Absolute rates of cholesterol synthesis in rat intestine in vitro and in vivo: a comparison of different substrates in slices and isolated cells

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Abstract In an effort to localize small intestinal sterol synthesis both along the length of the intestine and along the villus–crypt axis, we defined the optimal conditions to measure absolute rates of digitonin-precipitable sterol (DPS) synthesis in whole intestinal slices and in isolated epithelial cells. When [1-^{14}C]acetate and [1-^{14}C]octanoate were compared in midgut slices, [1-^{14}C]octanoate was preferentially metabolized to CO_2 and DPS. Because of its rapid intramitochondrial degradation, [1-^{14}C]octanoate effectively swamped-out other sources of acetyl CoA (C_2) and achieved rates of C_2 flux into DPS virtually identical to the absolute rates determined with [^{3}H]water. On the other hand, unlabeled acetate decreased the apparent C_2 flux from [1-^{14}C]octanoate into DPS, but not into CO_2, in a dose-dependent fashion up to 8 mM. Under comparable incubation conditions, intestinal epithelial cells isolated by the EDTA chelation technique differed from slices in several respects. First, DPS synthesis from the various substrates was proportional to time only for 30 min compared to 90 min in slices. Second, the preferred ^{14}C-labeled substrate for DPS and CO_2 synthesis was acetate rather than octanoate. Third, neither of the ^{14}C-labeled substrates achieved the rates of synthesis found with [^{3}H]water. Fourth, sterol synthesis from any of the substrates was essentially zero in the absence of glucose in the incubation medium. When the optimal rates of sterol synthesis in vitro were estimated using [^{3}H]water in villus and crypt cell fractions of the jejunum and ileum, all ileal fractions were more active. However, ileal villus cells exceeded the jejunal villus cells by 5.2-fold in their capacity to incorporate [^{3}H]water into DPS, whereas the crypt cell fractions differed by only 1.4-fold. The majority of the sterol synthetic capacity resided in the lower villus region in both the proximal and distal intestine and the crypts accounted for about 30% and 14%, respectively, of the total found in the jejunum and ileum. A similar distribution pattern along both the vertical and horizontal axes of the intestine was found after [^{3}H]water administration in vivo, although under these conditions the proportion recovered in the crypts increased to 38% and 31%, respectively, of the total [^{3}H]DPS found in the jejunum and ileum. These studies demonstrate that [^{3}H]water yields optimal rates of sterol synthesis both in intestinal slices and in isolated epithelial cells and is incorporated into DPS mainly in cells of the lower villus and crypt region both in vitro and in vivo.—**Stange, E. F., and J. M. Dietschy.** Absolute rates of cholesterol synthesis in rat intestine in vitro and in vivo: a comparison of different substrates in slices and isolated cells. *J. Lipid Res.* 1983. **24:** 72–82.

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While it has been appreciated for years that the intestine is an important site for cholesterol synthesis, the exact quantitative role of this organ in total body sterol synthesis has been difficult to define (1–10). Although early studies in the rat identified the intestine as the single most active extrahepatic site for sterol synthesis (1–3), under in vitro conditions hepatic slices still were 5- to 7-times more active than intestinal slices in incorporating various radiolabeled substrates into digitonin-precipitable sterols (DPS) (7). More recent investigations performed in vivo, however, have demonstrated that of the total amount of [^{3}H]water incorporated into DPS by the whole rat, about 50% had taken place in the liver and 25% in the intestine (9). Furthermore, in other species such as the rabbit, guinea pig, hamster, and squirrel monkey, it now appears that the intestine may synthesize as much or even more sterol than the liver under in vivo conditions (10).

The reasons for this uncertainty concerning the quantitative role of the small bowel in whole body cholesterol synthesis, as well as for the apparent discrepancies that exist in identifying the specific cells that carry out such synthesis, are primarily the result of technical problems associated with measurements of rates of sterol synthesis. For example, a large body of previous work focused on measurements of the activity of 3-hydroxy-3-methylglutaryl CoA reductase (HMG CoA reductase) (5, 6, 11, 12). This approach was complicated by several problems. First, HMG CoA reductase is present in the mucosa of the small bowel in an interconvertible form which is subject to regulation by phosphorylation and dephosphorylation under in vitro conditions (13–16). It is not clear whether the "active" or

Abbreviations: DPS, digitonin-precipitable sterols; HMG, 3-hydroxy-3-methylglutaryl; SA, specific activity.

"total" enzyme activity reflects in vivo rates of cholesterol synthesis (17). Second, villus cell HMG CoA reductase appears to be inactivated by using a scraping technique to isolate the mucosal cells (18). Third, after subcellular fractionation of the mucosa, recovery of the enzyme activity in the microsomal fraction is incomplete (5, 6, 14, 15). Fourth, various HMG CoA cleavage enzymes present in the enterocyte compete for the substrate so that very rigorous identification of the product may be required if serious errors are to be avoided (6, 15).

There are similar problems associated with the assessment of rates of sterol synthesis based upon the incorporation of various radiolabeled substrates into cholesterol by whole cell preparations of intestinal tissue. Precursor molecules such as acetate, octanoate, or glucose may vary in their abilities to penetrate cell membranes and to be metabolized to acetyl CoA, or may undergo variable degrees of dilution of their specific activities (SA) within the cells (7, 19, 20). The importance of each of these factors conceivably varies among the different cell types in a single tissue like the intestine.

These methodological problems probably account for the confusion that exists with regard to the sites of intestinal cholesterol synthesis. Originally it was suggested that the cells of the intestinal crypt are the predominant site of mucosal cholesterol synthesis based upon rates of incorporation of [^{14}C]acetate into sterols (1) or activities of HMG CoA reductase (5). This view was challenged when the EDTA-chelation technique was used to isolate cell fractions enriched with either villus or crypt cells (6, 21) or after preservation of the activity of HMG CoA reductase in villus cells isolated by a scraping technique in the presence of a trypsin inhibitor (18). More recent work using other techniques has yielded similarly conflicting views (22–25).

Utilizing recently improved methods to isolate cell fractions from different levels of the villus–crypt axis (26) and to measure absolute rates of cholesterol synthesis in vivo (8, 9) and in vitro (7), the present studies were undertaken to investigate four specific problems. *1*) Optimal incubation conditions were systematically defined in rat midgut intestinal slices to allow measurement of absolute rates of sterol synthesis in vitro. *2*) In order to characterize the intracellular sites of sterol synthesis, the degree of competition of different carbon sources with [^{14}C]acetyl CoA for incorporation into DPS and CO_2 was examined. *3*) A method for isolating viable jejunal and ileal cell fractions was devised, and absolute sterol synthesis rates were measured in enriched villus and crypt cell fractions under defined, optimal in vitro conditions. *4*) In a final set of experiments the content of sterol newly synthesized in vivo was measured in cells isolated from different areas along the villus–crypt axis.

