A New Aspect on the Mechanism of Intestinal Cholesterol Absorption in Rat

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Summary In order to study the mechanism of intestinal cholesterol absorption, the relationship between the amount of cholesterol administered and the rate of absorption was investigated by the dual isotope plasma ratio method *in vivo* and the ligated-loop method *in situ*. The energy requirement of cholesterol absorption was also observed by means of the ligated-loop method. The results obtained are summarized below.

1) Tri-phase absorption was observed by the dual isotope plasma ratio method. When less than 300 μg of cholesterol was administered, absorption increased linearly, with the coefficient of absorption being more than 80%. When the amount administered was between 300 and 500 μg , the absorption was constant. With the administration of more than 500 μg , absorption increased linearly, but the coefficient of absorption decreased to approximately 55%.

2) With the ligated-loop method, a second saturation profile was obtained when between 250 and 400 μg of cholesterol was administered to a segment. When 50 to 250 μg of cholesterol were administered, absorption increased in proportion to the increase in cholesterol dosage.

3) The mucosal uptake of cholesterol decreased to 40–60% of the control with the addition of metabolic inhibitors such as NaN_3 , KCN, 2,4-DNP and ouabain, whereas the uptake of palmitate showed no significant decrease. In addition, the uptake of cholesterol decreased remarkably to 25% of the control with the lowering of body temperature from 37°C to 27°C.

These results suggest the existence of an active transport system which has a limited capacity for cholesterol absorption and which requires energy for its operation in the physiological state.

Key Words cholesterol, β -sitosterol, intestine, inhibition, active transport, energy-dependent, cholesterol absorption, intestinal mucosa, mucosal uptake

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Cholesterol has been thought to be transported into the intestinal epithelial cells by a passive diffusion process, since cholesterol absorption in the intestine increases linearly corresponding to the amount of dietary cholesterol (1-3). Some phenomena contradictory to the passive diffusion theory of intestinal transport of cholesterol have, however, been observed. Chow and Hollander have reported that the free energy for the cholesterol uptake into the intestinal mucosa was much larger than the value which was calculated according to the simple diffusion theory (4). Sylvén and Borgström have demonstrated that the coefficient of cholesterol absorption is much higher than that of β -sitosterol, an analogue of cholesterol (5). Additionally, cholesterol absorption is competitively inhibited by β -sitosterol (6). Therefore, it is not yet clear whether the intestinal absorption of cholesterol is dependent on a passive diffusion mechanism or on an active transport process which requires energy and of limited capacity.

Recently, Zilversmit *et al.* have developed a dual isotope plasma ratio method as a new technique for the measurement of the intestinal absorption *in vivo* of cholesterol in rats (7, 8). Sylvén evaluated the *in situ* intestinal ligated-loop method as most convenient to study the mucosal uptake of cholesterol (9, 10). These two methods have been judged more reliable and useful than the everted gut sac and the lymph-duct fistula methods in determining the absorption and the uptake of cholesterol in the physiological state. Therefore, it would seem appropriate to study the mechanism of intestinal cholesterol absorption by using these methods.

The present paper describes the relationship between the amount of cholesterol administration and absorption and suggests the existence of an energy-dependent transport process for cholesterol absorption.

METHODS

1. *Chemicals and animals.* [7-³H]Cholesterol (21.5 Ci/mmol) and [1-¹⁴C]-palmitate (0.054 Ci/mmol) were obtained from New England Nuclear Co. (Boston, MA), and [4-¹⁴C]cholesterol (0.057 Ci/mmol) was obtained from Amersham/Searle Co. (Arlington Heights, IL). The purity of these radioactive compounds was tested by thin layer chromatography on Silica gel G (Merck Co., Darmstadt, Germany) with a solvent system of benzene:ethylacetate=2:1 (v/v). Compounds found to be more than 98% pure were used in all experiments.

Glycerol monooleate and glycochenodeoxycholate were obtained from Sigma Chem. Co. (St. Louis, MO), Omunifluor from New England Nuclear Co., and sodium azide (NaN₃) from Merck Co. Potassium cyanide (KCN) was purchased from Kishida Chem. Indust. Co. (Tokyo, Japan), 2,4-dinitrophenol (2,4-DNP) from Wako Pure Chem. Co. (Tokyo, Japan), and ouabain from Tokyo Kasei Co. (Tokyo, Japan). All other chemicals employed were of analytical grade.

2. *Preparation of micellar dose solution.* Micellar dose solutions containing [¹⁴C]cholesterol (0.01 to 1.0 μ Ci/0.5 ml) were prepared by dissolving appropriate amounts of non-radioactive cholesterol and 5 mM glycerol monooleate in 50 mM

glycochenodeoxycholic acid. [^{14}C]Palmitate was used instead of [^{14}C]cholesterol in some experiments. These solutions were incubated at 37°C overnight to produce stable micellar particles.

