

Puromycin inhibition of cholesterol absorption in the rat

George V. Vahouny, M. Ito,¹ E. M. Blendermann,² L. L. Gallo, and C. R. Treadwell

Department of Biochemistry, The George Washington University, School of Medicine and Health Sciences, Washington, D.C. 20037

Abstract The effect of puromycin on the intestinal absorption of cholesterol has been studied in rats with indwelling catheters in the left thoracic lymphatic duct. Puromycin administration to female rats produced a marked depression of cholesterol absorption under conditions where the absorption of simultaneously administered fatty acid was also dramatically inhibited. The same treatment of male rats also produced a significant depression in cholesterol absorption, but was without effect on absorption of the fatty acid. Despite the depressions of lipid absorption in puromycin-treated animals, there was no accumulation of either cholesterol or fatty acid in the intestinal mucosa of either sex. Actinomycin D treatment of fasting male and female rats, receiving constant infusions of saline, had no effect on the rate of lymph production. This suggests that altered lymph production was not responsible for the depressed lipid absorption observed in fed animals treated with protein synthesis inhibitors. The selective inhibition of cholesterol absorption in male rats also precludes the possibility that the major effect of the inhibitor is on delayed gastric emptying.

Supplementary key words protein synthesis inhibitors · fatty acid absorption

During the intestinal absorption of either endogenous or dietary lipids, the intestinal mucosal cells synthesize proteins that are associated with lipid transport particles. In addition to chylomicrons, there are transport particles comparable to the very low density lipoproteins, VLDL, and the low density lipoproteins, LDL (1–10). Considerable evidence, demonstrating the role of intestinal apoprotein synthesis in the assembly of these transport particles and their subsequent release into the lymph, has been derived through the use of various protein synthesis “inhibitors”; these include puromycin (11–14), cycloheximide and its derivatives (11, 14, 15), ethionine (16, 17), orotic acid (8), and actinomycin D (18). However, none of these studies has dealt directly with the effects of protein synthesis antagonists on the intestinal absorption and transport of dietary cholesterol.

The purpose of the present investigation was to determine the effect of puromycin administration to lymph duct-cannulated rats on the intestinal absorption of [7α - 3 H]cholesterol given in an intragastric emulsion. Because of a reported sex difference in the effect of ethionine on lipid absorption in rats (17), these studies were carried out with animals of both sexes. The simultaneous administration of [1 - 14 C]oleic acid provided information on gastric emptying and on triglyceride synthesis in the mucosa and release into lymph. Finally, the direct effect of actinomycin D on lymph flow in fasted rats of both sexes was determined.

MATERIALS AND METHODS

Materials

Puromycin and actinomycin D were obtained from Nutritional Biochemical Corporation, Cleveland, OH; the lipids were from Supelco, Bellefonte, PA; and the radioactive materials were purchased from Amer-sham-Searle Corporation, Arlington Heights, IL. The purity of oleic acid (99%) was checked by gas–liquid chromatography of the methyl ester; the purity of [1 - 14 C]oleic acid was determined by collection and counting of split fractions from gas–liquid chromatography after dilution with carrier oleic acid and transmethylation with boron trifluoride–methanol. Cholesterol and [7α - 3 H]cholesterol were purified through the dibromide and checked by thin-layer silicic acid chromatography.

Experimental procedure

Male and female rats of the Wistar strain (Carrworth Farms) were maintained on commercial chow

¹ Present address: Department of Medicine, Nagoya University School of Medicine, Nagoya, Japan.

² Present address: Office of Nutrition and Consumer Sciences, Food and Drug Administration, Washington, D.C.

and water ad libitum prior to use. Animals weighed 200–300 g and there were no statistically significant differences between groups. The left thoracic lymphatic was cannulated with polyethylene tubing (PE 10, Intramedic) cephalad to the cysterna chyli (19). The animals were placed in restraining cages (20) and fasted overnight.