MATERIALS AND METHODS

Animal preparations

Female Sprague-Dawley derived rats (Charles River Breeding Laboratories, Inc., Wilmington, MA), originally purchased in the weight range of 125–150 g, were kept in a room with alternating 12-hr periods of light (1500–0300 hr) and darkness (0300–1500 hr), and were fed plain rat chow (Wayne Laboratory Animal Diets, Allied Mills, Inc., Chicago, IL) and water ad libitum for at least 2 weeks before being used in experiments.

All studies were carried out using fed animals at the mid-dark phase of the light cycle. In the in vitro protocol, the animals were killed with ether and the small intestine was removed at the mesenteric border. In the in vivo protocol, 50 mCi of [^{3}H]water was injected intravenously into the femoral vein. The intestine was removed 60 min later after the animals were bled from the abdominal aorta for determination of the SA of plasma water (8).

Tissue and cell preparation

The small bowel was chilled and rinsed extensively with 0.9% saline to wash out luminal contents. The duodenum was discarded and the gut was divided into three segments designated midgut (5 cm), jejunum, or ileum. The midgut segment was sliced (0.8 mm thickness) and washed in Krebs' bicarbonate buffer gassed with 95% 0_2:5% CO_2, and weighed aliquots were taken for the incubation (7).

Enriched isolated epithelial cell fractions from along the villus–crypt axis were prepared according to Weiser (26) as described recently with some modifications. The jejunal segment was carefully everted on a glass rod, filled in the everted state with 0.9% saline through a syringe tied to its end (allowing continuous pressure adjustment) and immersed in the isolation buffers (previously gassed with O_2). In contrast, the ileal segment was directly filled with the respective gassed buffers and immersed in 0.9% saline. The plastic beakers with the gut loops were incubated at 37°C in a Dubnoff metabolic shaker at 80 oscillations per min in two different buffer systems: buffer A (pH 7.3) contained KCl (1.5 mM), NaCl (96 mM), sodium citrate (27 mM), KH_2PO_4 (8 mM), Na_2HPO_4 (5.6 mM), glucose (11.1 mM), and dithiothreitol (1 mM); and buffer B (pH 7.2) contained KCl (2.7 mM), NaCl (137 mM), KH_2PO_4 (1.5 mM), Na_2HPO_4 (4.3 mM), EDTA (1.5 mM), glucose (11.1 mM), and dithiothreitol (1 mM). Glucose was omitted in some experiments as indicated. The jejunal and ileal segments were immersed in or filled with buffer A for 15 min and, subsequently, with buffer B for various periods of time. These times were adjusted according

to the appearance of marker enzymes from released cells in the buffer (see Fig. 5). After collection of the cell fractions in buffer B in plastic tubes, they were immediately pelleted at 350 *g*, resuspended in ice-cold 0.9% saline and kept in ice until the final fraction, i.e., the crypts, was recovered. All fractions again were centrifuged at 350 *g* and resuspended in Krebs' bicarbonate buffer gassed with 95% O_2:5% CO_2. Aliquots were stained with 0.2% nigrosin to check cell viability, and fractions with at least 90% exclusion of the dye were incubated as described below. In the in vivo protocol, the cells containing the ^{3}H-labeled DPS were washed in 0.9% saline and taken directly for determination of ^{3}H-labeled DPS and protein content.

Incubations

Under standard conditions, 300 mg (wet weight) of midgut slices or 2–5 mg (protein) of epithelial cells were incubated in 5 ml of pre-gassed, Krebs' bicarbonate buffer at 37°C in a Dubnoff metabolic shaker (7). In specific experiments, Na acetate, Na octanoate, or glucose was present in the buffer and, in addition, either [^{3}H]water (20 mCi per flask), [1-^{14}C]acetate (2 μCi per flask), or [1-^{14}C]octanoate (2 μCi per flask) was added to the incubation medium. The amount of each of these substrates used in specific experiments is given in detail in the legends to the table and figures.

Enzyme assays

To define the enterocyte fractions isolated after various time intervals, alkaline phosphatase and sucrase were determined as marker enzymes for differentiated villus cells, whereas thymidine kinase was regarded as an indicator for crypt cells (26). For these assays the freshly prepared cell fractions were homogenized with a Braun Melsungen Sonicator (Melsungen, FRG) at 100 watts for 30 sec. Alkaline phosphatase was assayed as suggested by Weiser (26). Sucrase was measured according to Messer and Dahlqvist (27). The procedure for the thymidine kinase assay based on the method of Klemperer and Hayes (28) and Breitman (29) was described previously (6).

Chemical procedures

After completion of the incubations, the CO_2 was collected from those flasks containing ^{14}C-labeled substrates (30). Six ml of alcoholic KOH was added, the flasks were incubated on a steam bath for 2 hr to saponify the lipids, and the sterols were precipitated as the digitonides (30). In the experiments employing [^{3}H]water, the digitonides were split with pyridine and the free sterols were extracted into diethyl ether (7, 8). A small amount of radioactivity was also recovered in parallel incubations with 0.5 mg of carrier cholesterol and was subtracted as a background. Tissue or cell protein was estimated using the procedure of Lowry et al. (31).

Calculations

For the determination of intestinal sterol synthesis in vitro, corrections for the differential loss in radioactivity during the synthesis of DPS were made, allowing the calculation of "normalized" C_2 fluxes (7). The incorporation rates for the ^{14}C-labeled substrates were expressed as the nmol of C_2 units (i.e., acetyl CoA units) incorporated per g of tissue wet weight or per mg of cell protein per hr. Therefore, the incorporation rates were calculated using the specific activities of the substrates present in the incubation buffer and corrected by the factor of 1.5 for losses during sterol synthesis. With [1-^{14}C]octanoate, the result was also multiplied by 4 to correct for the number of C_2 units maximally generated from this 8-carbon fatty acid.[1] Assuming a H/C incorporation ratio of 0.69 in the intestine (7), we used the reciprocal value of 1.45 as the factor to calculate the theoretical C_2 flux from [^{3}H]water into DPS. In the in vivo experiments these same rates were normalized to mg of cell protein per hr. The specific activity of plasma water was calculated as previously described (8).

In all instances, the data are given as means ± 1 SEM.

