Determination of unidirectional uptake rates for lipids across the intestinal brush border

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Abstract An in vitro method is presented which measures valid, unidirectional uptake rates for lipids across the intestinal brush border. This method combines analysis by a newly devised, double isotope counting system for solubilized tissue with the use of a nonabsorbable marker to correct gross uptake determinations for contamination by adherent mucosal fluid. Of seven markers, only [^{3}H]inulin measured adherent mucosal fluid volumes as much as 20% greater than the other markers. Diffusion of the nonabsorbable marker, as well as of the compound being studied, into the unstirred layer made the time course of uptake critically important. The time lag for diffusion of marker invalidates the use of 1-min incubation periods; however, a linear time course of uptake that intercepts essentially at zero was found for taurocholate and octanoate for periods of from 2 to 5 min. Working within this critical time period with jejunum, it was shown that tissue dry weight was an appropriate measure of the amount of tissue and that uptake rates for taurocholic, octanoic, and lauric acids were linear with respect to concentration. Tissue binding of compounds was not significant. The results demonstrate that careful use of the described method yields accurate measurement of unidirectional uptake rates of lipids across the brush border that are of critical importance in defining the characteristics of membrane penetration and the rate-limiting steps in fat and sterol absorption.

Supplementary key words lipid absorption · fatty acid · bile acid · liquid scintillation counting · unstirred layer · intestinal absorption · inulin

Determination of passive and active absorption rates of a variety of substances across the intestinal mucosa has been the subject of numerous in vitro studies. Generally, three types of experimental tissue preparations have been utilized. In the first type the rate of absorption is quantified by determining the rate of disappearance of the test molecule from the mucosal perfusing solution (1, 2). With this technique there necessarily must be a measurable decrease in the concentration of the test molecule in the perfusate during the experimental period; hence, such a method has the inherent disadvantage that flux rates into the tissue must be measured under conditions where the concentration of the test molecule is decreasing continuously throughout the experimental period.

In a second type of preparation the rate of appearance of the test molecule on the serosal side of the intestine is measured (3). In this circumstance the assumption is made that, once a steady state is achieved, the rate of appearance of the test substance on the serosal surface accurately reflects the rate of absorption of the molecule across the rate-limiting membrane of the intestinal absorptive cell, i.e., presumably the luminal cell surface. While this technique is useful in determining absorption rates for a number of water-soluble molecules, it is invalid for quantification of absorption rates of lipids across the intestinal mucosa. Many lipids of biological importance, such as long-chain fatty acids and steroids, once absorbed, are metabolized and incorporated into specific lipoproteins within the cell; hence, the rate of appearance of these particles at the serosal surface does not reflect the rate of absorption of the constitutive lipids across the intestinal brush border.

In a third type of technique, absorption rates are measured by determining the uptake of a radiolabeled test molecule directly into the intestinal mucosa (4–6). This procedure, while apparently simple, has many possible sources of significant error that have never been evaluated adequately. Methods must be available, for

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example, for extracting the test molecule or, alternatively, for counting radioactivity in the presence of large amounts of tissue protein. Nonabsorbable markers must be utilized to correct for adherent mucosal fluid, and selection of such markers must take into consideration the presence of the unstirred water layers adjacent to the luminal cell membrane of the intestinal mucosal cell.

Ideally, any method for quantifying essentially instantaneous, unidirectional uptake rates of both water-soluble compounds and insoluble lipids across the intestinal brush border must have the following characteristics. *1*) Counting techniques must be available for simultaneously measuring ^{14}C and ^{3}H in the presence of solubilized whole tissue. This obviates the difficulty of attempting simultaneous quantitative extraction of compounds of widely differing polarity from the tissue. *2*) A marker of adherent mucosal fluid must be utilized to correct for contamination by extracellular test molecules. *3*) Incubation time should be sufficiently short so that significant intracellular concentrations of the test substance do not develop and so that none of the test molecules are lost from the tissue into the serosal compartment. *4*) The incubation time, on the other hand, must not be too short but must be of sufficient length to allow uniform labeling of the unstirred water layer adjacent to the mucosal cell surface by the nonabsorbable marker compound. *5*) Corrected values for tissue uptake should be demonstrated to be linear with respect to time. *6*) Micelles in the incubation medium should not alter either the adherent mucosal fluid volume or the inherent permeability characteristics of the lipid cell membrane.

In the present paper a technique is reported that meets these criteria. Using this method it is possible to measure with considerable accuracy unidirectional flux rates of both water-soluble compounds and insoluble lipids across the intestinal cell brush border. Such data are of considerable importance in defining the rate-limiting steps in fat and steroid absorption as well as in elucidating the more general features of cell membrane permeation.

METHODS

Incubation materials

Radiolabeled compounds used to measure adherent mucosal fluid volume, [^{14}C]- and [^{3}H]inulin, [^{14}C]- and [^{3}H]dextrans, and [^{14}C]polyethylene glycol, were obtained from the New England Nuclear Corp., Boston, Mass. Dextran compounds were purified by dialysis against distilled water. Other marker compounds were used as supplied.

[^{3}H]Taurocholic acid was also obtained from New England Nuclear Corp. and was purified by thin-layer chromatography. Unlabeled bile acids were obtained from Steraloids, Inc., Pawling, N.Y., and Calbiochem, Los Angeles, Calif. Unlabeled and 1-^{14}C-labeled fatty acids were obtained from Applied Science Laboratories Inc., State College, Pa. When necessary, radiopurity was established by thin-layer chromatography.

