Lipid synthesis in isolated intestinal cells

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Abstract Since the small intestine contributes significantly to serum cholesterol and very low density lipoprotein levels, acute regulation of lipid synthesis was investigated in isolated rat intestinal cells incubated in Krebs-Ringer bicarbonate buffer with 5 mM glucose and [^{14}C]acetate or $^{3}H_2O$. Incorporation of [^{14}C]acetate into cellular lipids was 6- to 8-fold greater in crypt than in villus cells. In both cell types the distribution of ^{14}C among the various lipid classes was as follows: 52.5% in triglycerides, diglycerides, and monoglycerides; 22.3% in cholesterol; 8.3% in cholesteryl esters; 1.9% in fatty acids; and 15.0% in phospholipids. In contrast, the medium lipids contained significantly higher amounts of tri-, di- and monoglycerides (61.1%) and lower amounts of cholesteryl esters (2.3%) and phospholipids (11.9%). After saponification, ⅔ of the recovered $^{3}H_2O$ was in fatty acids and ⅓ in cholesterol. Ethanol (10 mM) tripled $^{3}H_2O$ incorporation into cellular lipids but had no effect on [^{14}C]acetate incorporation. Epinephrine and norepinephrine (10 μM), glucagon (10 μM), dibutyryl cyclic AMP (1 mM), dexamethasone (1 mM and 1 μM), and cholera toxin (1 μg/ml) did not affect [^{14}C]acetate incorporation. We conclude that ethanol stimulates intestinal lipid synthesis; however, in sharp contrast to their inhibition of lipid synthesis in hepatocytes and adipocytes, catecholamines, glucagon, and dibutyryl cyclic AMP do not inhibit lipid synthesis in intestinal cells.

Supplementary key words cholesterol · triglycerides · phospholipids · villus and crypt cells · hormones · cyclic AMP

Liver, adipose tissue, and small intestine are the major contributors to the lipids and lipoproteins in the circulation. The liver is the most important site for the synthesis of cholesterol, fatty acids, and serum lipoproteins. Adipose tissue provides free fatty acids, a major energy source in the fasting state and a precursor of hepatic triglycerides secreted in very low density lipoproteins. In these two tissues considerable attention has been focused on the regulation of lipid synthesis by pituitary hormones, thyroxine, insulin, sex hormones, glucagon, and cyclic AMP (1–6).

Although the intestine is the sole extrahepatic site of plasma lipoprotein synthesis and second only to the liver in cholesterol synthesis (7, 8), little is known about the regulation of intestinal lipid synthesis. Significant advances have resulted from the use of isolated hepatocytes and adipocytes for studies of lipid synthesis (5, 9, 10); however, most of the limited studies on intestinal lipid synthesis have employed slices or segments of intestine (8, 11–13). The few investigators using isolated intestinal epithelial cells have prepared cells by a scraping technique (14) or by enzyme treatment (15–17). Weiser (18) has recently described the isolation of ultrastructurally intact intestinal cells by a method that does not involve the use of enzymes.

The main purpose of the present study was to investigate the suitability of isolated intestinal cells, prepared by Weiser's technique (18), for studies of the acute regulation of lipid synthesis in the small intestine. We have examined cell properties, the incorporation of [^{14}C]acetate and $^{3}H_2O$ into lipids, and the effects of various hormones and ethanol on label incorporation as a preliminary step toward understanding the regulation of lipid metabolism in rat small intestine by these substances (19).

MATERIALS AND METHODS

Materials

Sodium [1-^{14}C]acetate (2.55 mCi/mmol) and tritium-labeled water (100 mCi/g) were purchased from New England Nuclear (Boston, MA). Grade I sucrose, glucose oxidase, dianisidine, dithiothreitol, epinephrine, norepinephrine, *p*-nitrophenol, and *p*-nitrophenylphosphate were obtained from Sigma (St. Louis, MO). Horse radish peroxidase, grade D, was from Worthington Chemicals (Freehold, NJ). Tetrasodium salt of ethylene diamine tetraacetic acid (EDTA) and sodium acetate were from J. T. Baker Chemicals (Phillipsburg, NJ). Dibutyryl 3′:5′cyclic AMP, monosodium salt, and 3′:5′cyclic AMP were bought from Plenum Scientific Research, Inc. (Hackensack, NJ). Fatty acid-poor bovine albumin was obtained from Accurate Chemical and Scientific Corp. (Hicksville, NY). Glucagon was a gift from Dr. Otto

Abbreviation: cyclic AMP, cyclic adenosine 3′:5′-monophosphate.

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Preparation of intestinal cells

Male Sprague-Dawley albino rats, fed ad lib and weighing 175–250 g, were killed by cervical dislocation between 9 and 10 AM and the entire length of the small intestine was removed. Isolated intestinal cell preparations were made according to the method of Stern (20) as modified by Weiser (18). This method was changed slightly by using phosphate-buffered saline with 1 mM dithiothreitol for the initial rinsing of the small intestine. After thoroughly washing the intestine with this solution, the intestine was filled for 15 min with a phosphate buffer containing citrate. After discarding this solution, the intestine was filled for various periods of time with phosphate-buffered saline containing EDTA and dithiothreitol as described by Weiser (18). During these washings care was taken to avoid overmanipulation of the gut. By this method nine sequential fractions of intestinal cells were obtained. Cells were subsequently washed by gently suspending them in phosphate-buffered saline followed by centrifugation at 900 *g* for 3 min in an International Clinical centrifuge (Model CL). Subsequently the cells were either resuspended in phosphate-buffered saline for enzyme analysis or in Krebs-Ringer bicarbonate buffer for those experiments involving lipid synthesis. Cell fractions were pooled for studies of cellular lipid synthesis. Cell fractions 1, 2, and 3 were mixed together to examine upper villus cell function. Fractions 5, 6, and 7 were pooled as middle villus cells and fraction 9 was used for crypt cell activity. The cells were counted in a hemocytometer and cell viability was determined by exclusion of Nigrosin dye (21). Only those cells that excluded the dye were included in the cell counts for each incubation flask.

Enzyme analysis. Villus cells were distinguished from crypt cells by measuring the activities of alkaline phosphatase and sucrase. Both alkaline phosphatase and sucrase activities were assayed after disrupting the cells in a sonifier cell disruptor for 0.5 min at an output control of 3 (Model W 185 D-Heat Systems, Ultrasonics, Inc., Plainview, NY). The completeness of cell disruption was evaluated by microscopic examination of the cell suspension. The disrupted cell suspension was subsequently centrifuged at 2000 *g* for 10 min at 4°C in an International Clinical centrifuge. The supernatant fluid was used for protein determination by the method of Lowry et al. (22) and for enzyme assays. Sucrase activity was assayed by the method of Dahlqvist (23). Alkaline phosphatase was measured using *p*-nitrophenylphosphate as substrate as described by Weiser (18).