RESULTS

Studies with Intestinal Slices

Initial studies were undertaken to define the optimal incubation conditions for measuring rates of in vitro synthesis of sterols and CO_2 in midgut slices utilizing various radiolabeled substrates. Under standard conditions, as defined in the legend to **Fig. 1,** the incorporation of [^{3}H]water, [1-^{14}C]acetate, and [1-^{14}C]octanoate into both DPS and CO_2 was essentially a linear function with respect to the amount of tissue

[1] The factor of 4 assumes that the octanoate molecule is fully metabolized to four molecules of acetyl CoA so that the SA of the intracellular acetyl CoA pool equals one-fourth of the SA of the [1-^{14}C]octanoate. It is possible, however, that a fraction of the octanoate molecules may be metabolized only as far as the terminal 4-carbon unit. If this is the case then the SA of the acetyl CoA pool generated from the [1-^{14}C]octanoate would be somewhat greater than one-fourth of the SA of the [1-^{14}C]octanoate and the proper factor to be used in these calculations would be somewhat less than 4. However, in the liver cell it has been shown directly that the ^{3}H/C incorporation ratio equals 0.69 (7), a value that is only slightly higher than the value of 0.62 that would be expected if the octanoate were fully metabolized to acetyl CoA and the reductive H of NADPH were fully equilibrated with the ^{3}H of the [^{3}H]water. Thus, if a portion of the [1-^{14}C]octanoate is not fully metabolized, then that portion is so small as to be nearly undetectable so that the factor of 4 is appropriate. Unfortunately, no such data are available for the intestine so that it is conceivable that the proper factor in these calculations is slightly less than 4.

placed in each incubation flask between 100 and 400 mg (panels A, C and E, Fig 1) and with respect to the time of incubation of the flasks between 20 and 90 min (panels B, D and F). In other studies not shown in Fig 1, the thickness of the intestinal slices was varied between 0.3 and 3.0 mm. At 0.8 mm the incorporation of the radiolabeled substrates into DPS and CO_2 was maximal (100%) but the incorporation into DPS decreased to 39% and 42%, and into CO_2 declined to 72% and 69% of these values in slices that were 0.5 and 2.0 mm thick, respectively. These latter data were obtained with [1-^{14}C]octanoate. In still other studies it was shown that metabolic shaker rates either greater or slower than 120 oscillations per min resulted in less than optimal

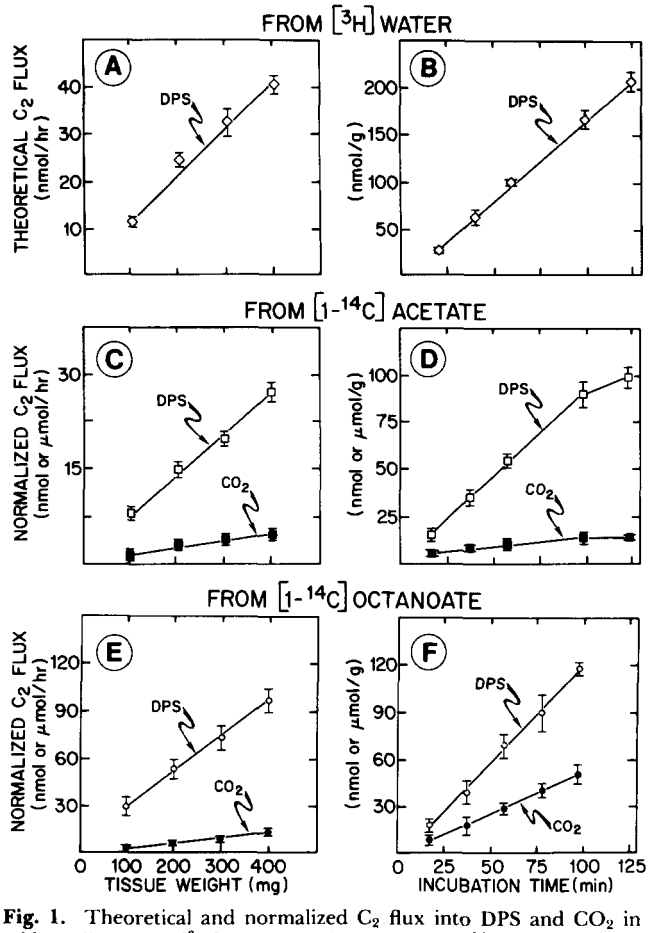

Fig. 1. Theoretical and normalized C_2 flux into DPS and CO_2 in midgut slices from [^{3}H]water (panels A and B), [1-^{14}C]acetate (C and D), or [1-^{14}C]octanoate (E and F) as a function of tissue weight (A, C, and E) or incubation time (B, D and F). In panels A, C, and E, varying amounts of tissue (0.8 mm slice thickness) were incubated for 90 min in 5 ml of Krebs' bicarbonate buffer with no glucose added using a shaker speed of 120 oscillations per min. Each flask contained 20 mCi of [^{3}H]water (A and B), 2 μCi of [1-^{14}C]acetate and 6 mM unlabeled acetate (C and D), or 2 μCi of [1-^{14}C]octanoate and 1.5 mM unlabeled octanoate (E and F). In panels B, D, and F, the incubation conditions were identical except that 300 mg of slices were incubated for varying periods of time. Mean values ± 1 SEM are shown for three different pools of intestinal slices.

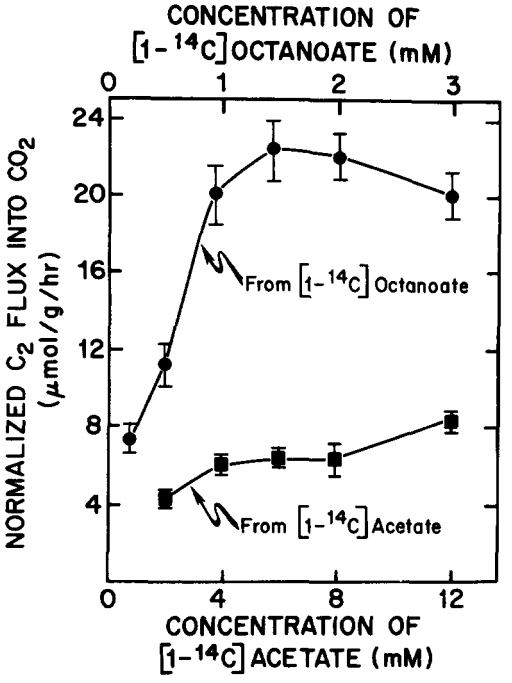

Fig. 2. Normalized C_2 flux into CO_2 (μmol/g per hr) from varying concentrations of [1-^{14}C]acetate and [1-^{14}C]octanoate. Three hundred mg of midgut slices were incubated for 90 min, otherwise the incubation conditions were identical as described in the legend to Fig. 1. Mean values ± 1 SEM are shown for three different pools of intestinal slices.

incorporation rates. Hence, in most subsequent experiments utilizing radiolabeled water, acetate, or octanoate as the precursor molecule, 300 mg of intestinal slices (0.8 mm thick) were incubated for 90 min at 37°C using a shaker rate of 120 oscillations per min.

