Stages of uptake and incorporation of micellar palmitic acid by hamster proximal intestinal mucosa

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Abstract The stages of uptake and incorporation of micellar palmitic acid by hamster proximal intestinal mucosa were investigated by incubation of everted sacs at 4°C and 37°C for 2, 5, 10, and 15 min in a micellar solution (10 μmoles of [1-^{14}C]palmitic acid, 10 μmoles of monoolein, and 100μ moles of sodium taurodeoxycholate) and subsequent serial rinsing of the sacs in ice-cold solutions as follows: one 20-sec rinse in unlabeled micellar solution, five 1-min rinses in Krebs-Ringer buffer (0.15 M, pH 6.3), and ten 2-min rinses in 2.5% albumin solution. The fatty acid-solubilizing capacity of all the rinsing solutions was always in excess of the amounts of radioactive palmitic acid released during each rinse. Radioactivity was determined in the tissue homogenates, rinsing solutions, and serosal fluids.

The results indicate that a significant proportion of radioactive palmitic acid taken up by the sacs during the short incubation was released into the rinsing solutions. Rinsing in Krebs-Ringer buffer resulted in release of 15.5 ± 2.4% of the labeled fatty acid, and this fraction was independent of the temperature of incubation. In contrast, the amounts of palmitic acid released in albumin were significantly greater and were markedly dependent on the temperature of incubation; a total of 48.6 ± 7.0% and 26.3 ± 5.1% was released from sacs incubated at 4°C and 37°C, respectively. While the proportion of radioactive palmitic acid in the free fatty acid fraction of the tissue after the rinsing sequence remained reasonably constant regardless of the temperature and duration of incubation, the radioactivity of the esterified palmitic acid in the tissue was much greater in the sacs incubated at 37°C and tended to increase linearly up to 10 min of incubation. A highly significant inverse relationship was found between the fraction of radioactive palmitic acid released by rinsing in albumin and the fraction of the label in the tissue esterified fatty acids.

The results suggest that the initial uptake of micellar fatty acid by intestinal mucosa may involve reversible binding to superficial sites with at least two strengths of binding: a weak, temperature-independent binding which could be easily dissociated by rinsing in Krebs-Ringer buffer, and a stronger, temperature-dependent binding which could be dissociated by rinsing in albumin, but not in Krebs-Ringer buffer. Analogous binding of micellar palmitic acid occurred in a brush border preparation of proximal intestine which was devoid of any fatty acid esterifying activity. This suggested that the reversible binding of fatty acid by the intestinal mucosa may be a property of its superficial components, namely the glycocalyx or microvillous membranes, and that it may be independent of the esterifying capacity of the tissue.

Supplementary key words micellar fatty acids · reversible binding · albumin · membrane receptors · [1-^{14}C]-palmitic acid

IT IS CURRENTLY ACCEPTED that fatty acid and monoglyceride, derived from the intraluminal hydrolysis of ingested triglyceride, undergo micellar solubilization before being absorbed by the intestinal epithelium (1, 2). The physicochemical laws governing micelle formation have been well defined (3, 4). Very little is known, however, about the sequence of events which take place once the micellar aggregate or its components come into contact with the outermost surface of the epithelial cell, namely the glycocalyx and microvillous plasma membrane. The nature of the association of the micelle with

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Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography; KRP, Krebs-Ringer phosphate; FFA, free fatty acid; EFA, esterified fatty acid.

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this region and the manner in which its components permeate the surface barrier to enter the cell interior are at present unknown.

Studies using electron microscopy alone or in conjunction with radioautography have been unsuccessful thus far in defining the mechanism of fatty acid uptake and transport. Strauss (5), using hamster jejunum incubated in micellar solutions of monoolein, linolenic acid, and taurodeoxycholate, was unable to detect the presence of micelles or oil droplets in the glycocalyx, the microvilli, or the terminal web. On the other hand, Ashworth and Lawrence, using rat jejunum (6), felt that they could visualize micelles of oleic acid, monoolein, and taurocholate in the glycocalyx and in contact with the outer plasma membrane of the microvilli. The interpretation of these results, however, and the conclusions that can be drawn about the mechanisms whereby micellar lipid is taken up and subsequently incorporated into the intestinal epithelial cell are seriously restricted by the limitations inherent in the techniques employed. During the fixation and dehydration of the tissue, lipid in the brush border region of the cell is removed and micellar aggregates are disrupted, since, by definition, micelles cannot exist without water.

In 1902, Overton (7) introduced the concept that the rate of penetration of a substance into a cell is largely determined by its lipoid solubility or its oil–water partition coefficient. Since that time it has generally been accepted that lipid molecules enter the intestinal epithelial cell by diffusing into and dissolving in the absorptive membrane (8–10). However, it was recently observed that the uptake of lipid by the rat intestine exhibited kinetic characteristics consistent with a saturation phenomenon (11). In addition, Bennett Clark and Holt (12) showed that trioctanoin inhibited the maximal absorption of triolein during steady state intestinal absorption in unanesthetized rats. These results suggested that the intestinal mucosa may not behave as a simple lipid interface and that a carrier-mediated transport mechanism might be operative in the absorption of lipids. These results also provide experimental support for the presumed existence of membrane receptors for lipids, the presence of which was postulated by Danielli in 1963 (13).

