1, pp 247–354, Plenum Press, New York.
Small, D. M., Bourges, M. C., & Dervichian, D. Y. (1966) *Biochim. Biophys. Acta 125*, 563–580.
Steck, T. L., & Kant, J. A. (1974) *Methods Enzymol. 31*, 177–178.
Szoka, F. C., Jacobson, K., & Papahadjopoulos, D. (1979) *Biochim. Biophys. Acta 551*, 295–303.
Tall, A. R., & Lang, Y. (1976) *Biochem. Biophys. Res. Commun. 80*, 206–212.
Tardieu, A., Luzzati, V., & Raman, F. C. (1973) *J. Mol. Biol. 75*, 711–733.
Weinstein, J. N., Yoshikami, S., Henkart, P., Blumenthal, R., & Hagins, W. A. (1977) *Science (Washington, D.C.) 195*, 489–490.
Wititsuwannakul, D., & Kim, K. (1977) *J. Biol. Chem. 252*, 7812–7817.
Zahler, W. L., Barden, R. E., & Cleland, W. W. (1968) *Biochim. Biophys. Acta 164*, 1–11.

Cholesterol Biosynthesis and Modulation of Membrane Cholesterol and Lipid Dynamics in Rat Intestinal Microvillus Membranes†

Thomas A. Brasitus and David Schachter*

ABSTRACT: Experiments were performed to test the hypothesis that cholesterol biosynthesis in the rat ileal enterocyte, the major absorptive cell lining the distal epithelium of the small intestine, can modulate the cholesterol content and the motional freedom of the plasma membrane lipids. Decreased sterol biosynthesis in vivo was elicited by feeding sodium taurocholate or by fasting the rats, whereas increased synthesis was induced by biliary ligation or feeding cholestyramine, a bile salt binding resin; these effects were monitored by assay of mucosal 3-hydroxy-3-methylglutaryl coenzyme A reductase. After each procedure, isolated microvillus membranes were examined to determine the lipid composition and the fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene. The results demonstrate that variations in cholesterol biosynthesis in vivo can modulate the cholesterol content and the motional freedom of the lipids of the microvillus membrane; similar effects were not observed on the basolateral membrane. The observations suggest that the normal pattern of decreased lipid motional freedom in microvillus membranes of the distal as compared to the proximal small intestine of the rat results from higher rates of cholesterol biosynthesis in the distal mucosa.

There is considerable evidence that many functions of biological membranes are influenced by the composition and physical state of the membrane lipids (Lee, 1975; Melchior & Steim, 1976; Sandermann, 1978). The functional significance of the lipids is further indicated by observations that membranes which differ in function also differ in lipid composition and lipid motional freedom or "fluidity".[1] Such differentiation is illustrated clearly by comparisons of the luminal (microvillus) and contraluminal (basolateral) portions of the plasma membrane of the rat enterocyte, the major absorptive cell of the small intestinal mucosa. These antipodal membranes, which regulate the exchange of substances between organism and environment, differ in ultrastructure (Bloom & Fawcett, 1968; Oda, 1976), enzyme and transport activities (Douglas et al., 1972; Lewis et al., 1975; Murer et al., 1974, 1976), electrophysiological properties (Rose & Schultz, 1971; Okada et al., 1977), and protein components (Fujita et al., 1973). Correspondingly, the membranes differ in lipid composition (Forstner et al., 1968; Douglas et al., 1972; Kawai et al., 1974; Lewis et al., 1975; Brasitus & Schachter, 1980), and lipid molecules of the basolateral as compared to the microvillus membrane have considerably greater motional freedom (Brasitus et al., 1980; Brasitus & Schachter, 1980; Gray et al., 1981).

Although the evidence suggests the existence of regulatory mechanisms which maintain the lipid composition and fluidity characteristic of specific membrane organelles, the precise nature of these mechanisms is largely unknown. Accordingly, the present studies were initiated to examine the role of membrane cholesterol, a component which decreases the motional freedom of bilayer lipids above their transition temperatures (Oldfield & Chapman, 1971). Specifically, we explored the hypothesis that the cholesterol content of rat enterocyte plasma membranes is modulated by the rate of cholesterol biosynthesis in the cell. Several lines of evidence point to this possibility. Studies of the steady-state fluorescence polarization of lipid-soluble fluorophores indicate that the lipid fluidity of rat microvillus membranes is least in the distal (ileal) segment of the intestine and increases in the proximal portion (Schachter et al., 1976; Schachter & Shinitzky, 1977). Corresponding to this distribution, it was found that both the rate of incorporation of precursors into cholesterol and the

† From the Departments of Physiology and Medicine, Columbia University College of Physicians and Surgeons, New York, New York 10032. *Received July 17, 1981.* Supported by National Institutes of Health Grants AM21238 and AM01483 and by Clinical Investigator Award AM00386 of the National Institute of Arthritis, Metabolism and Digestive Diseases (T.A.B.).