The next series of investigations was to define how incorporation rates into DPS and CO_2 varied with the concentration of the radiolabeled substrates in the incubation medium. As shown in **Fig. 2,** the normalized C_2 flux into CO_2 varied as a function of the concentration of both [1-^{14}C]acetate and [1-^{14}C]octanoate. The oxidation of radiolabeled acetyl CoA units derived from [1-^{14}C]octanoate increased with increasing octanoate concentrations in the medium until a maximum value was achieved at about 1.5 mM. In contrast, the oxidation of radiolabeled acetyl CoA units derived from [1-^{14}C]acetate took place at rates that were only about one-fourth of those seen with octanoate and, furthermore, the rates continued to rise throughout the range of acetate concentrations used in the incubation medium. Clearly, octanoate could penetrate the cell and mitochondrial membranes and be oxidized to CO_2 at rates that were far in excess of those achieved with acetate.

More important, however, was a comparison of the rates of incorporation of these substrates into DPS, a process that takes place exclusively in the cytosolic compartment, at least in the liver. As is evident in panel A

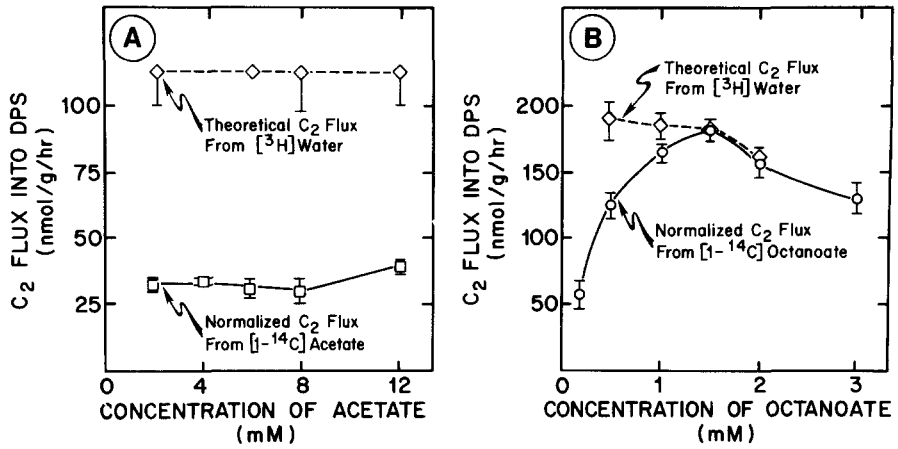

Fig. 3. Theoretical C_2 flux (nmol/g per hr) into DPS from [^{3}H]water (panels A and B) and normalized C_2 flux from [1-^{14}C]acetate (panel A) and [1-^{14}C]octanoate (panel B) at varying substrate concentrations. The incubation conditions were identical to those in Fig. 1 except that 300 mg of slices were incubated for 90 min. Mean values ± 1 SEM are shown for three different pools of intestinal slices.

of **Fig. 3,** the normalized C_2 flux into DPS from [1-^{14}C]acetate reached no more than 34% of the rates achieved with [^{3}H]water, regardless of the concentration of acetate added to the incubation medium between 2 and 12 mM. The high concentrations of acetate, however, were not toxic to the intestinal slices in that no suppression of [^{3}H]water incorporation into DPS was seen even at the highest concentration used. In contrast, as shown in panel B, the incorporation of radiolabeled acetyl CoA units derived from [1-^{14}C]octanoate into DPS increased with increasing concentrations of octanoate and, at 1.5 and 2.0 mM, these rates actually equalled the absolute rate of incorporation of C_2 units measured with [^{3}H]water. However, at concentrations

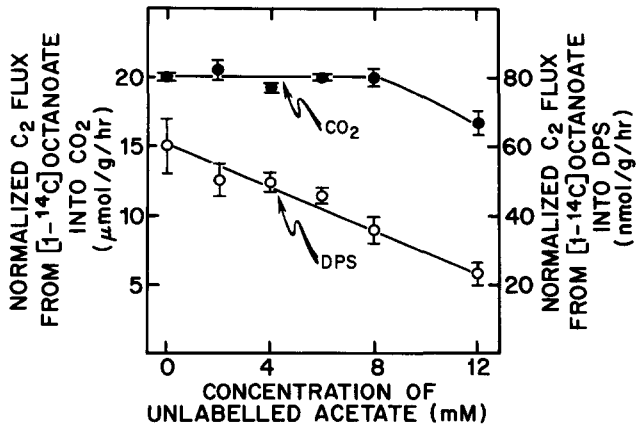

Fig. 4. The effect of varying concentrations of unlabeled acetate on the incorporation of [1-^{14}C]octanoate into CO_2 and DPS. The flasks contained 2 μCi of [1-^{14}C]octanoate and 1.5 mM unlabeled octanoate, and concentrations of Na acetate that varied from 0 to 12 mM. Three hundred mg of midgut slices were incubated for 90 min, otherwise the incubation conditions were identical to those described in the legend to Fig. 1. Mean values ± 1 SEM are shown for three different pools of intestinal slices.

of octanoate above 1.5 mM there clearly was inhibition of sterol synthesis from both substrates. This inhibition presumably was attributable to the detergent effect of this medium chain-length fatty acid.

In a final experiment with intestinal slices, the ability of unlabeled acetate to dilute out the flow of radiolabeled acetyl CoA units from [1-^{14}C]octanoate into DPS and CO_2 was examined. As seen in **Fig. 4,** the addition of increasing concentrations of unlabeled acetate, from 0 to 8 mM, had virtually no effect on the flux of labeled acetyl CoA units into CO_2. In contrast, the flux of C_2 units from [1-^{14}C]octanoate into DPS was progressively swamped-out as the concentration of unlabeled acetate in the medium was increased. As in liver (19, 20) these results are consistent with the view that exogenous acetate can be activated to acetyl CoA within the cytosolic compartment. This, in turn, dilutes the SA of radiolabeled acetyl CoA that is the substrate for the synthesis of DPS and that ultimately is derived from the metabolism of [1-^{14}C]octanoate within the mitochondrial compartment (19). Relatively little exogenous acetate, however, enters the mitochondrial compartment to dilute the SA of that acetyl-CoA pool. This result supports the earlier experimental finding (Fig. 2) that [1-^{14}C]acetate incorporation into CO_2 is a poor measure of enterocyte oxidative activity.

