivatives	0.9
7α-hydroxysitosterol	9.0
6β-hydroxysitosterol	3.0
7β-hydroxysitosterol	12.4
β-epoxysitosterol	11.8
α-epoxysitosterol	13.6
Sitostanetriol	2.5
6-ketositostanol	5.5
7-ketositosterol	21.1
Total oxidized sitosterol derivatives	78.9
Not fully identified compounds	8.3

obtained using successively methanol and acetone. Their composition was analyzed as described later and is presented in Table I.

2.2. Animals and diets

All studies were done within the guidelines set for the care and use of laboratory animals, in agreement with official French regulations. Thirty-two Golden Syrian five week old hamsters (Charles River, L'Arbresle, France) were used. During a pre-experimental period of a week, all animals received a purified diet [11] containing 5% in weight of soybean oil. Four groups of 8 animals weighing 61.9 ± 4.7 g were then fed during 2 weeks with the experimental diets. The groups differed only by the amount of OPS in their diet. Group A (control group) received the same control diet without OPS. The groups B, C and D received respectively 100 ppm, 500 ppm or 2500 ppm of OPS in their diet. The OPS were incorporated in soybean oil. The animals were fed ad libitum. At the end of the experiment, the animals were anesthetized using isoflurane.

2.3. Oxysterol analyses

This method allows to determine all the oxysterols (oxycholesterols and oxyphytosterols). Blood was collected from the aorta using EDTA. The plasmas was obtained by centrifugation and all the samples of the same group were then pooled and lipids were extracted using the method of Moilanen and Nikkari [12], modified by adding 0.05% BHT as the antioxidant. The aortas were dissected out, rinsed with NaCl 0.9% and the samples of the same group were pooled. The heart, kidneys and liver were also individually collected. All the samples of aorta, heart, kidneys and liver were placed in a blend of chloroform/methanol (2/1, v/v) containing 0.05% of BHT for lipid extraction [13]. For the livers, an aliquot of each lipid extract was analyzed. The rest of the lipid extracts were pooled by dietary treatments to obtain a sufficient quantity to improve the identification of the oxysterols, as described below. The lipids were saponified in the dark during 16 h at room temperature under argon. After saponification, the unsaponifiable components were extracted using dichloromethane. The purification of the oxysterols from unsaponifiables using solid phase extraction (SPE) on silica cartridges (Supelco, L'Isle d'Abeau, France), as well as their transformation in trimethylsilyl ether (TMSE) derivatives were described before (10). 5α-cholestane was added as the internal standard and the TMSE derivatives were analyzed by gas chromatography (GC) on a 30 m × 0.25 mm i.d. DB-5 column (J&W Scientific, Folsom, CA, USA). The column had a 0.25 μm film thickness. The column was placed in a 5890 Series 2 Hewlett- Packard

(Palo Alto, CA, USA) gas chromatograph including a needle falling injector (temperature 290 °C) and a flame-ionization detector (temperature 300 °C). Helium was the carrier gas. After 1 min at 50 °C, the temperature of the oven was raised at 20 °C/min until 270 °C, and then reached 290 °C at 1 °C·min^{-1}. The temperature was then maintained at 290 °C for 30 min. Quantitative data were obtained using a Chromjet SP 4490 integrator and a Winner software (Thermo Separation Products, Les Ulis, France).