Incubation conditions. In most experiments sodium [^{14}C]acetate (3 μCi/flask) incorporation was used to determine the rate of cellular lipid synthesis. An aliquot (0.6 ml) of suspended cells was added to 2.4 ml of Krebs-Ringer bicarbonate buffer, previously gassed for 10 min with 95% O_2 and 5% CO_2, to give a final concentration of 0.93–1.3 $\times$ 10^6 cells/ml of incubation medium. In experiments that used ^3H_2O to study cellular lipid synthesis, 0.5 ml of the cell suspension was added to 1 ml of Krebs-Ringer bicarbonate buffer containing 0.5 mCi of ^3H_2O to give a final cell concentration of 1.8–2.6 $\times$ 10^6 cells/ml. Incubations were carried out at 37°C in 25-ml plastic Erlenmeyer flasks with silicone stoppers in a water bath shaker at 125 oscillations/min. At the end of the incubation period the flasks were placed on ice and 0.2 ml of unlabeled 10% sodium acetate was added to each flask in experiments where [^{14}C]acetate was employed. Cells were separated from the medium by centrifugation at 2000 *g* for 10 min at 4°C. The incubation medium was carefully separated and dried in a lyophilizer (Virtis, Gardner, NY). Labeled lipids from both cells and lyophilized medium were extracted with chloroform–methanol 2:1 (v/v) by the method of Folch, Lees, and Sloane Stanley (24). Extraction of labeled lipids from cells by this method was at least 96% complete as determined by re-extraction of the cellular residues with chloroform–methanol. A measured aliquot of the lower phase containing labeled lipids was transferred to counting vials, gently dried on a hot plate, dissolved in 10 ml of scintillation fluid (complete L.S.C.; Yorktown Research, South Hackensack, NJ), and counted in a Beckman LS 133 model scintillation spectrometer. The efficiency of this instrument was 58% for ^{14}C and 54% for tritium.

Digitonin preparation

Digitonides were prepared by a modification of the method of Crawford (25). Aliquots of dried lipid extract were dissolved in 1 ml of a mixture of absolute ethanol–diethyl ether 3:1 in centrifuge tubes. An equal volume of unlabeled cholesterol (0.4 g/100 ml in the same solvent) was added and this was followed by the addition of 2 ml of 1% digitonin in 50% ethanol. After 15 min at room temperature the tubes were centrifuged at 2000 *g* for 10 min at 4°C. The precipitates were subsequently washed and recentrifuged twice with 1 ml of ethanol–diethyl ether 3:1, transferred to a counting vial in 1 ml of ether, dried under air, and counted. Controls, done with the addition of a known amount of labeled cholesterol to carrier

TABLE 1. Changes in cell viability and concentrations of alkaline phosphatase, glucose, and protein in the medium during incubation of intestinal cells

	Upper Villus Cells[a]					Crypt Cells[a]				
Time (min)	0	30	60	120	240	0	30	60	120	240
Cell viability %	78–85	78–87	78–84	70–75	58–62	70–76	70–74	70–74	64–69	50–55
Glucose (mg/dl)	100	94.8 ± 0.1	95.2 ± 0.8	93.9 ± 0.4	88.5 ± 0.6	100	97.7 ± 0.4	94.3 ± 0.9	91.9 ± 1.2	84.3 ± 1.8
Protein (μg)	22.1 ± 1.3	23.3 ± 1.1	24.8 ± 0.8	25.1 ± 1	26.7 ± 1.3	18.4 ± 0.5	19.8 ± 0.9	20.7 ± 1.0	21.6 ± 1.3	22.7 ± 0.7
Medium alkaline phosphatase[b]	0.05 ± 0.01	0.08 ± 0.03	0.11 ± 0.08	0.26 ± 0.04	0.31 ± 0.02					
Intracellular alkaline phosphatase	3.55 ± 0.2	3.57 ± 0.6	3.58 ± 0.3	3.55 ± 0.5	3.54 ± 0.8					

[a] Except for cell viability each value represents the mean± SEM of three experiments done in duplicate.
[b] The values for alkaline phosphatase are expressed as units (1 unit of activity equals 1 μmol of *p*-nitrophenol per 15 min).
Upper villus and crypt cells were incubated in a total volume of 3 ml at a cell concentration of 0.93–1.3 × 10^6 cells/ml (protein concentration 1.68–2.34 mg/3 ml) as described in Methods for the indicated periods of time. Incubation buffer was separated from the cells by centrifugation at 2000 *g* for 10 min. Intracellular alkaline phosphatase, assayed after sonification of cells, and cell viability were determined as described in Methods. Glucose concentration was determined by the method of Huggett and Nixon (27). The activity of alkaline phosphatase and the concentration of medium protein are expressed for 3 × 10^6.

cholesterol prior to digitonin addition, were tested to determine the recovery of cholesterol label by this method. The recovery of cholesterol was in the range of 86–92%.

Cell properties

Upper villus cells (fractions 1, 2, 3) accounted for 30–33% of the total cells isolated. Middle villus (fractions 5, 6, 7) and crypt (fraction 9) cells contributed 30–40% and 10–12% of the cell total, respectively. The upper villus cells were distinguished from crypt cells by measuring the activities of alkaline phosphatase and sucrase as we have reported previously (26). When examined with Giemsa stain, upper villus cell preparations contained 80% epithelial cells, 15% lymphocyte-monocytes, and 5% naked nuclei. The crypt cell preparation consisted of 70% epithelial cells, 23% lymphocyte-monocytes, and 7% naked nuclei. When cell counts of upper villus, middle villus, or crypt cells were compared prior to and after incubation for periods ranging from 30 to 240 min, there were no changes in cell number over the entire period. These findings confirm the absence of cell lysis during 4 hr of incubation. When any of the three cell types was incubated in Krebs-Ringer bicarbonate buffer, the pH of the incubation buffer did not change during 240 min of incubation. Immediately after isolation about 85% of the cell preparation consisted of single cells; the remainder were present in clumps of 4–8 cells. It was possible to count the clumped cells accurately when they were in groups of 4–8. During the first 60 min of incubation the degree of cell clumping did not change. However, after 90 min of incubation 25–28% of the cells were present in clumps of 4–8 cells; 30–34% and 35–37% of the cells were clumped after 120 and 240 min, respectively. The cells did not form clumps larger than 4–8 cells during 4 hr of incubation.