From these various experiments we concluded that the use of [1-^{14}C]acetate would lead to gross underestimation of the rates of synthesis of DPS and of CO_2 production in intestinal slices because of low rates of cell penetration or activation of this substrate to acetyl CoA within the cytosol and/or because of significant intracellular dilution of the SA of the cytosolic acetyl CoA pool. However, with [1-^{14}C]octanoate the flux of radiolabeled acetyl CoA units into DPS actually equalled

the theoretical flux determined with [^{3}H]water at an octanoate concentration of 1.5 mM. Hence, at this concentration the rates of cell and mitochondrial membrane penetration and of the oxidation to acetyl CoA of this substrate were apparently so rapid that other intracellular sources of unlabeled acetyl CoA were swamped-out and an absolute measure of sterol synthesis was achieved. This is in contrast to the situation in liver slices where the C_2 flux from [1-^{14}C]octanoate into DPS equals only about 75% of the absolute rate of sterol synthesis because, presumably, of higher rates of unlabeled acetyl CoA generation from other substrates in the hepatocyte (7, 19, 20).

Isolated intestinal cells

The second group of studies was undertaken to define these same substrate relationships in isolated enterocytes from different regions of the intestine. In order to obtain comparable cell fractions from both the jejunum and ileum, it was found necessary to vary both the times and conditions of incubation of the intestinal mucosa in the chelating buffer (see Materials and Methods). As shown in **Fig. 5,** incubation of the jejunum for 10- or 20-min intervals up to a total of 120 min (panel A) and of the ileum up to a total of 80 min (panel B) yielded successive cell fractions with varying activities of the three marker enzymes, alkaline phosphatase, sucrase, and thymidine kinase. The SA of alkaline phosphatase decreased gradually in cells progressively harvested from both areas of the intestine so that this enzyme was 16.3- and 6.4-fold more active in the initial cell fraction than in the final cell fraction obtained from the jejunum and ileum, respectively. The pattern was somewhat different with respect to sucrase in that this enzyme manifested a relatively constant level of activity in most of the initial five- or six-cell fractions, but then also markedly declined in the final three fractions. In contrast, the SA of thymidine kinase increased by 9.2- and 7.4-fold in the final cell fraction, relative to the initial fraction, in the jejunum and ileum, respectively.

In subsequent experiments these various cell fractions were combined into four separate pools of cells designated as upper, mid and lower villus cells, and as crypt cells. These terms represented only a functional classification based upon the enzyme content of these groups of enterocytes, and probably should not be taken as a literal indication of the anatomic sources for the various cells. As also shown in Fig. 5, the bulk of the cellular protein harvested by these techniques was found in those fractions designated as mid and lower villus cells.

Experiments were next undertaken to establish the optimal incubation conditions for measuring rates of sterol synthesis in such isolated cells. These studies were

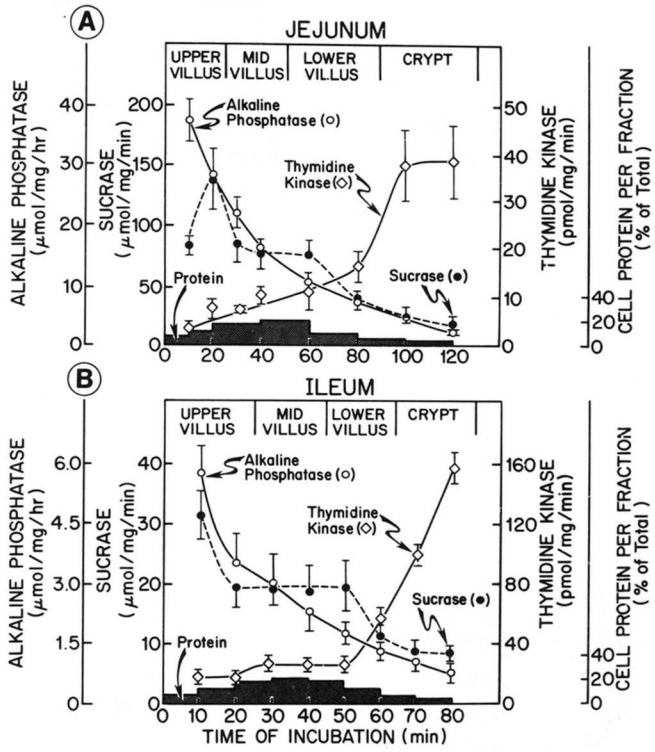

Fig. 5. The effect of varying the time of incubation on the types of cells obtained from the intestine by the chelation technique in the jejunum (panel A) and ileum (panel B). The specific activity of alkaline phosphatase and sucrase are given at the various time points and were used as marker enzymes for differentiated villus cells, while thymidine kinase activity was used as an indicator of crypt cells. The shaded areas at the bottom of the diagrams represent the amount of protein recovered in the sequentially obtained fractions expressed as a percentage of the total isolated protein. The detailed procedure for the fractionation technique is given in Materials and Methods. Mean values ± 1 SEM are shown for five rats.

undertaken using a large pool of isolated enterocytes obtained from both jejunal and ileal villi and crypts. As shown in **Fig. 6,** the incorporation of [^{3}H]water, [1-^{14}C]acetate, and [1-^{14}C]octanoate into DPS was linear with respect to the amount of cell protein in each flask up to 4.7 mg (panels A, C and E). However, in contrast to the slices of whole intestine (Fig. 1), the incorporation of these substrates into DPS was proportional to the time of incubation for only about 30 min (panels B, D, and F). Similarly, CO_2 production from the [^{14}C]substrates was not linear with respect to time except for only very short periods of incubation. Furthermore, in other studies it was shown that regardless of the substrate used, isolated cells were more vulnerable to vigorous shaking than intestinal slices so that the highest rates of CO_2 and DPS synthesis were found at shaker rates of 80, rather than 120, oscillations per min. Thus, in all subsequent experiments using isolated intestinal cells, up to 5 mg of enterocyte protein was incubated for 30 min at 37°C using a shaker rate of 80 oscillations per min.