2.4. Identification of oxysterols

The identification was effected on the pooled samples of liver lipids. After saponification and purification by SPE as already described, aliquots of the oxysterol fractions were diluted in hexane/TBME and separated by thin layer chromatography (TLC) on silica plates (Silica gel 60, 20 cm × 20 cm, Merck, Darmstadt, Germany). The method, adapted from Pie et al. [14], used hexane/TBME/ethyl acetate (33:33:33 v/v/v) as the migration solvent. After evaporation under nitrogen, the main part of the plates were protected using a piece of glass, and the two edges of the plate were revealed using dichlorofluorescein in ethanol. The bands were then delimited, scraped, extracted using TBME, transformed in TMSE derivatives using BSTFA containing 1% TMCS and analyzed by GC as already described and by gas chromatography coupled with electronic impact mass spectrometry (GC-EIMS). This was effected using a Hewlett-Packard 5890 gas chromatograph coupled to a 5970 mass selective detector. A 30 m × 0.25 mm i.d. HP-5 column (Hewlett-Packard) was used. The injection was made in the splitless mode and the other chromatographic conditions were the same as for flame-ionization detection GC. The transfer line was operated at 290 °C. The mass spectrometer was operated at an ionization energy of 70 eV. The identifications were effected using the commercial standards of oxycholesterols (Sigma, L'Isle d'Abeau Chesnes, France and Steraloids, Wilton, NH, USA), the standards of oxyphytosterols prepared in a precedent experiment [9], as well as the data recently published by Dutta [15].

2.5. Statistical analysis

The significance of the differences between groups was evaluated using the analysis of variance and the test of Newman-Keuls. A P value of less than 0.05 was considered as significant.

3. RESULTS

At the end of the experiment, not any differences of weight gain and the weight of the studied organs could be observed between the different groups.

The main objective of this study was to search if some oxyphytosterols could be observed in lipid tissues. These compounds were indeed observed in the lipid tissues of the hamsters. But the levels were very different in the different groups. Some differences were also observed between the tissues. In order to illustrate the separation of the studied compounds by GC, a chromatogram of the oxysterols (as TMSE) of the liver of group D is represented in Figure 3. We can see the residual cholesterol (the main part was removed during SPE fractionation), some oxycholesterols (7-ketocholesterol is represented as well as 4β-hydroxycholesterol which is very important in the hamster) and oxyphytosterols issued from sitosterol and campesterol. The results are presented in the Tables II to VI, corresponding respectively to the samples of plasma, aorta, liver, kidneys and heart. All the results presented in the liver, kidneys and heart were significantly different between the groups. However, the statistical significance of differences between plasma and aorta samples was not studied because only a pooled sample for each group was available, due to the necessity to have sufficient material for the analysis.

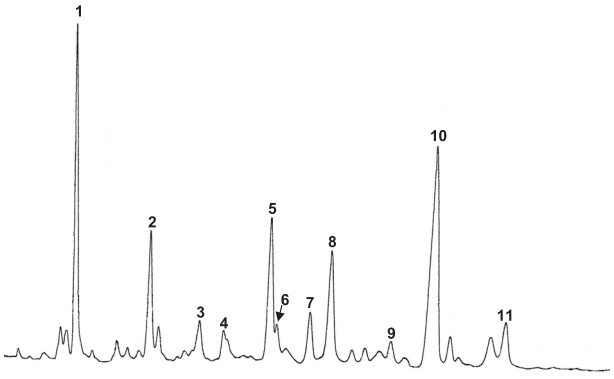

Figure 3. A part of the gas chromatogram of the oxysterol fraction (as TMSE) issued of a liver of a rat from group D. 1. Residual cholesterol. 2. 4β-hydroxycholesterol. 3. 7β-hydroxycampesterol. 4. β-epoxycampesterol. 5. 7β-hydroxysitosterol. 6. 7-ketocholesterol. 7. β-epoxysitosterol. 8. Campestanetriol. 9. 7-ketocampesterol. 10. Sitostanetriol. 11. 7-ketositosterol.

Table II. Oxyphytosterols in plasma lipids (pooled samples, ng·mL^{-1}).

	A	B[1]	C	D
7β-hydroxycampesterol			16.3	60.4
β-epoxycampesterol			8.2	25.0
Campestanetriol[2]	3.8		28.6	61.5
7-ketocampesterol			5.1	28.1
Total oxycampesterols	3.8		58.2	175.0
7α-hydroxysitosterol				13.5
7β-hydroxysitosterol			38.8	162.5
β-epoxysitosterol	15.1		67.3	203.1
Sitostanetriol	10.3		82.7	175.0
7-ketositosterol	3.4		17.3	77.1
Total oxysitosterols	28.8		206.1	631.3
Total oxyphytosterols	32.6		264.3	806.3

[1] This sample was lost during the analysis.
[2] The peak of campestanetriol also comprised a very small level of α-epoxysitoterol.

