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## Localization of Cholesterol Esterase and Cholesterol in Mucosal Fractions of Rat Small Intestine.* (28588)

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It has been reported that dietary cholesterol penetrates the intestinal mucosa in the free form but appears in the lymph almost entirely as ester(1). Also, it has been shown that lymph is not the site of cholesterol esterification in absorption(2). Therefore, the esterification of this cholesterol must occur between these two sites, i.e., in the intestinal mucosa. Moreover, the enzyme, cholesterol esterase, which catalyzes the synthesis of cholesterol esters and their hydrolysis has been demonstrated in the mucosa(3). The purposes of the experiments presented here were to determine, firstly, the subcellular location of the cholesterol esterase in the mucosa of the rat small intestine; secondly, to determine the levels of free and esterified cholesterol in the wall of the small intestine; and thirdly, to determine the distribution of free and esterified cholesterol in subcellular fractions of the mucosa.

*Experimental.* Male Carworth rats, weighing between 100 and 200 g, were fasted 18 hours before sacrifice. The intestinal mucosa was scraped from the previously washed and everted intestine (severed posterior to the common duct and at the ileo-cecal junction) and fractionated into brush border and supernatant fractions in .005 M EDTA according to the procedure of Miller and Crane(4) with modifications. Rat intestinal mucosa was used rather than hamster mucosa(4) and homogenization of the mucosa was carried out with a Potter-Elvehjem type homogenization tube fitted with a Teflon pestle by making 2 up and down passes at a pestle speed of 1200 rpm. This procedure was adequate for rupture of all the intact cells as observed by microscopic examination and at the same time left the brush border particles intact, as determined by invertase assay (Table I). It was also determined by invertase assay that repeated washing and centrifugation of the brush border did not result in its rupture (Table I). Brush border preparations which contained 90-100% of the invertase activity of the mucosal homogenate and which under microscopic examination were found free of intact cells and cell debris were considered adequate for further study. Invertase was measured by the method of Borgstrom and Dahlqvist(5).

Fractionation of the intestinal mucosa into subcellular fractions was carried out in 0.25 M sucrose, using the differential centrifugation procedure of Hogeboon and Schneider(6).

Cholesterol esterase activity was determined in the EDTA and sucrose homogenates of the intestinal mucosa and in the subfractions prepared as described above. (It should be noted that the term supernatant fraction is used to designate that fraction of the EDTA homogenate free of brush border, and that the term soluble fraction is used to designate that frac-

TABLE I. Recovery of Invertase Activity in Brush Border Preparations.

| Fraction | Invertase activity* (units) | Recovery (%) |
|---|---|---|
| Homogenate | 357 | — |
| Brush border | 343 | 96 |
| First supernatant | 21 | 6 |
| Washed brush border | 343 | 96 |

* Activity—μmoles reducing sugar/hr at 37°C.

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tion of the sucrose homogenate free of particulates of microsomal size and larger.) Levels of activity were determined as described below.

The procedure for preparation of the incubation media, the incubation conditions, and the molar ratio of the contents of the incubation flasks in the assay for cholesterol esterase activity were those of Swell(7). The incubation flasks for studying esterification contained free cholesterol (6.0 mg), oleic acid (13.1 mg), sodium taurocholate (17.7 mg) and albumin (6.0 mg). For determination of hydrolytic activity the flasks contained cholesterol butyrate equivalent to the amount of free cholesterol in the esterification system and the same amounts of sodium taurocholate and albumin. In all incubations, 3 ml of the enzyme source (subcellular fractions were restored to their original volume) were added to 1 ml of substrate mixture and incubated for 18-24 hr at 37°C in a Dubnoff shaker. All assays were run in triplicate. In control flasks the enzyme in the preparation was first inactivated by heating at 70°C for 10 min. and 3 ml of this dispersion was then added to the substrate in place of the active enzyme source. After the incubation period, the contents of each incubation flask were rehomogenized, sampled, the lipid extracted in acetone:alcohol (1:1) and free and total cholesterol determined.

Cholesterol levels in the wall of the small intestine were determined as follows: Samples of the proximal, central, and distal segments of the small intestine were excised, blotted, weighed (approximately 1 g total weight) and added to 25 volumes of acetone:alcohol (1:1) and homogenized for 2 minutes in a Servall homogenizer. The homogenate was brought to a boil, the supernatant decanted, and the tissue residue reextracted twice in 25 ml of boiling acetone:alcohol. The lipid extract was brought to volume and aliquots were taken for determination of free and total cholesterol. A mucosal homogenate was prepared from the remainder of the intestine from the same animal and fractionated into subcellular fractions. An acetone-alcohol extract was prepared from the mucosal homogenate and its fractions and aliquots taken for free and total cholesterol determination. In all cases cholesterol was determined by the method of Vahouny *et al.*(8).