Tissue preparation

Female Sprague-Dawley rats, 180–220 g, which had been fed rat chow ad lib., were killed by a blow on the head and bled. The proximal half of the intestine was removed, rinsed with cold saline, and immediately everted over a glass rod. After the intestine was filled with cold buffer, sacs 2 cm long from mid-jejunum (segments 2 to 4 [3]) were tied off sequentially, taking care to avoid Peyer's patches. Sacs were left with one long thread and were kept in cold buffer solution until incubation ($<$ 15 min).

All solutions were prepared using Krebs-Ringer bicarbonate buffer (7), which was altered only by the omission of Ca^{2+}. Solutions were oxygenated with 5% CO_2 in oxygen and had a pH of 7.4 at 37°C.

Incubation medium contained both the ^{14}C or ^{3}H compound whose uptake was being measured and a radiolabeled nonabsorbable marker to measure the volume of the adherent mucosal fluid. A ^{14}C-labeled marker was used when studying the uptake of a ^{3}H-labeled probe molecule, and a ^{3}H-labeled marker was utilized when measuring uptake of a ^{14}C-labeled test substance. Intestinal sacs were suspended by a long thread in a 24 × 150 mm test tube containing 15 ml of medium, which was vigorously stirred by means of a magnetic stirrer and a $^{5}/_{8}$-inch, round stirring bar (Roto-Stirrer, no. 2495/412, Chemical Rubber Co., Cleveland, Ohio). Speed of stirring was sufficient to produce a vortex half the depth of the medium.

Following incubation, sacs were quickly removed, rinsed vigorously for 3–5 sec in iced saline, and placed on moistened filter paper. Using a razor blade, the ends of the sacs were transected, and each central cylinder of tissue was placed in a previously weighed scintillation counting vial and dried overnight at 93°C in an oven. After cooling to room temperature, vials were reweighed for determination of tissue dry weight.

Radioactivity determinations

The dried tissue was saponified by adding 0.8 ml of 0.75 N NaOH to each vial and autoclaving at 230°F for 20 min. Subsequently, 15.8 ml of scintillation fluid was added and mixed vigorously with a Vortex mixer.

The scintillation fluid was a modified toluene–Triton X-100 solution that contained: 7 g of PPO (2,5-diphenyl-

oxazole, Arapahoe Chemicals, Boulder, Colo.); 0.1 g of POPOP (*p*-bis[2-(5-phenyloxazolyl)]-benzene, Pilot Chemicals, Inc., Watertown, Mass.); 1000 ml of toluene; 500 ml of Triton X-100 (Beckman Instruments, Inc., Fullerton, Calif.); and 80 ml of 2.5 N HCl. This solution, which cleared upon stirring, remained stable for at least several weeks. Chemiluminescence was minimal as the tritium background decreased from 50 to 25 cpm in 2–4 hrs and remained constant thereafter.

Counting was carried out in a Packard Tri-Carb liquid scintillation spectrometer (model 2311) with automatic external standardization. Discriminator settings appropriate for the degree of quenching were: channel 1, 60% gain, 25–300 window; channel 2, 13% gain, 175–300 window; channel 3, 2% gain, 250–1000 window. These settings gave energy ranges of 0.375–4.5 kev, 12.1–55.4 kev, and 112–450 kev for channels 1, 2, and 3, respectively. ^{3}H counting efficiency in channel 2 was less than 0.07%, and ^{14}C did not contribute to counts in channel 3. An external standard ratio (AES ratio) was computed for the external standard counts in channels 2 and 3.

Contribution of ^{14}C to channel 1 counts in each experimental vial was determined by relating that vial's AES ratio to a ^{14}C spill ratio versus AES ratio linear regression.

Calculations were as follows:

$$^{14}\text{C cpm} = \text{channel 2 cpm} - \text{channel 2 background cpm} \quad \text{(I)}$$

$$\text{AES ratio} = \frac{\text{AES cpm channel 2} - {}^{14}\text{C cpm}}{\text{AES cpm channel 3}} \quad \text{(II)}$$

$$^{14}\text{C spill ratio} = (\text{AES ratio})(\text{linear regression slope}) + (\text{linear regression intercept}) \quad \text{(III)}$$

$$^{14}\text{C cpm spill in channel 1} = {}^{14}\text{C cpm} \cdot {}^{14}\text{C spill ratio} \quad \text{(IV)}$$

$$^{3}\text{H cpm} = \text{channel 1 cpm} - \text{channel 1 background cpm} - {}^{14}\text{C cpm spill} \quad \text{(V)}$$

The volume of adherent mucosal fluid was calculated by dividing the amount of the marker in the tissue sample by its concentration in the incubation medium. The mass of the probe molecule present in this volume was subtracted from the total mass in the tissue sample, giving the amount of the test molecule taken up across the brush border. These calculations are described by the following formula:

$$J = \frac{P - (AMF)\,R}{P_s \cdot \text{time (min)} \cdot \text{tissue wt (mg)}} \times 100 \quad \text{(VI)}$$

This equation yields rates of uptake of the test molecule, J, expressed as nmoles/min/100 mg tissue dry weight when:

P = cpm of probe molecule in tissue sample

AMF = cpm of adherent mucosal fluid marker

$P_s = \frac{\text{cpm}}{\text{nmole}}$ of the probe molecule in incubation medium

$R = \frac{\text{cpm}/\mu\text{l compound}}{\text{cpm}/\mu\text{l AMF marker}}$ in incubation medium

RESULTS

Liquid scintillation counting system

Initial experiments involved development of the acid toluene–Triton X-100 scintillation fluid described in the Methods section that allows solubilization of greater than 30 mg dry weight of intestinal tissue. The characteristics of this solution with respect to ^{14}C and ^{3}H discrimination in the presence of solubilized tissue are shown in Fig. 1. The ratio of channel 2 to channel 3 external standard cpm (AES ratio), used to correct for variable quenching, increases with increasing quench. Fig. 1*A* shows that tissue weight is the major determinant of the change in the AES ratio for 95 experimental values.