All the tissues of hamsters fed with 2500 ppm of OPS (group D) contained noticeable amounts of 7β-hydroxycampesterol, β-epoxycampesterol, campestanetriol, 7-ketocampesterol, 7β-hydroxysitosterol, β-epoxysitosterol, sitostanetriol and 7-ketositosterol. However, the 7β-hydroxycampesterol and the β-epoxycampesterol were not quantified in the kidneys and heart, due to coelution with unknown compounds. 7α-hydroxysitosterol was detected only in the plasma of group D. A trace amount of α-epoxysitosterol was also detected in the peak of campestanetriol using mass spectrometry.

Table III. Oxyphytosterols in aorta lipids (pooled samples, ng·mg^{-1} of lipids).

	A	B	C	D
7β-hydroxycampesterol				6.4
β-epoxycampesterol				3.2
Campestanetriol[1]		0.9	nq[2]	8.6
7-ketocampesterol				1.6
Total oxycampesterols		0.9		19.8
7β-hydroxysitosterol			2.3	13.9
β-epoxysitosterol	1.8	3.6	4.8	6.1
Sitostanetriol	1.4	5.9	4.1	30.5
7-ketositosterol			1.9	5.4
Total oxysitosterols	3.2	9.5	13.1	55.9
Total oxyphytosterols	3.2	10.4	13.1	75.7

[1] The peak of campestanetriol also comprised a very small level of α-epoxysitosterol.
[2] Not quantified. (This compound was identified using mass spectrometry, but its levels were not quantified, due to coelution with an unknown compound.)

Table IV. Oxyphytosterols in liver lipids (n = 8, ng·mg^{-1} of lipids).

	A	B	C	D
7β-hydroxycampesterol			$0.6^{a} \pm 0.1$	$5.6^{b} \pm 1.3$
β-epoxycampesterol			$0.3^{a} \pm 0.1$	$4.6^{b} \pm 1.7$
Campestanetriol[1]			$1.1^{a} \pm 0.1$	$12.9^{b} \pm 2.3$
7-ketocampesterol[2]			$0.3^{a} \pm 0.2$	$4.0^{b} \pm 0.6$
Total oxycampesterols			$2.3^{a} \pm 0.3$	$27.1^{b} \pm 4.3$
7β-hydroxysitosterol			$1.9^{a} \pm 0.3$	$17.5^{b} \pm 1.9$
β-epoxysitosterol			$0.6^{a} \pm 0.2$	$5.8^{b} \pm 0.6$
Sitostanetriol	$0.24^{a} \pm 0.04$	$0.74^{b} \pm 0.05$	$2.8^{c} \pm 0.1$	$31.1^{d} \pm 3.0$
7-ketositosterol			$0.6^{a} \pm 0.1$	$8.9^{b} \pm 2.4$
Total oxysitosterols	$0.24^{a} \pm 0.04$	$0.74^{b} \pm 0.05$	$5.9^{c} \pm 0.2$	$63.4^{d} \pm 2.9$
Total oxyphytosterols	$0.24^{a} \pm 0.04$	$0.74^{b} \pm 0.05$	$8.3^{c} \pm 0.4$	$90.5^{d} \pm 7.3$

Different superscript letters indicate that the means are statistically different between groups.
[1] The peak of campestanetriol also comprised a very small level of α-epoxysitosterol.
[2] The peak of 7-ketocampesterol also contains a trace of an unknown compound.

However, it was not possible to quantify this compound using our chromatographic conditions. The oxidized derivatives of stigmasterol were present in the diet, but were not identified in the plasma and tissues.

Most of these compounds were also detected by MS in the tissues of hamsters fed with 500 ppm of OPS (group C), but their levels were clearly smaller than those of group D. In group C, with the exception of campestanetriol, the oxycampesterols were present in the kidneys and heart only as traces coeluting with other unknown compounds. In the aorta, the oxycampesterols were not detected, except for a trace of campestanetriol.