TABLE II. Esterification of Cholesterol and Hydrolysis of Cholesterol Butyrate by Rat Mucosal Fractions.

| | Enzyme distribution | | | |
|---|---|---|---|---|
| | Esterification | | Hydrolysis | |
| Fraction | (units)* | (%) | (units)† | (%) |
| Homogenate | 3.33 | 100 | 7.63 | 100 |
| Brush border | .57 | 17 | 1.80 | 24 |
| Supernatant | 2.67 | 80 | 5.66 | 74 |
| Sum of fractions | 3.24 | 97 | 7.46 | 98 |

* μmoles cholesterol esterified/hr/g of mucosa.
† μmoles cholesterol liberated by hydrolysis/hr/g of mucosa.

*Results. Cholesterol esterase activity in brush border and supernatant fractions:* Typical results from the esterification and hydrolysis studies are shown in Table II. Control flasks containing all the components of the system but lacking an active enzyme source did not synthesize or hydrolyze cholesterol ester. Analysis of the contents of the experimental flasks after incubation showed that 80% of the esterifying activity of the whole homogenate was in the supernatant fraction and 17% was in the brush border fraction. (Individual animals showed the following percentage partition of the enzyme between supernatant and brush border: 71, 18; 93, 16; 76, 18; 80, 17; 80, 15; and 74, 16). The same partition of hydrolytic activity was found as for esterifying activity. It was possible that the esterase activity recovered in the brush border was due to adsorbed pancreatic cholesterol esterase from the lumen of the intestine or residual contamination from the enzyme concentrated in the supernatant fraction. These possibilities were tested by using washed brush border preparations in the assay for hydrolytic activity of the enzyme. While unwashed brush border contained as much as 25% of the total activity, once-washed brush border was essentially free of cholesterol esterase activity. In 5 such experiments 10-25% of the total esterase activity was recovered in the wash.

*Cholesterol content of rat intestinal wall and intestinal mucosal fractions:* Cholesterol content of rat intestinal wall, intestinal mu-

TABLE III. Cholesterol Content of Rat Intestinal Wall and Mucosal Fractions.

| Tissue fraction | Cholesterol* (mg/g tissue) Total | Free | Ester |
|---|---|---|---|
| Intestinal wall | 2.15 ± .06 | 1.83 ± .11 | .32 ± .06 |
| " mucosa | | | |
| Homogenate | 2.23 ± .09 | 1.74 ± .06 | .49 ± .05 |
| Supernatant | 1.10 ± .08 | .07 ± .06 | .40 ± .05 |
| Brush border | 1.10 ± .05 | 1.02 ± .08 | .08 ± .03 |

* Avg of 7 experiments. ± = S.E. of mean.

cosa, and its fractions were determined on the same animals. This experiment was an attempt to determine if esterified cholesterol was concentrated in the same fraction of the mucosa as cholesterol esterase. Results are shown in Table III. Most of the cholesterol of the whole small intestine, free and ester, was concentrated in the mucosa. Of the total cholesterol in the mucosa, one-half or more occurred in the brush border in the free form. The remaining mucosal cholesterol which was present in the supernatant fraction contained 90-100% of the esterified cholesterol of the mucosa.

*Partition of cholesterol esterase activity among rat intestinal mucosal cell fractions:* These experiments were an attempt to further localize the site of action of cholesterol esterase. The results of esterification and hydrolysis studies on the partition of cholesterol esterase activity in mucosal subcellular fractions of the same rat are shown in Table IV.

TABLS IV. Esterification of Cholesterol and Hydrolysis of Cholesterol Butyrate by Subcellular Fractions of Mucosa.

| Fraction | Enzyme distribution Esterification* (units) | (%) | Hydrolysis† (units) | (%) |
|---|---|---|---|---|
| Homogenate | 1.90 | 100 | 3.87 | 100 |
| Nuclei and cell debris | .33 | 18 ± 2 | .64 | 16 ± 2 |
| Mitochondria | .01 | 1 ± 0.2 | .15 | 4 ± 2 |
| Microsomes | .26 | 12 ± 3 | .49 | 13 ± 2 |
| Soluble fraction | 1.19 | 63 ± 3 | 2.54 | 66 ± 5 |
| Sum of fractions | 1.79 | 94 | 3.82 | 99 |

* Values are averages of 4 experiments ± S.E. of mean. Activity = μmoles cholesterol esterified/hr/g mucosa. Flasks incubated 24 hr at 37°C.