It has been shown that a significant proportion of the fatty acid originally taken up by in vitro preparations is available for release into aqueous media (10, 14–16). We have recently shown that this is also true in the case of micellar palmitic acid taken up by hamster jejunum (17). On the basis of this finding, we postulated that the uptake of fatty acid is a dynamic process involving both influx and efflux and that the initial step in uptake might be mediated by the reversible association of fatty acids to superficial binding sites. Once bound in this region, fatty acid molecules could then proceed in either of two directions: they could penetrate deeper into the cell to become more tightly bound and eventually esterified, or, depending upon the existing conditions, they could be released back into the bathing fluid.

This investigation was undertaken to study the interaction between micellar fatty acid and the intestinal mucosa and to characterize further the reversible association of micellar palmitic acid with hamster jejunum under a variety of experimental conditions.

METHODS

Materials

Palmitic acid, monoolein (Hormel Institute, Austin, Minn.), and sodium taurodeoxycholate (NaTDC, Maybridge Research Chemicals, Tintagel, Cornwall, U.K.) were certified as at least 99% pure by the suppliers. [1-^{14}C]Palmitic acid, certified to be 98% pure, was supplied by the New England Nuclear Corp., Boston, Mass. Confirmatory analyses of these chemicals by GLC and TLC were in close agreement with the stated purities. Bovine serum albumin, fraction V, was obtained from Pentex Inc., Kankakee, Ill. Inherent fatty acids were removed by the method of Goodman (18). After dialysis of the extracted albumin against saline at 4°C, the free fatty acid still present was assayed by the method of Dole (19) and was found to be 0.01 mole/mole of albumin. All chemicals used were reagent grade and all organic solvents were doubly distilled.

Analytical

TLC was carried out on standard glass plates (20 × 20 cm) coated with silica gel G (E. Merck A.G., Darmstadt, Germany), 250 μ thick, containing 2′,7′-dichlorofluorescein; the solvent system was *n*-hexane–diethyl ether–acetic acid–methanol 90:20:2:3 (20). The various lipid fractions after separation were identified by comparison with standards (Hormel Institute) simultaneously chromatographed. The fractions were visualized by ultraviolet illumination, and the corresponding areas of silica gel and appropriate blank areas were scraped directly into counting vials that contained 12 ml of a scintillation solution (0.4% Omnifluor, in toluene; New England Nuclear).

Total radioactivity of the different incubation and washing solutions, as well as of the tissue homogenates and serosal fluid, was assayed in 1-ml aliquots added to 12 ml of Bray's solution (21). All samples were counted in a Tri-Carb liquid scintillation counter (model 3375, Packard Instrument Co., Downers Grove, Ill.) with a minimum efficiency of 85% and 45% for the toluene and Bray's solution, respectively. The samples were corrected

for quenching by the channels ratio method (22) or by internal standardization.

General procedure

Experiments using everted gut sacs. Female golden hamsters weighing 100–120 g (Quebec Breeding Laboratories, LaPrairie, Quebec) were fasted overnight and anesthetized with ether. The small intestine was rapidly removed and rinsed with ice-cold saline. The gut was then everted over a chilled glass rod and filled with Krebs-Ringer phosphate buffer (pH 6.3, 0.15 M, Ca^{2+} and Mg^{2+} omitted). Only the intestine lying between 4 and 16 cm from the pylorus was used, and sacs 3 cm long (250–350 mg wet wt) were prepared by cutting between double ligatures. This region of the small intestine exhibited uniform uptake, release, and esterification of [1-^{14}C]palmitic acid. Furthermore, 95.1% of the radioactivity taken up during incubation was present in the mucosal scrapings of this region. The sacs were randomized and incubated in duplicate for 2, 5, 10, and 15 min in separate Dubnoff shakers maintained at 4°C and 37°C in an atmosphere of 95% O_2 and 5% CO_2. The incubation medium consisted of 10 ml of a micellar solution which contained 10 μmoles of [1-^{14}C]palmitic acid (0.05 μCi/μmole), 10 μmoles of monoolein, 100 μmoles of NaTDC, and 10 μmoles of glucose made up in the KRP buffer (Ca^{2+} and Mg^{2+} omitted) (23, 24). After incubation, the sacs were transferred sequentially into a series of unlabeled washing solutions maintained at 4°C to ensure that the release of [1-^{14}C]palmitic acid was unaffected by the energy-requiring processes. The rinsing procedure was carried out in the following order: (*a*) a single 20-sec rinse in an identical but nonradioactive micellar solution; (*b*) five separate 1-min rinses in the KRP buffer (pH 6.3); and (*c*) ten 2-min rinses in 2.5% solutions of fatty acid-free albumin made up in saline (pH 7.4, 0.15 M). In some of the experiments, multiple 1-min rinses in KRP buffer or albumin were performed. Each successive rinse was carried out in a fresh 20-ml volume of rinsing solution, and the sacs were gently swirled every 30 sec. More vigorous shaking resulted in the fragmentation of the villous surface. The pH of the albumin was adjusted to 7.4, the point at which optimal fatty acid binding is achieved (25). Preliminary studies indicated that identical results were obtained by rinsing in volumes of 20, 50, and 100 ml of KRP buffer and albumin solution as well as with albumin concentrations of 1.0, 2.5, and 5.0%. The albumin concentration was set at 2.5% in anticipation of future experiments using higher concentrations of fatty acid.