In the experiments to study the effect of metabolic inhibitors on mucosal uptake of cholesterol, 40 mM sodium azide, 40 mM potassium cyanide, 20 mM 2,4-dinitrophenol, or 5 mM ouabain was dissolved in 0.5 ml of the micellar solution containing 10 μg or less of cholesterol or palmitate.

3. *Dual isotope plasma ratio method.* The measurement of intestinal cholesterol absorption was carried out *in vivo* by the dual isotope plasma ratio method of Zilversmit *et al.* with slight modifications (7). Under light anesthesia with ethylether, the abdominal cavity was opened by incision, and 1.0 μCi of [^{14}C]cholesterol in 0.5 ml of micellar solution was administered into the duodenum. 1.0 μCi of [^3H]cholesterol dispersed in 0.5 ml of serum obtained previously from the test animal was then immediately injected into the left external jugular vein. The rat was subsequently fed on standard diet and water *ad libitum*. Samples of blood (2 ml) were collected with heparinized syringes from the right external jugular vein of the rat at 48, 72 and 96 hr after the administration of cholesterol. The radioactive cholesterol was extracted from the plasma samples (1 ml each) with 3 ml of ethanol:hexane = 2:1 (v/v) after saponification with 50% potassium hydroxide at 50°C for 30 min. The radioactivities of [^3H]cholesterol and [^{14}C]cholesterol were then determined simultaneously in 7 ml of toluene-based scintillation cocktail containing 0.4% Omunifluor by a Packard Tri-Carb liquid scintillation spectrometer, Model 3380 (Packard Co., Downers Grove, IL). The counting efficiencies were 55% for ^{14}C and 40% for ^3H , respectively. Percent absorption of cholesterol was calculated (7, 11) as follows:

$$\% \text{ absorption} = \frac{\text{plasma } ^{14}\text{C (dpm)}/\text{dosed } ^{14}\text{C (dpm)}}{\text{plasma } ^3\text{H (dpm)}/\text{dosed } ^3\text{H (dpm)}} \times 100$$

4. *Intestinal ligated-loop method.* The measurements of mucosal uptake of cholesterol were carried out *in situ* by the ligated-loop method of Sylvén (9). After an animal was well anesthetized with nembutal (30 mg/kg of body weight), 4 to 6 segments of 4 cm length were made in the jejunum while maintaining the circulation of blood and lymph. One hundredth μCi of [^{14}C]cholesterol in 0.5 ml of dose solution was injected into each segment and then incubated in the body for 30 min, after closing the abdominal wall. To investigate the effect of metabolic inhibitors on cholesterol uptake, a micellar dose solution without the inhibitor (control) and a solution with one of the inhibitors were injected simultaneously into different segments of the same rat. In the experiments to compare the coefficient of absorption of cholesterol with that of palmitate, either [^{14}C]cholesterol or [^{14}C]palmitate was injected simultaneously into different jejunal segments of the same animal. After incubation for 30 min, each segment of the jejunal loop was removed from the body and washed with 2 ml of ice-chilled physiological saline solution. The tissue was further washed 5 times with 2 ml of ice-chilled 5 mM

sodium deoxycholate in saline solution to remove the radioactive materials remaining on the surface of the mucosa. Each sample of washed tissue weighed between 200 and 400 mg. The radioactive material in the tissue was extracted with 9 ml of chloroform:ethanol=2:1(v/v) as described by Folch *et al.* (12). After evaporating the chloroform, the radioactivity was determined in 10 ml of toluene-based scintillation cocktail by a liquid scintillation spectrometer. The counting efficiency was about 90%.

The amount of cholesterol or palmitate incorporated into the tissue was calculated and expressed as $\mu\text{g}/100\text{ mg tissue}/30\text{ min}$. The ratio of inhibition by the metabolic inhibitor was calculated by dividing the amount of cholesterol uptake in the presence of inhibitor by the amount of cholesterol uptake in the absence of inhibitor with the resulting value expressed as % of the control. The same procedure was used to compare the coefficient of uptake of cholesterol with that of palmitate.

In order to observe the effect of temperature on the mucosal uptake, the body temperature of the animal was kept at 27°C with crushed ice, and the rate of uptake was compared with that at 37°C .

RESULTS

1. Relationship between the amount of cholesterol administered and the coefficient of absorption of cholesterol

In order to observe the relationship between the amount of cholesterol

Fig. 1. The effect of varying amounts of administered cholesterol on the coefficient of cholesterol absorption *in vivo*. From $50\text{ }\mu\text{g}$ to $1,000\text{ }\mu\text{g}$ of $[4\text{-}^{14}\text{C}]$ cholesterol ($1.0\text{ }\mu\text{Ci}$ each) in 0.5 ml of micellar solution was injected intraduodenally and cholesterol absorption was measured by the dual isotope plasma ratio method. The values are the means of 5–9 determinations and expressed as μg of cholesterol absorption per rat.