Table V. Oxyphytosterols in kidneys lipids ($n = 8$, ng·mg^{-1} of lipids).

	A	B	C	D
7β-hydroxycampesterol			tr[1]	nq[2]
β-epoxycampesterol			tr[1]	nq[2]
Campestanetriol[3]			$12.9^{a} \pm 5.6$	$37.4^{b} \pm 15.8$
7-ketocampesterol[4]			tr.[1]	$23.1^{b} \pm 16.0$
Total oxycampesterols			$12.9^{a} \pm 5.6$	$60.5^{b} \pm 26.8$
7α-hydroxysitosterol				–
7β-hydroxysitosterol			$6.2^{a} \pm 1.9$	$38.5^{b} \pm 19.1$
β-epoxysitosterol			$15.8^{a} \pm 1.9$	$26.1^{b} \pm 13.8$
Sitostanetriol	$9.8^{a} \pm 5.4$	$19.5^{b} \pm 5.6$	$45.4^{c} \pm 8.8$	$104.0^{d} \pm 39.2$
7-ketositosterol			$8.0^{a} \pm 2.0$	$46.3^{b} \pm 23.9$
Total oxysitosterols	$9.8^{a} \pm 5.4$	$19.5^{b} \pm 5.6$	$75.3^{c} \pm 9.5$	$214.9^{d} \pm 84.6$
Total oxyphytosterols	$9.8^{a} \pm 5.4$	$19.5^{b} \pm 5.6$	$88.2^{c} \pm 14.2$	$275.4^{d} \pm 110.5$

Different superscript letters indicate that the means are statistically different between groups.
[1] Trace. (This compound was identified as a trace compound using mass spectrometry, but its level was not calculated, due to the coelution with an unknown compound.)
[2] Not quantified. (This compound was identified using mass spectrometry, but its level was not quantified, due to the coelution with an unknown compound.)
[3] The peak of campestanetriol also comprised a very small level of α-epoxysitosterol.
[4] The peak of 7-ketocampesterol also contains a trace of an unknown compound.

Table VI. Oxyphytosterols in heart lipids ($n = 8$, ng·mg^{-1} of lipids).

	A	B	C	D
7β-hydroxycampesterol			tr[1]	tr[1]
β-epoxycampesterol			tr[1]	tr[1]
Campestanetriol[2]			$8.5^{a} \pm 2.4$	$50.0^{b} \pm 30.7$
7-ketocampesterol[3]			tr[1]	14.8 ± 12.6
Total oxycampesterols			$8.5^{a} \pm 2.4$	$64.8^{b} \pm 42.5$
7β-hydroxysitosterol			$12.8^{a} \pm 3.5$	$70.9^{b} \pm 29.6$
β-epoxysitosterol			$2.0^{a} \pm 0.2$	$28.1^{b} \pm 13.2$
Sitostanetriol		$3.3^{a} \pm 0.4$	$24.8^{b} \pm 1.2$	$135.7^{c} \pm 92.3$
7-ketositosterol			$4.4^{a} \pm 0.5$	$25.8^{b} \pm 15.3$
Total oxysitosterols		$3.3^{a} \pm 0.4$	$44.0^{b} \pm 4.5$	$261.8^{c} \pm 140.6$
Total oxyphytosterols		$3.3^{a} \pm 0.4$	$52.5^{b} \pm 6.3$	$326.6^{c} \pm 178.6$

Different superscript letters indicate that the means are statistically different between groups.
[1] Trace. (This compound was identified as a trace compound using mass spectrometry, but its level was not calculated, due to the coelution with an unknown compound.)
[2] The peak of campestanetriol also comprised a very small level of α-epoxysitosterol.
[3] The peak of 7-ketocampesterol also contains a trace of an unknown compound.