[1] The term "lipid fluidity" as applied to model bilayers and natural membranes is used throughout this report to express the relative motional freedom of the lipid molecules or substituents thereof. When assessed by the estimation of steady-state fluorescence anisotropy of the fluorophore 1,6-diphenyl-1,3,5-hexatriene, changes in the fluorescence anisotropy may be due to alterations in the correlation time and/or maximal hindered anisotropy of the probe. As described previously (Brasitus & Schachter, 1980), we use the terms "lipid fluidity" or "motional freedom" to designate both kinds of alterations.

0006-2960/82/0421-2241$01.25/0

specific activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)[2] reductase, the rate-limiting enzyme of cholesterol synthesis, are greater in the distal as compared to the proximal intestinal segments (Dietschy & Siperstein, 1965; Merchant & Heller, 1977). Sinensky (1977, 1978) has described a Chinese hamster ovary cell mutant which is defective in the regulation of cholesterol synthesis by exogenous cholesterol. When the cholesterol of the ambient medium was increased, the mutant failed to reduce cholesterol biosynthesis, and the cholesterol content of the plasma membrane rose. Under similar circumstances, the parental cell line decreased the biosynthetic rate and maintained normal, control levels of membrane cholesterol. The author suggested that "the role of the cholesterol-synthesizing enzymes of the mammalian fibroblast is to regulate membrane fluidity" (Sinensky, 1978).

Our working hypothesis was tested by varying the rate of cholesterol biosynthesis of rat mucosal enterocytes in vivo, using procedures described by Andersen & Dietschy (1977). Decreased synthesis was elicited by feeding sodium taurocholate or by fasting the animals, whereas increased synthesis was induced by biliary ligation or by feeding cholestyramine, a resin which binds bile salts. The results below demonstrate that, in accord with the working hypothesis, variations in cholesterol biosynthesis in vivo can modulate the cholesterol content and the motional freedom of lipids in the microvillus membrane. Similar effects were not observed, however, on the basolateral membrane.

Experimental Procedures

Animal Experiments. Albino male rats of the Sherman strain weighing 200–300 g were fed a standard powdered diet of 20 g/day of Purina Rat Chow (Ralston Purina Co., St. Louis, MO) and water ad libitum. Rats were grouped in gang cages and maintained for at least 1 week in a controlled environment with alternating 12-h periods of light and darkness before use. In fasting studies, the animals were allowed free access to water; food was removed at the mid-light phase of the cycle, and the rats were killed 2 or 4 days later, again at the mid-light phase. In feeding experiments, each rat was allotted 18–20 g/day of the powdered, standard diet alone (controls) or the same amount containing either sodium taurocholate (Calbiochem-Behring Corp., La Jolla, CA; 97% pure)[3] or cholestyramine ("Questran", Mead Johnson & Co., Evansville, IN) in a final concentration of 2 g per 100 g of diet. The animals gained an average of 10 g over a 2-week period on these diets, with no significant differences in weight gain between the control and treatment groups. For ligation of the common bile duct, animals were anesthetized lightly with ether for a period of about 5–10 min, and the duct was doubly ligated and transected approximately 5 mm proximal to its entrance into the duodenum. Sham controls had a similar operation without actual ligation of the duct. Postoperatively the rats were pair-fed the powdered diet; at 2 days, the sham and ligated animals, respectively, exhibited mean weight losses of 18 and 23 g (P, not significant), and at 4 days, the corresponding weight losses were 22 and 49 g ($P < 0.01$). Following each of the foregoing treatments in vivo, the animals were maintained in the controlled environment with light cycling, and after appropriate intervals, they were fasted for 18 h and killed at the mid-light point of the cycle, and the small intestine was excised.[4]

Membrane Preparations. Unless indicated otherwise, the starting material was the distal 25 cm of the small intestine, and segments from 6–12 rats were pooled for the preparation of microvillus membrane suspensions as previously described (Brasitus et al., 1980). Purity and comparability of microvillus membrane suspensions were assessed by estimations of sucrase and *p*-nitrophenylphosphatase specific activities, and the preparations used were purified 10–20-fold as compared to the original homogenates. Basolateral membrane suspensions were prepared as previously reported (Brasitus et al., 1980), except that the time of the discontinuous sucrose density centrifugation was shortened to 2 h and the basolateral membrane fraction at the 30%/40% interface was collected and used. The final membrane suspensions were monitored by the marker enzymes ($Na^+ + K^+$)-dependent adenosinetriphosphatase and 5′-nucleotidase and were purified from 10- to 15-fold as compared to the starting homogenates. As noted previously (Brasitus et al., 1980), these plasma membrane preparations are relatively free of NADPH-cytochrome *c* reductase and succinate dehydrogenase, marker enzymes for microsomal and mitochondrial membranes, respectively.

Microvillus membrane vesicle suspensions were prepared and tested for sodium-dependent glucose transport as previously described (Brasitus et al., 1979).