Fig. 2. The effect of varying amounts of administered cholesterol on the coefficient of cholesterol uptake by jejunal segments. From 10 μg to 400 μg of [$4\text{-}^{14}\text{C}$]cholesterol (0.01 μCi each) dissolved in 0.5 ml of the dose solution was injected into a jejunal segment and the cholesterol uptake was measured *in situ* by the ligated-loop method. The values are the means of 9–13 determinations and are expressed as μg of cholesterol uptake per 100 mg tissue per 30 min.

administered and absorption, 100 to 1,000 μg of cholesterol in 0.5 ml of dose solution were directly injected into a rat jejunum, and the intestinal absorption was measured by the dual isotope plasma ratio method. In the lower range (less than 300 $\mu\text{g}/0.5\text{ ml}/\text{rat}$) of administration, absorption increased linearly according to the amount administered (Fig. 1). When the amount was between 300 μg and 500 μg , the absorption was constant. In rats given 300 μg of cholesterol, about 250 μg was absorbed. When the amount was increased to more than 500 $\mu\text{g}/0.5\text{ ml}/\text{rat}$, the absorption increased again linearly (Fig. 1). These results indicate that the intestinal absorption of cholesterol increases linearly at both low and high dosage levels but saturation takes place at intermediate dosage levels.

The mucosal uptake of cholesterol was also measured by means of the ligated-loop method with the administration of 10 μg to 400 μg cholesterol in 0.5 ml of dose solution. The mucosal uptake increased on linear relation to the dose level from 10 μg up to 250 μg cholesterol (Fig. 2). In the range of 250 μg through 400 μg of cholesterol, the mucosal uptake was clearly saturated.

From these results, it appears that the saturation of cholesterol absorption occurs at concentrations between 250 μg and 500 μg of cholesterol for both the dual isotope plasma ratio method *in vivo* and the jejunal ligated-loop method *in situ*. Therefore, it seems to be possible to investigate the mechanism of cholesterol absorption in the physiological state using the ligated-loop method.

Table 1. The effect of metabolic inhibitors on cholesterol uptake. Cholesterol uptake into the mucosa of jejunal segments was measured in the presence or the absence of inhibitors *in situ* by the ligated-loop method. Forty millimolar sodium azide, 40 mM potassium cyanide, 20 mM 2,4-dinitrophenol, or 5 mM ouabain was added to the dose solution containing less than 10 μ g of [4- 14 C]cholesterol in 0.5 ml. For the comparative experiment between cholesterol and palmitate, less than 10 μ g of [1- 14 C]palmitate were used instead of cholesterol. The values are the means of 8–10 determinations and are expressed as % of the control (that without inhibitor).

	Uptake	
	Cholesterol	Palmitate
Control	100	100
NaN ₃ (40 mM)	41.6 $\pm$ 3.2 ^a	80.9 ^c
KCN (40 mM)	38.4 $\pm$ 1.6 ^a	88.2 ^c
2,4-DNP (20 mM)	61.3 $\pm$ 20.0 ^b	85.5 ^c
Ouabain (5 mM)	60.2 $\pm$ 10.0 ^b	85.0 ^c

Significantly different from control ^a $p < 0.01$; ^b $p < 0.05$; ^c N.S.

Fig. 3. The effect of low temperature on cholesterol uptake. Body temperature of the rats was kept at $27 \pm 1.0^\circ\text{C}$ with ice and the cholesterol uptake was measured *in situ* by the ligated-loop method. A half milliliter of the dose solution containing less than 10 μ g of [4- 14 C]cholesterol was injected into the jejunal segment. For the comparative experiment to study the specificity of cholesterol uptake, less than 10 μ g of [1- 14 C]palmitate were used instead of [1- 14 C]cholesterol. The values are the means of 6 determinations and are expressed as % of the control (that at 37°C).

2. Effect of metabolic inhibitor on mucosal uptake of cholesterol

The saturable uptake of cholesterol suggests that cholesterol may not be transported by a simple diffusion process. Therefore, the effect of metabolic inhibitors on the mucosal uptake was studied using the ligated-loop method. As shown in Table 1, cholesterol uptake by the jejunal segments decreased significantly

with all metabolic inhibitors such as sodium azide, potassium cyanide, 2,4-dinitrophenol or ouabain. In contrast, the mucosal uptake of palmitate, which is thought to be transported by a simple diffusion process, showed no significant decrease with the addition of inhibitors (Table 1). These results suggest that an energy-dependent system is involved in the process of intestinal cholesterol uptake.

