FULL PAPER

Wildlife Science

Effects of feeding on plasma concentrations of vitamin A in captive African penguins (*Spheniscus demersus*)

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ABSTRACT. Vitamin A comprises vitamin A_1 and vitamin A_2; vitamin A_1 is retinol and its fatty-acid esters and vitamin A_2 is 3,4-didehydroretinol and its fatty-acid esters. Although vitamin A_1 is generally recognized as the major vitamin A, vitamin A_2 is found in some birds and mammals that eat fish containing vitamin A_2. Plasma concentration of retinyl esters, but not retinol, is known to increase postprandially in humans. The objectives of this study were to confirm the presence of vitamin A_2 in fish fed to penguins, and in penguin plasma, and the postprandial changes in vitamin A concentration in penguin plasma. Blood was collected from six male African penguins (*Spheniscus demersus*) before and after feeding on jack mackerels (*Trachurus japonicus*) along with a vitamin premix containing vitamin A_1. Vitamin A_1 concentration in fish was much higher than the requirement, and was 5-fold higher than the vitamin A_2 concentration. Vitamin A_2 was present in plasma but its concentration was at least 100-fold below that of plasma retinol, suggesting that vitamin A_2 is much less bioavailable than vitamin A_1 in penguins. Plasma retinol and retinyl palmitate concentrations were found to be stable after the meal. Plasma retinol concentration is suggested to be homeostatically controlled in penguins against the rapid flow of vitamin A_1 after meal. The absorbed vitamin A_1 is thought to be transported to the liver via the portal vein for storage in penguins, resulting in stable retinyl palmitate concentration in plasma after meal.

KEY WORDS: 3,4-didehydroretinol, penguin, plasma, retinol, retinyl palmitate

Vitamin A is important for vision, cell differentiation, hematopoiesis, reproduction, and immune functions. It consists of vitamin A_1 and A_2. The former is composed of retinol with its fatty-acid esters, and the latter is composed of 3,4-didehydroretinol with its fatty-acid esters. The relative activity of 3,4-didehydroretinol to retinol was reported as 40% [25] and 120% [24] in rats.

Fish contain many kinds of carotenoids, of which α-carotene, β-carotene, γ-carotene and β-cryptoxanthin are known as provitamin A. These compounds were reported to be present in extremely low amounts compared with those of vitamin A or below the limit of detection in the muscle and the liver of rainbow trout (*Oncorhynchus mykiss*) [19] and in the edible parts (the muscle with the skin) of jack mackerels (*Trachurus japonicus*) for humans [22]. Although vitamin A_1 is generally recognized as the major vitamin A, fish have a substantial amounts of vitamin A_2; in fact, vitamin A_2 is usually the predominant vitamin A in freshwater fish, and in marine fish, it makes up about 25% of the total vitamin A [20]. Consequently, a relatively high concentration of vitamin A_2 is observed in birds and mammals that eat freshwater fish. Retinol is the major vitamin A in the plasma of free-ranging birds that consume marine fish, whereas the plasma 3,4-didehydroretinol concentration is high in free-ranging osprey (*Pandion haliaetus*), which probably eats freshwater fish [17]. The concentration of vitamin A_2 in the liver and adipose tissue was much higher in free-ranging lacustrine seals (*Phoca hispida saimensis* and *Phoca hispida ladogensis*) than in free-ranging marine seals (*Phoca hispida botnica* and *Phoca hispida hispida*) [12]. Hepatic concentration of vitamin A_2 was remarkably higher in American minks *(Neovison vison)* fed with freshwater fish than in those nourished with marine fish [13]. On the other hand, the existence of vitamin A_2 has not been reported in penguins, which usually feed on marine fish [17].

In the foods of animal origin, vitamin A primarily exists as fatty-acid esters [29]. After dietary retinyl esters are hydrolyzed to retinol in the lumen of intestine and taken up by enterocytes, retinol is re-esterified with fatty acids and packed with lipids into chylomicron, and is secreted into the lymphatic system in mammals [10]. After entering the circulation, chylomicron is converted to chylomicron remnant without releasing a major part of retinyl esters, which are absorbed and stored in the liver [10]. Thus,

in humans, plasma concentration of retinyl palmitate, the major retinyl ester in circulation, is postprandially increased in the form of chylomicron and chylomicron remnant [14, 32]. On the other hand, as the lymphatic system is poorly developed in the gastrointestinal tracts of birds, portomicron, a homolog of chylomicron, is transported from the enterocytes to the liver via the portal vein [3, 18], which probably affect the postprandial changes in retinyl ester concentration in birds, including penguins.