The protocol for treating animals with puromycin was as described by Sabesin and Isselbacher (11). Rats were given four hourly intraperitoneal injections of 2.5 mg of puromycin (10 mg/ml), followed by five hourly injections of 1 mg of puromycin; the total dose was 15 mg of inhibitor. Control rats received similarly-timed injections of saline alone. At the time of the fifth injection, each rat was given, by gastric intubation, 3 ml of a 0.9% saline lipid emulsion containing 50 mg of albumin, 50 mg of [7α - ^3H]cholesterol (5 μCi), 292 mg of [1 - ^{14}C]oleic acid (2.5 μCi), and 288 mg of sodium taurocholate. These aqueous emulsions were prepared immediately before use as described earlier (21). Lymph was collected in 2-hr periods for 6 hr in graduated centrifuge tubes immersed in ice and containing 0.1 ml of heparin (1:1000).

At the end of the experiment, the animals were killed, and the entire small intestine was carefully removed and washed with 150 ml of cold physiological saline. The intestine was then homogenized in 25 ml of cold physiological saline in a glass homogenizing tube.

Lipid extraction and analysis

Aliquots of the lymph, and of intestinal homogenates, were immediately extracted with 20 volumes of chloroform–methanol 2:1 (v/v) according to Folch, Lees, and Sloane Stanley (22). After washing and separation of the solvent phases, the entire chloroform extract was evaporated to dryness under nitrogen, and the residual lipids were redissolved in hexane. Aliquots of the hexane extract were placed in scintillation vials and the solvent was evaporated under nitrogen. One ml of methanol and 10 ml of liquid scintillation mixture (500 mg of 1,4-di-2(5-phenyloxazolyl)-benzene and 4 g of 2,5-diphenyloxazole per liter of toluene) were added and radioactivity was measured by simultaneous counting of ^{14}C and ^3H in a Nuclear Chicago Mark I 3-channel liquid scintillation spectrometer using both external and internal standardization procedures.

Distribution of [7α - ^3H]cholesterol in free and esterified cholesterol, and of [1 - ^{14}C]oleic acid among the major lipid fractions in lymph and intestinal mucosa was determined as follows. Aliquots of the hexane extracts and standards of authentic lipids

were spotted on thin-layer silicic acid plates, and the major lipid classes were separated using a solvent system of hexane–diethyl ether–acetic acid 86:13:2 (by volume). The lipid classes were visualized by exposure of the plates to iodine vapors, and the silicic acid areas corresponding to cholesteryl esters, fatty acids, triglycerides, free cholesterol, minor glycerides, and phospholipids were individually scraped into scintillation vials. One ml of methanol and 10 ml of liquid scintillation mixture were added prior to liquid scintillation spectrometry. All isotope data were corrected to dpm. Apparent differences between means were analyzed for significance by the *t* test (23).

RESULTS

Absorption in female rats

The data on cumulative lymphatic absorption of [7α - ^3H]cholesterol and [1 - ^{14}C]oleic acid in female rats are summarized in **Fig. 1**. In the control group, the peak of absorption of both lipids occurred during the 2–4 hr period following administration of the test emulsion, as has been reported earlier (24). Approximately 24% of the administered cholesterol and 64% of the administered oleic acid were absorbed into the lymphatic system during the 6-hr experimental period (**Fig. 1**), again in accord with previous studies (24). For cholesterol, this represented a maximal absorption rate of 3.3 ± 0.7 mg/hr with a mean absorption rate for the 6-hr period of 2.0 ± 0.3 mg/hr. For oleic acid, the maximal absorption rate was 50.0 ± 9.0 mg/hr, and the mean absorption rate for 6 hr was 30.9 ± 4.8 mg/hr.

The absorption of both lipids in female rats was dramatically depressed by puromycin administration, and distinct peaks of absorption were not evident as was found with control rats. Cholesterol absorption was depressed from a value of 23.5% in control rats to approximately 7.5% of the administered dose in puromycin-treated females. The maximal absorption rate for cholesterol was 1.0 ± 0.15 mg/hr, and the mean absorption rate was reduced to 0.65 ± 0.15 mg/hr (from 2.0 ± 0.3 mg/hr).

Oleic acid absorption in puromycin-treated females represented only 25% of the fed dose compared to 64% in controls; the maximal absorption rate was depressed from 49 mg/hr to 14 ± 2.8 mg/hr, and the mean absorption rate was 12.0 ± 1.5 mg/hr or approximately 40% of that in control rats.