After completion of the rinsing sequence, the sacs were drained of serosal fluid, weighed, and homogenized in 10 ml of normal saline in an all-glass Potter-Elvehjem homogenizer. 1-ml aliquots of incubation medium, rinsing solutions, tissue homogenate, and serosal fluid were directly counted in Bray's solution as described. The remaining samples were extracted with chloroform–methanol 2:1 (v/v) (26), and aliquots of the lipid extract were taken for TLC to determine the distribution of radioactivity among the free and esterified fatty acid fractions.

The total uptake of [1-^{14}C]palmitic acid (μmoles $\times 10^{-3}$/100 mg wet wt) was calculated by adding the amounts of [1-^{14}C]palmitic acid released into all the rinsing solutions to that which remained in the tissue at the end of the rinsing sequence. As no direct measurements of free fatty acid mass were made, all calculations assume that the specific activity of [1-^{14}C]-palmitic acid released into the washing solutions was similar to that present in the initial incubation medium. This assumption was based on results from preliminary experiments which showed that over 90% of the fatty acid was palmitic acid and that the specific activity remained within reasonably narrow limits throughout the rinsing procedures. Experiments in which the sacs were extracted before the rinsing sequence yielded values for total uptake which were within one standard deviation of the mean value obtained by the method described above. The amount of esterified [1-^{14}C]palmitic acid at the end of the incubation and that after rinsing for 25 min at 4°C remained within very narrow limits, indicating that the labeled palmitic acid released during the rinsing procedure had not been derived from the lipolysis of this fraction. Furthermore, the substitution of [9,10-^{3}H]-palmitic acid for the ^{14}C-labeled acid in the incubation medium did not change the results obtained. This finding is consistent with the conclusion that oxidation of the terminal carboxyl group had not occurred to any significant extent under our experimental conditions. In control experiments, the identical incubation and rinsing sequence was carried out with boiled (2 min in boiling KRP buffer) everted sacs or with silk strings ($\approx 3.0 \times 0.5$ cm) made up of 36 strands of surgical silk (Deknatel, surgical silk 2–0, J.A. Deknatel & Son, Inc., Queens Village, N.Y.), entwined and tied together at the ends. Because of the friability of the boiled sacs, each incubation medium and rinsing solution was filtered in the same sequence as the rinsing, and the tissue fragments were collected over the same filter paper, which was then rinsed thoroughly in the medium used for homogenization of the sacs. No appreciable radioactivity was retained on the filter paper.

Experiments using intestinal brush borders. Brush borders were prepared from the proximal 15 cm of hamster small intestine according to the method of Miller and Crane (27), as modified by Donaldson, Mackenzie, and Trier (28). The resultant preparation was found by electron microscopy to be morphologically pure. The isolated

brush borders were taken up without disruption into 10 ml of KRP buffer (pH 6.3, 0.15 M). Aliquots (2 ml) of this suspension were added to glass centrifuge tubes which contained 8 ml of micellar solution adjusted so that the final concentration in 10 ml would be identical to that of the incubation media used for the everted gut sacs. In control tubes, 2 ml of KRP buffer was substituted for the brush border suspension. Incubations were carried out for 15 min at 4°C and 37°C in separate Dubnoff metabolic incubators with continuous shaking and gassing with 95% O_2 and 5% CO_2. The reactions were terminated by addition of 4 ml of ice-cold KRP buffer to each flask; the mixture was centrifuged at 800 *g* for 10 min, and the supernatant was decanted. The brush border pellet was washed three times in 10 ml of ice-cold KRP buffer (pH 6.3) and 5 times in 10 ml of ice-cold 2.5% fat-free albumin (pH 7.4). Each wash was carried out by resuspending the pellet with vigorous stirring on a Vortex mixer for 1 min. After these procedures, the mixture was centrifuged for 10 min at 800 *g* and the supernatant was decanted. The control tubes were rinsed in an identical manner. Upon completion of the rinsing sequence, the pellet of brush borders was taken up in 5 ml of KRP buffer and homogenized in a Potter-Elvehjem homogenizer, and the resulting suspension was made up to 10 ml.

Total radioactivity, as well as the distribution of the ^{14}C among the lipids, was determined in all incubating and rinsing solutions and in the homogenate of the brush borders in the manner described earlier. Protein determinations were carried out by the method of Lowry et al. (29).

