Unidirectional flux rate of cholesterol and fatty acids into the intestine of rats with drug-induced diabetes mellitus: effect of variations in the effective resistance of the unstirred water layer and the bile acid micelle

A. B. R. Thomson[1,2]

Division of Gastroenterology, Department of Medicine, University of Alberta, Edmonton, Canada

Abstract A previously validated in vitro technique was used to study the unidirectional flux rate (J_d) of cholesterol and a homologous series of saturated fatty acids (FA) into the jejunum and ileum of rats with alloxan or streptozotocin-induced diabetes mellitus (DM), under conditions of variable resistance of the intestinal unstirred water layer (UWL), and under conditions of varying concentrations of taurodeoxycholic acid (TDC). When the bulk phase was stirred and UWL was low, the J_d of FA 2:0, 4:0, 10:0, and 12:0 were greater in the jejunum and ileum of DM than controls, both 3 and 14 days after alloxan or streptozotocin; the incremental free energy change of transfer of FA was thus higher in DM. The J_d of FA 16:0, 18:0, and cholesterol were increased at 14 but not 3 days after induction of DM; the J_d of cholesterol was higher in DM over a wide range of concentrations of cholesterol, TDC, and both cholesterol and TDC. The J_d of all probe molecules was lower when the bulk phase was unstirred and UWL was high; the J_d of cholesterol but not FA remained greater in DM than in controls. The results suggest that *a*) the passive permeability of the diabetic intestine to fatty acids is increased in DM but this difference is lost as the animals age; *b*) the J_d of cholesterol is greater in the jejunum and ileum in DM under conditions of variable concentrations of both cholesterol and bile acids; and *c*) the bile salt micelle functions in both DM and in controls to solubilize cholesterol and provide the source from which the cholesterol partitions prior to its uptake by the intestinal mucosal membrane.—**Thomson, A. B. R.** Unidirectional flux rate of cholesterol and fatty acids into the intestine of rats with drug-induced diabetes mellitus: effect of variations in the effective resistance of the unstirred water layer and the bile acid micelle. *J. Lipid Res.* 1980. **21:** 687–698.

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Abnormalities in cholesterol metabolism have been described in man and animals with diabetes mellitus (1–3), but the role of the intestine in these abnormalities has not been evaluated. Changes in the intestinal absorption of carbohydrates, amino acids, and conjugated bile salts have been demonstrated in animals with drug-induced hyperglycemia (4–8), but the significance of these observations must be questioned since none of these studies accounted for the potential effect of the intestinal unstirred water layer (9–13). The effective resistance of the unstirred water layer may be of sufficient magnitude so as to lead to serious underestimation of passive permeability coefficients, and overestimation of apparent Michaelis affinity constants of carrier-mediated processes (14–21). For some lipophilic molecules such as long-chain length fatty acids and cholesterol, the unstirred water layer, rather than the microvillus membrane, may become rate-limiting to absorption (18). Bile salts are required for the absorption of cholesterol (22), and it is likely that they serve to solubilize cholesterol and aid in overcoming the effective resistance of the unstirred layer (23, 24). There are several mechanisms by which cholesterol may be taken up into the intestinal mucosal cell from the micelle, but recent experimental and theoretical considerations support the suggestion that cholesterol first partitions from the lipid phase in the micelle

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Abbreviations: All, alloxan-injected diabetic animals; d, effective thickness of the intestinal unstirred water layer; D, free diffusion coefficient of probe molecule; DM, diabetes mellitus, diabetic animals; FA, saturated fatty acid; $\delta\Delta F_w \rightarrow 1$, incremental free energy change; J_d, unidirectional flux rate; P_d, passive permeability coefficient of intestinal membrane; SEM, standard error of the mean; St, streptozotocin-injected diabetic animals; TDC, taurodeoxycholic acid; UWL, unstirred water layer, effective resistance of the unstirred water layer.

[1] With the technical assistance of B. Philips and M. Yuen.
[2] Send reprint requests to: A. B. R. Thomson, 8-104 Clinical Sciences Bldg., University of Alberta, Edmonton, Canada T6G 2G3.

into an aqueous phase, and that mucosal uptake then occurs from this aqueous phase (23).

The effective resistance of the unstirred water layer can never be eliminated experimentally, but an in vitro technique has previously been validated (25) as useful for the measurement of unidirectional flux rates under conditions known to yield variable effective resistance of the unstirred layer (18). This technique was used to assess the effect of alloxan and streptozotocin on the uptake of a homologous series of saturated fatty acids and cholesterol into the jejunum and ileum of rats, under conditions of low and high effective resistance of the unstirred water layer, and under conditions of varying concentrations of cholesterol and bile acid.

METHOD OF PROCEDURE

Probe and marker compounds

The compound used to measure the adherent mucosal fluid volume ([G-^{3}H]dextran, mol wt approximately 15,000 to 17,000) was obtained from New England Nuclear Corporation and was used as supplied by the manufacturer.

Unlabeled and labeled short-chain fatty acids, cholesterol, and bile acids were 99% pure and were used as supplied by Applied Science Laboratories Incorporated, State College, PA, by Sigma Chemical Corporation, St. Louis, MO, and New England Nuclear Corporation.

Preparation of micellar incubation solutions

An appropriate amount of both a ^{14}C-labeled and unlabeled probe molecule was dissolved in an exact volume of chloroform–methanol 2:1 (v:v) in an incubation beaker, and the chloroform–methanol phase was then evaporated to ensure complete removal of the organic solvents. Seventy-five ml of a 40 mM taurodeoxycholate solution in Krebs-bicarbonate buffer (with calcium omitted) was added to the beaker and the solution was stirred with a magnetic bar for 2 hr. The solution was then further diluted by the addition of 75 ml of Krebs-bicarbonate buffer to give a final volume of 150 ml and a final taurodeoxycholate concentration of 20 mM. The beaker was then gassed with 5% carbon dioxide in oxygen for 2 hr at 37°C and, if necessary, the pH was readjusted to 7.4. A trace amount of radiolabeled volume marker [G-^{3}H]dextran was then added and the solution was ready to be used for determination of tissue uptake rates. This technique for preparation of micellar incubation solutions has been published elsewhere (23).

Production of experimental drug-induced diabetes mellitus

Drug-induced glucose intolerance was produced in female albino Wistar rats weighing 150–175 g by the intraperitoneal or intravenous administration of Beta-cell cytotoxic agents, alloxan (200 mg/kg) or streptozotocin (80 mg/kg). Unidirectional flux rates were measured 3, 14, or 83 days after the induction of diabetes; when diabetes was produced in old rats, the animals weighed over 375 g and were approximately 9–12 months of age. The induction of glucose intolerance was assessed by the presence of glucosuria and hyperglycemia, using the glucose oxidase method for measurement of blood sugar. All diabetic animals had a blood glucose of greater than 175 mg/dl, averaging 369 ± 27 mg/dl, and none of the animals had ketonuria.