Stored retinyl esters are hydrolyzed to retinol in the liver and released into the blood stream [10]. In birds and mammals, retinol is transported as a complex with retinol binding protein 4 (RBP4) and transthyretin [10, 11]. In humans, as the retinol concentration in plasma is homeostatically controlled, it is stable even after consuming meals rich in vitamin A_1 [29]. However, there have been no reports investigating postprandial changes of plasma vitamin A in penguins.

The objective of this study was to verify the existence of vitamin A_2 in dietary fish and in plasma of penguins. Additionally, the study was also aimed at describing the changes, if any, in plasma concentration of vitamin A after meal in penguins.

MATERIALS AND METHODS

Ethics statement

Animal care and experiments were approved by the Animal Care Committee, Kyoto University (Approved number 28-80). All animal experiments were conducted in accordance with the approved guidelines.

Animals and feeding

Six adult male African penguins (*Spheniscus demersus*) with 4.7 ± 1.9 years of age and 3.2 ± 0.2 kg body weight (mean ± standard deviation) were randomly selected from the colony maintained at Kyoto Aquarium. These were routinely hand fed with about 360 g/day of whole jack mackerels three times a day, and given one tablet of a vitamin premix (Mazuri Vita-Zu Small Bird Tablet 5M25, PMI Nutrition International, St. Louis, MO, U.S.A.) that contains vitamin A_1 as retinyl acetate at 840 IU/tablet, with only the morning meal.

Blood sampling

Blood was collected from the medial metatarsal vein of the penguins in a heparinized plastic tube, just before the morning meal, and 2, 4, and 6 hr after the meal. The last blood collection was performed prior to the second meal. The plasma was separated immediately by centrifugation at $2,000 \times g$ at 4°C and stored at −80°C until analyses.

Analyses

3,4-Didehydroretinol was purchased from Toronto Research Chemicals (North York, ON, Canada) and the other reagents were purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan).

Ten dietary fish were randomly selected and each one was separately minced on the whole, for further analyses. Moisture content in the fish samples was determined using the method recommended by the Association of Official Analytical Chemists (AOAC, 950.46) [4], from which the dry weight was calculated.

As per a standard method for determination of vitamin A concentration in food [23], fish samples were saponified with potassium hydroxide and extracted by n-hexane, and vitamin A_1 (retinol and its fatty-acid esters) concentration was determined by HPLC (LC-10AD, Shimadzu, Kyoto, Japan) with a spectrophotometer (SPD10A, Shimadzu), at 325 nm, a reversed-phase column (UG120, 150 mm × 4.6 mm, 5 μm, Shiseido, Tokyo, Japan), and methanol-ethanol (80/20 v/v) as the mobile phase at a flow rate of 1.0 m*l*/min. Concentration of vitamin A_2 (3,4-didehydroretinol retinol and its fatty-acid esters) was determined by the aforementioned method but at a wavelength of 350 nm, and the mobile phase of methanol-water (90/10 v/v) [28].

Plasma retinol and retinyl palmitate concentrations were determined by HPLC, after *n*-hexane extraction [1]. Since retinyl palmitate is the major retinyl ester in plasma [7], its plasma concentration was determined followed by studies in penguins [2, 8, 30]. Concentration of 3,4-didehydroretinol in plasma was determined following the aforementioned method for plasma samples with the same measuring wavelength and the mobile phase as for the determination of vitamin A_2 in fish samples. In addition, plasma samples were saponified with potassium hydroxide and extracted using *n*-hexane [23]. Subsequently, vitamin A_2 concentration was determined by the same method as for 3,4-didehydroretinol in plasma samples.

The existence of 3,4-didehydroretinol in fish and plasma samples was confirmed by its absorption spectrum between 260 and 380 nm in HPLC using a diode array detector (DAD-3000, Thermo Fisher Scientific, Waltham, MA, U.S.A.).