Cell viability and changes in glucose, protein, and alkaline phosphatase in the incubation medium are given in **Table 1**. Initial cell viability was 78–85% for upper villus cells and 70–76% for crypt cells. However, by the end of 4 hr cell viability had fallen to 58–62% and 50–55% for the upper villus and crypt cells, respectively. After 4 hr of incubation medium glucose concentrations decreased by 11.5% with upper villus cells and 15.7% with crypt cells.

The protein concentration in the incubation buffer increased from 22.1 to 26.7 μg for the upper villus cells by the end of 4 hr of incubation and from 18.4 to 22.7 μg for the crypt cells. The medium protein increased only from 1.2% of the protein content of upper villus cells at zero time to 1.5% of cell protein at the end of 4 hr. Medium proteins were 0.96% of the total crypt cell protein at zero time and 1.1% at 4 hr. Although the mean alkaline phosphatase in the medium increased by 0.26 units after 4 hr of incubation, the intracellular concentration of alkaline phosphatase did not change significantly during this period.

Effects of incubation media on acetate incorporation into lipids

[^{14}C]Acetate incorporation into cellular lipids was compared in Krebs-Ringer bicarbonate buffer with

TABLE 2. Effect of incubation media on [^{14}C]acetate incorporation into cellular lipids

Buffer	Additions	Relative Incorporation[a]	
		Upper Villus Cells	Crypt Cells
		%	%
Krebs-Ringer bicarbonate buffer	glucose (5.5 mM)	100	100
	glucose (11.0 mM)	124.4 ± 3.9	129.1 ± 3.6
	sucrose (5.5 mM)	75.1 ± 3.1	74.0 ± 2.7
	sucrose (11.0 mM)	64.4 ± 3.0	70.7 ± 2.2
	glucose (5.5 mM) + 1% albumin	64.3 ± 4.4	64.5 ± 2.2
	glucose (5.5 mM) + 2% albumin	70.9 ± 6.6	71.3 ± 5.6
	glucose (5.5 mM) + 3% albumin	71.3 ± 5.6	80.1 ± 1.4
	none	9.1 ± 1.1	12.3 ± 0.5
Phosphate buffered saline	glucose (5.5 mM)	5.3 ± 1.0	5.9 ± 0.8
	none	1.7 ± 0.5	2.3 ± 0.5

[a] Each value represents the mean ± SEM of three experiments done in duplicate.

Upper villus and crypt cells (0.93–1.3 × 10^6/ml) were incubated in a total volume of 3 ml of Krebs-Ringer bicarbonate buffer with 3 μCi of labeled acetate. The incorporation of labeled acetate into cellular lipids was determined as described in Methods.

varying concentrations of glucose, sucrose, and fatty acid-poor albumin (**Table 2**). Acetate incorporation in Krebs-Ringer bicarbonate buffer was about 25% greater with 11 mM glucose than with 5.5 mM glucose. With equimolar concentrations of sucrose, cellular lipid synthesis decreased by about 30%. Addition of albumin (1–3%) produced a similar reduction in labeled acetate incorporation into cellular lipids. Cellular lipid synthesis was negligible when glucose and sucrose were omitted or Krebs-Ringer bicarbonate buffer was replaced with phosphate-buffered saline. Effects of these changes in the incubation medium were essentially identical in upper villus and crypt cells.

TABLE 3. Effect of adding unlabeled acetate on the incorporation of [^{14}C]acetate into cellular lipids of crypt cells

Medium Acetate Concentrations	CPM		Acetate Incorporation[a]	
	Mean	Range	Mean	Range
μM			*nmole*	
1.2	23872	22600–25143	4.3	4.0–4.6
2.4	20400	19800–21000	7.4	7.2–7.6
3.6	10072	9200–10944	5.5	5.0–5.9
6.0	4659	4518–4800	4.3	4.1–4.4
10.8	1950	1800–2100	3.4	2.9–3.8

[a] Each value represents the mean of two separate experiments.

Incubation conditions and concentrations of cells were as described in Table 2 and in Methods. Increasing concentrations of unlabeled acetate were added to give the final indicated acetate concentrations. All flasks contained 3.0 μCi of labeled acetate.

Experiments were also conducted to determine whether the addition of albumin to the incubation buffer changed the release of labeled lipids into the medium. When upper villus cells were incubated in the presence of 2% albumin, label in medium lipids was reduced in a manner similar to that seen in the cellular lipids (data not shown). **Table 3** shows the effects of adding increasing concentrations of unlabeled acetate on the incorporation of acetate into cellular lipids of crypts cells. When medium acetate concentration was doubled, the incorporation of acetate into cellular lipids increased by 72%; however, with a 9-fold increase in medium acetate concentration, incorporation decreased by 20%. Similar results were obtained with upper villus cells (data not shown).

Relationship between cell number and cellular lipid synthesis

As seen in **Fig. 1**, cellular lipid synthesis was linear for upper villus, middle villus, and crypt cells over a concentration range of 0.5–8 × 10^6 cells.

Time course of [^{14}C]acetate incorporation into lipids (Fig. 2)

Upper villus cells incorporated labeled acetate into cellular lipids at a linear rate during the first 15 min of incubation. Continued lipid synthesis was observed, although at a slower rate, throughout the entire 240-min period. Cellular lipid synthesis was linear with middle villus and crypt cells during the first 30 min, but subsequently the rate of label incorporation fell progressively. Compared with upper villus cells, acetate incorporation into cellular lipids was 2.7 and 3.2 times greater in middle villus cells and 5.6 and 7.9 times greater in crypt cells at 15 and 240 min of incorporation, respectively.

Experiments were also conducted to determine whether cell fragments or enzymes released into the incubation medium incorporated [^{14}C]acetate into labeled lipids. Upper villus cells were incubated in Krebs-Ringer bicarbonate buffer. After 30 min the medium was separated from the cells and incubated with labeled acetate. The incorporation of labeled acetate into lipids in the absence of cells was negligible.