Tissue preparation

Albino Wistar rats were anesthetized with Nembutal. As is outlined in detail elsewhere (18, 25), a short segment of proximal jejunum or ileum was rapidly removed, rinsed with 50 ml of cold saline, opened along its mesentery border, and the mucosal surface was carefully washed with a stream of cold saline from a syringe to remove visible mucus and debris. Circular pieces of intestine were cut from a segment, mounted as a flat sheet in incubation chambers, and clamped between two plastic plates so that the mucosal and serosal surfaces were exposed to separate incubation solutions, with apertures in the plates exactly 0.5 cm in diameter. To the serosal compartment was added 1.0 ml of Krebs-bicarbonate buffer, and the chambers were transferred to beakers containing oxygenated Krebs-bicarbonate buffer at 37°C for a pre-incubation period of 30 min. The chambers were then transferred to other beakers for specific experiments. The pre-incubation and incubation solutions were mixed at identical stirring rates with circular magnetic bars, and the stirring rates were precisely adjusted by means of a strobe light. Stirring rates were reported as the revolutions per minute (rpm) at which the stirring bar was driven. Stirring rates were altered in a systematic and reproducible manner to yield different values of the effective thickness and surface area of the unstirred water layer. These previous measurements were made in rabbits, and accordingly, the effective resistance of the unstirred layer was estimated in control and diabetic rats, according to the technique outlined below.

Determination of unidirectional flux rates

After pre-incubation, the chambers were transferred to other beakers containing [^{3}H]dextran and

various ^{14}C-labeled probe molecules in oxygenated Krebs-bicarbonate buffer at 37°C. Validation experiments had demonstrated a linear rate of cholesterol uptake between 4 and 10 min in both control and diabetic animals, and accordingly a 6-min incubation period was arbitrarily chosen. After incubation for 6 min, the experiment was terminated by removing the chamber and quickly rinsing the tissue in cold saline for approximately 5 sec. The exposed mucosal tissue was cut out of the chamber with a circular steel punch, blotted on filter paper, after which it was placed in a tared counting vial. The tissue was dried overnight in an oven and the dry weight determined. The sample was then saponified with sodium hydroxide, scintillation fluid was added, and radioactivity was determined by means of an external standardization technique to correct for variable quenching of the two isotopes (26).

Expression of results

The unidirectional flux rate, J_d, was calculated after correcting the total tissue ^{14}C-radioactivity for the mass of the probe molecule present in the adherent mucosal fluid. These rates were expressed as either the nanomoles of the probe molecule taken up into the mucosa per minute per 100 mg dry weight of tissue, or as nmol/min. In the latter calculations, the uptake rate was uncorrected for the tissue dry weight, and the values represented uptake into the tissue exposed through the 0.5 cm diameter aperture into the transport chamber.

The statistical significance of the difference between the mean values of groups was determined using Student's t-test. When calculating the incremental change in free energy (Fig. 1), the method of least squares was used to determine the slope and intercept for the lines describing the relationship between the number of carbon atoms in the fatty acids, and the uptake rate, expressed as ln J_d/D. The slopes were determined for fatty acids 6:0 to 12:0; one value for each fatty acid was obtained from each animal, and 12 animals were used in the study. The slopes of the least square regression lines for the homologous series of saturated fatty acids was tested, and the diabetic slope was found to be significantly different from the control slope, $P < 0.05$.

Validation of technique

Certain criteria must be fulfilled in order to obtain valid estimates of unidirectional flux rates. First, it was shown that the nonpermeant marker [G-^{3}H]-dextran had equilibrated into the extracellular fluid space over the 6-min time period used in this study. Second, there was a linear rate of uptake of choles-

terol, as well as acetic, hexanoic and lauric acids, between 4 and 12 min incubation, and the rate of uptake extrapolated to zero uptake at zero time. Finally, there was a linear relationship noted between the concentration of probe molecule in the bulk phase, and the uptake of the solute, when examined for FA 2:0, 6:0, 12:0, as well as cholesterol (Fig. 2).

Pieces of rat jejunum from six control and six diabetic animals were mounted in the chambers and incubated in Krebs-bicarbonate buffer, at 37°C and pH 7.4, for 30 min. The tissue was fixed in Bouin's solution, imbedded in paraffin, and cut longitudinally.

Delineation of diffusion barriers and morphology

Morphological assessment demonstrated approximation of the villi after the 30-min preincubation period, as described previously in the rabbit (18). The dimensions of the unstirred water layer diffusion barrier, and a number of parameters of the morphology of the jejunum were determined in control and diabetic animals. Segments of jejunal tissue were weighed wet and after drying overnight at 70°C to a constant weight, and the proportion of the tissue comprised of water was thereby calculated. The mean weight of jejunum per unit serosal surface area was determined by weighing the full thickness intestinal wall exposed in a fixed aperture of the transport chambers. In both groups of rats the mucosa was scraped with a glass slide, and the proportion of the full intestinal thickness comprised of mucosa was determined. The mg dry weight of the jejunal mucosa per unit serosal surface area was then calculated.

The effective resistance of the intestinal unstirred water layer was assessed by determining, at two rates of stirring of the bulk phase (0 rpm and 600 rpm), the relationship between the rates of uptake of a homologous series of saturated fatty alcohols and the number of carbon atoms in each compound (18). Preliminary experiments showed that maximum rates of passive uptake of aliphatic alcohols (10:0 and 12:0) were achieved in the control and diabetic rats at the two stirring rates. At these rates of stirring, the unstirred water layer became absolutely rate-limiting to absorption, and from those values of the rate of diffusion-limited uptake of lauryl alcohol (12:0) it was possible to determine the effective resistance of the unstirred water layer (18, 24).