In the liver, heart and kidneys coming from control animals (group A) and the animals receiving 100 ppm of OPS (group B), only sitostanetriol was observed. Moreover, in the hearts of group A, sitostanetriol was not identified with certitude. The pool of plasma from the control group showed the presence of campestanetriol, β-epoxysitosterol, sitostanetriol and 7-ketositosterol. However, the pool of plasma of group B was unfortunately lost during the analysis. The same compounds but 7-ketositosterol were observed in the aorta of group B. In the aorta of control animals, sitostanetriol and β-epoxysitosterol were also observed in little amounts.

4. DISCUSSION

The animals of group D were fed 2 500 ppm of OPS, which is a very high level largely superior to the amounts eventually present in the diets. However, this group was very interesting: it indicates what can happen with these compounds. In this case, the oxycampesterols represented 20 to 30% of the identified OPS in the tissues: this is a greater proportion than in the diet (12.7%) and is probably due to a better absorption. The 7α-hydroxyderivatives were not observed in the tissue lipids, with the exception of plasma. It is possible that 7α-hydroxyphytosterols could form biliary acids similar to those issued from 7α-hydroxycholesterol. The α-epoxyphytosterols were present only as trace components. This suggested that these compounds could be better metabolized than the β-epoxyphytosterols. Aringer and Eneroth observed in vivo that the α-epoxyphytosterols could be preferentially transformed into triols [16]. The 7-ketophytosterols, which are main compounds of OPS of the diet, present smaller levels in the tissues. Lyons et al. [17] observed that 7-ketocholesterol was readily metabolized. It is possible that the 7-ketophytosterols could be metabolized in the same manner. Moreover, the intestinal absorption of 7-ketophytosterols was lower in the rat than those of epoxides [9]. So, the main OPS in tissues are the triols and the 7β-hydroxyphytosterols. There are no data on the physiological effects and the metabolism of these compounds. However, the analogue compounds issued from cholesterol were studied: 7β-hydroxycholesterol is considered as a good marker of the risk of cardiovascular disease [18] and cholestanetriol was described as cytotoxic [19] and as involved in atherosclerosis [20, 21]. For some oxycholesterols, many studies have demonstrated several in vitro biological effects related to the atherosclerotic process and that the systemic administration of some of these compounds to animals induce the formation of atherosclerotic lesions, which indicates that these compounds may play an important role in the initiation and progression of atherosclerosis [22–24]. It would be interesting to know if these phytosterol-derived compounds could present the same effects. In group D, the liver presented less OPS in its lipids, than the kidneys or the heart. This probably reflects a higher metabolic rate in this organ.

The same compounds were observed in group C, fed with 500 ppm of OPS, but with very lower levels and it has not been possible to quantify some compounds. However, group B (fed with 100 ppm of OPS) looked more like the control group (A) than the other groups receiving OPS in their diet (C and D). With the exception of the plasma and aorta, the only observed oxyphytosterol in groups A and B was sitostanetriol. But, there was a significant difference in the amount of this compound between groups A and B. It is surprising to observe some oxyphytosterols in the tissues of control animals which were not fed with OPS. However, the same compounds were also observed recently in human plasma [10]. It seems that these compounds are not artifacts. Moreover, a very recent experiment showed that these oxyphytosterols are not produced in vivo and that a long time is necessary to eliminate these compounds from plasma: after 4 weeks of a diet without sterols and OPS, sitostanetriol decreased, but

was still observed in rat plasma [25]. It is then possible that these OPS are issued from the diet of the hamsters, before the experiment. The fact that the amount of sitostanetriol is higher in group B than in group A strengthens the hypothesis of the dietary origin for sitostanetriol. This may be the compound which is metabolized with the lowest rate among the OPS. However, we cannot exclude that some oxyphytosterols were present in the control diet, even if no epoxyphytosterols and triols were observed in the soybean oil used for the experiment.

In conclusion, this study shows that the dietary oxyphytosterols can enter into all the tested lipid tissues. When the levels of OPS are rather small in the diet, main OPS were not observed in the tissues, showing that these compounds can probably be eliminated by the animals. But we also observed that sitostanetriol is always observed, even in the control group. It is probable that the metabolism of this compound is slow.

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