# EXTRACTION OF ABSORBED LIPID (LINOLEIC ACID-1-$^{14}$C) FROM RAT INTESTINAL EPITHELIUM DURING PROCESSING FOR ELECTRON MICROSCOPY

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## INTRODUCTION

Intestinal epithelial cells absorb ingested neutral lipids, apparently as water-soluble micelles of bile salts, monoglycerides, and/or fatty acids (3, 7). Electron microscopy of intestinal cells fixed in osmium tetroxide reveals some of the absorbed lipid as electron-opaque droplets in the apical cytoplasm distal to the microvilli and subjacent terminal web (10). Experiments by Strauss and Ito (17) and Strauss (18) suggest that these lipid droplets in the apical cytoplasm are the higher glyceride products synthesized from absorbed fatty acids and monoglycerides. The absorbed precursors of the higher glycerides must pass through the microvilli and/or terminal web to the apical cytoplasm. A few investigators (5, 11, 13) have reported electron-opaque particles (lipid) in the microvilli and/or terminal web of intestinal cells from fat-fed animals. However, these observations have not been as consistent or as frequent as expected when there is ample ultrastructural evidence of lipid absorption in the cellular area distal to the microvilli and terminal web. We believe that extraction during tissue preparation could be the explanation for inconsistent and infrequent evidence of lipid in the microvilli and terminal web. Recent reports of lipid extraction from cells (amoeba (4), lung (9), liver (14), mammary glands (15), heart (16)) during preparation for electron microscopy have led us to the hypothesis that some absorbed lipid is likewise extracted from intestinal epithelial cells. Our experiments were designed to explore this possibility.

## MATERIALS AND METHODS

*Solution A* was composed of Kreb's-Ringer bicarbonate buffer (2), pH 7.3, without $Ca^{++}$ and $Mg^{++}$. Glucose and sodium taurocholate (Mann Research Laboratory, Inc., New York) were added to give concentrations of 16.7 mM and 4 mM, respectively.

*Incubation medium* for each experiment was prepared by placing 1 ml of a benzene solution containing 0.2 $\mu$M linoleic acid-1-$^{14}$C (Volk Radiochemical Co., Chicago, specific activity = 10 mc/mmole, radiopurity = 98%) into a 25-ml Erlenmeyer flask; the benzene was evaporated under $N_2$. Then 5 ml of solution A were added to the flask to form a micellar solution of linoleic acid-1-$^{14}$C, and the flask was gassed with 5% $CO_2$/95% $O_2$ (v/v).

Male albino rats (Holtzman Co., Madison, Wisc.) weighing approximately 200 g were starved for 24 hr and sacrificed by a blow to the head; two everted jejunal sacs per experiment were prepared by the method of Wilson and Wiseman (19).

A 25-ml Erlenmeyer flask containing 5 ml of solution A was put into a Dubnoff shaker at 37°C and gassed with 5% $CO_2$/95% $O_2$. A pair of everted sacs was placed in the flask for 5–10 min. Then the pair of sacs was transferred to the incubation medium for 30 min. After incubation, one ligated end of each sac was excised, and the serosal fluid voided. The two sacs were washed by agitating them in three successive vials containing 4 ml of solution A at 0°C for 15 sec each. The washed sacs were randomly designated *control tissue* and *experimental tissue*, and each was

weighed (average wet weight = 166 mg) after excising the remaining ligated end.

The control tissue was put into a glass homogenizer tube containing 5 ml of hydroxide of Hyamine 10-X (Rohm & Haas, Philadelphia). The tissue was homogenized for 5 min, and then the tube containing the homogenate was stoppered and placed in a 60°C oven for 12 hr. After cooling to room temperature, the homogenate was diluted in a volumetric flask to 10 ml with methanol washes of the homogenizer. Since a slight precipitate occurred the diluted material was centrifuged, and a 1-ml sample of the supernatant was added to a scintillation vial containing 10 ml of Bray's solution (1) (0.6% PPO, 0.03% dimethyl-POPOP, w/v). The contents of the scintillation vial were neutralized by the addition of two drops of HCl (conc).

The experimental tissue was put into a 15- by 50-mm glass-stoppered weighing vial containing approximately 2 ml of Millonig's osmium tetroxide fixative (8) without glucose at 0°C. The tissue remaining in the weighing vial was fixed, dehydrated, and infiltrated in approximately 2 ml of the following solutions in the sequence listed for the time and at the temperature given: Millonig's osmium tetroxide fixative, 0°C, 2 hr; three water washes, 0°C, 5 min each; 50, 75, 95, and 100%-I ethanol, 0°C, 5 min each; 100%-II ethanol, 5 min at 0°C, then 5 min at room temperature; propylene oxide I and II, room temperature, 10 and 20 min, respectively; propylene oxide:Luft's Araldite mixture (6) (1:1), room temperature, 1 hr; previous mixture doubled in volume in the weighing vial with Luft's Araldite mixture, room temperature, 6 hr. At the end of the time stated for each solution except the osmium tetroxide fixative, the liquid was decanted to a scintillation vial containing 10 ml of Bray's solution. The fixative was diluted to 10 ml, and a 1-ml sample was put into a scintillation vial containing 10 ml of Bray's solution.

Finally, the experimental tissue was put into a glass homogenizer tube containing 5 ml of hydroxide of Hyamine and subjected to the same treatment as the control tissue.

The radioactivity in each scintillation vial was measured in a Packard Spectrometer (Model 314EX-2); an internal standard was used to monitor efficiency.