Absorption in male rats

Data on cumulative absorption of [7α - ^3H]cholesterol and [1 - ^{14}C]oleic acid in male rats are summarized

in Fig. 2. Approximately 14% of the administered cholesterol (compared to 24% in females) and 48% of the administered oleic acid (compared to 64% in females) appeared in thoracic duct lymph during the 6-hr experimental period (see Fig. 3). This represented a maximal rate of cholesterol absorption of 1.34 ± 0.21 mg/hr with a mean absorption rate for the 6-hr period of 0.94 ± 0.15 mg/hr (compared to 2.0 ± 0.3 in females). For oleic acid, the maximal absorption rate was 30.5 ± 4.1 mg/hr (compared to 49 mg/hr in females), and the mean absorption rate for 6 hr was 23.3 ± 2.3 mg/hr (compared to 30.9 ± 4.8 in females).

From the data in Fig. 2, it is apparent that puromycin administration to male rats resulted in a significant depression of cholesterol absorption, although the effect was less than in female rats. Overall, puromycin-treated males absorbed only about 7% of the fed dose compared to approximately 13% in control animals. The mean rate of cholesterol absorption during the entire experimental period was 0.60 ± 0.11 mg/hr, which was significantly less ($P < 0.05$) than that in controls (0.94 ± 0.15 mg/hr).

Oleic acid absorption in male rats was unaffected by treatment with puromycin (Fig. 2). The maximal rate of fatty acid absorption occurred during the same period as in controls and was 31.5 ± 2.2

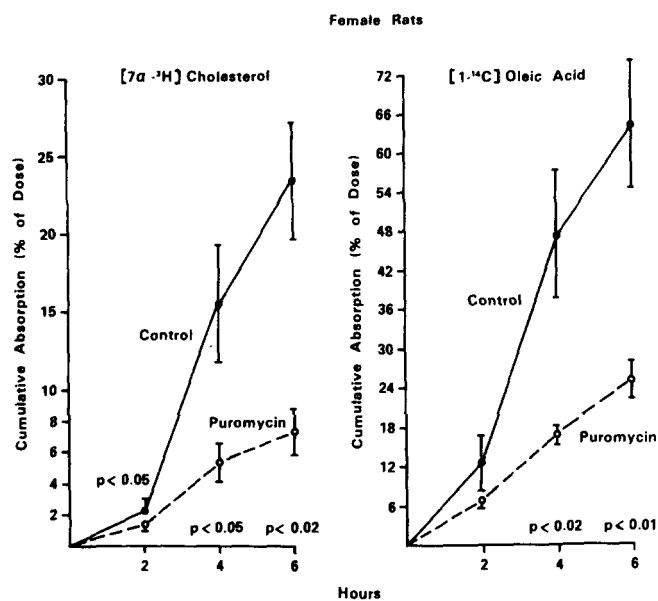


Fig. 1. Cumulative appearance of $[7\alpha\text{-}^3\text{H}]$ cholesterol and $[1\text{-}^{14}\text{C}]$ oleic acid into thoracic duct lymph of female rats. Closed circles represent means \pm SEM from four rats receiving an intragastric test emulsion containing 50 mg of albumin, 50 mg of $[7\alpha\text{-}^3\text{H}]$ cholesterol, 288 mg of sodium taurocholate, and 292 mg of $[1\text{-}^{14}\text{C}]$ oleic acid per 3 ml of saline. Open circles represent means \pm SEM from four female rats treated with 15 mg of puromycin, as described in the text, prior to receiving the test emulsion.