To correct for the adsorption of micellar palmitic acid to the glass surface of the centrifuge tubes and the subsequent removal of this radioactivity by our washing procedures, the amount of radioactive palmitic acid released into simultaneous "control" washings (0.8–2.1% of the total uptake) was subtracted from the amounts present in the corresponding solutions which had been exposed to the brush border. The total uptake of [1-^{14}C]-palmitic acid (μmoles $\times$ 10^{-3}/mg of protein) was derived by adding the amounts of [1-^{14}C]palmitic acid released into the KRP and albumin washes to that present in the brush border pellet after rinsing. The amount of labeled palmitic acid in the brush border preparation before rinsing was very close to the amount determined by adding the radioactivity released into the rinsing solutions to that remaining in the brush border pellet after the rinsing sequence.

Statistical analysis

Student's *t* test for paired values was used for comparing individual differences. Only *P* values of < 0.01 were considered to be significant.

RESULTS

Effect of duration and temperature of incubation on the uptake of micellar [1-^{14}C]palmitic acid

The total uptake of [1-^{14}C]palmitic acid (Fig. 1) during incubation at 4°C and 37°C increased linearly during the first 10 min, after which the uptake leveled off. Although the values obtained at 37°C were always greater than those at 4°C, the differences were not found to be statistically significant.

The amount of [1-^{14}C]palmitic acid released during the 20-sec rinse in nonradioactive micellar solution comprised between 15 and 30% of the total uptake (lower portion of Fig. 1) and was not significantly affected by the duration of incubation. Although the sacs incubated at 4°C released greater amounts of radioactivity, this difference was statistically significant only for the sacs incubated for 5 and 10 min. It was found that 94.4 $\pm$ 1.1% of the released radioactivity was in the form of unesterified fatty acid, and this was independent of the duration and temperature of incubation. The amount of radioactive palmitic acid present in the tissue after this short rinse ("net uptake") showed the same pattern of change with time and temperature as did the total uptake (Fig. 1). The temperature-related differences in the net uptake were found to be statistically significant only at 5 and 10 min of incubation.

The amount of radioactivity in the serosal fluid was very small and it was unaffected by the incubation conditions employed. The mean content was 1.1 $\pm$ 0.9% of the total uptake, and in all subsequent experiments this amount was considered as part of the radioactivity taken up by the tissue.

In the control experiments, the total uptake by boiled everted sacs and by strings varied considerably but never exceeded 38 μmoles $\times$ 10^{-3}/100 mg wet wt, and it was independent of the temperature and duration of incubation. A single 20-sec rinse in the nonradioactive micellar solution removed 82.3 $\pm$ 4.8% and 91.1 $\pm$ 5.4% of the total uptake by boiled sacs and strings, respectively. Approximately 8% of the residual radioactivity of the sacs was accounted for by the radioactivity in the serosal fluid, reflecting the greater permeability of the boiled sacs.

Characterization of the reversibly bound [1-^{14}C]palmitic acid

In order to determine the fraction of the net uptake of [1-^{14}C]palmitic acid that was reversibly bound to the tissue and to characterize the nature of the association between medium palmitic acid and the tissue, the preincubated sacs, after the 20-sec exposure to unlabeled micelles, were sequentially rinsed in KRP buffer or in albumin as described in Fig. 2. The results obtained in-