Statistical analyses

The data obtained was expressed as mean ± standard deviation. Linear regression of vitamin A_1 and A_2 concentrations on dry weight in fish, and the Pearson correlation between the two vitamin concentrations were evaluated by PROC REG and PROC CORR of SAS (version 9.1, SAS Institute, Cary, NC, U.S.A.), respectively. The effect of blood sampling time was evaluated by MIXED PROC of SAS (version 9.1, SAS Institute). $P<0.05$ was considered statistically significant.

RESULTS

Presence of vitamin A_2 in dietary fish and penguin plasma

On comparison of the absorption spectra of the authentic peak of 3,4-didehydroretinol and the peak in fish samples corresponding to the retention time of the authentic peak in the chromatograms, it was found that the absorption spectrum did not

differ between the peaks (Fig. 1A, 1B). We found the peak in plasma samples corresponding to the retention time of the authentic peak of 3,4-didehydroretinol (Fig. 1C), and the absorption spectrum was similar between these peaks. However, the plasma levels of 3,4-didehydroretinol were lower than 1.6 μg/dl that is the lower limit of quantification based on the signal-to-noise ratio of 5. Concentration of vitamin A_2 in saponified plasma samples was below the level of quantification (data not shown), indicating that, fatty-acid esters of 3,4-didehydroretinol was also negligible in penguin plasma. These results indicate that vitamin A_2 exists in dietary fish and penguin plasma but its concentration is remarkably low in plasma of penguins.

Vitamin A concentration in dietary fish

Dry weight content was, on average, 29.1 ± 2.8% in 10 fish samples. Vitamin A_1 concentration increased with fish weight and significant regression was observed (r^2=0.583, *P*=0.010, Fig. 2A). Vitamin A_1 concentration ranged between 3,970 and 15,210 μg/kg dry weight (13,233 and 50,700 IU/kg dry weight), with an average of 7,700 ± 3,550 μg/kg dry weight (25,667 ± 11,833 IU/kg dry weight). The lowest concentration of vitamin A_1 in dietary fish was much more than the requirement of vitamin A for penguins (3,500 IU/kg dry weight) [9]. The average vitamin A_1 intake from fish and the vitamin premix was 2,690 and 840 IU/day, respectively. The dietary vitamin A_1 concentration was calculated as 33,690 IU/kg dry weight, as the weight of the vitamin premix was negligible. Further, the penguins were provided with fish, containing vitamin A_1 at 280 IU/kg body weight with fortified retinyl acetate at 262 IU/kg body weight, as the morning meal.

Vitamin A_2 concentration also tended to increase with fish weight (r^2=0.343, *P*=0.075, Fig. 2B), and was positively correlated with vitamin A_1 concentrations (r=0.889, *P*<0.001, Fig. 2C). Vitamin A_2 concentration ranged between 770 and 2,260 μg/kg dry weight, and averaged at 1,410 ± 430 μg/kg dry weight. Thus, the average vitamin A_2 concentration was approximately 5-fold lower than the average vitamin A_1 concentration in dietary fish.