Male Rats

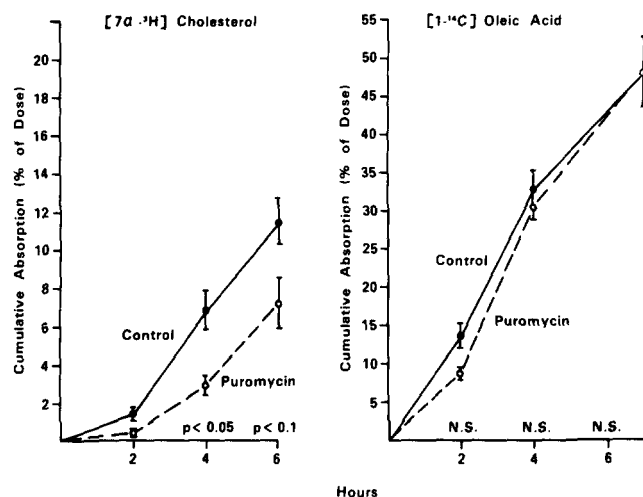


Fig. 2. Cumulative absorption of $[7\alpha\text{-}^3\text{H}]$ cholesterol and $[1\text{-}^{14}\text{C}]$ oleic acid into thoracic duct lymph of male rats. Closed circles represent means \pm SEM from five rats receiving an intragastric test emulsion containing 50 mg of albumin, 50 mg of $[7\alpha\text{-}^3\text{H}]$ cholesterol, 288 mg of sodium taurocholate, and 229 mg of $[1\text{-}^{14}\text{C}]$ oleic acid per 3 ml of saline. Open circles represent means \pm SEM from three male rats administered 15 mg of puromycin, as described in the text, prior to receiving the test emulsion.

mg/hr; the mean rate of oleic acid absorption for the 6-hr period was 23.1 ± 2.0 mg/hr, which was also comparable to that in controls.

Mucosal and lipid levels

Comparative data on lymphatic absorption and mucosal levels of the administered radioactive lipids in both sexes are summarized in Fig. 3. For female rats, approximately 15% and 13% of the administered cholesterol and oleic acid, respectively, were found associated with the intestinal mucosa at the termination of study. Similar data were obtained with male rats (13% and 11% for cholesterol and oleic acid, respectively).

Thus, despite the marked inhibition of the lymphatic transport of both lipids in puromycin-treated female rats, and of cholesterol in puromycin-treated male rats, there was no significant "accumulation" of these lipids in the intestinal mucosa. The data with oleic acid confirm the findings of others (12, 13), although it is in contrast to the earlier report of Sabesin and Isselbacher (11).

Distribution of labeled lipids in intestinal mucosa and lymph

The distribution of absorbed $[7\alpha\text{-}^3\text{H}]$ cholesterol between the free and esterified forms in the intestinal mucosa, and of oleic acid among the major lipid fractions of the mucosa are summarized in Table I.

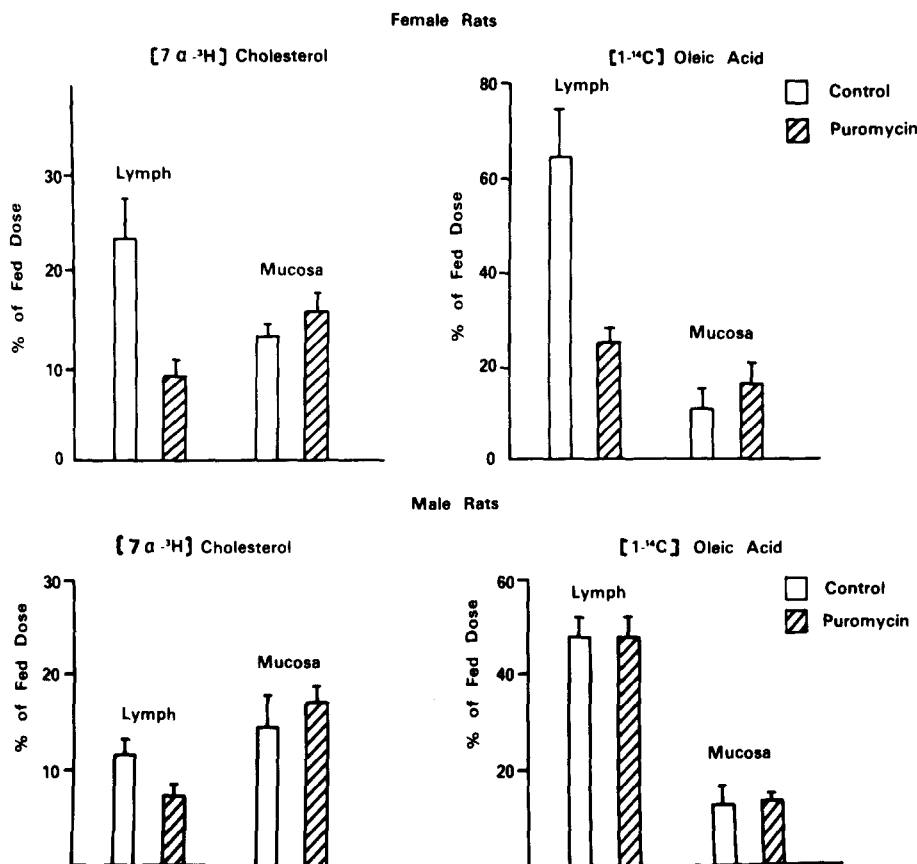


Fig. 3. Total lymphatic absorption and mucosal content of [^3H]cholesterol and [^{14}C]oleic acid in control and puromycin-treated rats receiving the intragastric test emulsion. Solid bars represent means \pm SEM from four female and five male control rats, and hatched bars represent means \pm SEM from four female and three male rats administered puromycin, as described in the text.