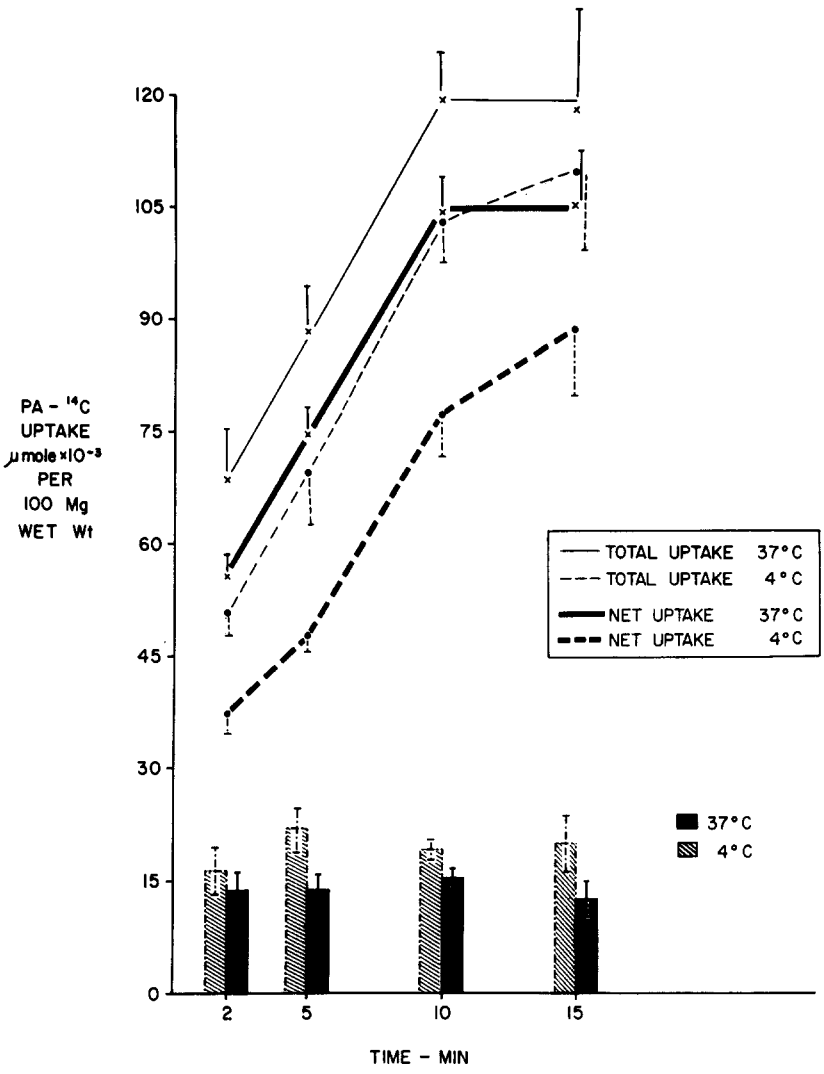

FIG. 1. Effect of duration and temperature of incubation on the uptake of micellar [1-^{14}C]palmitic acid (PA-^{14}C) by hamster everted jejunal sacs. The incubation medium consisted of 10 ml of a micellar solution containing 10 μmoles of [1-^{14}C]palmitic acid, 10 μmoles of monoolein, and 100 μmoles of sodium taurodeoxycholate made up in KRP buffer (0.15 M, pH 6.3). The continuous and dashed lines represent incubation at 37°C and 4°C, respectively. The upper, thinner lines indicate the "total uptake" of radioactive palmitic acid, which was the amount of radioactivity present in the tissue at the end of the incubation. The lower, thicker lines represent the "net uptake" of [^{14}C]palmitic acid, which was defined as the amount of [^{14}C]palmitic acid remaining after the 20-sec rinse in the unlabeled micellar solution. The amounts of [^{14}C]-palmitic acid released during this rinse is shown by the bars in the lower portion of the graph. All the values shown represent the mean ± SEM of four experiments.

dicate that although the pattern of [1-^{14}C]palmitic acid release was similar for both rinsing solutions, the amount released into albumin always exceeded that released into KRP. During 25 min of rinsing, 27.4 ± 2.8% and 53.1 ± 3.9% of the [^{14}C]palmitic acid taken up (net) was released into KRP buffer (pH 6.3) and albumin (pH 7.4), respectively. Furthermore, 93.1 ± 1.1% and 91.6 ± 1.0% of the radioactivity released into these respective solutions was in the form of unesterified fatty acids. The greater amount of radioactive palmitic acid released into albumin could not be attributed to a difference in the pH of the solutions, as identical results were obtained when the pH of the rinsing solutions was adjusted to either 6.3 or 7.4.

The theoretical possibility existed, however, that exposure of the tissue to albumin may have resulted in an alteration of its binding properties so that palmitic acid could subsequently be more easily released into any rinsing solution, be it albumin or KRP. This possibility was tested as follows. The usual sequence of albumin rinsing was interrupted at different times during the 25-min rinsing period, and the sacs were then transferred into a series of KRP buffer washings for the duration of the rinsing time. The amounts of radioactive palmitic

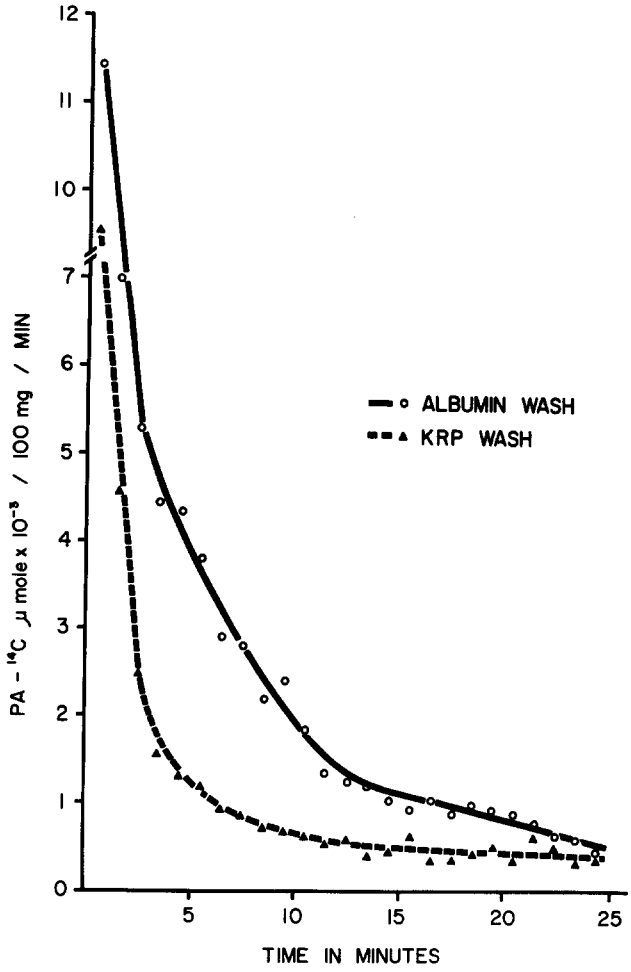

FIG. 2. Release of [1-^{14}C]palmitic acid (PA-^{14}C) by sequential rinsing in KRP buffer (- - - -) or 2.5% albumin solution (——): The everted sacs had been preincubated for 10 min at 37°C in the micellar incubation medium described. After the 20-sec rinse in the unlabeled micellar solution, the sacs were rinsed sequentially for 1 min in separate 20-ml volumes of KRP buffer or albumin solution. Each value shown is the mean of four experiments (eight sacs).