Counting efficiencies for ^{14}C and ^{3}H were determined for acetone-quenched standards and are shown as solid circles in Fig. 1*B*. As is apparent, changes in efficiency of counting are minor in the range of quenching found in these experiments. The change in the ^{14}C spill ratio, however, is of major importance for the double isotope analysis. The ^{14}C spill ratio, defined as the ratio of ^{14}C cpm in channel 1 (^{3}H window) to cpm in channel 2 (^{14}C window), was determined for acetone-quenched (open circles) and tissue-quenched (triangles) standards. Quenching by acetone is indistinguishable from quenching by tissue. Using the linear regression determined by least squares fit of results from ^{14}C standards, the ^{14}C spill ratio may be estimated for each vial from the relationship:

$$^{14}\text{C spill ratio} = 0.15521(\text{AES ratio}) - 0.14660$$

This linear regression applies to values for AES ratios between 1.95 and 2.40. Accuracy of estimation of the ^{14}C spill ratio is approximately ± 0.005. Experiments were designed to yield approximately twice as many ^{3}H cpm as ^{14}C cpm to improve the accuracy of the double isotope analysis.

Adherent mucosal fluid

With an in vitro technique for measuring tissue uptake during very short incubation periods, incubation

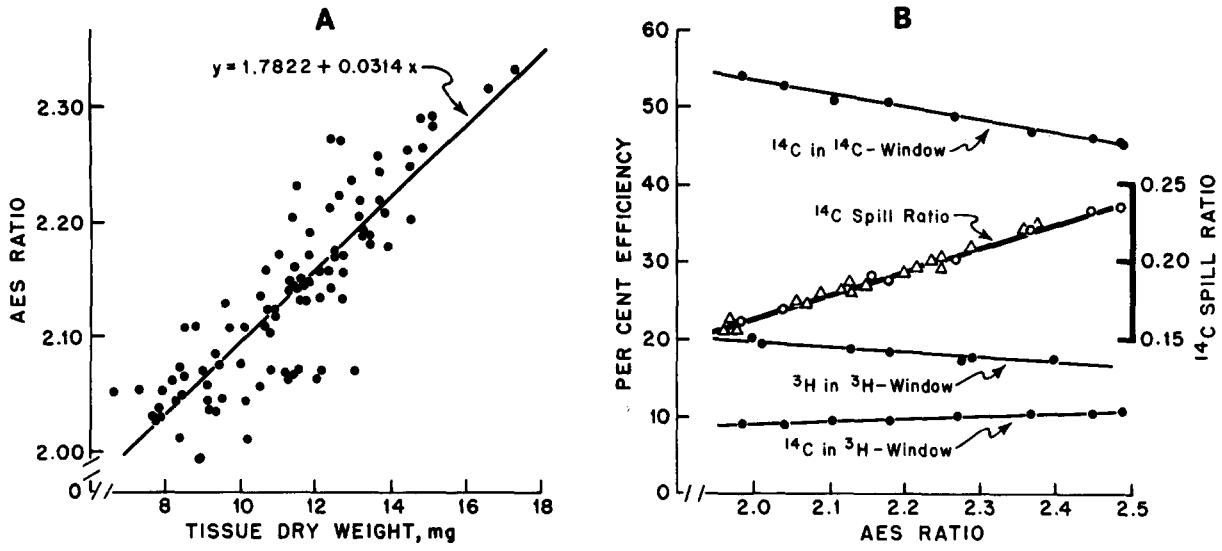

FIG. 1. Characteristics of the liquid scintillation counting system for solubilized tissue. Panel *A* shows the relationship of the external standard (AES) ratio to the amount of solubilized tissue. Panel *B* shows the relationship of the AES ratio to counting characteristics for acetone-quenched (circles) and tissue-quenched (triangles) standards. The ^{14}C spill ratio is related to the AES ratio by the linear regression, $y = (0.15521)$ (AES ratio) $- 0.14660$, when the AES ratio is between 1.95 and 2.40.

medium adherent to the tissue and carried over into the counting vial may result in significant errors in determination of uptake rates. It is mandatory in such experiments, therefore, to provide a correction for the adherent mucosal fluid. In order to evaluate the various nonabsorbable markers available, the studies shown in Tables 1 and 2 were undertaken. In Table 1 are listed the values for the adherent mucosal fluid volumes determined using seven different marker compounds. All markers yielded similar values except for [^{3}H]inulin, which appears to measure a slightly larger volume.