In a further group of eight diabetic and control rats, the intestinal mucosa was scraped, homogenized, and the content of cholesterol and cholesteryl esters was determined by an enzymatic method as well as by high performance thin-layer chromatography (27, 28). Similar results were obtained by each method, and the results were expressed as total cholesterol,

TABLE 1. Unidirectional flux rate of a homologous series of saturated fatty acids in control and diabetic rat jejunum

Saturated Fatty Acid	Stirring Rate, 600 rpm: Low Unstirred Layer Resistance						Stirring Rate, 0 rpm: High Unstirred Layer Resistance					
	2:0	4:0	6:0	8:0	10:0	12:0	2:0	4:0	6:0	8:0	10:0	12:0
	J_d, nmol/min/100 mg											
Control	32 ± 2	31 ± 2	50 ± 4	56 ± 5	73 ± 5	114 ± 16	9 ± 1	7 ± 1	6 ± 1	9 ± 1	9 ± 1	12 ± 2
Alloxan												
3 day	29 ± 1	29 ± 2	63 ± 12	43 ± 1	73 ± 1	169 ± 15	7 ± 1	6 ± 2	12 ± 1	2 ± 1	11 ± 3	14 ± 2
14 day	32 ± 2	26 ± 7	40 ± 7	55 ± 3	32 ± 8	78 ± 13	25 ± 3	10 ± 3	12 ± 1	13 ± 2	7 ± 2	9 ± 1
Streptozotocin												
3 day	35 ± 4	39 ± 6	46 ± 6	62 ± 5	98 ± 12	228 ± 23	12 ± 2	8 ± 1	15 ± 1	10 ± 1	8 ± 1	12 ± 1
14 day	49 ± 6	38 ± 4	46 ± 5	69 ± 6	101 ± 23	204 ± 20	8 ± 1	16 ± 4	8 ± 3	8 ± 1	11 ± 4	11 ± 2

The flux rates are expressed as nmoles/100 mg/min. The uptake rates were determined from solutions containing 0.2–5.0 mM concentrations of the test molecules, but were all normalized to a concentration of 1.0 mM. The values represent the mean ± SEM of the results from 8 to 12 animals. The diabetic animals were given intraperitoneal alloxan, 200 mg/kg, or intravenous streptozotocin, 80 mg/kg, 3 or 14 days before study. The bulk phase was stirred at either 600 rpm, or was unstirred, 0 rpm.

mg/g wet weight of mucosal homogenate, and as the ratio of free cholesterol/cholesteryl esters.

When the bulk phase was stirred at 600 rpm to minimize the effective resistance of the intestinal unstirred water layer (18), the rate of uptake, J_d, of fatty acid 2:0 (acetic acid) was greater in the 14-day streptozotocin diabetic rats than in the control animals, and the J_d of fatty acid 4:0 (butyric acid) was higher in both the 3- and 14-day streptozotocin animals, whereas the J_d of FA 6:0 and 8:0 (hexanoic and octanoic acid) were similar in diabetic and control animals, and those of FA 10:0 and 12:0 (decanoic and lauric acid) were greater in the 3- and 14-day streptozotocin diabetic than the control animals (**Table 1**). This greater uptake of FA 10:0 and 12:0 was also demonstrated in 3- but not in 14-day alloxan-diabetic animals, as well as in old rats (over 375 g) rendered diabetic with streptozotocin 14 days before study, but not in long-term (83-day) streptozotocin-diabetic animals (**Table 2**). When the bulk phase was unstirred and the effective resistance of the unstirred layer was high (18), the uptake of each fatty acid was much less than when the bulk phase was stirred (Table 1). Under these conditions of high unstirred layer resistance, the rate of uptake of each short- and medium-chain length fatty acid was similar in control and diabetic animals (Table 1).

As the chain length of the homologous series of fatty acids increased and when unstirred layer resistance was low, there was a linear increase in the value of ln J_d/D for FA 6:0 through 12:0 (**Fig. 1**). The slope of this line was greater in diabetic than in control animals. The apparent incremental free energy change ($\delta\Delta Fw \rightarrow 1$) of the transfer of these medium-chain length fatty acids was -142 cal/mol in the control animals, and -246, -234, and -203 cal/mol in the 3-day alloxan and streptozotocin rats, and in the 14-day streptozotocin animals, respectively (**Table 3**). When the values of the apparent passive permeability coefficients were corrected for unstirred layer effects, the true values of the incremental free energy change was even higher in both control and diabetic rats, -246, and -382 cal/mol, respectively. This compared with a value of -258 cal/mol in the rabbit jejunum

TABLE 2. Unidirectional flux rate of a homologous series of saturated fatty acids in control and diabetic jejunum of animals of varying ages and duration of hyperglycemia[a]

Saturated Fatty Acids	2:0	4:0	6:0	8:0	10:0	12:0
	nmol/min/100 mg					
Control	44 ± 8	47 ± 9	84 ± 13	35 ± 5	73 ± 9	335 ± 35
Long term, 83 day	49 ± 14	53 ± 8	100 ± 12	33 ± 5	69 ± 9	251 ± 37
Control	19 ± 2	17 ± 2	36 ± 4	26 ± 3	53 ± 5	200 ± 19
Old, 14 day	12 ± 3	18 ± 3	40 ± 4	36 ± 7	64 ± 12	572 ± 70

[a] Stirring rate, 600 rpm: low unstirred layer resistance.

In the first group, the diabetic animals had been injected with streptozotocin 83 days before study. The control animals had been given saline, and were the same sex and age as the diabetic rats. In the second group, both sets of animals were approximately 9 months old and weighed over 375 g. The control rats were injected with saline, while the diabetic animals were given streptozotocin 14 days before study. The flux rates were expressed as nmol/100 mg/min. The uptake rates were determined from solutions containing 0.2–5.0 mM concentrations of the test molecules, but all were normalized to a concentration of 1.0 mM. The values represent the mean ± SEM of the results from six to nine animals. The bulk phase was stirred at 600 rpm to reduce the effective thickness of the unstirred water layer (18).

under similar conditions of stirring of the bulk phase (18). The apparent value of $\delta\Delta Fw \rightarrow 1$ rose as the control animals aged (Table 3), so that the difference between control and diabetic values was obscured as the animals grew older (-382 and -371 cal/mol in the control and 83-day streptozotocin animals, respectively), but if old rats were rendered diabetic, the apparent value of $\delta\Delta Fw \rightarrow 1$ rose even further from -351 to -460 cal/mol. Similar results were obtained when J_d was expressed as nmoles/min. Note that the value of $\ln J_d/D$ for FA 2:0 and 4:0 in the diabetic rats was above a linear extrapolation of the component of the line between FA 6:0 to 12:0 (Fig. 1); this phenomenon has been described previously (18, 29). Thus, the passive permeability properties of the intestine were greater in the diabetic than in the control animals, increased with the duration of diabetes, and were higher in the older than in the younger animals. When the bulk phase was unstirred and unstirred layer resistance was high, there was a gradual increase in the value of $\ln J_d/D$ with increasing chain length (Fig. 1) and the values were similar in the diabetic and in the control animals (Table 1).