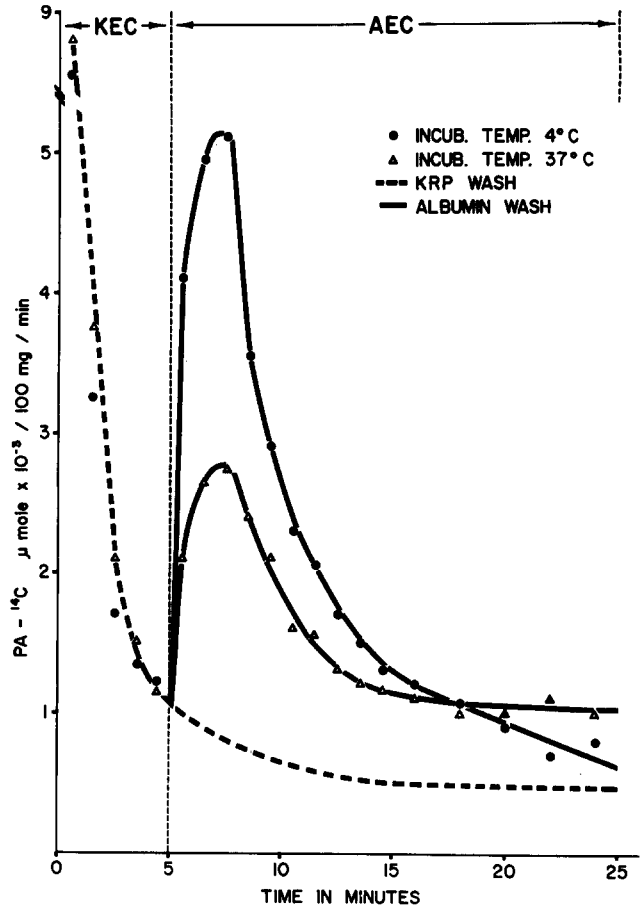

FIG. 3. Effect of temperature of incubation on the amount of [1-^{14}C]palmitic acid (PA-^{14}C) released during each rinsing period into KRP buffer (- - - -) and albumin (——). The everted sacs had been preincubated for 10 min at 4°C (●) and 37°C (Δ) in the micellar solution described. After this, the sacs were rinsed for 20 sec in the unlabeled micelle solution. They were then subjected to five 1-min rinses in separate 20-ml volumes of KRP buffer (pH 6.3) and then to twenty 1-min rinses in separate 20-ml volumes of 2.5% albumin (pH 7.4). Each value represents the mean of four experiments (eight sacs). Continuation of the dashed line beyond 5 min indicates the pattern of [^{14}C]palmitic acid release in KRP buffer with sequential rinsing for 25 min as shown in Fig. 2. *KEC*, KRP-extractable compartment; *AEC*, albumin-extractable compartment.

acid released upon exposure to KRP buffer fell immediately to the values predicted by the KRP curve shown in Fig. 2, indicating that the binding properties of the tissue had not been altered during exposure to albumin. Altering the sequence of rinsing from KRP to albumin as shown in Fig. 3 also resulted in the immediate release of larger amounts of radioactivity. These results indicate that irrespective of the sequence of exposure of the tissues to the rinsing solutions, albumin always removed a significantly greater amount of radioactivity which could not be removed by KRP alone.

Opposite to the above experiments, exposure of the boiled sacs and the strings to a single rinse in KRP or albumin removed most of the residual radioactivity after the initial rinse in nonradioactive micellar solution. After the complete sequence of rinsing, less than 1.5% of the total uptake was found in the boiled sacs, and virtually no radioactivity was found in the strings.

The finding that [1-^{14}C]palmitic acid from the intact sacs was more readily released into albumin compared with KRP buffer is consistent with the strong binding affinity and large binding capacity of albumin for palmitic acid (23, 30) and the very limited solubility of palmitic acid in KRP (31). However, the amount of radioactive palmitic acid released into any given 20-ml rinse with KRP buffer or albumin was much less than the theoretical capacity for solubilization or binding, or both, of palmitic acid by the respective solutions. Furthermore, the use of rinsing volumes of 50 or 100 ml as well as

albumin concentrations of 1.0, 2.5, and 5.0% did not alter the amounts of radioactivity released. This would indicate that under the conditions of our experiments, the fatty acid-solubilizing capacity of the rinsing solutions used could not have been a limiting factor in the release of radioactive palmitic acid. Differences in the rates of solubilization in the rinsing solutions and in the rates of diffusion of palmitic acid out of the tissue cannot completely explain the findings. It is seen in Fig. 3 that the amount of labeled palmitic acid released during the initial rinses in KRP was comparable to that released after the exposure to albumin. This would indicate that the amount of releasable radioactive palmitic acid on or into the tissue was well within the solubilizing capacity of KRP and was in concentrations sufficient to maintain a favorable gradient for diffusion from tissue to rinsing solution. The fact that the additional amount of labeled palmitic acid could be released only after rinsing in albumin suggests the possibility that the palmitic acid which could not be removed by KRP was bound to the tissue with an affinity comparable to that of albumin.