A more precise comparison is possible, as shown in Table 2, using the double isotope technique, in which the volume of the adherent mucosal fluid can be measured by two markers simultaneously in the same intestinal segment. A comparison of values for each determination shows that [^{3}H]dextran, [^{14}C]dextran, [^{14}C]polyethylene glycol, and [^{14}C]inulin yield essentially identical values for the volume of the adherent mucosal fluid. [^{3}H]Inulin again measures a slightly larger volume than any of the ^{14}C compounds. This small overestimate of the volume of the adherent mucosal fluid is of minor importance when tissue uptake is very rapid, but becomes of considerable importance when uptake is slow.

TABLE 1. Comparison of results obtained using different markers for measurement of the volume of adherent mucosal fluid in intestinal mucosa in single isotope experiments

Nonabsorbable Marker	Approximate Molecular Weight	Adherent Mucosal Fluid Volume
		μl/100 mg tissue dry wt
[^{3}H]Inulin	5,000–5,500	99.8 ± 16.1
[^{14}C]Inulin	5,000–5,500	83.4 ± 11.4
[^{3}H]Dextran	60,000–90,000	71.6 ± 11.1
[^{14}C]Dextran	60,000–90,000	67.2 ± 8.6
[^{3}H]Dextran	15,000–17,000	85.7 ± 8.1
[^{14}C]Dextran	15,000–17,000	68.9 ± 9.5
[^{14}C]Polyethylene glycol	4,000	75.3 ± 8.0

In this study seven sacs were prepared from each animal and one of these sacs was placed in an incubation medium containing one of the radiolabeled markers listed above. After incubation for 4 min at 37°C, tissue was prepared as described in Methods but was counted using a wide window. Values are the means ± SEM for six determinations.

Other characteristics of the adherent mucosal fluid volume measurement are shown in Fig. 2. First, the volume of adherent mucosal fluid measured by [^{14}C]-inulin as a function of time of incubation (Fig. 2*A*) shows that the inulin does not achieve equilibrium within the adherent mucosal fluid until after 3 min. Thus, at shorter incubation times the marker would underestimate the adherent fluid volume.

That high concentrations of bile acids do not alter the volume measured by [^{14}C]inulin is shown in Fig. 2*B*. Even with taurocholate concentrations as high as 20 mM, the measured adherent mucosal fluid volume remained constant, thus indicating that bile acid micelles did not influence the microvillous membrane or the extramembranous structure of the tissue. Similar studies with taurodeoxycholate gave values of 92.2 ± 5.3, 91.2 ± 3.8, and 91.1 ± 4.2 μl/100 mg tissue dry weight at 0, 10, and 20 mM concentrations, respectively (n = 18–40).

In order to explore further this important point, the permeability rates of a water-soluble fatty acid were measured in the presence and absence of bile acid micelles. In the absence of bile acid, heptanoic acid has an uptake rate of 39.4 ± 5.2 nmoles/min/100 mg tissue

TABLE 2. Comparison of results obtained using different markers in pairs for measurement of the volume of adherent mucosal fluid in intestinal mucosa in double isotope experiments

Nonabsorbable Marker		Adherent Mucosal Fluid Volume		Marker Comparison
^{3}H Label	^{14}C Label	^{3}H Label	^{14}C Label	$^{3}H - ^{14}C$
		μl/100 mg tissue dry wt		
Inulin	Inulin	105.6 ± 6.5	88.1 ± 5.3	17.5 ± 1.9 (31)[a]
Inulin	Dextran (A)	102.3 ± 11.5	76.5 ± 8.7	25.8 ± 3.4 (5)[a]
Inulin	Dextran (B)	117.0 ± 17.2	86.9 ± 13.0	30.1 ± 6.3 (5)[a]
Inulin	Polyethylene glycol	112.8 ± 8.8	92.7 ± 7.6	20.1 ± 4.1 (5)[a]
Dextran (A)	Inulin	100.9 ± 20.7	105.2 ± 22.2	−4.3 ± 3.4 (4)
Dextran (B)	Inulin	106.8 ± 17.1	107.4 ± 17.9	−0.6 ± 1.5 (5)
Dextran (A)	Dextran (A)	99.4 ± 14.2	91.9 ± 12.9	7.5 ± 1.7 (5)*[a]

In this study sacs were incubated for 4 min at 37°C in medium containing both ^{3}H- and ^{14}C-labeled markers as shown in the first two columns. Each of these markers was then used to calculate the apparent volume of the adherent mucosal fluid in the same intestinal segment. Dextran (A) and dextran (B) have molecular weights of 15,000–17,000 and 60,000–90,000, respectively. Overall means for [^{3}H]inulin and [^{14}C]inulin were 107.3 ± 5.1 (46) and 92.2 ± 5.3 (40), respectively.

[a] $P < 0.01$ for comparison of sample means on paired values for each determination, using the t test (Student distribution).

dry weight (n = 10). The comparable rate of 34.4 ± 3.4 obtained in the presence of 20 mM taurodeoxycholate provides further evidence that the presence of bile acid has not adversely altered the permeability characteristics of the microvillous membrane under the conditions of these experiments.

Characteristics of tissue uptake across the brush border

Using a bile acid and several fatty acids as test molecules, the characteristics of absorption across the brush border in this preparation with respect to tissue weight, time, and substrate concentration were next investigated. That tissue dry weight is a measure of the amount of tissue effective for uptake is verified in Fig. 3*A*. Taurocholate (*TC*) uptake is plotted as open circles, and octanoic acid (*FA 8:0*) is plotted as closed circles. These determinations may be described by linear regressions which have essentially zero intercepts. Thus, dividing tissue uptake by tissue dry weight is an appropriate method for normalizing determinations.