## RESULTS AND DISCUSSION

The data reported are the results of six experiments. The fraction of absorbed linoleic acid-1-$^{14}$C extracted from the experimental tissue during its preparation for electron microscopy was 26%. The distribution of the extracted linoleic acid-1-$^{14}$C among the solutions used for tissue preparation is given in Fig. 1. The major loss occurred

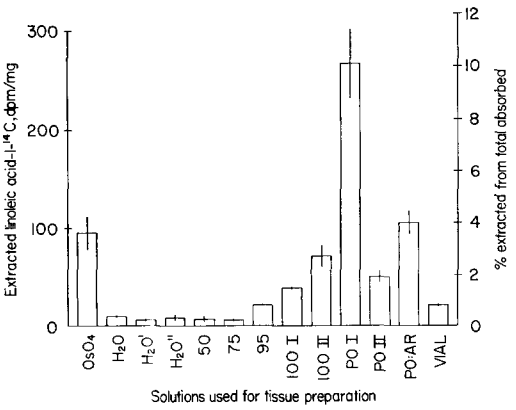


FIGURE 1 The extraction of absorbed linoleic acid-1-$^{14}$C by solutions used for tissue preparation of intestine for electron microscopy. In each experiment, the tissue was subjected to each solution listed on the abscissa from left to right for the time and at the temperature stated in Materials and Methods. The columns represent the quantity of linoleic acid-1-$^{14}$C recovered from the solutions designated under the columns. The column heights are the average results of six experiments, and the vertical lines represent the standard errors. The left ordinate shows the quantity of radioactivity extracted by each solution per mg of experimental tissue processed. The right ordinate shows the percentage of the total absorbed linoleic acid-1-$^{14}$C extracted. The total absorbed was measured by the radioactivity recovered from the control tissue which was 2646 ± 145 (SE) dpm/mg. The experimental tissue contained 1828 ± 131 (SE) dpm/mg after passing through the solutions. Average recovery of radioactivity from the experimental tissue plus the processing solutions was 95% of the radioactivity found in the control tissue.

in the solutions containing propylene oxide maintained at room temperature; this loss amounted to 17.0% of the absorbed linoleic acid-1-$^{14}$C. The remaining loss was equally divided between the cold aqueous fixative (3.6%) and water washes (1.0%), on the one hand, and the cold ethanol solutions on the other (4.6%).

The tissue was subjected for 7.50 hr to the propylene oxide solution, for 2.25 hr to the fixative and subsequent washes, and for 0.50 hr to the ethanol solutions. We are currently studying the effects of time and temperature on the extraction of absorbed linoleic acid-1-$^{14}$C by the solutions used for tissue preparation.

Luft (6) introduced propylene oxide as an excellent transitional solvent between dehydration and epoxy embedding. It has strong solvent properties, and it can infiltrate tissue readily

because of its low viscosity. We believe that this solvent would easily extract lipids, especially those not fixed by osmium tetroxide. Fixed lipids might also be susceptible to extraction by propylene oxide because of the solvent's reactive properties. Indeed, we often observed the extraction of reduced osmium from fixed tissue in propylene oxide, albeit the reduced osmium might not be chemically associated with lipid extracted simultaneously. A reduction in the loss of lipid into propylene oxide might be obtained if the process is carried out at a low temperature and for less time, but this alteration in procedure might also affect embedding.

We would expect the extraction of water-insoluble linoleic acid-1-$^{14}$C by the aqueous solution of osmium tetroxide if the fatty acids were in the form of water-soluble micelles or salts after absorption. We also detected the extraction of linoleic acid-1-$^{14}$C by an aqueous medium when we washed the tissue ten times after incubation. Analysis of radioactivity in the ten washes showed that the first three contained exponentially decreasing amounts of radioactivity. The fourth to tenth washes each contained the same amount of radioactivity. We, therefore, conclude that the radioactivity carried over from the incubation medium was washed from the tissue during the first three washes, as indicated by the exponential decrease in radioactivity from wash 1 to 3. Further washing permitted absorbed lipid to continue diffusing from the tissue, as indicated by the constant amount of radioactivity in each of the washes 4 to 10. Further evidence that fatty acids diffuse from intestinal cells after absorption is given by Porte and Entenman (12).

In order to elucidate the relation between the lipids extracted during tissue preparation and their state in the intestinal cell, we initiated a new series of experiments. We based these on methods and results reported by Strauss and Ito (17) and Strauss (18) who found that incubation at 0°C prevents the formation of apical cytoplasmic lipid droplets distal to the terminal web. We expect that under these conditions all of the absorbed lipid remains in the state of initial absorption. Preliminary data clearly demonstrate a greater loss of lipid into the osmium tetroxide fixative and subsequent water washes when the tissue is incubated at 0°C instead of 37°C. These data support the possibility that lipid absorbed into the microvilli and terminal web is significantly extracted during tissue preparation for electron microscopy.

Studies on the amount of lipid extracted after incubation at 0°C as well as electron microscopic observations on tissue incubated at 37° and at 0°C are in progress.

## SUMMARY

Everted sacs of rat jejunum were incubated in a bile salt solution of linoleic acid-1-$^{14}$C. After a 30-min absorptive period the tissue was fixed, dehydrated, and embedded for electron microscopy. Analysis of the solutions used in tissue preparation showed that 26% of the absorbed lipid was lost to the solutions. Preliminary studies reported support the possibility that lipid absorbed into the microvilli and terminal web of intestinal epithelial cells is significantly extracted during tissue preparation for electron microscopy.

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