These results suggested the possibility that labeled palmitic acid had been reversibly bound to the tissue with at least two different strengths of binding: loosely bound [1-^{14}C]palmitic acid which could be easily removed by rinsing in KRP buffer and the more tightly bound labeled palmitic acid which could be dissociated by albumin but not by KRP buffer.

In order to obtain a meaningful estimate of the amount of radioactive palmitic acid releasable by rinsing in KRP buffer ("KRP-extractable compartment") and the amount releasable by rinsing in albumin ("albumin-extractable compartment"), and to determine the effect of the temperature of incubation on the distribution of [1-^{14}C]palmitic acid in these compartments, the following experiments were performed. Sacs incubated for 10 min at 4°C and 37°C were sequentially rinsed in KRP buffer for a total of 5 min, as it was found that during this time approximately 70% of the loosely bound radioactive palmitic acid was removed (Fig. 2). After this, the sacs were exposed to albumin for the remaining 25 min of rinsing (i.e., for a total of 20 min) to approximate the amount of [1-^{14}C]palmitic acid which could be released by rinsing in albumin solution. Rinsing the sacs for longer than 25 min resulted in the histological deterioration of the intestinal epithelium.

Fig. 3 shows that, after the sequence of five 1-min rinses in KRP buffer, exposure of the sacs to albumin produced a significant and consistent rise in the amount of [1-^{14}C]palmitic acid released. The amount of radioactive palmitic acid released from sacs incubated at 4°C was always greater than the amount released from sacs incubated at 37°C. This relationship between the amount of radioactive palmitic acid released by rinsing in albumin solution and the temperature of incubation of the sacs is also seen in Fig. 4. The amount of [1-^{14}C]-palmitic acid which could be released by rinsing in KRP, however, was independent of the temperature at which the sacs were incubated (Figs. 3 and 4).

The radioactive palmitic acid which could not be removed by our washing techniques was distributed in the free and esterified fatty acid compartments of the tissue (Fig. 4). It can be seen that while the amount of labeled palmitic acid contained in the tissue unesterified fatty acid compartment was unaffected by the temperature of incubation, the amount of esterified [1-^{14}C]palmitic acid was much greater at 37°C. It is further seen that an inverse relationship existed between the amounts of radioactive palmitic acid releasable by rinsing in albumin solution and the tissue esterified fatty acid compartment.

Effect of duration and temperature of incubation on the compartmental distribution of [1-^{14}C]palmitic acid

The effect of incubation of the sacs for 2, 5, 10, and 15 min at 4°C and 37°C on the compartmental distribution of radioactive palmitic acid is shown in Figs. 5 and 6. The labeled palmitic acid content of the KRP-extractable compartment was unaffected by the temperature of incubation. It increased linearly up to 10 min of incubation, after which a leveling off occurred (Fig. 5, *A*). In addition, the fraction of the net amount of [1-^{14}C]palmitic acid taken up which was present in

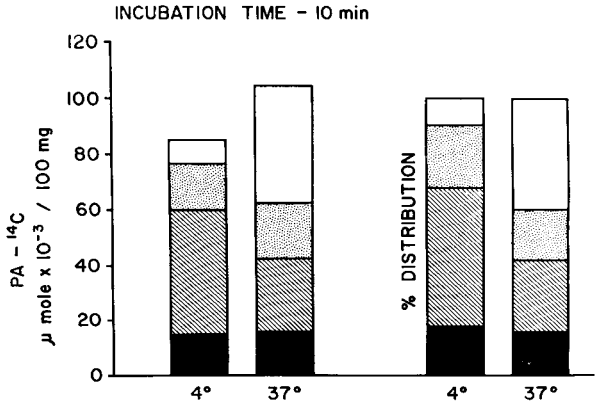

Fig. 4. Effect of temperature of incubation on the compartmental distribution of [1-^{14}C]palmitic acid (PA-^{14}C). Sacs were preincubated for 10 min at 4°C and 37°C as described and were rinsed for 20 sec in the unlabeled micelle solution. The left-hand portion represents the absolute amounts of [^{14}C]palmitic acid, and the right-hand portion represents the percentage of the net uptake in each compartment. Black bars represent the KEC (see Fig. 3); hatched bars, AEC; stippled bars, tissue FFA compartment; and open bars, tissue EFA compartment. Each value is the mean of four experiments (eight sacs). SEM is shown in Figs. 5 and 6.