Enzyme Assays. Homogenates of ileal mucosa were assayed for HMG-CoA reductase. The distal 25 cm of each small intestine was excised and the mucosa scraped from the underlying coats and suspended in an ice-cold "homogenizing" buffer consisting of 25 mM Tris, pH 7.5, 10 mM ethylenediaminetetraacetate, pH 7.5, 10 mM dithiothreitol, and 50 mM NaF. The suspensions were homogenized for 20 s in a Polytron apparatus (Brinkmann Instruments) at a setting of 4–5, and the resulting material was tested immediately, in triplicate, by the mixed phase assay of Phillipp & Shapiro (1979). Preliminary studies showed that the assays were linear with respect to the quantity of homogenate protein added and to time. Methods used to assay the marker enzymes of the microvillus and basolateral membranes have been described previously (Brasitus et al., 1979, 1980).

Chemical Estimations. Total lipids were extracted from membrane preparations by the method of Folch et al. (1957), and the composition of the lipid extracts was examined by thin-layer chromatography according to the procedure of Katz et al. (1976). Total phospholipid was estimated by the method of Ames & Dubin (1960). Cholesterol in serum and in tissue lipid extracts was quantified by the method of Zlatkis et al. (1953). Protein was estimated by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Fluorescence Polarization. Membranes were treated with the lipid fluorophore 1,6-diphenyl-1,3,5-hexatriene (DPH), and estimations of the steady-state fluorescence polarization were made in a dual-channel polarization spectrofluorometer (SLM Instruments, Urbana, IL) as previously described (Schachter & Shinitzky, 1977; Brasitus & Schachter, 1980). The polarization of fluorescence was expressed as the fluorescence anisotropy, r, and as the anisotropy parameter, $[(r_0/r) - 1]^{-1}$, where r_0, the maximal limiting anisotropy of the probe, was taken as 0.362 (Shinitzky & Barenholz, 1974). The anisotropy parameter varies directly with the rotational relaxation time of the fluorophore and inversely with the motional freedom

[2] Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; DPH, 1,6-diphenyl-1,3,5-hexatriene; $[(r_0/r) - 1]^{-1}$, fluorescence anisotropy parameter, where r is the fluorescence anisotropy and r_0 is the maximal limiting anisotropy of the probe.

[3] We could not obtain similar results with a less pure preparation obtained from ICN Pharmaceuticals (Cleveland, OH) and stated to contain approximately 65–70% taurocholic acid.

[4] When rats were killed at the mid-dark point of the cycle, the changes to be described below were less consistent and less marked.

Table I: Composition of Lipid Extracts of Rat Intestinal Microvillus Membranes[a]

component	% by wt of total lipid[b]		% by wt of total lipid[c]		
	proximal fourth of intestine	distal fourth of intestine	diet alone	diet + taurocholate	diet + cholestyramine
cholesterol	14.9 ± 1.7	18.8 ± 1.7[d]	19.8 ± 2.3	19.0 ± 2.1	24.3 ± 1.5[e]
cholesterol esters	2.5 ± 0.3	2.6 ± 0.3	1.1 ± 0.7	1.1 ± 0.5	2.4 ± 1.0
triglycerides	0.3 ± 0.1	2.3 ± 0.2[d]	1.0 ± 0.6	0.2 ± 0.1	2.0 ± 0.7[e]
fatty acids	16.0 ± 1.6	7.6 ± 0.7[d]	8.8 ± 1.5	8.4 ± 0.8	9.4 ± 0.4
total phospholipids	40.7 ± 1.9	37.4 ± 1.5	49.7 ± 2.4	47.4 ± 2.7	44.6 ± 2.6
lecithin	14.3 ± 1.2	12.6 ± 0.8	17.0 ± 2.5	14.0 ± 3.1	15.4 ± 1.4
lysolecithin	5.7 ± 2.8	2.5 ± 2.0	7.2 ± 1.5	8.2 ± 2.0	5.0 ± 1.4
phosphatidylethanolamine	9.2 ± 2.7	5.3 ± 1.7	1.7 ± 0.5	1.5 ± 0.3	1.9 ± 0.3
glycolipids	26.5 ± 3.7	31.2 ± 4.0	20.0 ± 3.1	23.7 ± 2.6	18.0 ± 3.4
cholesterol:phospholipid molar ratio	0.83 ± 0.10	1.01 ± 0.10[d]	0.81 ± 0.10	0.80 ± 0.10	1.10 ± 0.10[e]

[a] Values are means ± SE, expressed as the percent by weight of total lipid, except for the cholesterol:phospholipid molar ratio. Sphingomyelin is not included because of poor resolution in the solvent system. [b] Rats on the standard pellet diet; three membrane preparations and eight determinations. [c] Rats on the powdered diet ± 2% taurocholate or 2% cholestyramine for 2 weeks; three ileal membrane preparations and six determinations of each. [d] $P < 0.05$ for the difference between proximal and distal preparations (paired t test). [e] $P < 0.05$ for the difference between cholestyramine-treated and either the control or the taurocholate-treated preparations (paired t test).