Vitamin A_1 concentration in plasma

The plasma retinol and retinyl palmitate concentrations were almost stable after the morning meal that had a relatively high concentration of vitamin A_1 (Fig. 3). The plasma retinol concentration was approximately 180 μg/dl and the retinyl palmitate concentration was about 10 μg/dl. These results indicated that retinol concentration was much higher than the retinyl palmitate concentration in the plasma, which was consistent with our previous study in captive African penguins [2], and the study of Wallace *et al*. [31] in free-ranging Humboldt penguins (*Spheniscus humboldti*). Further, captive Humboldt penguins had almost comparable serum retinol and retinyl palmitate concentrations to the levels in the present experiment, without any signs of toxicity, even when the dietary vitamin A_1 level was about 2-fold higher than in the present experiment [8].
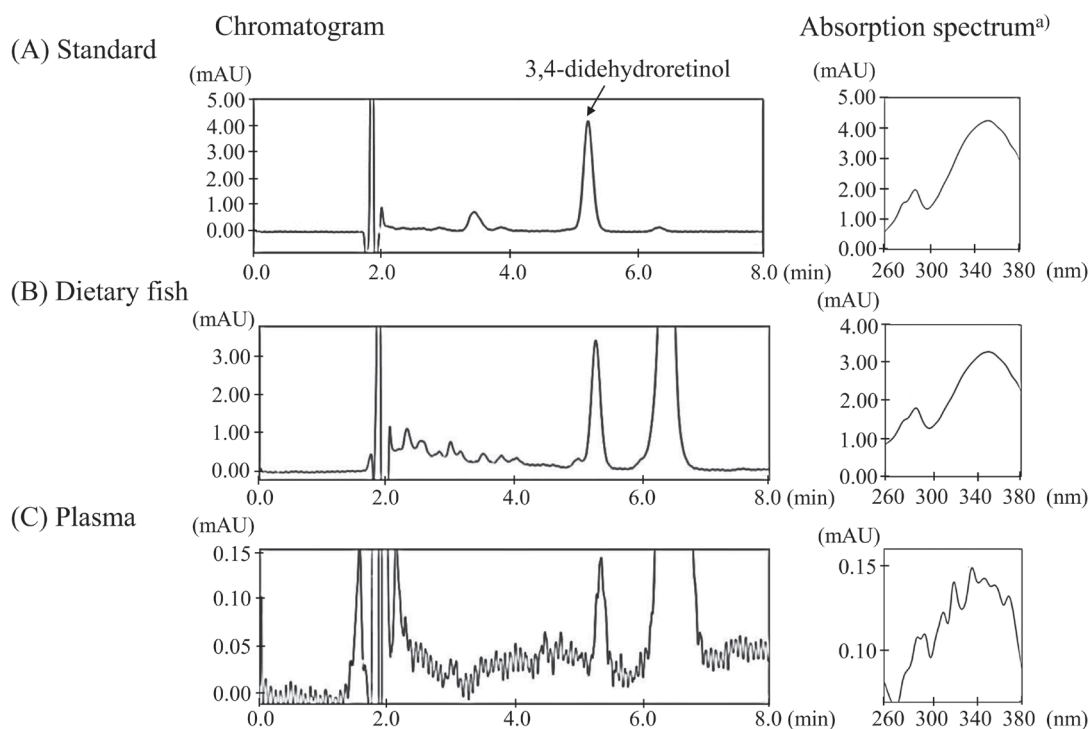

Fig. 1. The representative chromatograms in authentic standard (A), dietary fish (B) and penguin plasma (C). a) Absorption spectrum of the authentic peak of 3,4-didehydroretinol and the corresponding peaks in the dietary fish and the plasma of penguins.
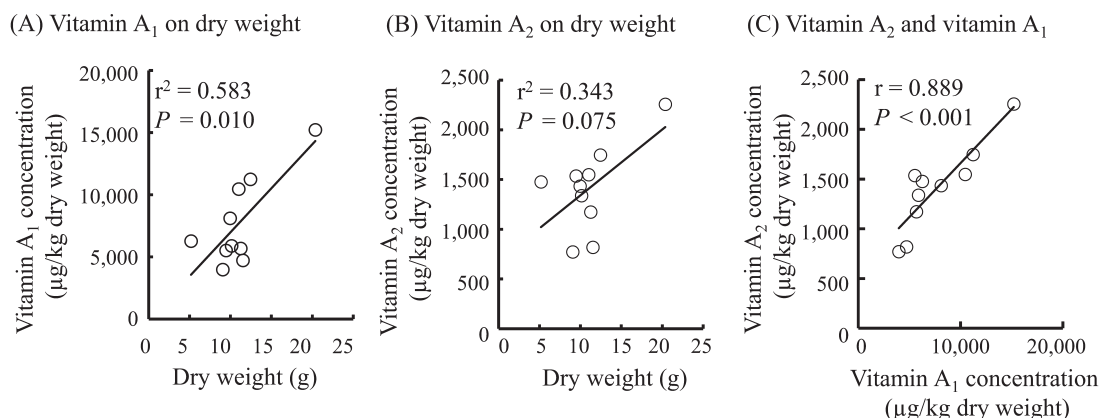
(A) Vitamin A_1 on dry weight (B) Vitamin A_2 on dry weight (C) Vitamin A_2 and vitamin A_1