† Values are averages of 5 experiments ± S.E. of mean. Activity = μmoles cholesterol liberated by hydrolysis/hr/g mucosa. Flasks incubated 18 hr at 37°C.

The data show a close agreement between the distribution of hydrolytic and esterifying activity among the fractions. Sixty-three per cent or more of the activity of the whole homogenate was in the soluble fraction with lesser amounts of activity in the nuclei and cell debris, microsomal, and mitochondrial fractions.

*Discussion.* Cholesterol esterase activity was concentrated in the supernatant fraction of the intestinal mucosa as opposed to the brush border fraction. Eighty per cent of the synthetic and hydrolytic activity was present in the supernatant fraction. However, in hydrolysis experiments carried out on washed brush border, an additional 10-25% of the total esterase activity was recovered in the supernatant in each of 5 experiments. Washed brush border was practically free of cholesterol esterase activity and the invertase assay showed that esterase removed in the washing step was not derived from rupture of the brush border. This residual activity in the brush border was probably due to contamination from the intestinal cholesterol esterase in the supernatant. Hence, these data suggest that the processes of cholesterol esterification and cholesterol ester hydrolysis do not take place in the brush border but occur deeper in the mucosa.

It was also found that the cholesterol esters of the mucosa were concentrated in the supernatant fraction. These data agree with the recovery of the cholesterol esterase in the supernatant fraction. The ester would be expected to be concentrated near the site of action of the enzyme.

Cholesterol determinations on rat intestinal wall and mucosa demonstrated that most of the cholesterol of the intestinal wall is concentrated in the mucosa. The esterified fraction, which was almost entirely localized in the supernatant fraction of the mucosa, is probably newly formed ester resulting from the action of cholesterol esterase.

When the intestinal mucosa was fractionated into subcellular fractions, an average of 63% and 66% of the synthetic and hydrolytic activities, respectively, were in the soluble fraction. An average of 18% of the esterifying activity and 16% of the hydrolytic activity was in the nuclei and cell debris with

smaller amounts of activity in the microsomes and mitochondria. There was no evidence for a separation of hydrolytic and esterifying activity and hence, no evidence for 2 separate enzymes catalyzing the 2 different processes of esterification and hydrolysis.

The occurrence of a major part of the esterase activity in the soluble fraction and nuclei-cell debris also suggests that esterification occurs in the mucosal cells rather than in the brush border. It is likely that cholesterol passes through the brush border and enters the mucosal cells in the free form(1). Then, the cholesterol is esterified within the mucosal cells and rapidly passes into the lymph as a part of the chylomicron(1).

*Summary.* A modified procedure for brush border preparation has been developed which gives 90-100% yields of invertase activity in the brush border. It was determined that the esterifying and hydrolytic activities of cholesterol esterase are concentrated in the supernatant fraction of the mucosa as opposed to the brush border. Further fractionation of the mucosal cells into nuclei and cell debris, mitochondria, microsomes, and soluble fraction, and assay of these fractions for esterase activity demonstrated that 60-66% of the esterase activity of the whole homogenate was in the soluble fraction. The remaining activity was distributed among the other fractions. The data suggest that esterification of cholesterol occurs in the mucosal cell rather than in the brush border. Cholesterol levels in rat intestinal wall, intestinal mucosa, and mucosal subcellular fractions are also reported.

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## Immunological Specificity of the Secondary Response with Dinitrophenylated Proteins.* (28589)

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Haptens may be coupled to different carrier proteins to produce complete antigens. If the antibodies are assayed with an antigen made from the hapten coupled to an unrelated carrier protein, only the anti-hapten specificity is tested. Taking advantage of this principle, the role of the carrier protein in the secondary response may be examined. Employing dinitrophenylated proteins, it was seen that a secondary response was produced only with the same hapten-carrier protein combination as used for the primary immunization.

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*Materials and methods.* 1. *Animals:* Random-bred albino rabbits weighing approximately 3-4 kg were used for immunization. Hartley-strain albino guinea pigs, 250 ± 50 g, were used for passive cutaneous anaphylaxis (PCA) reactions.

2. *Antigens:* 2,4-Dinitrophenylsulphonic acid (DNPS) (Eastman Kodak) was twice recrystallized from water and used for coupling to 3 different carrier proteins, *i.e.,* bovine gamma globulin (BGG) (Armour Pharmaceuticals, Lot #T 30203); crystalline bovine serum albumin (BSA) (Armour Pharmaceuticals, Lot #W 69312); crystallized egg albumin (EA) (Worthington Biochemical Corp., Lot #A 598).