As has been shown earlier (24), about 80% of the labeled cholesterol in the mucosa was unesterified in the control animals. Also, about 65% of the fatty acid label in the mucosa of female rats was present as triglyceride with lesser percentages in phospholipids, unesterified fatty acids, cholesteryl esters, and minor glycerides, respectively.

Despite the absence of an accumulation of mucosal

lipids in female rats treated with puromycin, there was an effect of puromycin on the distribution of the labeled cholesterol between the free and esterified forms, i.e., a significant increase in the percentage of ^3H associated with esterified cholesterol. However, the amount of labeled oleic acid in this fraction was reduced in puromycin-treated rats. This apparent difference might be explained by esterification of the

TABLE 1. Distribution of [$7\alpha\text{-}^3\text{H}$]cholesterol and [$1\text{-}^{14}\text{C}$]oleic acid in intestinal mucosa

Group ^a	% [$7\alpha\text{-}^3\text{H}$]Cholesterol		% [$1\text{-}^{14}\text{C}$]Oleic Acid					
	Free	Esterified	TG ^c	DG	MG	FA	PL	CE
Female rats								
Control (4)	78.7	21.3	65.6	4.9	1.2	9.7	11.2	7.3
Puromycin (4)	67.9 ^b	32.1 ^b	68.2	7.2	2.2	14.5	4.4 ^b	4.5 ^b
Male rats								
Control (4)	77.9	22.1	86.4	2.2	0.7	4.4	3.9	2.4
Puromycin (4)	82.0	18.0	74.0 ^b	3.9 ^b	2.4 ^b	12.7 ^b	4.6	2.9

^a The aqueous emulsion for intragastric feeding contained 50 mg of [$7\alpha\text{-}^3\text{H}$]cholesterol, 292 mg of [$1\text{-}^{14}\text{C}$]oleic acid, 288 mg of sodium taurocholate, and 50 mg of albumin in 3 ml of saline per rat.

^b $P < 0.05$.

^c TG, triglyceride; DG, diglyceride; MG, monoglyceride; FA, fatty acid; PL, phospholipid; CE, cholesteryl ester.

labeled sterol by an unlabeled pool of fatty acids in the mucosa.

A second effect of puromycin treatment was the significant decrease of fatty acid label associated with the mucosal phospholipid fraction. This is in accord with the data of O'Doherty, Yousef, and Kuksis (25) who reported a significant depression of phospholipid synthesis in isolated mucosal cells from puromycin-treated female rats.

There was no effect of puromycin on the distribution of labeled cholesterol between the free and esterified forms in the mucosa of male rats, which is in contrast to the effect seen in female rats. This may be related to the less dramatic effect of puromycin on total lymphatic transport of cholesterol in the male. Despite the apparent lack of effect of puromycin on fatty acid absorption into lymph in male rats, there was decreased radioactivity in the triglyceride fraction of the mucosal lipids, with increases in radioactivity associated with lower glycerides and unesterified fatty acids.

The distribution of absorbed cholesterol between the free and esterified forms in lymph, and of oleic acid among the major lipid fractions of lymph was also determined. With both sexes, 80–86% of the absorbed cholesterol recovered in lymph was in the esterified form, and this was not altered despite the effect of puromycin on the net absorption of cholesterol. Of the oleic acid transported into lymph, 90–94% was recovered in the triglyceride fraction; 5% was found as esterified cholesterol and lesser amounts were associated with the remaining lipid fractions. Again, these distributions were unaffected by puromycin treatment of either sex.