Labeled lipids, detected in the incubation medium at the time of first sampling (7.5 min), were released at a linear rate for 30 min by all cell types. Continued release of labeled lipids was observed during the entire 240 min of incubation. Although the time course of release of labeled lipids was similar for intestinal cells from the three fractions, the proportion of total labeled lipids released into the medium was considera-

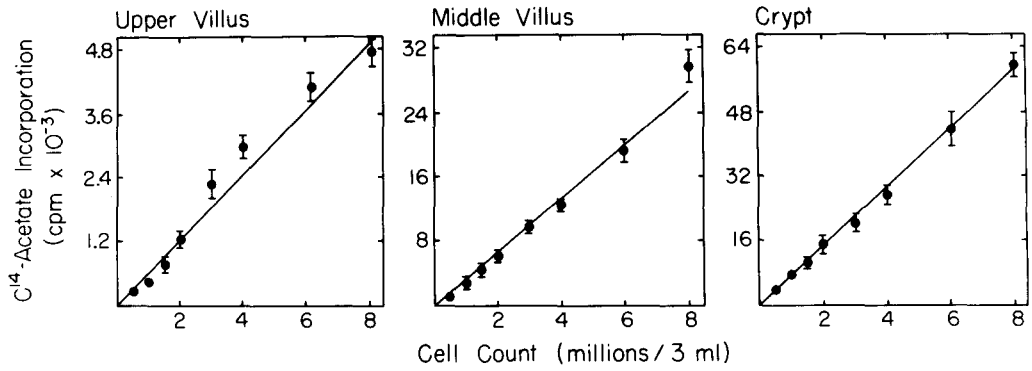

Fig. 1. Relationship between cell number and [^{14}C]acetate incorporation into cellular lipids. Krebs-Ringer bicarbonate buffer (3 ml) containing the indicated concentrations of upper villus, middle villus, and crypt cell were incubated at 37°C. After 30 min of incubation [^{14}C]acetate incorporation was determined as described in Methods. Each point represents the mean of three experiments ± SEM.

bly lower with the crypt cell fraction. Thus, between 30 and 240 min of incubation, the percent of labeled lipids released into the incubation medium changed from 26.7% to 22% with upper villus cells, from 21.4% to 25.3% with middle villus cells, and from 8.3% to 10.9% with crypt cells.

The time course for [^{14}C]acetate incorporation with added unlabeled acetate was investigated with crypt cells. As seen in Fig. 2*C*, the time course was similar when these cells were incubated with or without unlabeled acetate.

The incorporation of [^{14}C]acetate into isolated lymphocytes was investigated to determine whether lymphocytes present in intestinal cell preparations contributed significantly to lipid synthesis. For this purpose, lymphocytes were isolated from rat spleen by a teasing technique. Cell viability, determined by the exclusion of trypan blue, ranged from 90 to 95%. When lymphocytes were incubated under the same conditions as intestinal cells, average incorporation by 10^6 cells in two experiments was only 84 cpm after 30 min and 210 cpm after 4 hr of incubation. Based on the fraction of lymphocytes in the upper villus preparation (15%) and the acetate incorporation by these cells (Fig. 2*A*), the lymphocytes contributed less than 1.4% of the total counts in the upper villus cells. These data strongly suggest that the lymphocytes present in the intestinal cell preparations did not contribute significantly to lipid synthesis.

Time course of ^3H_2O incorporation (Fig. 3)

Both upper and middle villus cells incorporated ^3H_2O into cellular lipids at a linear rate for 15 min; the rate of ^3H_2O incorporation was linear for 30 min of incubation with crypt cells. Continued lipid synthesis was observed with all cell fractions for the entire period of incubation.

Compared with upper villus cells, ^3H_2O incorporation into cellular lipids was 2.3 and 2.1 times greater in middle villus cells and 4 and 3.3 times greater in crypt cells at 15 and 240 min of incubation, respectively. Thus, the differences between the various cell types in the incorporation of label into lipids were less marked with ^3H_2O than with [^{14}C]acetate.

Distribution of label among lipid classes

As seen in **Table 4**, the pattern of [^{14}C]acetate incorporation into various lipid classes was almost identical after 30 or 180 min of incubation in both the villus and crypt cells. In both cell types the distribution of ^{14}C among the various lipid classes was as follows: 52.5% in triglycerides, diglycerides, and monoglycerides; 22.3% in cholesterol; 8.3% in cholesteryl esters; 1.9% in fatty acids, and 15% in phospholipids. In contrast the incubation media contained a significantly lower percentage of cholesteryl esters (2.3%) and phospholipids (11.9%) than the cellular lipids. Moreover, the percent of label in total glycerides (61.1%) was significantly higher in the incubation medium than in cells.

Since 1,3-diglycerides and cholesterol had identical mobilities with the solvent system employed for thin-layer chromatography, the incorporation of [^{14}C]-acetate into cholesterol was corroborated by digitonin precipitation (25). In three separate experiments the percent of total lipids recovered as cholesterol by digitonin precipitation was 24% ± 0.4 SEM in upper villus cells and 25.6% ± 0.6 SEM in crypt cells. The corresponding values for cholesterol in these experiments by thin-layer chromatography were 20.7 ± 0.5 SEM for upper villus cells and 22.1% ± 0.6 SEM for crypt cells.

The incorporation of [^{14}C]acetate and ^3H_2O into fatty acids (saponifiable lipids) and cholesterol (non-saponifiable fraction) was examined in the upper villus and crypt cell fractions. As seen in **Table 5**,