The time course (Fig. 3*B*) for taurocholate (*TC*) as well as octanoic acid (*FA 8:0*) is linear from 2 to 5 min and has an intercept very near zero. Other experiments have shown that with longer times tissue uptake rates are underestimated as radioactivity is lost into the serosal compartment.

Uptake rates for fatty acids and bile acids in the jejunum are perfectly linear with respect to concentration. Fig. 3*C* shows this relationship for octanoic acid (*FA 8:0*) and taurocholate (*TC*) up to 4 mM and for lauric acid (*FA 12:0*) up to 0.6 mM, its solubility limit in Krebs-Ringer bicarbonate buffer. Finally, Fig. 4 presents a time course for uptake of the long-chain fatty acid, palmitic acid (*FA*); in addition, the amount of the fatty acid actually incorporated into tissue tri-

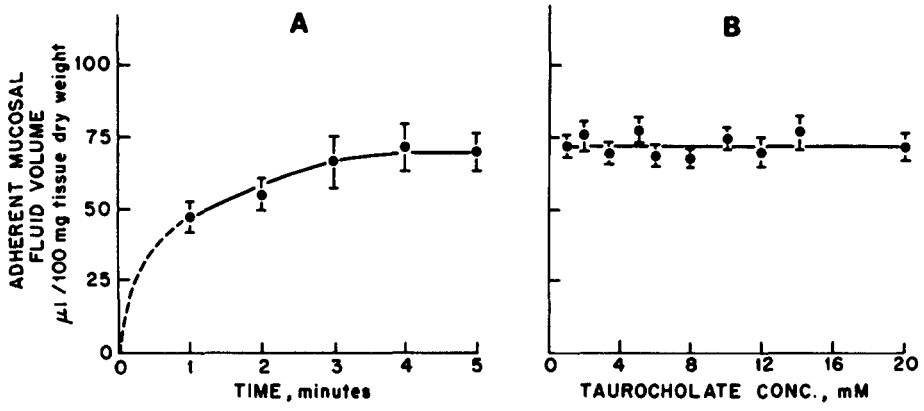

FIG. 2. Characteristics of the adherent mucosal fluid volume in intestinal mucosa. Everted sacs were incubated in Krebs-Ringer bicarbonate buffer containing [^{14}C]inulin as the marker. Time of incubation was 3 min in panel *B*. Values are means ± SEM for 5–26 determinations.

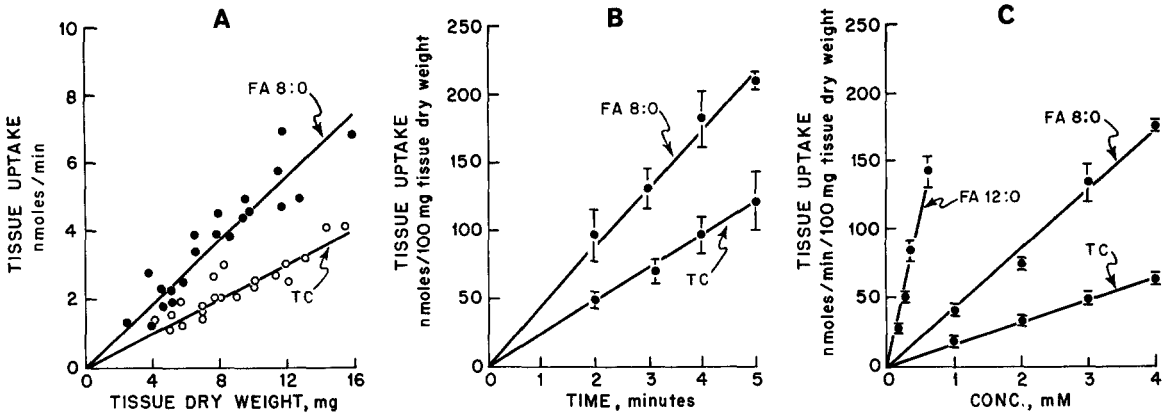

FIG. 3. Characteristics of intestinal mucosal uptake of fatty acids and bile acids. Everted sacs were incubated in Krebs-Ringer bicarbonate buffer containing [^{3}H]taurocholate (*TC*), [^{14}C]octanoic acid (*FA 8:0*), or [^{14}C]lauric acid (*FA 12:0*). Conditions for incubation were: panel *A*, 1 mM TC for 3 min, 1 mM FA 8:0 for 4 min; panel *B*, 1 mM TC, 1 mM FA 8:0; panel *C*, TC, 3 min, FA 8:0 and FA 12:0, 4 min. Values in panels *B* and *C* are means ± SEM for 5–25 determinations.

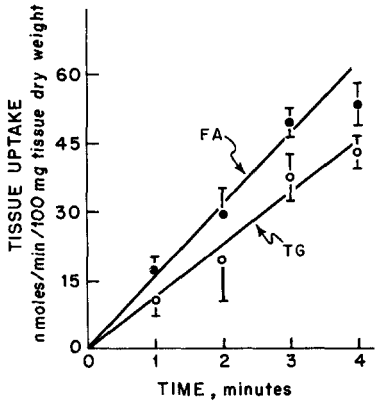

FIG. 4. Tissue uptake and esterification of palmitic acid. Everted sacs were incubated in Krebs-Ringer bicarbonate buffer containing 0.5 mM [^{14}C]palmitic acid in 20 mM taurodeoxycholate. Net tissue uptake of palmitic acid (*FA*) was determined by double isotope counting of tissue. ^{14}C recovered in tissue triglyceride (*TG*) after thin-layer chromatography was calculated as the equivalent amount of fatty acid present as triglyceride. Values are means ± SEM for three determinations.

glyceride is also shown (*TG*). Since the fatty acid is almost all in the form of triglyceride, these essentially linear relationships together with experimental temperature coefficients of 1.2 to 1.4 show that measured fatty acid uptake in this sytem is, in fact, uptake of the test molecule into the cell.