Lymph flow

As shown in Fig. 4, the depressed lipid absorption resulting from puromycin treatment of lipid-dosed, female rats was accompanied by a significant reduction in lymphatic flow rates and total lymph volume for the 6-hr experimental period. In order to determine whether depressed lymph flow was a direct result of the protein antagonist or was related indirectly to depressed lipid absorption, the effect of a second protein synthesis antagonist on lymph flow in rats given no exogenous lipid was investigated. It had been reported (18) that administration of actinomycin D, at a level of 1 mg/kg, depressed lymph flow in lipid-dosed, female rats, despite a constant intraduodenal infusion of saline to maintain lymph flow. As shown in Fig. 5, a single injection of actinomycin D to fasting female or male rats maintained on infusions of 0.9% saline–5% glucose, had no effect on the rate of production of thoracic duct lymph.

DISCUSSION

The present report represents our initial study on the effects of the protein synthesis antagonist, puromycin, on the intestinal absorption of exogenous cholesterol. It is also one of the few studies comparing the effects of puromycin on lipid absorption in male and female rats, although it is generally recognized that, with respect to lipid transport systems, female animals are more responsive to protein antagonists than are males (e.g., ref. 17). Finally, our studies on puromycin inhibition of fatty acid

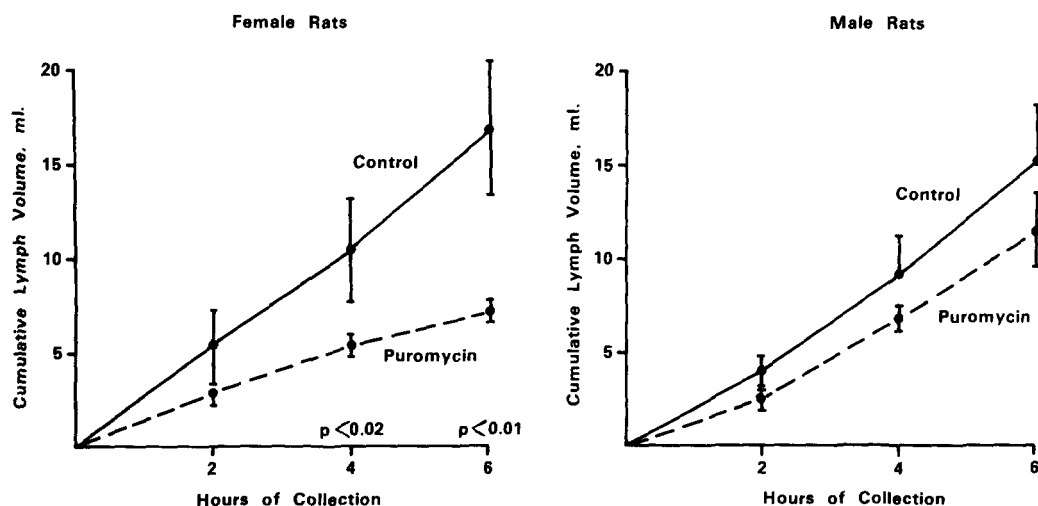


Fig. 4. Cumulative lymph flow in female and male control rats and rats administered 15 mg of puromycin, as described in the text.

absorption, which are like those reported by others (11–14), provide comparative and complementary data to those on cholesterol absorption in the same animals; such data have not been reported previously.

The effect of puromycin and similar protein synthesis antagonists on the net absorption of lipid from the gastrointestinal tract into lymph has been attributed to a variety of mechanisms, including inhibition of intestinal protein synthesis. These include decreased gastric emptying time and intestinal motility (12), decreased lymph flow (12, 18), decreased mucosal uptake (26), decreased triglyceride (13) or phospholipid (25) synthesis, and decreased synthesis of cellular membranes, including endoplasmic reticulum (27).

In the present studies with female rats, puromycin administration resulted in a dramatic reduction in both the rate and extent of cholesterol and fatty acid absorption into lymph. This effect confirms the original observations of Sabesin and Isselbacher (11) on the inhibition of neutral fat absorption by puromycin, as reflected by alterations in plasma lipid levels. It also complements the limited data obtained by Kayden and Medick (13) on puromycin inhibition of lymphatic absorption of oleic acid and triolein in female rats.

In the female animals treated with puromycin, the depressed absorption of oleic acid and cholesterol was accompanied by a significant reduction in lymph flow. In addition, the data on the level and distribution of labeled oleic acid in the intestinal mucosa at the termination of the experiment showed that there was no measurable accumulation of lipids despite an apparent inhibitory effect of puromycin on fatty acid esterification (e.g., phospholipids and cholesteryl esters). In a preliminary experiment in which [^{14}C]leucine was administered intraduodenally 2 hr after the lipid dose, puromycin treatment of female rats resulted in a 54% inhibition of isotope incorporation in a 4-hr study. Thus, the data with female rats do not allow a differentiation between the various proposed mechanisms by which puromycin might depress lipid absorption, as enumerated above.