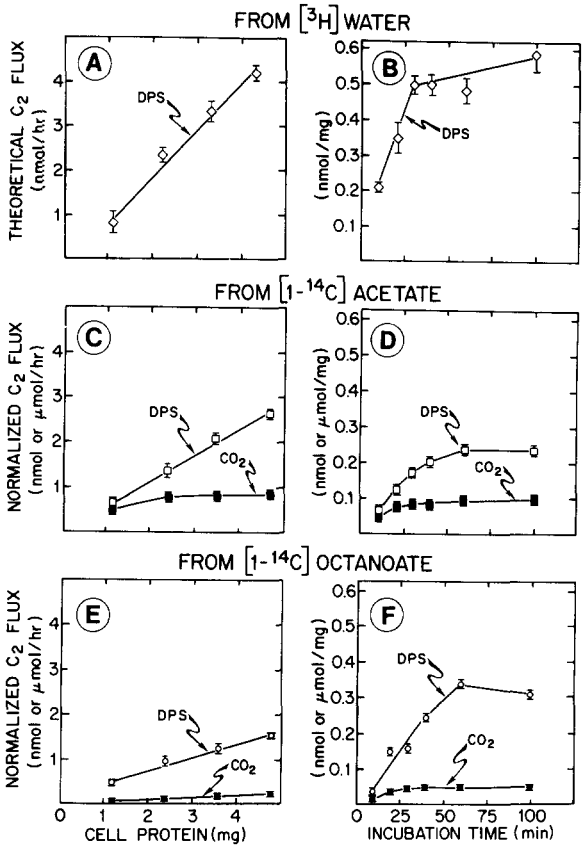

Fig. 6. Theoretical and normalized C_2 flux into DPS (nmol/mg protein per hr) or CO_2 (μmol/mg protein per hr) in pooled jejunal and ileal epithelial cells from [^{3}H]water (panels A and B), [1-^{14}C]acetate (C and D), and [1-^{14}C]octanoate (E and F) as a function of the amount of cell protein (A, C, and E) or the incubation time (B, D, and F). In panels A, C, and E, varying amounts of cells were incubated for 30 min in 5 ml of Krebs' bicarbonate buffer at a shaker speed of 80 oscillations per min. Each flask contained 20 mCi of [^{3}H]water (panels A and B), 2 μCi of [1-^{14}C]acetate and 6 mM unlabeled acetate (C and D), or 2 μCi of [1-^{14}C]octanoate and 1.5 mM unlabeled octanoate (E and F). In panels B, D, and F, incubation conditions were identical, except that 4 mg of cell protein were incubated for various periods of time. Mean values ± 1 SEM are shown for three different pools of cells.

Using these incubation conditions, experiments were next performed to characterize rates of DPS synthesis and CO_2 production by this pool of isolated enterocytes at various concentrations of the two ^{14}C-labeled substrates. Furthermore, in order to be able to directly compare these rates (expressed per mg protein of isolated cells) with the rates obtained in slices (expressed per g wet weight of tissue) it was determined that the average protein content of 1 g (wet weight) of intestinal cells equalled 37 mg.

As seen in **Fig. 7,** the isolated intestinal cells behaved very differently from the intestinal slices, with regard to both sterol synthesis and CO_2 production. Unlike intestinal slices (Fig. 2), CO_2 production from [1-^{14}C]acetate by the enterocytes was very dependent upon the concentration of acetate present in the incubation medium and greatly exceeded the rates of oxidation seen with [1-^{14}C]octanoate (panel A). Furthermore, CO_2 production from both substrates was greatly enhanced by the addition of 11.1 mM glucose to the incubation medium: this occurred, it should be noted, despite the possibility that such unlabeled glucose might generate unlabeled acetyl CoA in the cells and so dilute out the flow of radiolabeled acetyl CoA units into CO_2.

When assayed under the most optimal conditions (1.17 mg of enterocyte protein incubated for 30 min in the presence of glucose), the normalized C_2 flux into CO_2 in the isolated cells was equivalent to 20.85 ± 0.55 and 2.11 ± 0.44 μmol per g wet weight of intestinal cells per hr from [1-^{14}C]acetate and [1-^{14}C]octanoate, respectively. Thus, the isolated cells behaved very differently from the intestinal slices and yielded rates of CO_2 production from [1-^{14}C]acetate (about 21 μmol/g per hr) that were 3.5-fold higher than those seen in the slices (about 6 μmol/g per hr, Fig. 2) and that, in fact, essentially equalled the C_2 flux into CO_2 from [1-^{14}C]octanoate (about 22 μmol/g per hr). In contrast, the rates of oxidation of [1-^{14}C]octanoate by the isolated cells occurred at only one-tenth the level seen in the slices.

The "protective" effect of glucose on rates of DPS synthesis was even more pronounced. In its absence there was no measurable incorporation of any of the precursors, including [^{3}H]water, into DPS (panel B, Fig. 7). In the presence of glucose, acetyl CoA generated from [1-^{14}C]acetate was again preferentially utilized over that generated from [1-^{14}C]octanoate for the synthesis of DPS, regardless of the concentration of acetate of octanoate present in the incubation medium. Nevertheless, even at the optimal concentration of [1-^{14}C]acetate, the absolute C_2 flux into DPS equalled only about 77% of the absolute rate of DPS synthesis measured with [^{3}H]water.

When the rates of DPS synthesis in the cells were again normalized to the equivalent units used with the slices, the C_2 flux into DPS equalled 11.86 ± 0.53 nmol/g per hr from [1-^{14}C]octanoate, 21.61 ± 5.09 nmol/g per hr from [1-^{14}C]acetate, and 28.08 ± 1.85 nmol/g per hr from [^{3}H]water. These rates equalled only 6.7%, 64.2%, and 26.9%, respectively, of the rates found with the same substrates in midgut slices. These direct comparisons between synthesis rates in cells and slices were calculated by relating rates found in the isolated cells with the respective rates obtained in slices at the same substrate concentrations (Fig. 3). However, the rate of DPS synthesis from [^{3}H]water by intestinal slices was determined in a separate experiment in which tissue from four rats was incubated in the presence of 11.1 mM glucose and equalled 104.5 ± 7.4 nmol/g per hr. It is noteworthy that this value was not different from the rate found in the absence of glucose (Fig. 1).