3. *The effect of body temperature on mucosal uptake of cholesterol*

The effect of body temperature on cholesterol uptake was observed by means of the ligated-loop method. When the body temperature of an animal was lowered to 27°C, cholesterol uptake decreased significantly compared to the control (that at 37°C) though the uptake of palmitate was not inhibited at the low temperature (Fig. 3). These results also support the hypothesis that an energy-dependent system is involved in the mucosal uptake of cholesterol.

DISCUSSION

It has been generally accepted that cholesterol is absorbed *via* simple passive diffusion in the intestine (1-3). Our results have, however, suggested that the intestinal absorption of low levels of cholesterol occurs by an active transport process requiring energy.

It has been previously demonstrated by balance studies and lymph-collection techniques that the coefficient of cholesterol absorption varies widely from 7.5% to 97% (13, 14). However, the acceptable value of the coefficient is 40-60% as Zilversmit *et al.* have demonstrated by means of the dual isotope plasma ratio method (8). In these papers, it has been generally believed that the coefficient of cholesterol absorption was a constant independent of the amount administered.

In our results, cholesterol absorption did not simply increase according to the increment of cholesterol dosage, but instead showed a tri-phase relationship (Fig. 1). When cholesterol amounting to more than 500 µg/0.5 ml/rat was administered, the coefficient of absorption was about 55%, independent of the amount administered. This value agrees acceptably with that reported by others (8, 15). The coefficient of absorption was, however, greater than 80% for low levels of administration (less than 300 µg/0.5 ml/rat), and was remarkably different from that observed with high levels of administration (more than 500 µg/0.5 ml/rat). Thus, our results suggest that the coefficient of absorption varies substantially when the amount of cholesterol administration exceeds the physiological dose level. In other papers reporting a linear increase in cholesterol absorption independent of the amount of administration (1-3), more than 10 mg of cholesterol was given to each animal. This dose level is 10-20 times greater than the dosage in our experiments. Therefore, it appears that the saturable phase in cholesterol absorption was not detected by other groups, because the phenomenon occurs only at low dosage levels. Dosages of more than 10 mg of cholesterol are excessively large in comparison with the normal daily dietary intake in the physiological state of animal and would

appear inappropriate for studying the absorption process.

Hollander and Muralidhara have demonstrated that the intestinal absorption of retinol, one of the water-insoluble materials, saturates once at low concentrations and then is enhanced again above a certain level with increasing levels of administration (16). The mucosal uptake of β -sitosterol, an analogue of cholesterol, however, showed no saturation throughout a wide range of administration (from 10 μ g to 500 μ g in 0.5 ml of dose solution) (17). Therefore, saturation at low dose levels appears to be a phenomenon characteristic of the intestinal absorption of only certain materials such as cholesterol and retinol.

Using the intestinal ligated-loop method, Sylvén has indicated that the mucosal uptake of cholesterol decreases remarkably when the blood flow to the ligated-loop segments is interrupted (10). This suggests that the cholesterol uptake system is energy-dependent. In our observations, the uptake of palmitate showed no significant decrease with the addition of metabolic inhibitors or with the lowering of body temperature, whereas cholesterol uptake significantly decreased. This was the case even when palmitate was simultaneously injected into different segments of the same animal while the cholesterol was administered. Present results strongly support Sylvén's suggestion that the mucosal uptake of cholesterol requires energy. At the same time, the energy-dependent absorption seems to be a phenomenon specific to cholesterol, but not to β -sitosterol or palmitate.

Vahouny *et al.* have reported that the lymphatic transport of cholesterol is prevented by puromycin, an inhibitor of protein synthesis, and that about 80% of cholesterol in the lymph fluid is in the form of free cholesterol. They suggested that the inhibitory effect of puromycin was related to chylomicron synthesis, and further that the cholesterol incorporated into intestinal epithelial cells was released in the form of free cholesterol rather than the esterified form (18). In our experiments, when the mucosal uptake of cholesterol in the absence of metabolic inhibitor was measured by the ligated-loop method, a saturable uptake was observed, and the radioactive cholesterol incorporated could not be detected in the blood during incubation for 30 min. Furthermore, the radioactive cholesterol in the extract from intestinal mucosa was mostly of the free form in thin layer chromatography. Therefore, our results indicate that the step of mucosal uptake is one of the limiting stages in the process of cholesterol absorption, but the esterification of cholesterol is not, and that the decrease in uptake by metabolic inhibitors is not related to the process of transport into the lymph.

In our previous paper (6), β -sitosterol showed a varying inhibitory effect on the intestinal absorption of cholesterol according to the stage of mucosal uptake. Additionally, it was demonstrated in the present report that the process of cholesterol uptake is saturable and requires energy. These observations strongly suggest that there is a specific carrier in the intestinal mucosa for cholesterol as well as some other nutrients. The carrier involved in this active transportation of cholesterol may be a specific binding protein.

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