The puromycin treatment of male rats resulted in a significant depression of the absorption of exogenous cholesterol but was without effect on the absorption of oleic acid administered simultaneously. As in the case of female rats, the decreased absorption of cholesterol was not accompanied by an increase in mucosal levels of the labeled sterol. Since mucosal levels of the administered lipids were only determined at the termination of study, it is

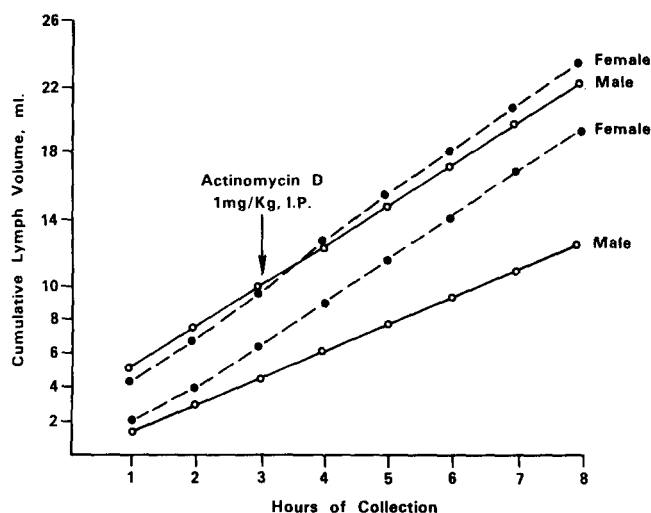


Fig. 5. Effect of actinomycin D on lymph flow in male and female rats. Actinomycin D (1 mg/kg) was injected intraperitoneally in 0.8 ml saline–5% ethanol at the times indicated, and all animals were given a continuous duodenal infusion of saline–5% glucose at a rate of 3 ml/hr. Each curve represents cumulative lymph volumes from a single animal.

not possible to determine whether transient lipid “accumulations” may have occurred at earlier times after the lipid dose.

The data of the studies on male rats clearly demonstrate that the depressive effects of puromycin on intestinal lipid absorption cannot be explained entirely by effects of the agent on gastric emptying. The use of lipids in an aqueous emulsion, in contrast to administration of corn oil (18), precludes the problem of delayed gastric emptying typical of feeding bulk lipid to “normal” animals. This type of intragastric feeding was employed by us earlier (e.g., 24), and the rate of absorption of lipids from this preparation is identical to that observed after administering lipids intraduodenally.³ Furthermore, as is shown clearly in **Fig. 6**, puromycin treatment of male rats produced a significant effect on cholesterol absorption without significantly affecting the absorption of the large dose of oleic acid given in the test meal. Any effect of puromycin on gastric emptying, whether profound or negative, might be expected to affect the absorption of both lipids if this were a major mechanism of drug action.

It also appears unlikely that puromycin and comparable antagonists of protein synthesis have a direct and significant effect on lymph flow per se. We (24) and others (e.g., 28) have demonstrated a direct response of lymph flow to the rate and extent of lipid absorption from the intestine, and have con-

³ Blendermann, E. M., and G. V. Vahouny. Unpublished results.