Finally, to ascertain the completeness of removal of

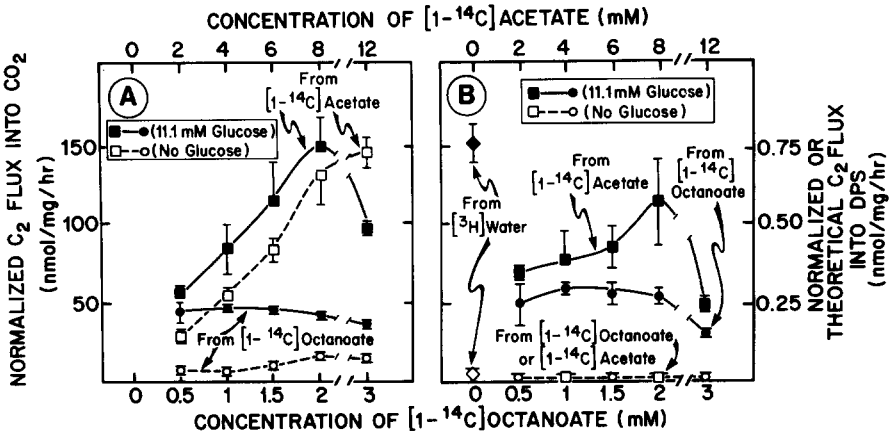

Fig. 7. Normalized C_2 flux (nmol/mg protein per hr) from [1-^{14}C]acetate and [1-^{14}C]octanoate into CO_2 (panel A) and the theoretical and normalized C_2 flux from [^{3}H]water and [1-^{14}C]acetate or [1-^{14}C]octanoate into DPS (panel B) by pooled jejunal and ileal epithelial cells at varying substrate concentrations. Four mg of cell protein were incubated for 30 min at a shaker rate of 80 oscillations per min. Mean values ± 1 SEM are shown for three different pools of cells.

the mucosal cells by this chelation technique, the theoretical C_2 flux into DPS by the remnant of the intestine were measured and was found to equal 12.6 and 2.4 nmol/g per hr (means of two experiments) in the jejunum and ileum, respectively. These rates equal only 4.3% and 0.8% of those found in midgut slices and suggest that the chelation technique nearly completely denuded the gut wall of mucosal cells.

On the basis of the information obtained from these various experiments, it was apparent that neither [1-^{14}C]acetate nor [1-^{14}C]octanoate gave rates of synthesis in isolated intestinal cells that were equal to those absolute rates achieved with [^{3}H]water. Hence, the final set of studies designed to localize the sites of sterol synthesis in the intestine under both in vitro and in vivo conditions was undertaken using this radiolabeled precursor. As shown in **Table 1**, in fractions of enterocytes incubated with [^{3}H]water in vitro, the synthesis of DPS, expressed per mg of cell protein, increased slightly in going from the cells of the upper villus to those of the crypt in the jejunum. However, when multiplied by the mass of protein recovered in each of these fractions, nearly 70% of the synthetic activity was found in cells from the lower villus and crypt region. In these same cell fractions isolated from the ileum and incubated in vitro with [^{3}H]water, the rates of DPS synthesis were approximately 4-fold higher in cells harvested from all levels of the villus while the rate of incorporation of [^{3}H]water into DPS in the crypt cells more closely approximated that rate found in the crypts of the jejunum.

TABLE 1. In vitro and in vivo rates of sterol synthesis in villus and crypt cell fractions from the jejunum and ileum

Cell Preparation	Jejunum		Ileum	
	In vitro	In vivo	In vitro	In vivo
	nmol/mg protein per hr		*nmol/mg protein per hr*	
Upper villus	0.183 ± 0.026 (10.17%)	1.13 ± 0.43 (9.36%)	0.652 ± 0.212 (7.97%)	2.89 ± 0.34 (3.98%)
Mid villus	0.187 ± 0.031 (21.74%)	1.42 ± 0.37 (21.05%)	0.978 ± 0.252 (24.97%)	4.06 ± 0.46 (11.50%)
Lower villus	0.217 ± 0.039 (38.60%)	1.85 ± 0.66 (31.53%)	0.828 ± 0.093 (53.13%)	6.43 ± 0.37 (53.39%)
Crypt	0.243 ± 0.062 (29.47%)	2.69 ± 0.66 (38.05%)	0.334 ± 0.088 (13.91%)	5.93 ± 0.86 (31.13%)

In these studies the rates of sterol synthesis were determined both in vitro and in vivo. In the in vitro studies the various cell fractions were first harvested, and the isolated cells were incubated with 20 mCi of [^{3}H]water per flask in Krebs' bicarbonate medium containing 11.1 mM glucose. In the in vivo experiments the animals were first administered 50 mCi of [^{3}H]water intravenously and the cells were then harvested 60 min later. Each value represents the mean ± 1 SEM for results obtained in cells harvested from four animals. The percentage of the total synthetic activity found in each fraction is also given.

For comparison, the incorporation of [^{3}H]water into DPS next was allowed to take place in vivo, and then the same cell fractions were isolated. The results of these experiments also are shown in Table 1. Two major points warrant emphasis with regard to these data. First, at any level of the intestinal villus the absolute rates of [^{3}H]water incorporation into DPS were from 4- to 6-times higher in vivo than in vitro. Thus, as with most isolated cells or tissues, rates of synthesis measured in vitro are gross underestimations of the actual rates of synthesis occurring in the live animal (7–9). Second, despite these large differences, however, the distribution of relative synthetic activity among the different cell fractions was remarkably similar in the in vitro and in vivo studies. Thus, for example, in vivo from 70 to 84% of sterol synthetic activity was still found in the cells isolated from the region of the lower villus and crypt.

DISCUSSION

The anatomical and physiological structure of the bowel is complex with marked differences in cell morphology and function both horizontally along the length of the intestine and vertically along the axis of the villus. This raises the possibility that cholesterol synthesis in these different regions subserves different functions. The rates of sterol synthesis in the mature villus cells, for example, might be related to the amount of chylomicrons being synthesized (32), while in the crypt cells it may be the rate of cell division that dictates the rate of cholesterol biosynthesis (33).

The first group of studies in the present investigation characterized rates of sterol synthesis in slices of whole intestine. Previous work from this laboratory has documented the major quantitative errors that result from the use of ^{14}C-labeled substrates to measure rates of cholesterol synthesis in such tissue slices. In liver and in major extrahepatic tissues, for example, [1-^{14}C]acetate is incorporated into DPS at velocities that vary from 4% to 62% of the absolute rates, while [1-^{14}C]octanoate gives similar underestimates equal to 6% to 91% of the absolute rates, depending upon the tissue studied (7). After establishing optimal conditions for performing such studies in small intestinal slices (Fig. 1), similar findings were observed in the present studies. At most concentrations of octanoate and, particularly of acetate, the flux of C_2 units from the ^{14}C-labeled substrates into DPS and CO_2 grossly underestimated actual rates of sterol synthesis and CO_2 production (Figs. 2 and 3). Only when the measurements were made using [1-^{14}C]octanoate at a concentration of 1.5 mM were rates of sterol synthesis equal to those obtained with [^{3}H]water achieved (Fig. 3). It should be noted, however, that these absolute rates of sterol synthesis, whether determined with [1-^{14}C]octanoate or [^{3}H]water, equalled only about 120 to 180 nmol/g per hr, whereas rates in excess of 400 nmol/g per hr are routinely found under in vivo conditions (9, 10). Thus, as with nearly all other tissues, even absolute rates of sterol synthesis measured under in vitro conditions equal only a fraction of the rates observed in the same tissues in vivo (8–10).