Fig. 2. Regression of vitamin A_1 (A) and vitamin A_2 (B) concentrations on dry weight, and correlation between vitamin A_2 and vitamin A_1 concentrations (C) in the dietary fish (n=10). Vitamin A_1 and vitamin A_2 concentrations were determined as retinol and 3,4-didehydroretinol, respectively, after the saponification.
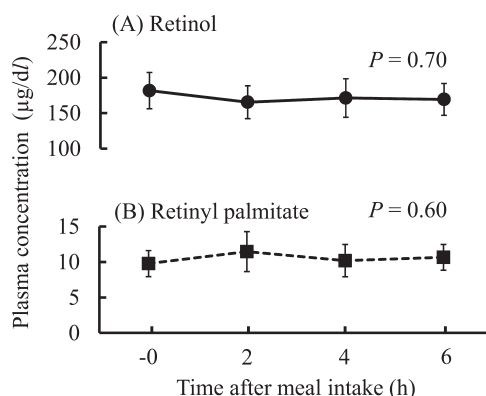

Fig. 3. Concentrations of retinol (A) and retinyl palmitate (B) in penguin plasma before (0 hr) and after meal intake. Data are expressed as means ± standard deviation (n=6).

DISCUSSION

In the present study, it was seen that vitamin A_1 and A_2 concentrations were largely variable among dietary jack mackerels, and increased with weight of the fish. Penguins feed on a variety of fish, in which the concentration of vitamin A_1 vary widely [8, 30]. The results of the present study indicated that even within the same species of fish, the concentration of vitamin A_1 fluctuated extensively. Vitamin A_1 is mainly stored in the liver of the mammals [10]. On the other hand, high vitamin A_1 concentration was reported in the pyloric cecum of the arrowtooth halibut (*Atheresthes evermanni*) [33]. Further, both vitamin A_1 and A_2 concentrations were higher in the pyloric cecum than in the liver of the rainbow trout (*Salmo gairdneri*), and the heavier fish had more vitamin A_1 and A_2 in these organs [6]. As penguins are usually hand fed with several whole fish in which vitamin A_1 concentrations are widely variable, controlling the vitamin A_1 intake is difficult. Therefore, the supplementation of vitamin A_1 might be reasonable for avoiding its deficiency. However, the reported death of a captured Rockhopper penguins (*Eudyptes cresfutus*) [5] was probably due to vitamin A or vitamin D toxicosis caused by the supplements containing very high amounts of vitamin A_1 and D. The lowest level of vitamin A_1 concentration in jack mackerels was more than the requirement of vitamin A for penguins [9]. Therefore, vitamin A_1 supplementation is not necessary for penguins fed on jack mackerels.

The present study showed that jack mackerel, a marine fish, contained 5-fold less vitamin A_2 than vitamin A_1, and, in sardine (*Clupea pilchardus*), the vitamin A_2 concentration was almost half of the vitamin A_1 concentration in the liver [26]. It was also reported that the vitamin A_2 concentration was 25% of total vitamin A in some marine fish [20]. However, concentration of vitamin A_2 was much higher than that of vitamin A_1 in the liver and the pyloric cecum of rainbow trout [6]. Although these observations imply that freshwater fish have more vitamin A_2 than marine fish, considerable amount of vitamin A_2 exists even in marine fish, as is shown in the present study. On the other hand, in penguins, plasma 3,4-didehydroretinol and vitamin A_2 concentrations were below the level of quantification, which was 100-fold lower than plasma retinol concentration. Riabroy *et al*. [24] fed almost equal amounts of retinyl acetate and 3,4-didehydroretinyl acetate separately to two groups of vitamin A-deficient rats, and found that

vitamin A_2 concentrations in serum and the liver were 3-fold and 9-fold lower in rats that were given vitamin A_2 compared with serum retinol and hepatic vitamin A_1 concentrations in those given vitamin A_1. Shantz and Brinkman [25] also reported similar results in the liver of the vitamin A-deficient rats, upon feeding with 3,4-didehydroretinol or retinol. Although the relative activity of 3,4-didehydroretinol to retinol was inconsistently reported as 40% [25] and 120% [24] in rats, these results suggest that vitamin A_2 is less bioavailable than vitamin A_1 in rats and in penguins fed on fish rich in vitamin A_1. However, the relative bioavailability of vitamin A_2 to vitamin A_1 was likely to be much lower in penguins than in rats. The large amount of dietary vitamin A_1 possibly decreases the bioavailability of vitamin A_2 in penguins, because penguins were fed with dietary fish containing not only vitamin A_2, but also a large amount of vitamin A_1 in the present experiment, while in case of rats, vitamin A_2 was the sole vitamin A in their diet [24, 25]. Otherwise, species difference could be the reason for the remarkably lower bioavailability of vitamin A_2 observed in penguins as compared with that in rats. Further research is necessary for understanding the effect of vitamin A_1, if any, on the bioavailability of vitamin A_2. Although the difference of vitamin A activity between vitamin A_1 and A_2 has not been clarified in penguins, vitamin A_1 is probably the major component of vitamin A, and the nutritional value of vitamin A_2 can be ignored in penguins fed with marine fish.