When the bulk phase was stirred and the effective resistance of the unstirred water layer was low, the unidirectional flux rate of FA 16:0 (palmitic acid) and 18:0 (stearic acid) as well as cholesterol was increased in 14-day alloxan- and streptozotocin-diabetic animals, in the presence of both 5 and 20 mM taurodeoxycholic acid (**Table 4**). In the 3-day diabetic animals, uptake was not consistently changed:
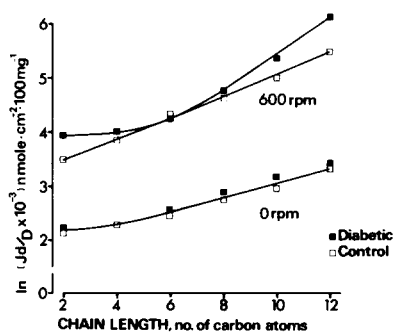

Fig. 1. The unidirectional flux rate of a homologous series of saturated fatty acids into jejunum of control and diabetic rats. This figure shows the effects of increasing the chain length, N, on the uptake of each fatty acid, $\ln J_d/D$. Each point represents the mean of the results of 12 animals; the magnitude of the SEM is given in Table I, and in most instances was smaller than the value of a point shown on the graph. The diabetic rats had been given streptozotocin 14 days before study. The difference between the means of the control and diabetic groups were statistically significant ($P < 0.05$) for fatty acids 2:0, 4:0, 10:0, and 12:0. The incremental free energy change, $\delta\Delta Fw \rightarrow 1$, was calculated from the linear portion of the relationship between $\ln J_d/D$ and N for FA 6:0, 8:0, 10:0, and 12:0. Those values marked with an asterisk (*) are significantly different from the control value, $P < 0.05$.

TABLE 3. Incremental free energy changes in the control and different groups of diabetic animals

	$\delta\Delta Fw \rightarrow 1$, Incremental Free Energy Change	
	Without Correction for Unstirred Layer	With Correction for Unstirred Layer
	cal/mol	
Control	-142	-246
3 day:Alloxan	$-246*$	
Streptozotocin	$-240*$	
14 day:Alloxan	-90	
Streptozotocin	$-210*$	$-382*$
Long Term, 83 day		
Control	-380	
83 day		
Streptozotocin	-370	
Old, Control	-359	
14 day: Streptozotocin	$-460*$	

Animals were rendered diabetic by the intraperitoneal injection of alloxan, or the intravenous administration of streptozotocin. The young animals (150–175 g) were diabetic for 3, 14, or 83 days. The old animals (heavier than 375 g) were injected 14 days before study. The values in the first column were obtained by dividing the unidirectional flux rate J_d, obtained when the bulk phase was stirred at 600 rpm, by C_1. Those values marked with an asterisk (*) are significantly greater than the control value, $P < 0.05$. The effective resistance of the unstirred layer was determined in the control and 14-day streptozotocin diabetic rats, and was very close in value (Table 5). The values for $\delta\Delta Fw \rightarrow 1$ shown in the second column were obtained by dividing J_d/C_2, where C_2 was obtained by correcting C_1 for the unstirred layer effect ($J_d \cdot d/Sw \cdot D$).

J_d was increased for FA 16:0 in 20 mM taurodeoxycholate (TDC) in alloxan rats, for FA 18:0 in 5 mM TDC in alloxan and streptozotocin animals, and for FA 18:0 in 20 mM TDC in alloxan rats. Cholesterol uptake was enhanced only in the 3-day alloxan but not the 3-day streptozotocin rats (Table 4). Qualitatively similar results (not shown) were obtained when the uptake rates were expressed as nmol/min. When the effective resistance of the unstirred water layer was high, there was a lower unidirectional flux rate of FA 16:0, 18:0, and cholesterol into the intestine. The J_d of FA 18:0 and cholesterol was significantly higher in 14-day alloxan diabetic animals than in controls; the uptake of cholesterol was also increased into the jejunum of 14-day streptozotocin rats (Table 4), when the bulk phase was unstirred.

Since the differences in uptake of cholesterol were greatest in 14-day animals and when the bulk phase was stirred, these experimental conditions were used to examine further the effect of diabetes mellitus on cholesterol absorption. When the concentration of taurodeoxycholic acid (TDC) was constant at 20 mM, there was a linear relationship between increasing concentrations of cholesterol in the bulk phase, and cholesterol uptake into the jejunum and ileum (**Fig. 2**); uptake was over twofold greater in the diabetic than in the control animals.

TABLE 4.   Unidirectional flux rate of long-chain length fatty acids and cholesterol into the jejunum of control and diabetic animals

Probe Molecule	Bile Acid mM	Control		3 day Diabetes Mellitus				14 day Diabetes Mellitus			
				Alloxan		Streptozotocin		Alloxan		Streptozotocin	
		600 rpm	0 rpm	600 rpm	0 rpm	600 rpm	0 rpm	600 rpm	0 rpm	600 rpm	0 rpm
						J_d, nmol/100 mg/min					
FA 16:0	5	32 ± 4	8 ± 0.8	47 ± 10	7 ± 2	28 ± 6	10 ± 1	136 ± 23	6 ± 2	72 ± 6	5 ± 0.8
	20	43 ± 4	6 ± 0.5	90 ± 17	5 ± 0.9	31 ± 2	7 ± 0.5	58 ± 11	4 ± 1	76 ± 2	3 ± 0.3
FA 18:0	5	76 ± 8	6 ± 0.6	223 ± 48	10 ± 1	111 ± 19	8 ± 0.6	237 ± 48	35 ± 4	104 ± 12	4 ± 0.4
	20	64 ± 10	4 ± 0.4	206 ± 75	9 ± 1	27 ± 4	6 ± 0.2	131 ± 17	50 ± 10	149 ± 19	6 ± 1
Cholesterol	5	373 ± 37	0.2 ± 0.2	925 ± 195	4 ± 0.7	77 ± 13	3 ± 0.08	833 ± 167	4 ± 0.3	850 ± 50	1 ± 0.1
	20	29 ± 5	0.15 ± 0.1	—	—	13 ± 3	1 ± 0.2	73 ± 12	4 ± 0.3	132 ± 30	0.7 ± 0.2

The diabetic animals were given alloxan or streptozotocin 3 or 14 days before study. The concentration of the taurodeoxycholic acid in the bulk phase was either 5 mM or 20 mM; the uptake rates were determined from solutions containing 0.2–1.0 mM concentrations of the test molecules, but all were normalized to a concentration of 1.0 mM. Therefore, these values for cholesterol uptake are different from those shown in Figs. 2–4. The bulk phase was either stirred at 600 rpm, or was unstirred (0 rpm). The values represent the mean ± SEM of the results from 8–12 animals. The flux rates were expressed as nmol/100 mg/min.