DISCUSSION

Numerous techniques have been utilized to study fat absorption in vitro, yet, for the most part, these methods have failed to take into account the unusual complexity of this process. For example, the bulk incubation medium seldom contains a single chemical species but, rather, may contain ionized and protonated fatty acids present in both a dispersed monomer phase and in bile acid micelles. In addition, as recently demonstrated in work from this laboratory (8), under certain circumstances the rate of absorption of lipid is determined primarily by the presence of an unstirred water layer adjacent to the mucosal surface of the intestinal cell and not by the lipid cell membrane. Hence, to date no method has been reported that allows quantification of essentially instantaneous, unidirectional uptake rates of both water-soluble compounds and lipids across the brush border of the intestinal mucosal cell.

As outlined in the Introduction, such a technique must include counting methods that allow discrimination of two isotopes and quench correction for counting in the presence of solubilized tissue; must involve a correction for test molecules contained in adherent mucosal fluid; and must take cognizance of the presence of a significant unstirred water layer adjacent to the epithelial cell brush border.

In the present study, the counting solution allows solubilization of significant amounts of intestinal tissue after an initial saponification step. No stability problems were encountered with this system, which allows solubilization of more tissue at less than half the cost of most other reported systems (9, 10). In addition, as shown by the data in Fig. 1, appropriate techniques allow for correction of the quenching effects of the tissue. It should be emphasized that this method, which involves solubilization of whole intestinal tissue, allows use of this technique for measurement of uptake rates of a variety of test molecules where quantitative extraction might be difficult or impossible.

The use of a marker for adherent mucosal fluid is another important part of this procedure, and the relative importance of this correction is shown by the theoretical curves presented in Fig. 5. Since only a portion of the mass of labeled test material analyzed with the tissue actually has penetrated the microvillous

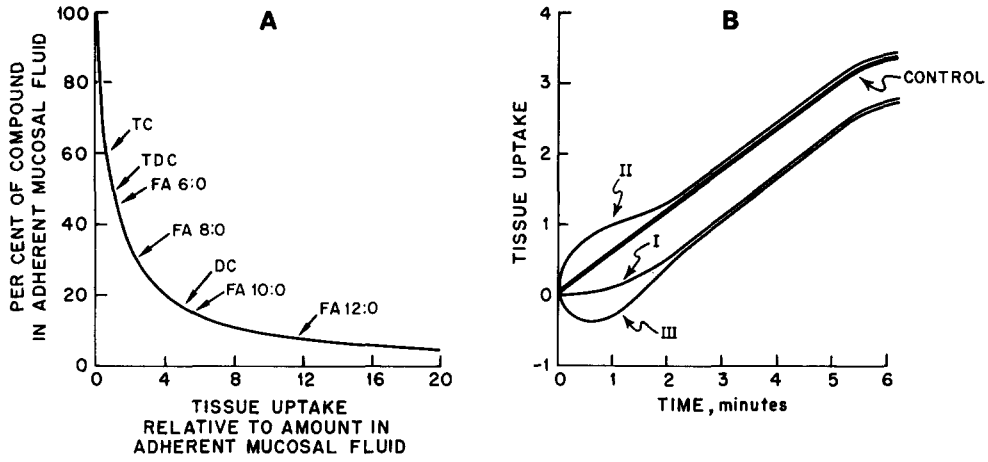

FIG. 5. Theoretical curves showing characteristics of the correction for adherent mucosal fluid. In panel *A* the amount of compound in the adherent fluid volume, calculated as the percentage of the total amount of compound determined in the tissue sample, has been plotted as a function of the rate of tissue uptake. This diagram demonstrates that the more rapidly a compound is taken up, the less important the correction for the adherent fluid. Conversely, for compounds taken up only slowly, analyzed compound in contaminating adherent fluid may represent more than 50% of the total amount in the tissue sample. To plot the approximate position of several compounds on this curve, uptake for a 3-min incubation period from a 1 mM solution was considered relative to an adherent mucosal fluid volume of 75 μl/100 mg tissue dry weight. Panel *B* shows the time courses expected when markers and test compounds of different sizes are paired for measuring uptake corrected for adherent mucosal fluid. These hypothetical curves were drawn using diffusion constants in the range of those appropriate for the nonabsorbable markers and test molecules used in these studies. The control line represents the theoretical uptake with no unstirred layer, while curve *I* describes tissue uptake in the presence of an unstirred layer. Curve *II* represents experimentally measured uptake when the test compound taken up is very small but the fluid marker is very large. Curve *III* represents experimental uptake for a very large test compound but a relatively small marker.

membrane, the amount of material in the adherent mucosal fluid introduces an error into the uncorrected uptake rate measurement. The magnitude of this error, in turn, depends on the actual rate at which the molecule penetrates the cell membrane. When uptake is very rapid, the amount of the compound in the adherent fluid represents only a small percentage of the total amount analyzed in the sample (e.g., *FA 10:0* and *FA 12:0* in Fig. 5*A*), but when uptake is low, more than 50% of the total compound in the sample actually may be due to contamination of the tissue with incubation fluid, despite vigorous rinsing for several seconds (e.g., *TC* and *TDC* in Fig. 5*A*). Thus, some experiments may not require correction if the uptake rate is very high, but tissue uptake rates below 100 nmoles/min/100 mg tissue dry weight for a substrate concentration of 1 mM demand a correction for adherent mucosal fluid. Even at this rate during an incubation period of 3 min, approximately 20% of the total amount of compound analyzed in the tissue will be due to adherent fluid contamination.