With these issues defined, the second set of experiments was designed to evaluate these same parameters in isolated enterocytes. These studies were undertaken using cells isolated by the citrate-EDTA chelation technique since it provided the means for obtaining pools of enterocytes enriched with either mature villus cells or crypt cells (Fig. 5) in a very reproducible manner. In our hands, however, the standard technique reported by Weiser (26) was only successful in the ileum of the rat. Crypt cells from the jejunum were only incompletely released into the incubation medium. Thus, the technique was modified in that the jejunum was everted prior to incubation in the chelation buffers, as suggested by Raul et al. (34). Both methods provided "viable" isolated cells as judged by the conventional dye exclusion test, although for our purposes it was important to evaluate more critically the biochemical integrity of these isolated intestinal cells. In this regard, it should be noted that the metabolic parameters of CO_2 production and DPS synthesis measured in the isolated cells could be related to similar data obtained in the intestinal slices since essentially the same incubation conditions were employed in both.

From such comparisons it is evident that the isolated cells had very different characteristics from the cells of the intestinal epithelial surface present on the intestinal slices. First, the isolated cells were more fragile and performed metabolically relatively poorly under in vitro conditions. Thus rates of CO_2 production and DPS synthesis were linear with respect to time for only about 30 min (Fig. 6) and were inhibited by more vigorous mechanical shaking. While the decline in the rates of incorporation of the substrates into DPS could reflect inactivation of HMG CoA reductase when the isolated cells were incubated in the presence of bicarbonate ion (13), the parallel fall in the rates of oxidation of the ^{14}C-labeled substrates to CO_2 suggested a more generalized loss of cell viability under these conditions of incubation. Second, in the isolated cells, but not in the slices, the rates of ^{14}C-labeled substrate incorporation into both CO_2 and DPS were markedly dependent upon the presence of glucose (Fig. 7). While the reasons for this finding were not further explored, it is likely that during the preparation of the isolated enterocytes the cells became depleted of their stores of glycogen and glucose.

Under these conditions there presumably would develop a relative deficiency of oxalacetate (derived from pyruvate) intracellularly which, in turn, would severely limit the ability of the cells to oxidize [1-^{14}C]acetyl CoA to CO_2 within the mitochondria and to shuttle this radiolabeled acetyl CoA into the cytosolic compartment (via citrate) where cholesterol synthesis takes place. If this is the case, then incubation of the tissue with glucose (or with pyruvate) should restore oxalacetate concentrations to more normal levels and greatly enhance the incorporation of the ^{14}C-labeled substrates into both CO_2 and DPS, as was observed in these studies.

Finally, even under optimal conditions, there were also striking differences in the ability of isolated enterocytes and the intestinal slices to utilize acetate and octanoate. Unlike the intestinal slices, the highest rates of CO_2 production and DPS synthesis in the isolated cells were achieved with [1-^{14}C]acetate rather than with [1-^{14}C]octanoate. Whether this was due to a subtle toxic effect of octanoate on the isolated cells or to a specific, acquired defect in the mitochondrial oxidation of this fatty acid is not known. However, even the maximal rates of synthesis achieved with [^{14}C]acetate were only about 70% to 80% of the rates found with [^{3}H]water. Hence, in the isolated cells, unlike the intestinal slices, there was no ^{14}C-labeled substrate that yielded absolute rates of sterol synthesis, so only [^{3}H]water could be used for this purpose. Such absolute rates determined in isolated cells in vitro, however, again equaled only about one-tenth to one-fifth of the rates at which these same cells synthesized sterols in vivo (Table 1).

From this information it is apparent that any studies designed to measure the rates of sterol synthesis at different levels of the intestine, first, had to utilize [^{3}H]water and, second, had to be undertaken both in vitro and in vivo. When such studies were done, as summarized in Table 1, a number of important points became apparent. First, regardless of whether the [^{3}H]water was incorporated into DPS under in vitro or in vivo conditions, sterol synthesis in the cells isolated from the ileum was 3- to 4-times more active than in cells isolated from the jejunum. This confirms earlier reports in which rates of radiolabeled acetate or octanoate incorporation into DPS were measured in intestinal slices or biopsy specimens obtained from the rat, monkey, and man (1, 3, 35). Thus, these ^{14}C-labeled substrates gave relative rates of sterol synthesis that were correct even though they underestimated, to a variable degree, the absolute rates of synthesis. Second, the higher rates of sterol synthesis (per mg of cell protein) that were seen in cells from the distal half of the intestine were primarily attributable to much higher rates of synthesis in cells isolated from the intestinal villi than from the crypts. This would be consistent with the view that sterol synthesis in the mature villus cells of the jejunum was inhibited by dietary and biliary cholesterol that was being absorbed across the epithelial cell surface in this region of the bowel. In contrast, the rate of synthesis in the crypt cell layer was relatively uniform down the length of the intestine. However, when the amount of cell protein harvested from each area of the villus was taken into consideration, the vast majority of total intestinal sterol synthesis still occurred in the cells of the lower villus and crypt regions in both the jejunum and ileum. Third, in absolute terms, the incorporation of [^{3}H]water into DPS by enterocytes in vivo was 5- to 10-times higher than when these same cell fractions were incubated with [^{3}H]water under in vitro conditions. Despite these large differences in absolute values, however, the relative distribution of synthetic activity down the axis of the villus was very similar whether the measurements were carried out in vitro or in vivo.

In summary, these studies point out many potential sources of error in measuring rates of cholesterol synthesis under both in vitro and in vivo conditions using intestinal slices and isolated enterocyte fractions. The use of ^{14}C-labeled substrates in tissue slices and in isolated cells under in vitro conditions can lead to serious errors in both the absolute and relative values for rates of synthesis. The rates at which [^{3}H]water is incorporated into sterols appear to give similar relative values both in vitro and in vivo and probably represent the most reliable means for studying in detail the complex mechanisms of regulation of intestinal cholesterol synthesis.

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