A reciprocal decrease in cholesterol uptake was observed with increasing concentrations of TDC in the bulk phase, but the cholesterol concentration constant held constant at 0.2 mM (**Fig. 3**); uptake into diabetic intestine was much greater than into jejunum or ileum of control animals. When the concentrations of both cholesterol and TDC in the bulk phase were increased, but the ratio of TDC/cholesterol was maintained constant at 100/1, there was little change in the unidirectional flux rate of cholesterol (**Fig. 4**); the J_d was once again higher in diabetic jejunum and ileum than in control intestine.

The effective resistance of the unstirred water layer was estimated from the rate of uptake of a diffusion-limited alcohol, lauryl alcohol; this value was found to be much higher in the control than in the diabetic rats (500 ± 39 versus 143 ± 12 min.100 mg/cm³, respectively) when the bulk phase was unstirred. When the bulk phase was stirred at 600 rpm to reduce the effective resistance of the unstirred layer, there was a marked reduction in the values, to 50 ± 5 and 34 ± 3 min.100 mg/cm³ in the control and diabetic animals, respectively (**Table 5**). The mean weight of the jejunal wall per unit serosal surface area was significantly lower in 3-day streptozotocin rats than in control animals (1.80 ± 0.02 versus 1.72 ± 0.02 mg, respectively) but was much higher in the 14-day diabetic rats (2.0 ± 0.02 mg). The percentage of the jejunal wall
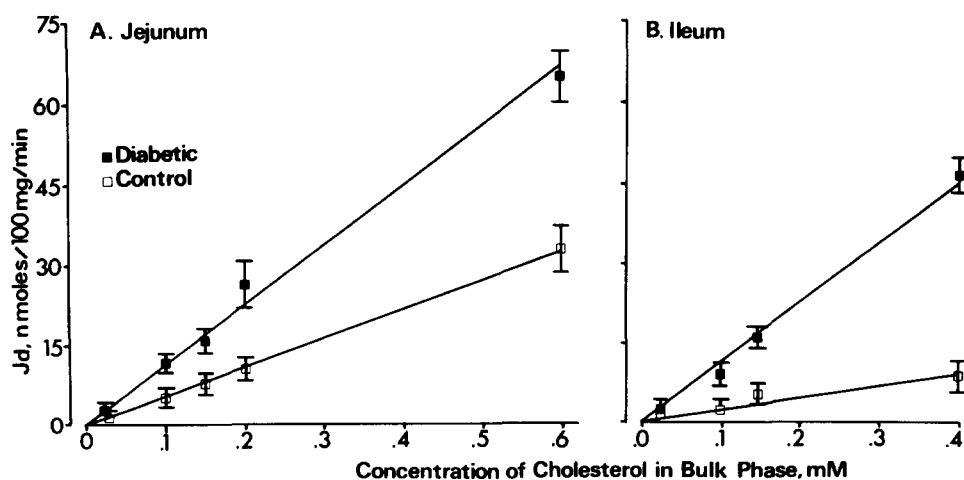

Fig. 2.   Unidirectional flux rate of varying concentrations of cholesterol into intestine of control and diabetic rats. This figure shows the rate of cholesterol uptake when the concentration of taurodeoxycholic acid in the bulk phase was held at 20 mM, but the concentration of cholesterol was increased from 0.025 to 0.2 mM. Each point represents the mean ± SEM of the results of 8 to 12 animals. The diabetic rats were given streptozotocin 14 days before study. The bulk phase was stirred at 600 rpm in order to reduce the effective resistance of the unstirred layer (18). "Jejunum" represents the 10 cm of small intestine distal to the ligament of Treitz, and "Ileum" represents the 10 cm of distal small intestine.
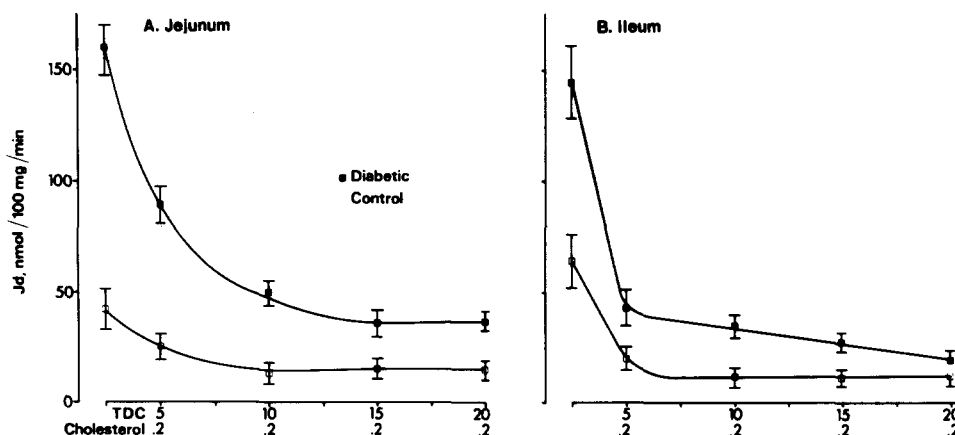
Fig. 3. Unidirectional flux rate of cholesterol into intestine of control and diabetic rats when the concentration of bile acids was varied. The effect of increasing the concentration of bile acid relative to that of cholesterol on the mucosal uptake of cholesterol in control and 14-day streptozotocin rats. This figure shows the rate of uptake of 0.2 mM cholesterol when the concentration of taurodeoxycholic acid was varied from 2.5 to 20 mM. Each point represents the mean ± SEM of the results from 8 to 12 animals. The diabetic rats were given streptozotocin 14 days before study. The bulk phase was stirred at 600 rpm in order to reduce the effective resistance of the unstirred layer.

comprised of mucosa was greater in the 14-day streptozotocin rats than in the control animals (76.5 ± 1.0% versus 59.1%, respectively). This difference was associated with a greater dry weight of jejunal mucosa per unit serosal area. Miller and co-workers (30) have previously reported that the height of the jejunal villi is about 25% higher in ad libitum-fed diabetic rats. The height of the villi was not measured in this study, but the greater proportion of the scraped mucosa in the diabetic than in the control animals was presumably a reflection of the longer villi in the diabetic.