It should be emphasized further that this correction is of critical importance in studies of lipid absorption where the bulk phase concentration of the test lipid in micellar solution may be several thousand times the concentration of the compound in solution in monomer phase. Since uptake across the brush border depends primarily on the monomer concentration, uptake relative to the total bulk phase concentration will be very low, so that the amount of adherent mucosal fluid will account for a very large percentage of the total mass analyzed in the sample.

An ideal marker of adherent fluid volume should be totally excluded from the cell, i.e., membrane penetration should approximate zero and should not be susceptible to metabolic or physical alteration during the course of a study. As detailed in Tables 1 and 2, several markers of differing molecular weights are available that are satisfactory for measuring the adherent fluid volume. The exception is [^{3}H]inulin, which consistently gives a higher apparent volume. This is very likely due to rapid exchange of labeled tritium with tissue protons; in any event, this substance is not a satisfactory marker because of this problem.

In the presence of an unstirred water layer, use of a nonabsorbable marker becomes somewhat complex, and careful evaluation of time courses is required to avoid serious artifacts in the data. The importance of these possible artifacts is illustrated diagrammatically by the curves in Figs. 2 and 5. As shown in Fig. 2*A*, for example, it is apparent that 3 min is required for inulin to label the adherent mucosal fluid uniformly. The shape of this curve probably is determined by the time required for the marker to diffuse uniformly throughout the unstirred water layer on the tissue. For an unstirred layer thickness of 200 μm, it will take 1.27 min to achieve a concentration of inulin at the membrane surface of one-half the bulk phase concentration,

so that only after a 3- to 4-min incubation will the measured adherent mucosal fluid volume equal more than 90% of the true volume.[1] After a 1-min incubation, the measured volume is only about 50% of the true volume.

As shown in Fig. 5*B*, the size of the marker molecule relative to that of the test substance must also be taken into consideration. The heavy line represents the estimate of uptake anticipated in the absence of an unstirred layer where the only deviation from linearity is the falloff at long time periods due to efflux of the compound into the serosal compartment. Curve *I*, on the other hand, shows the theoretical uptake anticipated in the presence of an unstirred layer. There is a delay in time before the curve becomes linear that is directly proportional to the thickness of the unstirred layer and inversely proportional to the free diffusion constant of the test molecule.

Curves *II* and *III* represent the extreme situations that may be encountered experimentally that result from grossly dissimilar rates of diffusion between the marker and the test material. If the marker does not diffuse through the unstirred layer at a rate identical with that of the test compound, an inappropriate correction may be made. Curve *II* describes the situation in which uptake of a rapidly diffusing compound is measured using a slowly diffusing adherent fluid marker. During short time periods the apparent volume of adherent mucosal fluid is greater for the test compound than for the marker, and the true correction is underestimated. Uptake rates determined at short incubation times, therefore, will be artifactually high. In contrast, a slowly diffusing test molecule paired with a rapidly diffusing marker (curve *III*) will give negative rates of uptake at short incubation times. These artifacts are important to consider, since only certain time periods are appropriate for measuring uptake. With the present method, incubation periods of at least 3 min are necessary so that the adherent mucosal fluid volume will be accurately measured. On the other hand, incubation periods of greater than 5 min should be avoided, since estimates of uptake rates decrease as the compound leaks into the serosal fluid.

Since we have occasionally seen both artifactually low and high uptake rates at very short incubation times (< 2 min), it is critical to include time-course validation studies with each uptake experiment, especially when short incubation periods are used. Again, this becomes particularly important when studying uptake of a lipid from a large, expanded, mixed micelle that only slowly diffuses across the unstirred layer.

With in vitro uptake studies, micellar solutions with high concentrations of bile acids often are used. The question arises whether such solutions might alter the permeability properties of the membrane. This effect has been reported but always after much longer incubation times than were used here (12). Two direct pieces of information from the present studies show that short periods of exposure to high concentrations of bile acids do not damage the membrane. First, Fig. 2*B* shows that the distribution volume of inulin does not depend on the taurocholate concentration, even up to 20 mM. Similar results have been obtained with taurodeoxycholate. The second piece of evidence is that the uptake rate of heptanoic acid, a water-soluble, short-chain fatty acid, is not altered by the presence of 20 mM taurodeoxycholate. Thus, the presence of the bile acid neither induces membrane penetration of inulin nor alters the permeation rate of fatty acid.

Finally, as shown in Fig. 3, using the described counting techniques and correction for adherent fluid volume, uptake rates were determined using a bile acid and several fatty acids as test molecules. The experiments illustrated in these three panels demonstrate that under the conditions of these experiments tissue uptake is linear with respect to tissue weight, to time, and to the concentration of the test molecule in the mucosal medium. These three results verify that experimental conditions appropriate for measurement of tissue uptake rates have been established.