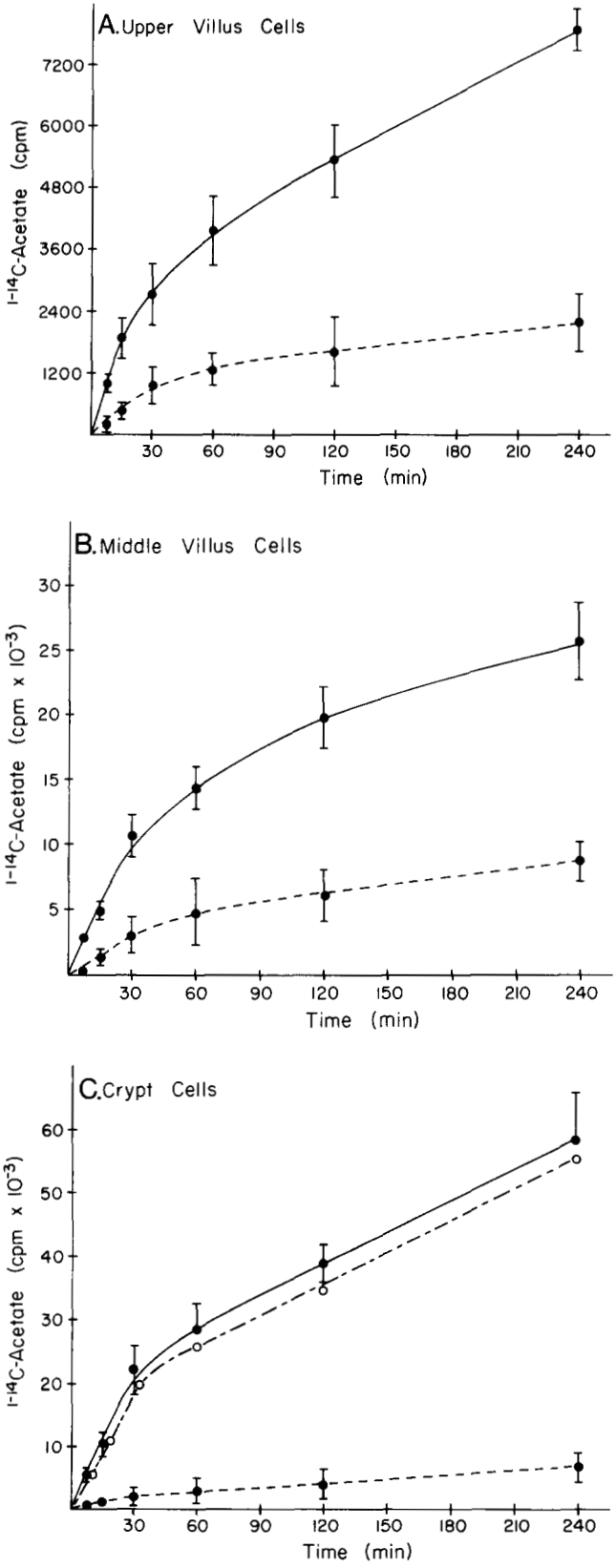

Fig. 2. Time course of [^{14}C]acetate incorporation into cellular and medium lipids. Incubation conditions are as described in Methods. Upper villus (*A*), middle villus (*B*), crypt cells (*C*) (0.93–1.3 × 10^6 cells/ml) were incubated for various periods of time and the labeled lipids in the medium and the cells were determined as described in Methods. Each point represents the mean of four experiments ± SEM. [^{14}C]Acetate incorporation is expressed per 1 × 10^6 cells. ● —— ●, Cellular lipids; ● – – – ●, medium lipids; ○ –·–·– ○, cellular lipids—incubation conditions are exactly the same with the exception of adding 1 μmole of unlabeled acetate to the incubation medium.

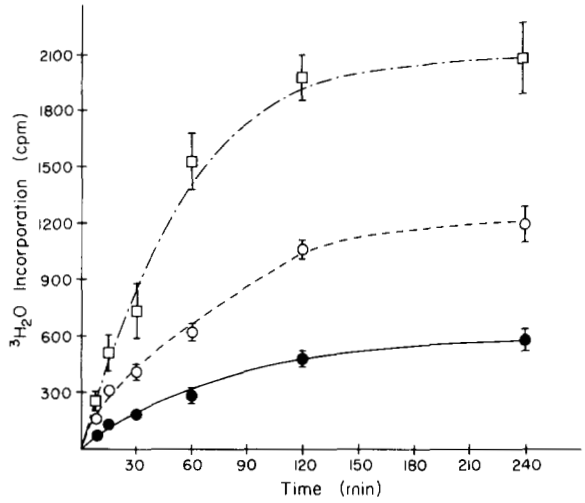

Fig. 3. Time course of ^3H_2O incorporation into cellular lipids in upper and middle villus and crypt cells. Upper villus, middle villus, and crypt cells were incubated at a concentration of 1.8–2.6 × 10^6 cells/ml. The incorporation of ^3H_2O into cellular lipids was determined as described in Methods. Each point represents the mean of three experiments ± SEM done in duplicate. Label incorporation is expressed per 1 × 10^6 cells. ● —— ● —— ●, Upper villus cells; ○ – – – ○ – – – ○, middle villus cells; □ –·–·□ □ –·–·□, crypt cells.

72% of the ^{14}C label was in the saponifiable fraction and 28% in the nonsaponifiable fraction with the upper villus cells. The crypt cells showed essentially the same distribution. These values agree well with the results obtained by thin-layer chromatography and by digitonin precipitation. With ^3H_2O, about 68% of the label was detected in the saponifiable fraction and 32% in the nonsaponifiable fraction in both upper villus and crypt cells (**Table 6**). In contrast with labeled acetate, only about half of the tritium label was recovered in the lipid fractions; the remainder of the label was shown to be in the aqueous phase following saponification.

Effect of homones and ethanol

Glucagon, epinephrine, norepinephrine, dexamethasone, dibutyryl cyclic AMP, cyclic AMP, theophylline, and cholera toxin did not significantly alter the rate of labeled acetate incorporation into cellular lipids (**Table 7**). Also examined were the effects of dibutyryl cyclic AMP and epinephrine on the release of labeled lipids from upper villus cells into the incubation medium. Neither of these com-

TABLE 4. Distribution of labeled acetate among various lipid classes

Lipid Class Time (min)	Upper Villus Cells[a]			Crypt Cells[a]		
	Cellular Lipids		Medium Lipids	Cellular		Medium Lipids
	30[b]	180	180	30	180	180
	%	%	%	%	%	%
Cholesteryl esters[c]	7.2 ± 0.3	7.6 ± 0.4	1.8 ± 0.3	8.1 ± 0.2	8.3 ± 0.5	2.3 ± 0.4
Triglycerides[c]	24.4 ± 0.5	25.5 ± 0.7	29.9 ± 0.8	24.9 ± 0.8	25.6 ± 0.3	29.3 ± 0.7
Fatty acids	1.8 ± 0.3	2.5 ± 0.2	1.4 ± 0.1	2.3 ± 0.4	1.9 ± 0.1	1.9 ± 0.4
Cholesterol	21.6 ± 0.6	19.0 ± 1.4	18.9 ± 0.4	21.8 ± 1.1	22.3 ± 0.7	22.8 ± 0.7
Diglycerides[c]	20.9 ± 0.5	23.1 ± 0.9	27.5 ± 0.6	23.6 ± 0.8	22.5 ± 0.4	25.2 ± 0.6
Monoglycerides[c]	6.4 ± 0.9	5.5 ± 0.8	8.6 ± 0.3	4.3 ± 0.8	4.4 ± 0.3	6.6 ± 0.5
Phospholipids[c]	17.7 ± 1.3	16.8 ± 0.6	11.9 ± 1.3	15.0 ± 2.1	15.0 ± 1.0	11.9 ± 0.6
Recovery	76.3 ± 1.1	78.0 ± 1.0	81.3 ± 2.2	76.0 ± 2.9	75.7 ± 3.6	79.3 ± 2.5

[a] Mean ± SEM; each value is the mean of three experiments done in duplicate.

[b] Time of incubation.

[c] The differences between the sum total glycerides in cells and medium were significant ($P < 0.001$ for upper villus cells and $P < 0.005$ for crypt cells). Similarly the differences between cholesteryl ester and phospholipids in cells and medium were significant (for cholesterol ester: villus cells $P < 0.001$, crypt cells $P < 0.02$, and $P < 0.05$ for villus and crypt cells).