Table II: Effects of Biliary Ligation[a]

days after surgery	group	HMG-CoA reductase activity[b] (pmol min^{-1} mg^{-1})	DPH anisotropy parameter,[c] $[(r_0/r) - 1]^{-1}$	serum cholesterol (mg/dL)
2	sham	95 ± 10 (8)	3.72 ± 0.17 (9)	67 ± 13 (12)
	ligated	142 ± 16 (8)	4.19 ± 0.24 (9)	150 ± 15 (12)
	P	<0.05	<0.01	<0.01
4	sham	101 ± 12 (6)	4.08 ± 0.09 (4)	65 ± 10 (8)
	ligated	168 ± 16 (6)	4.66 ± 0.25 (4)	170 ± 15 (8)
	P	<0.01	<0.05	<0.01

[a] Values are means ± SE; number of determinations in parentheses. Nine groups of rats (three per group) were examined at 2 days and four groups at 4 days after surgery. [b] Enzyme assays of ileal mucosal homogenate. [c] Fluorescence anisotropy studies of isolated ileal microvillus membranes. P values are for the paired t test.

("fluidity")[1] of the membrane lipids. Inasmuch as the changes in the DPH anisotropy parameter to be described below are in the range of 5–26%, it is noteworthy that the reproducibility of the fluorescence anisotropy measurement in this laboratory is ±2.5%. Moreover, an empirical relationship, DPH $[(r_0/r) - 1]^{-1}$ at 24 °C = 1.11 ± 1.53 (cholesterol:phospholipid molar ratio), has been established in our laboratory, by using samples of human erythrocyte membranes of varying cholesterol content (M. Flamm and D. Schachter, unpublished experiments). From this relationship and the cholesterol:phospholipid molar ratios reported below, one would predict changes in DPH $[(r_0/r) - 1]^{-1}$ of 7–20%, in agreement with the relative changes observed.

Results

Microvillus Membrane Lipids. Prior evidence indicates that the rate of cholesterol biosynthesis is greater in rat enterocytes of the distal (ileal) as compared to the proximal segments of the small intestine (Dietschy & Sipirstein, 1965). Thus, the pattern observed of decreased lipid fluidity in the microvillus membranes of the distal gut (Schachter et al., 1976; Schachter & Shinitzky, 1977) could result from the increased cholesterol content of the ileal membranes. For examination of this possibility directly, lipid extracts were prepared from microvillus membranes isolated from the proximal and distal quarters of the small intestine, and the lipid compositions were determined by thin-layer chromatography. The values in Table I indicate that both the cholesterol:phospholipid molar ratio and the cholesterol percentage by weight of total lipid are significantly greater ($P < 0.05$) in the distal membranes. Inasmuch as these results support a possible relationship between cholesterol biosynthesis and microvillus membrane cholesterol, further evidence was sought by experimental manipulation of the biosynthetic rate in ileal enterocytes.

Biliary Ligation. Decreasing the bile salt content of the intestinal lumen in vivo by interruption or diversion of bile flow enhances cholesterol biosynthesis and HMG-CoA reductase activity in rat ileal mucosa (Dietschy, 1968; Shefer et al., 1973; Andersen & Dietschy, 1977). Accordingly, groups of three to six rats were compared at 2 and 4 days following ligation of the common bile duct or a sham operation. Serum cholesterol values were estimated, and ileal mucosa was obtained to assay HMG-CoA reductase and to prepare isolated microvillus membranes for determination of the DPH fluorescence anisotropy and the cholesterol:phospholipid molar ratio. At both 2 and 4 days after surgery, the ligated animals showed marked increases in the serum cholesterol and in the specific activity of ileal mucosal HMG-CoA reductase (Table II). Concomitantly, the mean DPH $[(r_0/r) - 1]^{-1}$ values of the ileal microvillus membranes were increased by 13% at 2 days ($P < 0.01$) and by 14% at 4 days ($P < 0.05$). The cholesterol:phospholipid molar ratios of microvillus membranes prepared from the sham and ligated animals, respectively, were 1.12 ± 0.03 and 1.29 ± 0.05 (mean ± SE; $N = 9$, $P < 0.05$) at 2 days.

Feeding Experiments. The bile salt content of the intestinal lumen was also varied by feeding groups of six rats a diet supplemented with either 2% sodium taurocholate or 2% cholestyramine. The values in Table III show that after 2 weeks on the diets cholestyramine treatment increased ($P < 0.05$) and taurocholate feeding decreased ($P < 0.05$) the ileal HMG-CoA reductase activity, with trends in these directions apparent after 1 week. Corresponding to the effects on ileal sterol synthesis, after 2 weeks of treatment, cholestyramine significantly increased the DPH $[(r_0/r) - 1]^{-1}$ value of the microvillus membranes by 11% ($P < 0.01$) whereas tauro-