Several other points warrant emphasis, however. First, since we have demonstrated a significant unstirred layer in the intestine (8), one would anticipate that the curve describing tissue uptake as a function of time would not extrapolate linearly to zero but would show an initial curvilinear portion with displacement of the line to the right as shown by curve *I*, Fig. 5*B*. However, the diffusion constants for small molecules such as fatty acids and bile acids are high enough so that the unstirred layer reaches equilibrium quickly, and it can be calculated that little displacement of the curve would be anticipated.[2] This is precisely what is found in the experimental data shown in Fig. 3*B*, where the linear curves essentially do extrapolate to zero. Thus, in this circumstance it is appropriate to measure tissue uptake at some arbitrary time, 3 or 4 min, and divide by time to obtain values for apparent permeability coefficients with the units nmoles/min/

[1] This calculation was made (11) assuming a free diffusion coefficient for inulin at 37°C of 2×10^{-6} cm²/sec.

[2] For a 200-μm unstirred layer, a compound with a free diffusion coefficient of 1.4×10^{-5} cm²/sec will achieve one-half the bulk phase concentration at the membrane after 11 sec. This short time for diffusion will result in a time lag of uptake of only about 15 sec. This short delay is insignificant for an uptake measurement period of 3 or 4 min.

100 mg of tissue (dry wt) when the tissue is exposed to a substrate concentration of 1 mM.

Conversely, if the compound whose uptake is being measured diffuses through the unstirred layer very slowly, such as a large mixed micelle, there will be a significant displacement of the uptake time course to the right (as in curve *I*, Fig. 5*B*). Under these conditions, uptake must be determined at several times, allowing determination of the actual slope of the linear portion of the time course. In this circumstance, use of the double isotope method to correct measurements for adherent mucosal fluid, while theoretically not essential, is still of significant value. In our hands, the volume of adherent mucosal fluid in experimental determinations has a standard deviation of greater than 30% of the mean; thus, eliminating this source of variation in each experimental value allows a much improved accuracy of rate measurement.

Finally, it should also be pointed out that this method does measure transmembrane movement of the test molecule and not merely adsorption onto the cell surface. The observations that support this conclusion are that the time course does extrapolate to zero and does not have a positive intercept, as would be anticipated for binding, and that the temperature coefficients for the uptake of both fatty acids and bile acids are in the range of 1.2–1.4. This latter finding, again, is consistent with the passive movement of molecules across a lipid membrane but would not be expected for a simple binding phenomenon. In addition, the rate of uptake of long-chain fatty acids (as shown in Fig. 4) approximates the rate of appearance of fatty acid in intracellular triglyceride, and, further, the relative rates of uptake of different bile acids measured by this technique are identical with those determined by measuring the appearance of the bile acids in serosal fluid using other experimental procedures.[3]

[3] Schiff, G., and J. Dietschy. Unpublished observation.

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REFERENCES

1. Schedl, H. P., D. L. Miller, H. D. Wilson, and P. Flores. 1969. α-Aminoisobutyric acid transport and tissue concentration at various intestinal sites. *Amer. J. Physiol.* **216:** 1131–1138.
2. Gallagher, N. D., and M. R. Playoust. 1969. Absorption of saturated and unsaturated fatty acids by rat jejunum and ileum. *Gastroenterology*. **57:** 9–18.
3. Dietschy, J. M., H. S. Salomon, and M. D. Siperstein. 1966. Bile acid metabolism. I. Studies on the mechanisms of intestinal transport. *J. Clin. Invest.* **45:** 832–846.
4. Bennett Clark, S. 1971. The uptake of oleic acid by rat small intestine: a comparison of methodologies. *J. Lipid Res.* **12:** 43–55.
5. Schultz, S. G., P. F. Curran, R. A. Chez, and R. E. Fuisz. 1967. Alanine and sodium fluxes across mucosal border of rabbit ileum. *J. Gen. Physiol.* **50:** 1241–1260.
6. Schultz, S. G., and C. K. Strecker. 1971. Cholesterol and bile salt influxes across brush border of rabbit jejunum. *Amer. J. Physiol.* **220:** 59–65.
7. Umbreit, W. W., R. H. Burris, and J. F. Stauffer. 1957. Manometric Techniques. 3rd ed. Burgess Publishing Co., Minneapolis, Minn. 149.
8. Wilson, F. A., V. L. Sallee, and J. M. Dietschy. 1971. Unstirred water layers in intestine: rate determinant of fatty acid absorption from micellar solutions. *Science.* **174:** 1031–1033.
9. Stevens, A., E. Estrada, M. Pollay, and R. Kaplan. 1970. Efficient and rapid solubilization of biological material. *Anal. Biochem.* **37:** 1–10.
10. Pollay, M., and F. A. Stevens. 1970. Solubilization of animal tissues for liquid scintillation counting. *In* The Current Status of Liquid Scintillation Counting. E. D. Bransome, Jr., editor. Grune & Stratton, New York. 207–211.
11. Diamond, J. M. 1966. A rapid method for determining voltage-concentration relations across membranes. *J. Physiol.* (London). **183:** 83–100.
12. Feldman, S., and Gibaldi. 1969. Bile salt-induced permeability changes in the isolated rat intestine. *Proc. Soc. Exp. Biol. Med.* **132:** 1031–1033.