Incubation conditions are as described in Methods. Upper villus and crypt cells were incubated in a total volume of 3 ml Krebs-Ringer bicarbonate buffer at a cell concentration of 1.8–2.6 × 10^6 cells/ml with 6 μCi of labeled acetate. Cells and medium were separated, extracted, and washed as described in Methods. The cellular and medium lipids were resolved by thin-layer chromatography using hexane–diethyl ether–glacial acetic acid 70:20:1 into the above fractions. The spots were identified by exposure to iodine and scraped from the plate; the labeled lipids were extracted from the scrapings with chloroform–methanol 2:1 which was transferred to counting vials, dried and counted as described in Methods.

pounds significantly changed the release of labeled lipids (data not shown). Although ethanol did not affect acetate incorporation into lipids, the incorporation of ^3H_2O into cellular lipids by both crypt and villus cells was approximately doubled by 5 mM ethanol and tripled by 10 mM ethanol (**Table 8**). Saponification data showed that the ^3H_2O incorporation was increased to a similar degree in the saponifiable and nonsaponifiable fractions as well as in the aqueous phase.

TABLE 5. Labeled acetate incorporation in saponifiable and nonsaponifiable lipids

Cell Type	30-min Incubation[a]		180-min Incubation[a]	
	Saponifiable Fraction	Non-saponifiable Fraction	Saponifiable Fraction	Non-saponifiable Fraction
	%	%	%	%
Upper villus	71.6 ± 1.8	28.4 ± 1.8	72.3 ± 1.9	27.7 ± 1.8
Crypt	73.4 ± 0.9	26.6 ± 0.8	70.4 ± 1.2	29.6 ± 1.2
Recovery	86.2 ± 2.7		84.0 ± 0.9	

[a] Each value is a mean ± SEM of three experiments done in duplicate. The percent values for both saponifiable and nonsaponifiable lipids were calculated for the label recovered.

Incubation conditions and lipid extraction are as described in Methods. The chloroform–methanol mixture containing the labeled lipids was dried under a gentle stream of nitrogen in a water bath at 45°C. Water (1 ml) was then added to the dried lipid extract and subsequently 2.0 ml of 95% ethanol and 0.5 ml of 60% (w/v) KOH were added; the resulting mixture was saponified at 80°C for 90 min. Nonsaponifiable lipids were extracted into heptane and backwashed once with water. The heptane phase was transferred to counting vials and these samples were counted as described in Methods. The original aqueous phase was washed once with heptane and acidified; fatty acids were then extracted into heptane and counted.

DISCUSSION

Isolated intestinal cells have several advantages for metabolic studies. They are less complex than intestinal segments or slices, and the cell surfaces are better exposed to oxygen, substrates, and other constituents of the incubation buffer. Moreover, the availability of a number of aliquots of specific cell types from

TABLE 6. ^3H_2O incorporation in saponifiable and nonsaponifiable lipids

Cell Type	30-min Incubation[a]		180-min Incubation[a]	
	Saponifiable Fraction	Non-saponifiable Fraction	Saponifiable Fraction	Non-saponifiable Fraction
	%	%	%	%
Upper villus	67.7 ± 1.0	32.3 ± 1.0	70.7 ± 0.6	29.4 ± 1.3
Crypt	66.0 ± 1.2	34.0 ± 1.2	71.5 ± 2.7	28.5 ± 2.7
Recovery	51.7 ± 3.9		48.6 ± 1.0	

[a] Each value represents the mean ± SEM of three experiments done in duplicate.

Incubation conditions are as described in Methods; saponifiable and nonsaponifiable lipids were fractionated as described in Table 5.

TABLE 7. Effect of various hormones, dibutyryl cyclic AMP, theophylline, and cholera toxin on [^{14}C]acetate incorporation into cellular lipids

Additions	Final Concentration	Relative Incorporation into Cellular Lipids[a]	
		Upper Villus Cells	Crypt Cells
		%	%
None		100.0 ± 0.0	100.0 ± 0.0
Norepinephrine	10 μM	96.2 ± 1.4	109.2 ± 3.7
Epinephrine	10 μM	100.7 ± 1.0	105.1 ± 3.4
Dexamethasone[b]	1 μM	105.4 ± 2.1	105.6 ± 3.8
Glucagon	10 μM	107.3 ± 2.4	113.3 ± 9.7
Dibutyryl cyclic AMP[c]	1 mM	95.6 ± 2.1	97.5 ± 1.6
Theophylline	1 mM	98.1 ± 3.2	96.8 ± 2.8
Cholera Toxin	1 μg/ml	101.3 ± 3.9	104.7 ± 4.1

[a] Each value is the mean of three experiments done in duplicate.
[b] Similar results were obtained with 1 mM dexamethasone.
[c] Similar results were obtained with 1 mM cyclic AMP.
The various additions were made separately to incubation flasks containing either upper villus or crypt cells. Incubations were carried out for 30 min as described in Methods.

one intestine allows the investigator to examine the effect of multiple substances on the metabolic activities of the different cell types.

The intactness of the intestinal cells used in the present study is demonstrated by the exclusion of Nigrosin dye and by the absence of cell lysis or significant increases in the protein content of the incubation medium. The appearance of alkaline phosphatase in the medium, unassociated with changes in intracellular enzyme content, probably results from enzyme secretion into the incubation buffer. However, we have not investigated this possibility in detail. Although the isolated cells were sufficiently organized to synthesize the major lipid classes in a physiological buffer, the rate of lipid synthesis was linear for only a short time. Considerable incorporation of label continued for 4 hr, but the rate of incorporation during the final 2 hr was only 25% of the initial rate. Finally, the ethanol-induced enhancement in lipid synthesis reveals responsiveness of these cells to an external stimulus.

Although Stern (20) used sucrose to investigate cellular respiration in isolated suspensions of intestinal cells, we found that glucose supported lipid synthesis somewhat better than sucrose. Most other investigators have also utilized glucose in their studies of lipid synthesis in slices or everted sacs of intestine (12, 28). In the present study the presence of glucose or sucrose in the incubation buffer was essential for lipid synthesis by intestinal cells. Adipocytes also require glucose for lipid synthesis,[1] but acetate incorporation in isolated hepatocytes is unaffected by the presence or absence of glucose.[1] The addition of bovine serum albumin from a single supplier slightly reduced acetate incorporation into lipids by intestinal cells.