In the present study, plasma retinol concentration was much higher than that of retinyl palmitate in penguins, and this result was consistent with previous studies in captured African and Humboldt penguins [2, 8] and in free-ranging Humboldt penguins [31]. However, in felines, vitamin A_1 is transported from the liver as retinyl esters, and retinyl palmitate concentration was as high as that of retinol in plasma [21]. Present study confirmed that vitamin A_1 is mainly transported as retinol in the circulation of penguins.

Plasma retinol and retinyl palmitate concentrations were stable in the penguins after the morning meal, while in humans, plasma retinyl palmitate concentration was reported to increase after a meal fortified with vitamin A_1 [14, 32]. Krasinski *et al.* [14] reported increased retinyl ester concentration in plasma of humans 3 hr after consumption of a meal containing retinyl palmitate (133 IU/kg body weight). Penguins were fed with the morning meal containing vitamin A_1 at 280 IU/kg body weight with fortified retinyl acetate at 262 IU/kg body weight. In humans, the absorbed vitamin A_1 is transported from the intestine through the lymphatic system and blood vascular system to the liver as retinyl esters in chylomicron and chylomicron remnant [10]. Therefore, meal intake increased the plasma retinyl palmitate concentration in the form of chylomicron and chylomicron remnant in humans [14, 32]. On the other hand, the lymphatic system is poorly developed in the gastrointestinal tract of birds, and portomicron, a homolog of chylomicron is transported from the intestine to the liver via the portal vein [3, 18]. We believe that retinyl palmitate is directly transported from the intestine to the liver as portomicron, resulting in stable plasma retinyl palmitate concentration even after meal with a relatively high amount of vitamin A_1.

Vitamin A_1 is transported from the liver to peripheral tissues via blood vascular system as the retinol complex with RBP4 and transthyretin in mammals and birds [10, 11]. Although severe vitamin A_1 deficiency decreases plasma retinol concentration, it is homeostatically controlled through RBP4 synthesis in vitamin A_1-sufficient animals, and thus plasma retinol concentration is relatively stable over a wide range of liver vitamin A_1 concentration [29]. Although plasma retinyl palmitate concentration was postprandially increased, plasma retinol concentration was stable after meal with vitamin A_1 supplement in humans [32]. The present study indicates that penguins also possess homeostatic control of plasma retinol concentration against the rapid flow of vitamin A_1 after a meal. Chronic exposure to high level of vitamin A_1 increased the plasma retinyl ester concentration, but not retinol concentration, in fasting humans [15] and rats [16]. On the other hand, it was reported that plasma retinol concentration was positively correlated to vitamin A_1 intake in fasting captive Humboldt penguins [30]. Crissey *et al.* [8] also reported that serum retinol concentration increased in captive Humboldt penguins fed on fish containing 15-fold more vitamin A_1 than the vitamin A requirement of penguins [9] for 12 months. Plasma retinol concentration increased in chicken too, after being fed with extremely high level of vitamin A_1 for 8 weeks [27]. These results indicate the possibility that chronic exposure to very high level of vitamin A_1 disturbs the homeostatic control of retinol, resulting in the rise of plasma retinol concentration because the intake of vitamin A_1 was much more in the studies using penguin [8] and chicken [27] than in the human study [15] and rat study [16].

In conclusion, vitamin A_2 is present in both, diet and plasma of penguins, but its nutritional value is likely to be negligible because of its extremely low bioavailability. Plasma retinyl palmitate concentration does not increase postprandially, probably because the absorbed retinyl esters are transported to the liver via the portal vein and stored there. Plasma retinol concentration does not postprandially increase because retinol efflux from the liver is homeostatically controlled.

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