Table III: Effects of Feeding Sodium Taurocholate or Cholestyramine[a]

weeks on diet	diet group	HMG-CoA reductase activity[b] (pmol min^{-1} mg^{-1})	DPH anisotropy parameter,[c] $[(r_0/r) - 1]^{-1}$	serum cholesterol (mg/dL)
1	control	99 ± 9 (10)	3.91 ± 0.17 (12)	66 ± 5 (12)
	taurocholate	90 ± 13 (10)	3.77 ± 0.18 (12)	75 ± 6 (12)
	cholestyramine	123 ± 17 (10)	3.97 ± 0.15 (12)	59 ± 9 (12)
	P	<0.05	<0.05	ns
2	control	102 ± 7 (12)	4.13 ± 0.17 (14)	66 ± 4 (18)
	taurocholate	81 ± 10 (12)	3.63 ± 0.16 (14)	85 ± 8 (18)
	cholestyramine	131 ± 11 (12)	4.59 ± 0.21 (14)	51 ± 6 (18)
	P	<0.01	<0.001	<0.001

[a] Values are means ± SE; numbers of determinations are shown in parentheses. [b] Enzyme assays of ileal mucosal homogenate. [c] Fluorescence anisotropy studies of isolated ileal microvillus membranes. P values are for the differences between the cholestyramine- and taurocholate-treated groups (paired t test). ns means not significant.

Table IV: Effects of Fasting[a]

days fasted	group	HMG-CoA reductase activity[b] (pmol min^{-1} mg^{-1})	DPH anisotropy parameter,[c] $[(r_0/r) - 1]^{-1}$	serum cholesterol (mg/dL)
2	fed	105 ± 12 (6)	3.40 ± 0.14 (5)	64 ± 9 (6)
	fasted	89 ± 11 (6)	3.57 ± 0.13 (5)	58 ± 11 (6)
	P	ns	ns	ns
4	fed	99 ± 14 (6)	3.68 ± 0.21 (5)	67 ± 13 (6)
	fasted	51 ± 18 (6)	3.45 ± 0.25 (5)	61 ± 15 (6)
	P	<0.01	<0.025	ns

[a] Values are means ± SE; the number of groups of rats compared is indicated in parentheses. [b] Enzyme assays of whole ileal mucosal homogenate. [c] Fluorescence anisotropy studies of isolated ileal microvillus membranes. P values are based on the paired t test.

cholate decreased the value by 12% ($P < 0.001$); trends in these directions were also observed after 1 week. Lipid composition studies of the isolated membranes are summarized in Table I. Cholestyramine treatment, as compared to the controls or taurocholate-fed animals, increased the cholesterol content and the cholesterol:phospholipid molar ratio ($P < 0.05$); the control and bile salt fed groups, on the other hand, did not differ significantly from each other. The data of these experiments were also evaluated by calculating correlation coefficients. Significant correlations were found for membrane DPH $[(r_0/r) - 1]^{-1}$ as a function of HMG-CoA reductase activity (correlation coefficient, 0.82; $P < 0.001$), for membrane cholesterol:phospholipid molar ratio as a function of HMG-CoA reductase activity (correlation coefficient, 0.53; $P < 0.05$), and for cholesterol:phospholipid molar ratio as a function of DPH $[(r_0/r) - 1]^{-1}$ (correlation coefficient, 0.83; $P < 0.01$).

The effects of feeding sodium taurocholate or cholestyramine on enterocyte basolateral membranes were also explored by maintaining groups of rats on the appropriate diets for 2 weeks. Thereafter, ileal basolateral membranes were isolated, and the DPH $[(r_0/r) - 1]^{-1}$ values (mean ± SE) of the control, cholestyramine-treated, and taurocholate-treated groups were, respectively, 2.84 ± 0.05, 2.66 ± 0.05, and 3.01 ± 0.16. The apparent differences between the values are neither statistically significant nor concordant with the changes in cholesterol biosynthesis.

Effects of Starvation. Fasting is reported to decrease cholesterol synthesis in rat intestinal mucosa (Andersen & Dietschy, 1977). As shown in Table IV, 4 days of fasting significantly decreased both the HMG-CoA reductase activity of the ileal mucosa ($P < 0.01$) and the DPH $[(r_0/r) - 1]^{-1}$ value of the microvillus membranes ($P < 0.025$). In addition, the cholesterol:phospholipid molar ratios of the microvillus membranes prepared from the fed and starved animals, respectively, were 1.33 ± 0.05 and 1.19 ± 0.05 ($P < 0.05$).

Membrane Vesicle Studies. The foregoing results demonstrate that variations in cholesterol biosynthesis can modulate the content of microvillus membrane cholesterol and thereby the motional freedom of the bilayer lipids. Since the relative changes in DPH $[(r_0/r) - 1]^{-1}$ in our experiments are in the range of 5–26%, the question arises whether the changes are sufficient to influence membrane functions. Evidence on this point was obtained by examining the sodium-dependent influx of D-glucose into ileal microvillus membrane vesicles. Membrane vesicles were prepared from groups of rats following maintenance for 2 weeks on control and taurocholate- and cholestyramine-supplemented diets as described above. As shown in Table V, in the presence of added Na^+ the flux of D-glucose into vesicles prepared from the taurocholate-treated animals significantly exceeded ($P < 0.05$) the flux values of the control or cholestyramine-treated groups. In the absence of added Na^+, no significant difference in flux was observed.