With knowledge therefore of the effective resistance of the unstirred water layer, it was possible to calculate the true membrane permeability P_d for the various probe molecules. The experimentally determined rates of uptake J_d obtained at a stirring rate of 600 rpm, shown in Table 1, were corrected for unstirred layer resistance. The value of D/Sw used in column two of **Table 6** was calculated from the diffuse-limited rate of uptake of lauryl alcohol. The value of P_d was calculated from J_d/C_2; the value of C_2 was obtained by correcting C_1 for the unstirred layer effect, $(J_d)(D)/(Sw)(d)$. Note that the difference in the rate of uptake of the medium chain-length fatty acids, shown
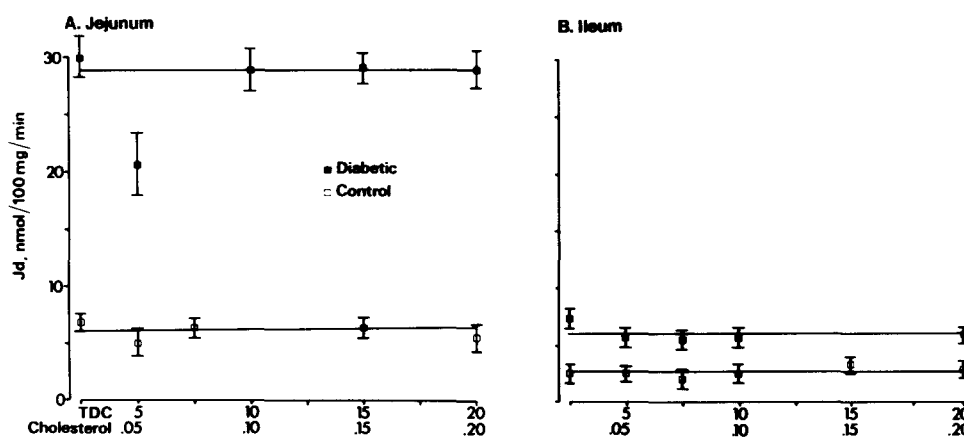

Fig. 4. Unidirectional flux rate of cholesterol under conditions of varying concentrations of both cholesterol and bile acids. The effect of increasing the concentration of both the bile acid and the cholesterol on the mucosal uptake of cholesterol in control and 14-day streptozotocin rats. This figure shows the rate of uptake of cholesterol when the concentration of cholesterol was varied from 0.025 to 0.2 mM, the concentration of taurodeoxycholic acid (TDC) was varied from 2.5 to 20 mM, but the ratio of TDC/cholesterol was kept constant at 100/1. Each point represents the mean ± SEM of the results from 8 to 12 animals. The diabetic rats were given streptozotocin 14 days before study. The bulk phase was stirred at 600 rpm to reduce the effective resistance of the unstirred layer.

TABLE 5. Dimensions of unstirred layer diffusion barrier and morphology of jejunum in control and diabetic rats

Parameter of Jejunal Structure	Control	Streptozotocin	
		3 Day	14 Day
Mg dry weight of jejunum/ unit serosal surface area	1.80 ± 0.02	1.72 ± 0.02	2.0 ± 0.02
Proportion of total tissue dry weight comprised of scraped mucosa, %	59.1 ± 1.8	61.4 ± 1.3	76.5 ± 1.1
Ratio of jejunal wet/dry tissue weight	19.4 ± 0.6	14.7 ± 0.4	12.7 ± 0.2
Mg dry weight of jejunal mucosa/unit serosal surface area	1.06 ± 0.01	1.06 ± 0.01	1.53 ± 0.02
Effective resistance of the jejunal unstirred water layer for lauryl alcohol, min. 100 mg/cm³			
600 rpm	50 ± 5		34 ± 3
0 rpm	500 ± 39		143 ± 12

The techniques used to obtain the morphological parameters are given in the Methods section. The effective resistance of the jejunal unstirred water layer was obtained from the rate of uptake of lauryl alcohol; the uptake of this probe is limited by the effective resistance of the unstirred water layer, and the value of ln J_d/D for this molecule may be used to assess unstirred layer resistance (18). Note that the magnitude of the resistance of the unstirred layer is given by $d/Sw.D$, where d and Sw are the effective thickness and surface area of the unstirred layer, respectively, and D is the free diffusion coefficient of the probe molecule. The value of d/Sw may be obtained from the measurement of the rate of uptake of a diffusion-limited probe, but this technique does not discriminate between the individual values for d and Sw. Since D varies with each probe, then the values of the effective resistance of the unstirred layer will differ from one probe to another. The values for the effective resistance of the unstirred layer, shown in Table 5, were for lauryl alcohol, but different values of $d/Sw.D$ were used to calculate P_d of each fatty acid, reported in column 3 of Table 6.

in Table 1, became even more marked after correction for unstirred layer effects, indicating an even greater difference in the incremental free energy change, $\delta\Delta Fw \to 1$. These values of $\delta\Delta Fw \to 1$ obtained after correction for unstirred layer effects, are shown in Table 3. Three points warrent emphasis. First, the apparent values for $\delta\Delta Fw \to 1$ calculated from the values of the permeability coefficient uncorrected for unstirred layers (J_d/C_1) were much lower than those values of $\delta\Delta Fw \to 1$ corrected for unstirred layer effect (J_d/C_2). Second, the values of $\delta\Delta Fw \to 1$ were similar in the jejunum of nondiabetic (control) rats and in rabbits (18). Third, both the apparent and true values of $\delta\Delta Fw \to 1$ were much higher in diabetic than in control animals.

The total cholesterol content in the jejunum of diabetic rats was significantly higher than in control jejunum, 2.91 ± 0.10 versus 2.44 ± 0.44 mg/g wet weight of tissue, respectively ($P < 0.05$). Similarly, the total cholesterol content was higher in the ileum of diabetic than control animals (3.06 ± 0.59 versus

2.29 ± 0.33 mg/g wet weight of tissue, respectively, $P < 0.05$). However, the ratio of free to esterified cholesterol was similar in control and diabetic animals, 2.71 ± 0.2 versus 2.88 ± 0.2 respectively.

DISCUSSION

The rate of intestinal uptake of a passively absorbed molecule is determined by three parameters; the effective resistance of the unstirred water layer, the concentration of the probe molecule at the aqueous-membrane interface, and the permeability properties of the microvillus membrane. Any difference in the uptake of passively absorbed molecules in diabetes mellitus must be explained in terms of these factors. When the effective resistance of the unstirred layer (d/SwD) was minimized by vigorously stirring the bulk phase, the rate of uptake of FA 10:0 and 12:0 was greater in the 3-day and 14-day young streptozotocin animals than in control rats (Table 2 and Fig. 1).