In intestinal slices Dietschy and Siperstein (11) found that labeled acetate incorporation into lipids by villus cells was less than 1% of that observed in crypt cells. Recently, Muroya, Sodhi, and Gould (29) showed that intestinal villus cells from the rat synthesize sterols as rapidly as the crypt cell fractions. In the present study upper villus cells incorporated about 12% as much acetate into lipids as the crypt cells, and the distribution of label among the various lipid classes was identical in the two cell types. The incorporation of [^{14}C]acetate or $^{3}H_2O$ into cellular lipids by upper villus cells could result from the presence of lymphocytes or crypt cells in the villus cell fraction. Because they incorporate very small amounts of labeled acetate into lipids, lymphocytes contribute insignificantly to lipid synthesis by isolated villus cells. By measuring thymidine kinase, a crypt cell enzyme, and by injecting rats with labeled thymidine prior to cell isolation, Weiser (18) previously found little or no contamination of upper villus cell fractions with crypt cells. Since we followed the procedure described by Weiser, it is unlikely that our upper villus cell preparations contained significant numbers of crypt cells. Although we recovered only about one-quarter of the incorporated acetate in cholesterol, in earlier studies with intestinal slices [^{14}C]acetate was incorporated almost equally into digitonin-precipitable sterols and fatty acids (11). These discrepancies may reflect differences in metabolic properties between isolated cells and slices.

The appearance of labeled lipids in the incubation medium could result from cell lysis, pinching off of cell membranes, leakage of cellular lipids, or secretion of lipoproteins. Cell lysis and loss of cell membranes

TABLE 8. Effect of ethanol on [^{14}C]acetate and $^{3}H_2O$ incorporation into cellular lipids

Ethanol Concentration	Relative Incorporation into Cellular Lipids[a]			
	[^{14}C]Acetate		$^{3}H_2O$	
	Upper Villus Cells	Crypt Cells	Upper Villus Cells	Crypt Cells
	%	%	%	%
None	100	100	100	100
5 mM	101.4 ± 3.3	105.3 ± 2.4	206.6 ± 6.1	214.6 ± 9.4
10 mM	105.2 ± 4.2	101.6 ± 1.0	292.3 ± 9.5	296.0 ± 9.1

[a] Each value is the mean ± SEM of three experiments done in duplicate.
Intestinal cells (cell concentrations were 0.93–1.3 × 10^6/ml for labeled acetate experiments and 1.8–2.6 × 10^6/ml for $^{3}H_2O$) were incubated as described in Methods.

[1] Margolis, S. Unpublished observation.

were minimal, based on the failure to observe a decrease in cell number, an increase in Nigrosin uptake, or significant increases in medium protein during incubation. The small changes in medium protein, which are far more soluble than lipids, suggest that leakage of cellular lipids was insignificant. Furthermore, the distribution of labeled lipids in the medium was different from that of cellular lipids. In particular, the medium was relatively enriched in less polar lipids (glycerides) and contained smaller quantities of the more polar lipids (phospholipids) which are major constituents of cell membranes.

The reason for the relatively low concentration of label in the medium cholesteryl ester fraction is not clear, especially since lecithin-cholesterol acyltransferase has been recently found in intestinal lymph (30). Our results suggest that the labeled lipids in the incubation medium are bound to lipoproteins secreted by intestinal cells. Further support for this interpretation is provided by experiments that have documented the release of chylomicrons by isolated cells of rat intestinal mucosa (15).

Prior investigations have examined several aspects of the regulation of intestinal lipid synthesis. Although bile salts inhibited lipid synthesis in intestinal slices (31), neither cholesterol feeding nor fasting (8) affected lipid synthesis. The addition of insulin to intestinal slices did not alter the incorporation of [^{14}C]acetate into fatty acids (12). The present investigation indicates that lipid synthesis in rat intestinal cells is unaffected by catecholamines or glucagon, which stimulate adenyl cyclase activity and inhibit lipid synthesis in hepatocytes and adipocytes (6, 10). Cholera toxin, an agent previously shown to increase adenyl cyclase activity in intestinal epithelial cells (32), did not alter the rate of acetate incorporation by intestinal cells. The absence of hormonal response in these cells may be due to loss of ultrastructural integrity, loss of cell receptors during cell isolation, or unresponsiveness of these cells to hormones. Although we did not repeat electron microscopic examination of these cells, a number of other investigators (33, 34) have used Weiser's technique for the isolation of intestinal cells. The loss of intestinal cell membrane receptors seems unlikely, since this rather gentle technique of cell isolation does not utilize proteolytic enzymes. Furthermore, neither dibutyryl cyclic AMP nor theophylline, which do not require cell receptors for their effects, altered the rate of lipid synthesis. Taken together, these results strongly suggest that, unlike liver and adipose tissue, intestinal cell lipid synthesis is not regulated by cellular levels of cyclic AMP.

Although most studies on the effects of ethanol on lipid metabolism have been directed towards liver (35, 36), ethanol is largely absorbed in the small intestine, which contains alcohol dehydrogenase and can metabolize ethanol (37). Thus, high luminal concentrations of ethanol might influence intestinal lipid metabolism. Middleton et al. (38) observed enhanced cholesterol synthesis in rat intestine after ethanol administration. Mistilis and Ockner (39) showed that intraduodenal administration of ethanol increased mucosal triglyceride content and stimulated the production of endogenous triglyceride-rich lipoproteins by small intestine. In the present study ethanol concentrations similar to blood levels in man after moderate ethanol ingestion clearly increased the incorporation of tritiated water into lipids of both upper villus and crypt cells. The absence of an ethanol effect of [^{14}C]acetate incorporation might be related to a dilution of the intracellular acetate pool by the conversion of ethanol to acetate. The problems produced by such changes in the pool of acetate are overcome by the use of $^{3}H_2O$ in these cells, as has been suggested previously for liver cells (40). However, we cannot explain the discrepancies in the effects of ethanol on labeled acetate and $^{3}H_2O$ incorporation into cellular lipids. The present study shows that isolated intestinal cells are suitable for studies of the rapid regulation of lipid synthesis. In contrast to their known inhibiting effects on lipid synthesis in hepatocytes and adipocytes, dibutyryl cyclic AMP, epinephrine, and glucagon had no effect on lipid synthesis in intestinal mucosa cells. Isolated intestinal cells should prove useful for elucidation of the mechanism for the ethanol stimulation of lipid synthesis.

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REFERENCES

1. Bortz, W. M. 1973. On the control of cholesterol synthesis. *Metabolism.* **22:** 1507–1524.
2. Ho, K. J., and C. B. Taylor. 1970. Control mechanisms of cholesterol biosynthesis. *Arch. Pathol.* **90:** 83–92.
3. Dietschy, J. M., and J. D. Wilson. 1973. Regulation of cholesterol metabolism. *N. Engl. J. Med.* **282:** 1128–1138.
4. Steinberg, D., and M. Vaughan. 1965. Release of free fatty acids from adipose tissue in vitro in relation to rates of triglyceride synthesis and degradation. *In* Handbook of Physiology, Section 5, Adipose Tissue. A. E. Renold and G. F. Cahill, editors. American Physiological Society, Washington, D.C. 345–346.