Table V: D-Glucose Flux into Ileal Microvillus Membrane Vesicles[a]

flux period (s)	group	D-glucose uptake (μmol/mg of protein) with Na^+	D-glucose uptake (μmol/mg of protein) without Na^+
15	control	0.28 ± 0.04 (8)	0.16 ± 0.02 (8)
	taurocholate	0.45 ± 0.05 (8)[b]	0.15 ± 0.01 (8)
	cholestyramine	0.31 ± 0.06 (8)	0.12 ± 0.02 (8)
30	control	0.46 ± 0.05 (12)	0.30 ± 0.01 (12)
	taurocholate	0.69 ± 0.13 (12)[c]	0.30 ± 0.02 (12)
	cholestyramine	0.37 ± 0.02 (12)	0.31 ± 0.01 (12)

[a] Values are means ± SE; number of determinations is shown in parentheses. [b] $P < 0.02$ for difference from the controls. [c] $P < 0.025$ for difference from cholestyramine-treated preparations.

Discussion

The experimental results indicate that cholesterol biosynthesis in the rat enterocyte can modulate the cholesterol content and thereby the motional freedom of the microvillus membrane lipids. This conclusion interrelates two sets of observations reported previously: cholesterol biosynthesis is greater in the distal as compared to the proximal intestinal mucosa (Dietschy & Siperstein, 1965); the motional freedom of microvillus membrane lipids is decreased in the distal as compared to the proximal mucosa (Schachter et al., 1976; Schachter & Shinitzky, 1977). As the conclusion also implies, the observed

cholesterol content of the microvillus membrane lipid is higher in the distal as compared to the proximal mucosa of the rat (Table I).

Merchant & Heller (1977) pointed out that the increased cholesterol synthetic rate and HMG-CoA reductase activity of the distal mucosa may result from the greater absorption of luminal sterols in the proximal small intestine (Slyvên & Nordstrom, 1970). In accord with the idea that intracellular sterol content regulates the biosynthetic rate (Dietschy & Wilson, 1970; McIntyre & Isselbacher, 1973), greater absorption would lower selectively the proximal HMG-CoA reductase activity. Whether or not this is the predominant mechanism, the fact that dietary sterols are absorbed mainly in the proximal intestine whereas sterol synthesis predominates in the distal region makes it feasible to ascertain whether the dietary or the endogenously synthesized sterol is the major precursor pool determining the content of microvillus membrane cholesterol. Our finding (Table I) that the ileal membrane has a higher cholesterol content indicates that the biosynthetic mechanism predominates in this respect. Additional support for this conclusion comes from the studies of rats made hypercholesterolemic by a high cholesterol diet (Lasser et al., 1973). The intestinal microvillus membranes prepared from these animals showed no significant change from the controls in cholesterol:phospholipid molar ratio or in lipid fluidity (T. A. Brasitus and D. Schachter, unpublished experiments).

In contrast to the ileal microvillus membranes, basolateral membranes of the enterocytes showed no significant alterations of the DPH anisotropy parameter with changes in endogenous sterol synthesis. The results could imply that the two antipodal membranes derive their cholesterol from different intracellular pools. Alternatively, the subsequent turnover of sterol may differ in the two membranes. It is noteworthy that the basolateral membranes, for example, are more intimately exposed to lipid-associated lipoproteins transported out of the enterocyte or present in the extracellular fluid.

While the physiological consequences of modulating microvillus membrane cholesterol are unknown, our observations of the Na^+-dependent flux of D-glucose into ileal membrane vesicles suggest that this transport mechanism operates normally below its full capacity and can be stimulated by decreasing the DPH $[(r_0/r) - 1]^{-1}$ (Table V). This effect is not due to a general increase in membrane permeability, inasmuch as the D-glucose flux independent of Na^+ is unaffected (Table V). Moreover, similar results have been obtained by studies of the rapid mucosal uptake of D-glucose, both Na^+ dependent and independent, by segments of rat ileum in vitro (H. N. Nellans et al., unpublished experiments). The effects of microvillus membrane cholesterol on D-glucose transport could account, in part at least, for the observations that active absorption of hexose is greatest in the proximal intestine and diminishes in the distal segment of the rat gut (Finkelstein & Schachter, 1962).