The value of ln J_d/D for FA 2:0 in the diabetic rats was higher than that value anticipated from the linear extrapolation of the linear relationship between lnJ_d/D and N for FA 6:0 to 12:0 (Fig. 1). Similar observations have been made before (29), but the mechanism of this higher uptake is not known. While some workers suggest that there may be active transport of some short-chain length fatty acids (31), most would

TABLE 6. Calculation of corrected permeability coefficients

Test Molecule Fatty Acid	$\dfrac{Jd}{D} \times 10^{-3}$		$\dfrac{(Jd)(d)}{(Sw)(D)}$		P_d	
	Control	Diabetic	Control	Diabetic	Control	Diabetic
	nmol/cm²/100 mg		mM		nmol/100 mg/min/mM	
2:0	32.0	49.0	0.07	0.07	34.4	52.7
4:0	42.7	52.3	0.10	0.08	47.4	56.8
6:0	79.4	73.0	0.18	0.12	96.8	83.0
8:0	98.4	121.3	0.23	0.19	127.8	149.8
10:0	144.8	200.4	0.33	0.32	216.1	294.7
12:0	250.5	448.4	0.58	0.71	596.4	1546.2

To calculate the true membrane permeability P_d for the various probe molecules, the experimentally determined J_d obtained at a stirring rate of 600 rpm, shown in Table 1, was corrected for unstirred layer resistance. The value of d/Sw used in column 2 was calculated from the diffusion-limited rate of uptake of lauryl alcohol. The permeability coefficient P_d was normalized to tissue weight, and was obtained from the ratio J_d/C_2. The value of C_2 was obtained by correcting C_1 for the unstirred layer effect, $(Jd)(d)/(Sw)(D)$. Note that the difference in the rate of uptake of the medium-chain length fatty acids, shown in Table 1, became even more marked after correction for unstirred layer effects, indicating an even greater difference in the incremental free energy change, $\delta\Delta Fw \to 1$; $\delta\Delta FW \to 1$ was -246 cal/mol in control rats and -382 cal/mol in the 14-day streptozotocin diabetic animals.

agree that it is likely that they are absorbed by passive diffusion (18, 29). If this is the case, then it may be speculated that their unexpectedly high rate of uptake might be due to the availability of leaky aqueous pores through which these water-soluble probe molecules may diffuse and gain greater access to the intestinal tissue, or to altered properties of intestinal membrane itself.

It is clear that this enhanced absorption of FA 2:0 in diabetes is not a generalized effect of hyperglycemia on the intestine since the absorption of FA 4:0, 6:0, and 8:0 is unaffected (Fig. 1) and since the uptake of FA 2:0 and 4:0 was unaffected in the 3- and 14-day alloxan diabetic animals, and in the 3-day streptozotocin rats, despite comparable degrees of hyperglycemia. Furthermore, the change in absorption of these fatty acids is unlikely to be due to a "toxic" effect of the drugs alloxan or streptozotocin on the intestine, because there was no change observed in the uptake of any of the fatty acids when the occasional animal injected with alloxan or streptozotocin failed to become hyperglycemic.

The rate of uptake of cholesterol into the intestine was much greater in 14-day diabetic than in control animals, under conditions of increasing concentrations of cholesterol (Fig. 2), increasing concentrations of TDC (Fig. 3), increasing concentrations of both cholesterol and TDC (Fig. 4), and in both jejunum and ileum (Figs. 2–4). This difference must be explained by the effect of the resistance of the unstirred water layer, the role of the bile acid micelle, a change in the permeability properties of the microvillus membrane, a change in intramucosal esterification rates, or a change in the surface area of the mucosal membrane. The enhanced uptake of cholesterol in the diabetic animals was unlikely due entirely to a difference in the effective resistance of the unstirred layer because the estimates of the effective resistance of the unstirred layer yielded close values in the control and diabetic intestine, when the bulk phase was stirred (Table 5). The uptake of the medium- and long-chain length fatty acids was also increased in the diabetic animals (Tables 1–3). The passive permeability properties of the diabetic intestine were much greater than in control animals (Table 6): after correcting for unstirred layer effects, the incremental free energy change was −246 cal/mol in control rats, and −382 cal/mol in the diabetic animals (Table 3). Thus, the greater uptake of cholesterol in the diabetic animals was likely due to the greater membrane permeability of the diabetic intestine towards lipophilic compounds, including cholesterol.

The increased uptake of fatty acids and cholesterol in the diabetic animals is unlikely to be due simply to the known changes in jejunal morphology. Like others, we have found increased gut weight after alloxan and streptozotocin treatment (Table 5). Three days after the production of chemically-induced diabetes mellitus, the mean jejunal tissue weight (per unit serosal surface area of tissue contained in the transport chamber) fell to values significantly below those observed in control animals. Thereafter, the dry weight of the jejunum of the diabetic animals rose to exceed the control values. The uptake of lauric acid into the jejunum of 3-day and 14-day streptozotocin rats was increased (Table 1), so it is unlikely that the observed enhanced rates of uptake were simply due to changes in jejunal weight, since those weights were decreased on day 3, increased on day 14, yet the rates of uptake of FA 12:0 were enhanced to a similar degree in both groups. In addition, the same qualitative changes in uptake of fatty acids and cholesterol in the diabetic rats were observed when the uptake rates were expressed as nmol/min/unit serosal surface area, thereby obviating the potential problem of changes in flux rates due solely to changes in tissue morphology. Nonetheless, the height of the villus-crypt column was higher in the diabetic animals, and the number of columnar cells per column was greater (31), and in this study, the proportion of the total tissue dry weight comprised of scraped mucosa changed. The dry weight of the total jejunal wall per unit serosal surface area increased from a control value of 1.80 ± 0.02 mg to 2.0 ± 0.02 mg in the 14-day streptozotocin animals, and the dry weight of the scraped jejunal mucosa per unit serosal surface area also increased from 1.06 ± 0.01 mg to 1.53 ± 0.01 mg (Table 5). It must be appreciated however, that theoretical considerations have emphasized that different portions of the villus may be responsible for the uptake of different probe molecules (32), and in view of this likely functional heterogeneity of the villus, a change in villus height per se does not necessarily provide an adequate explanation for a change in a functional absorption parameter. If the morphological alterations in the diabetic intestine were the explanation for the differences in uptake rates, then the uptake of each fatty acid should have been increased, and to the same extent. This was not observed (Table 1): the J_d of FA 4:0 and 6:0 were unchanged in the 14-day streptozotocin animals, and the percentage change in uptake of FA 2:0, 53%, was less than the 79% increase in J_d of FA 12:0. In addition, from unpublished theoretical considerations as well as those apparent from the examination of equation 13, pg. 41, of reference 24, the change in the slope of the relationship between the chain length of fatty acids, and

uptake expressed as $\ln J_d/D$ (Table 4 and Fig. 1) is compatible with a change in the permeability properties of the diabetic intestinal membrane, rather than a change in functional membrane surface area. Finally, the calculation of $\delta\Delta Fw \rightarrow 1$, has the units cal/mol, obviating any potential influence of a difference in the morphology of the villus. Therefore, while alterations in the gut anatomy do occur in the diabetic animal, these morphological changes are unlikely to be the basis for the enhanced uptake of fatty acids and cholesterol in these animals.