5. Rodbell, M. 1965. The metabolism of isolated fat cells. *In* Handbook of Physiology, Section 5, Adipose Tissue. A. E. Renold and G. F. Cahill, editors. American Physiological Society, Washington, D.C. 478–482.
6. Galton, D. J. 1971. The Human Adipose Tissue. Appleton Century Crofts. New York. 110, 115–121.
7. Dietschy, J. M., and J. D. Wilson. 1968. Cholesterol synthesis in the squirrel monkey: relative rates of synthesis in various tissues and mechanisms of control. *J. Clin. Invest.* **47:** 166–174.
8. Dietschy, J. M., and M. D. Siperstein. 1967. Effect of cholesterol feeding and fasting on sterol synthesis in seventeen tissues of the rat. *J. Lipid. Res.* **8:** 97–104.
9. Capuzzi, D. M., and S. Margolis. 1971. Metabolic studies in isolated rat liver cells. *Lipids.* **6:** 601–608.
10. Capuzzi, D. M., V. Rothman, and S. Margolis. 1974. The regulation of lipogenesis by cyclic nucleotides in intact hepatocytes prepared by a simplified technique. *J. Biol. Chem.* **249:** 1286–1294.
11. Dietschy, J. M., and M. D. Siperstein. 1965. Cholesterol synthesis by the gastrointestinal tract: Localization and mechanisms of control. *J. Clin. Invest.* **44:** 1311–1327.
12. Franks, J. J., E. M. Riley, and K. J. Isselbacher. 1966. Synthesis of fatty acids by rat intestine in vitro. *Proc. Soc. Exp. Bio. Med.* **121:** 322–327.
13. Porte, D., Jr., and C. Entenman. 1965. Fatty acid metabolism in segments of rat intestine. *Amer. J. Physiol.* **208:** 607–614.
14. Mansbach, C. M., 2nd. 1973. Complex lipid synthesis in hamster intestine. *Biochim. Biophys. Acta.* **296:** 386–402.
15. O'Doherty, P. J. A., I. M. Yousef, and A. Kuksis. 1973. Effect of puromycin on protein and glycerolipid biosynthesis in isolated mucosal cells. *Arch. Biochem. Biophys.* **156:** 586–594.
16. O'Doherty, P. J. A., I. M. Yousef, G. Kakis, and A. Kuksis. 1975. Protein and glycerolipid biosynthesis in isolated intestinal epithelial cells of normal and bile-fistula rats. *Arch. Biochem. Biophys.* **169:** 252–261.
17. O'Doherty, P. J. A., and A. Kuksis. 1975. Glycerolipid biosynthesis in isolated intestinal epithelial cells. *Can. J. Biochem.* **53:** 1010–1019.
18. Weiser, M. M. 1973. Intestinal epithelial cell surface membrane glycoprotein synthesis. *J. Biol. Chem.* **248:** 2536–2541.
19. Shakir, K. M. M., S. G. Sundaram, and S. Margolis. 1977. Regulation of lipid synthesis in isolated intestinal cells. *Federation Proc.* **36:** 1115. (Abstract.)
20. Stern, B. K. 1966. Some biochemical properties of suspensions of intestinal epithelial cells. *Gastroenterology.* **51:** 855–864.
21. Kaltenbach, J. P., M. H. Kaltenbach, and W. B. Lyons. 1958. Nigrosin as a dye for differentiating live and dead ascites cells. *Exp. Cell. Res.* **15:** 112–117.
22. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
23. Dahlqvist, A. 1964. Method for assay of intestinal disaccharidases. *Anal. Biochem.* **7:** 18–25.
24. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
25. Crawford, N. 1958. An improved method for the determination of free and total cholesterol using the ferric chloride reaction. *Clin. Chim. Acta.* **3:** 357–367.
26. Shakir, K. M. M., S. Margolis, and S. Baylin. Localization of histaminase in rat small intestinal mucosa: site of release by heparin. *Biochem. Pharmacol.* **26:** 2343–2348.
27. Huggett, A. St. G., and D. A. Nixon. 1957. Use of glucose oxidase, peroxidase and *o*-dianisidine in determination of blood and urinary glucose. *Lancet.* **273** (2)**:** 368–370.
28. Breckenridge, W. C., and A. Kuksis. 1975. Diacylglycerol biosynthesis in everted sacs of rat intestinal mucosa. *Can. J. Biochem.* **53:** 1170–1183.
29. Muroya, H., H. S. Sodhi, and R. G. Gould. 1977. Sterol synthesis in intestinal villi and crypt cells of rats and guinea pigs. *J. Lipid. Res.* **18:** 301–308.
30. Clark, S. B., and K. R. Norum. 1977. The lecithin-cholesterol acyl transferase activity of rat intestinal lymph. *J. Lipid Res.* **18:** 293–300.
31. Dietschy, J. M. 1968. The role of bile salts in controlling the rate of intestinal cholesterogenesis. *J. Clin. Invest.* **47:** 286–300.
32. Weiser, M. M., and H. Quill. 1975. Intestinal villus and crypt cell responses to cholera toxin. *Gastroenterology.* **69:** 479–482.
33. Bouhours, J. F., and R. M. Glickman. 1976. Rat intestinal glycolipids. II. Distribution and biosynthesis of glycolipids and ceramide in villus and crypt cells. *Biochim. Biophys. Acta.* **441:** 123–133.
34. Bouhours, J. F., and R. M. Glickman. 1977. Rat intestinal glycolipids III. Fatty acids and long chain bases of glycolipids from villus and crypt cells. *Biochim. Biophys. Acta.* **487:** 51–60.
35. Ontko, J. A. 1973. Effects of ethanol on the metabolism of free fatty acids in isolated liver cells. *J. Lipid Res.* **14:** 78–86.
36. Lieber, C. S., R. Teschke, Y. Hashmura, and L. M. Decarli. 1975. Differences in hepatic and metabolic changes after acute and chronic alcohol consumption. *Federation Proc.* **34:** 2060–2074.
37. Spencer, R. P., K. R. Brody, and B. M. Lutters. 1964. Some effects of ethanol on gastrointestinal tract. *Amer. J. Dig. Dis.* **9:** 599–604.
38. Middleton, W. R. J., E. A. Carter, G. D. Drummey, and K. J. Isselbacher. 1971. Effects of oral ethanol administration on intestinal cholesterogenesis in the rat. *Gastroenterology.* **60:** 880–887.
39. Mistilis, S. P., and R. K. Ockner. 1972. Effects of ethanol on endogenous lipid and lipoprotein metabolism in small intestine. *J. Lab. Clin. Med.* **80:** 34–46.
40. Dietschy, J. M., and M. S. Brown. 1974. Effect of alterations of the specific activity of the intracellular acetyl CoA pool on apparent rates of hepatic colesterogenesis. *J. Lipid Res.* **15:** 508–516.