In our experiments, variations in HMG-CoA reductase were induced mainly by varying the luminal content of the bile salts. Accordingly, it is possible that the changes in bile salt concentration affected the microvillus membranes by mechanisms independent of sterol biosynthesis. For example, Roy et al. (1975) reported that bile salt infusions increase the rate of renewal of the intestinal epithelium, and it is conceivable that our results could be influenced by altered enterocyte populations. If, for example, feeding taurocholate increased the rate of migration of cells from the crypt to the tip of the intestinal villus, and if the younger cells (closer to the crypt) contained microvillus membranes of lower cholesterol content and lower DPH anisotropy parameter values, our results would be anticipated. To test this possibility, we used the method of Weiser (1973) to isolate two populations of ileal enterocytes, older cells closer to the villus tip and younger cells from the mid-villus region. Microvillus membranes were prepared, and estimations of the DPH anisotropy parameter revealed higher values in the younger cells. These results, therefore, exclude more rapid renewal as an explanation of our observations. It is significant, moreover, that starvation, which decreases HMG-CoA reductase activity without necessarily altering bile salt metabolism, also lowered the microvillus membrane cholesterol content and DPH anisotropy parameter (Table IV). The totality of the evidence thus supports the working hypothesis that sterol biosynthesis modulates microvillus membrane cholesterol.

Lastly, it is instructive that the changes in membrane lipid motional freedom and cholesterol content documented in our experiments were detectable initially because of the sensitivity and precision of the steady-state fluorescence polarization methods. These techniques are particularly useful in exploratory studies which can be confirmed by conventional chemical methods.

Acknowledgments

We are grateful to Eugenia Dziopa for expert technical assistance in all aspects of this investigation.

References

Ames, B. N., & Dubin, D. T. (1960) *J. Biol. Chem. 235*, 769–775.

Andersen, J. M., & Dietschy, J. M. (1977) *J. Biol. Chem. 252*, 3646–3651.

Bloom, W., & Fawcett, D. W. (1968) *A Textbook of Histology*, pp 560–569, W. B. Saunders, Philadelphia, PA.

Brasitus, T. A., & Schachter, D. (1980) *Biochemistry 19*, 2763–2769.

Brasitus, T. A., Schachter, D., & Mamouneas, T. G. (1979) *Biochemistry 18*, 4136–4144.

Brasitus, T. A., Tall, A. R., & Schachter, D. (1980) *Biochemistry 19*, 1256–1261.

Dietschy, J. M. (1968) *J. Clin. Invest. 47*, 286–300.

Dietschy, J. M., & Siperstein, M. D. (1965) *J. Clin. Invest. 44*, 1311–1327.

Dietschy, J. M., & Wilson, J. D. (1970) *N. Engl. J. Med. 282*, 1128–1138.

Douglas, A. P., Kerley, R., & Isselbacher, K. J. (1972) *Biochem. J. 128*, 1329–1338.

Finkelstein, J. D., & Schachter, D. (1962) *Am. J. Physiol. 203*, 873–880.

Folch, J., Lees, M., & Sloane-Stanley, G. H. (1957) *J. Biol. Chem. 226*, 497–509.

Forstner, C. G., Tanaka, K., & Isselbacher, K. J. (1968) *Biochem. J. 109*, 51–59.

Fujita, M., Kawai, K., Asano, S., & Nakao, M. (1973) *Biochim. Biophys. Acta 307*, 141–151.

Gray, J. P., Henderson, G. I., Dunn, G. D., Swift, L. L., Wilson, F. A., & Hoyumpa, A. M. (1981) *Gastroenterology 80*, 1162.

Katz, S. S., Shipley, G. G., & Small, D. M. (1976) *J. Clin. Invest. 58*, 200–211.

Kawai, K., Fujita, M., & Nakao, M. (1974) *Biochim. Biophys. Acta 369*, 222–233.

Lasser, N. L., Roheim, P. S., Edelstein, D., & Eder, H. A. (1973) *J. Lipid Res. 14*, 1–8.

Lee, A. G. (1975) *Prog. Biophys. Mol. Biol. 29*, 3–56.