The enhanced rate of uptake of fatty acids and cholesterol in the diabetic intestine, and their accumulation in the mucosa (Table 1 and reference 33) may have been influenced by intramucosal esterification rates. The rate of penetration of the mucosal membrane is influenced by the difference in the concentration of the probe molecule just outside (C_2) and just inside (C_3) the membrane. If the concentration of the probe just inside the membrane were lower in the diabetic than in the control rats, then the concentration drop across the membrane would have been greater, and the rate of uptake would have been higher. Since the lipid molecule is quickly metabolized within the cell (22, 34), it has always been assumed that the fatty acids and cholesterol are rapidly esterified in the mucosal cell, and that these probe molecules never accumulate as unesterified lipids adjacent to the inner surface of the membrane. By this argument, $C_2 \gg C_3$, the concentration gradient across the membrane, C_2-C_3 approaches C_2, and the unstirred layer and the microvillus membrane become rate-limiting for diffusion (24). However, the diabetic intestine contributes significantly to cholesterogenesis (35) and the intestinal mucosa of the diabetic rat contained significantly more total cholesterol. Since the ratio of free to esterified cholesterol was similar in the diabetic and control animals, it is unlikely that the rate of mucosal esterification was higher in the diabetic animals. It is also unlikely that the mucosal cholesterol content exerted any major influence on the rate of cholesterol uptake, because the rate of cholesterol uptake was higher in the diabetic jejunum, even though the mucosa contained more cholesterol. It is likely, therefore, that the greater mucosal cholesterol content was the result of the enhanced rate of uptake.

There are several possible mechanisms by which the bile salt micelle may enhance the uptake of cholesterol. The cholesterol and the micelle may both be taken up together into the mucosa, but there is abundant evidence that this thesis is untenable (20, 29, 36, 37). Alternatively, the micelle may make contact with the membrane, with the cholesterol entering the mucosa directly from the micelle; or the cholesterol may partition from the micelle into the aqueous phase, and thence into the mucosa. Previous theoretical and experimental evidence (23) supports the concept of cholesterol uptake from an obligatory aqueous phase. The results of the present study also support the suggestion that cholesterol uptake in control and diabetic animals occurs from an aqueous phase after partitioning of the cholesterol from the bile acid micelle: the increase in the unidirectional flux rate with increasing concentrations of cholesterol in the bulk phase (Fig. 2), the reciprocal decrease in cholesterol uptake with increasing concentrations of taurodeoxycholic acid (Fig. 3), and the lack of increase in cholesterol uptake when the concentrations of both cholesterol and TDC were increased but their ratio was held constant (Fig. 4). Furthermore, the uptake of cholesterol into the intestine of diabetic animals is quantitatively greater than in controls, but the qualitative patterns are similar. Thus this difference in the rate of cholesterol uptake was likely due to a change in the properties of the membrane itself, rather than due to a change in the basic mechanism by which cholesterol is absorbed.

Changes in cholesterol metabolism occur in diabetes mellitus: the serum cholesterol and triglyceride levels are increased, as are hepatic and biliary cholesterol levels (1, 3). The expanded cholesterol pool in diabetic animals is partially due to an increased rate of delivery of exogenous cholesterol to the lymph, and partly due to increased cholesterol conversion into bile salts (1). Cholesterol production in the ileum is normally regulated by the amount of bile salts absorbed into the mucosa, whereas hepatic cholesterogenesis is influenced by the amount of both bile acid and cholesterol absorbed by the intestine (38, 39). Although the absorption of some bile salts (7, 8) as well as of cholesterol (Figs. 2–4) is increased in the diabetic rat, the ileal and hepatic synthesis has been reported to be accelerated (40), normal (41), or depressed (42) in this metabolic disorder. Usually, the bile salt synthesis is regulated by a negative feed-back mechanism that depends on the rate of bile salts returning to the liver (43). In the diabetic rat, bile salt absorption is increased (7, 8) and the mean biliary excretion of bile salts is high enough to inhibit completely bile salt synthesis in normal animals (3). The diabetic rat's synthesis of trihydroxycholanic acid is even greater than in control animals (1, 44) and it thus appears that the feed-back inhibition mediated by the enterohepatic circulation of bile salts is partly blocked, and the liver cell settles its synthesis of bile salts at a new level. Inasmuch as the major steps in cholesterol absorption are obligatorily mediated by bile salts (23), and because the size

of the bile salt pool has a determinant effect on the rate of the absorptive process (45, 46), then the higher biliary excretion of bile salts observed in the diabetic rat probably facilitates a faster micellarization, intestinal esterification, and delivery of cholesterol in the lymph. Thus, the markedly enhanced rate of intestinal uptake shown in this study (Table 4, and Figs. 2–4), in conjunction with the demonstration of increased delivery of cholesterol into the lymph (3), suggests that the expansion of the cholesterol pool in the diabetic animal is related in part to a greater input into the system. To prevent the indefinite accrual of body cholesterol, the hepatic cell then unchains a faster production of bile acids (1, 3, 45), expansion of the bile acid pool size, and significantly increased excretion of bile salts in the diabetic animal (1, 3). Thus, the underlying fault may rest with the enhanced absorption of cholesterol due to increased intestinal membrane permeability in the diabetic animal.

These studies may have some bearing of clinical relevance. Aberrations in lipid metabolism are known to occur in patients with diabetes mellitus, and possible complications of these aberrations are common (47). Cholesterol absorption has not been reported to be increased in human patients with diabetes mellitus, but the report of the accumulation of lipid in the small bowel mucosa of children with poorly-controlled juvenile-onset diabetes mellitus (33) is consistent with the possibility of increased uptake of fat. The effective resistance of the unstirred water layer is likely higher in vivo (48–50) than in vitro, but even when unstirred layer resistance was high, cholesterol uptake was greater in diabetic than in control animals (Table 4), and it would be anticipated that cholesterol absorption would also be higher in vivo in the diabetic individual. The incremental free energy change of the intestine increased as the animal aged (Table 3), but the difference in fatty acid uptake between control and diabetic rats became obscured as the duration of the diabetes was prolonged (Table 2). Thus it is possible, though unproven, that as a result of the effect of the normal process of aging on the intestine, there is a diminution in the relatively greater uptake of cholesterol in the young diabetic. The therapeutic potential of treatment with insulin, and diets of varying fat and cholesterol content must now be explored.■

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