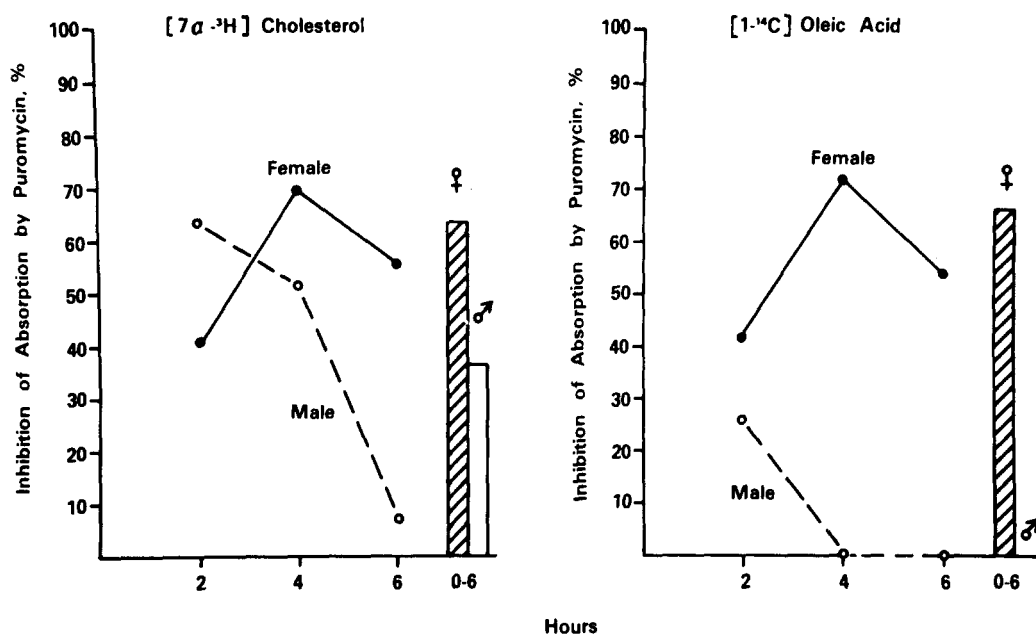


Fig. 6. Comparative puromycin inhibition of the lymphatic absorption of oleic acid and cholesterol in female and male rats. The inhibition by puromycin is plotted as percentage of the appropriate control values for each 2 hr of time during the 6-hr experiment. The vertical bars represent the inhibition of oleic acid and cholesterol absorption in female and male rats for the entire 6-hr period.


sidered this an effect of, rather than a determinant of, lipid transport into lymph. In the present study, as well, reduced lipid absorption due to puromycin in female rats was accompanied by reduced lymph flow. The earlier suggestions that protein synthesis inhibitors depress fat absorption via a direct inhibitory effect on lymph production was a result of studies on actinomycin D administration to fat-fed animals (18). However, as shown in Fig. 5, when actinomycin D was administered to either male or female rats that had not been given a lipid dose, there was no measurable effect on normal lymph flow.

Finally, in puromycin-treated males, the differential effect of the drug on the absorption of cholesterol and oleic acid suggests a major difference in either mucosal uptake or lymphatic transport of the two lipids. Although there is no direct evidence on the first of these possibilities, there is information regarding the latter.

In 1958, we (29) reported that absorbed cholesterol was associated largely with a chylomicron-free lipoprotein fraction of rat lymph. Similar findings were subsequently reported in dogs (30) and rabbits (31). Ochner et al. (6) determined that passage of endogenous cholesterol in the lymph of fasting rats was associated with a transport particle having the same flotation, chemical, and immunological properties as plasma very low density lipoproteins. Thus, it has become evident that a large

fraction of lymph cholesterol either of exogenous or endogenous origin is associated with one or more non-chylomicron, lipoprotein fractions. We have recently found³ that approximately 47% of the cholesterol and 17% of the triglycerides of exogenous origin are transported in a chylomicron-free lipoprotein fraction (d 1.019 g/ml) of lymph. In these animals, in which lipids were administered by duodenal infusion and lymph flow was maintained by constant infusions of saline, puromycin administration resulted in a small but significant inhibition of chylomicron protein synthesis but was without effect on the extent of absorbed lipids associated with this transport particle. In contrast, leucine incorporation into the d < 1.019 lipoprotein was inhibited by 88%, and this was accompanied by a significant depression of absorbed lipids associated with this fraction. However, since only 17% of the absorbed oleic acid was associated with the d < 1.019 lipoprotein fraction, the overall result of puromycin treatment was an insignificant effect on oleic acid absorption into lymph and a significant depression of cholesterol transport.

The results of the present studies demonstrate a major difference, probably dose-related, between the response of male and female rats to a protein synthesis antagonist. These studies do not elucidate the mechanism of this difference; however, comparative data on lipid absorption in both groups of rats suggest that a major effect of the drug on

cholesterol absorption is associated with inhibition of intestinal protein synthesis rather than to nonspecific effects on gastric emptying and/or lymph production. As suggested above, further studies are directed at the effects of the inhibitor on the specific transport particles in lymph. 

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