Lewis, B. A., Gray, G. M., Coleman, R., & Michell, R. H. (1975) *Biochem. Soc. Trans. 3*, 752–753.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
McIntyre, N., & Isselbacher, K. J. (1973) *Am. J. Clin. Nutr. 26*, 647–656.
Melchior, D. L., & Steim, J. M. (1976) *Annu. Rev. Biophys. Bioeng. 5*, 205–238.
Merchant, J. L., & Heller, R. A. (1977) *J. Lipid Res. 18*, 722–733.
Murer, H., Hopfer, U., Kinne-Saffran, E., & Kinne, R. (1974) *Biochim. Biophys. Acta 345*, 170–179.
Murer, H., Ammann, E., Biber, J., & Hopfer, U. (1976) *Biochim. Biophys. Acta 433*, 509–519.
Oda, T. (1976) in *Recent Progress in Electron Microscopy of Cells and Tissues* (Yamada, E., Mizuhira, V., Kurosumi, K., & Nagano, T., Eds.) pp 13–17, University Park Press, Baltimore, MD.
Okada, Y., Irimajiri, A., & Inouye, A. (1977) *J. Membr. Biol. 31*, 221–232.
Oldfield, E., & Chapman, D. (1971) *Biochem. Biophys. Res. Commun. 43*, 610–616.
Philipp, B. W., & Shapiro, D. J. (1979) *J. Lipid Res. 20*, 588–593.
Rose, R. C., & Schultz, S. G. (1971) *J. Gen. Physiol. 57*, 639–663.
Roy, C. C., Laurendeau, G., Doyon, G., Chartrand, L., & Rivest, M. R. (1975) *Proc. Soc. Exp. Biol. Med. 149*, 1000–1004.
Sandermann, H., Jr. (1978) *Biochim. Biophys. Acta 515*, 209–233.
Schachter, D., & Shinitzky, M. (1977) *J. Clin. Invest. 59*, 536–548.
Schachter, D., Cogan, U., & Shinitzky, M. (1976) *Biochim. Biophys. Acta 448*, 620–624.
Shefer, S., Hauser, S., Lapar, V., & Mosbach, E. H. (1973) *J. Lipid Res. 14*, 400–405.
Shinitzky, M., & Barenholz, Y. (1974) *J. Biol. Chem. 249*, 2652–2657.
Sinensky, M. (1977) *Biochem. Biophys. Res. Commun. 78*, 863–867.
Sinensky, M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1247–1249.
Slyvén, C., & Nordstrom, C. (1970) *Scand. J. Gastroenterol. 5*, 57–63.
Weiser, M. M. (1973) *J. Biol. Chem. 248*, 2536–2541.
Zlatkis, A., Zak, B., & Boyle, A. J. (1953) *J. Lab. Clin. Med. 41*, 486–492.

Transient Kinetics of Transfer Ribonucleic Acid Binding to the Ribosomal A and P Sites: Observation of a Common Intermediate Complex†

Wolfgang Wintermeyer* and James M. Robertson

ABSTRACT: The mechanism of tRNA–ribosome complex formation has been studied in fluorescence stopped-flow as well as slow kinetic experiments. As a fluorescent probe, proflavin was inserted into the anticodon loop of yeast $tRNA^{Phe}$ ($tRNA^{Phe}_{Prf37}$). Complex formation of $tRNA^{Phe}_{Prf37}$ or *N*-Ac-Phe-$tRNA^{Phe}_{Prf37}$ with poly(U)-programmed, vacant *Escherichia coli* ribosomes results in a biphasic change of both intensity and polarization of fluorescence characterized by relaxation times in the 100-ms (τ_{fast}) and 1-s (τ_{slow}) time range. The data are consistent with a sequential two-step mechanism of tRNA–ribosome complex formation, in which a rapid association–dissociation equilibrium is followed by a rearrangement step. From the dependence of the inverse relaxation time upon ribosome concentration, the following parameters are obtained (20 °C): $k_{12} = (1.5 \pm 0.2) \times 10^7$ M^{-1} s^{-1}, $k_{21} = 0.5 \pm 0.2$ s^{-1}, $k_{23} = 0.3 \pm 0.2$ s^{-1}, and $k_{32} \leq 0.1$ s^{-1}. Neither the amplitude of the fluorescence change nor the rate constants of the fast second-order step are affected by the presence or absence of poly(U) or by preoccupancy of the P site with nonfluorescent tRNA. This suggests that the fast step leads to an intermediate complex in which the tRNA is bound to a site other than the P site in a codon-independent manner. An activation energy of 9 ± 2 kcal/mol indicates that the apparent second-order step in fact represents a fast rearrangement that is coupled to the probably diffusion-limited formation of a weak encounter complex. The first-order rearrangement (k_{23}) is related to the binding of the tRNA into the P site. It is completely depressed by blocking the P site with nonfluorescent tRNA. Furthermore, codon–anticodon interaction takes place during this step. Provided the P site is occupied, the intermediate tRNA–ribosome complex very slowly (minute time range) rearranges to the A-site complex. The presence of poly(U) is required for this rearrangement to occur. Experiments with Phe-$tRNA^{Phe}_{Prf37}$ show that elongation factor Tu and GTP strongly accelerate the rearrangement but not the formation of the intermediate complex.

Protein synthesis in *Escherichia coli* cells proceeds at a rate of 15 amino acids incorporated into protein s^{-1} ribosome^{-1}, implying elementary steps of the elongation cycle in the time range of milliseconds (Gouy & Grantham, 1980). Although the steady-state rates of protein synthesis in the current in vitro systems are much slower—for instance, with *E. coli* ribosomes and factors more than 100 times—these figures nevertheless indicate that fast kinetic techniques have to be applied in order to determine the rates of individual steps and to isolate short-lived intermediates of ribosomal protein synthesis. In the past, kinetic techniques like stopped flow, pressure jump, or temperature jump have been utilized only scarcely for studies in the ribosome system, the well-documented examples being limited to studies on the association–dissociation equi-

† From the Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie der Universität München, 8000 München 2, Federal Republic of Germany. *Received August 20, 1981.* This work was supported by Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 51.

0006-2960/82/0421-2246$01.25/0