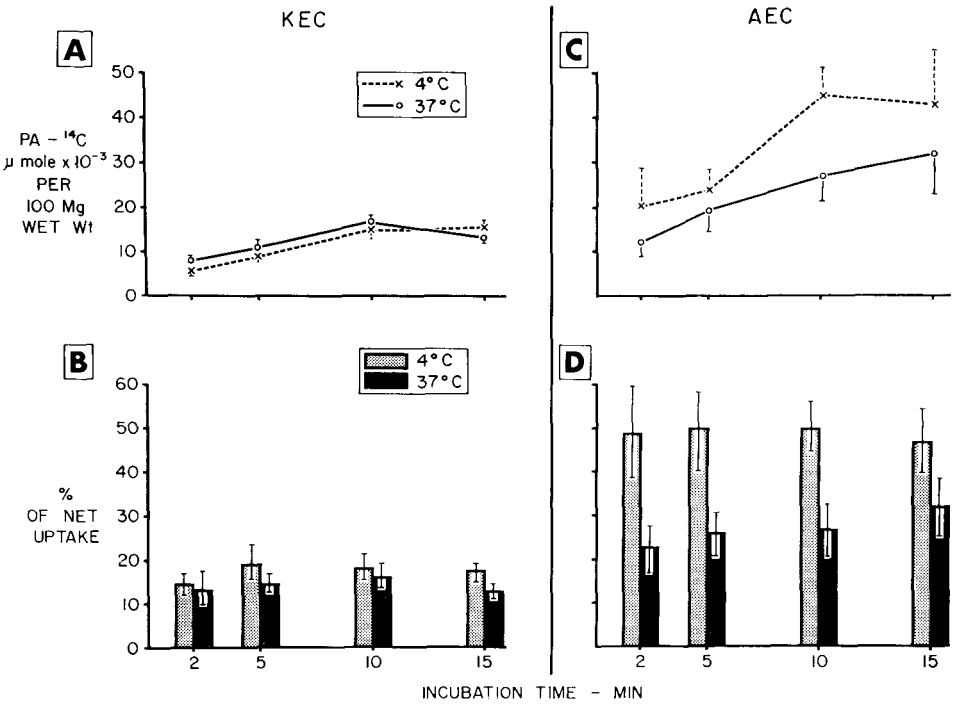

FIG. 5. Effect of duration and temperature of incubation on the distribution of [1-^{14}C]palmitic acid (PA-^{14}C) in the KRP-extractable (*KEC*) and the albumin-extractable (*AEC*) compartments. *A*, absolute amount of [^{14}C]palmitic acid in the KEC; *B*, % of the net uptake of [^{14}C]palmitic acid in the KEC; *C*, absolute amount of [^{14}C]palmitic acid in the AEC; and *D*, % of the net uptake of [^{14}C]palmitic acid in the AEC. Each value is the mean of four experiments (eight sacs) ± SEM.

the KRP-extractable compartment was independent of both the duration and the temperature of incubation and was between 12.3 and 19.1% of the net uptake (Fig. 5, *B*). The amount of labeled palmitic acid contained in the albumin-extractable compartment, however, was always greater after the incubation at 4°C, with the maximum values occurring after 10 min of incubation (Fig. 5, *C*). After incubation at 4°C, a constant proportion of the net amount of palmitic acid taken up, ranging between 46.7 and 50.0%, was found in the albumin-extractable compartment (Fig. 5, *D*). The corresponding values obtained after incubation at 37°C were always smaller and showed a tendency to increase linearly with time. Analysis of the released radioactivity indicated that 93.1 ± 1.1% and 91.6 ± 1.0% of the labeled palmitic acid released by rinsing in KRP or albumin solution, respectively, was unesterified, irrespective of the experimental conditions used.

The amount of radioactive palmitic acid which could not be removed by the rinsing procedure (tissue unesterified fatty acid compartment) was unaffected by the temperature of incubation (Fig. 6, *A*). Whereas the absolute amount of labeled palmitic acid in this compartment showed a tendency to increase with time, the relative amount of [1-^{14}C]palmitic acid present, expressed as the percentage of the net uptake, remained within very narrow limits (Fig. 6, *B*). The amount of labeled palmitic acid in the tissue esterified fatty acid compartment was very markedly temperature-dependent and was significantly inhibited during incubation at 4°C (Fig. 6, *C*). Incubation at 37°C stimulated the esterification of [1-^{14}C]palmitic acid, which increased linearly up to 10 min. After this, a falling off in activity occurred which may have been due to deterioration of the tissue preparation or activation of intracellular lipolysis (32). Light and electron microscopy of the sacs after the 15-min incubation at 4°C and 37°C and after the rinsing sequence in KRP and albumin solution showed no significant alterations. It was further shown that reincubation for 10 min in a radioactive micellar solution of sacs previously subjected to the rinsing sequence resulted in an uptake and esterification of palmitic acid analogous to that of sacs before rinsing. The possibility of activation of intracellular lipolysis would also seem very unlikely, as the decrease in the amount of esterified [1-^{14}C]palmitic acid at 15 min of incubation was not accompanied by a corresponding increase in the amount of radioactivity released by rinsing in KRP or albumin solution or in the amount of labeled palmitic acid in the tissue free fatty acid compartment. The percentage of radioactive palmitic acid present in the tissue esterified fatty acid compartment was rather constant for a given

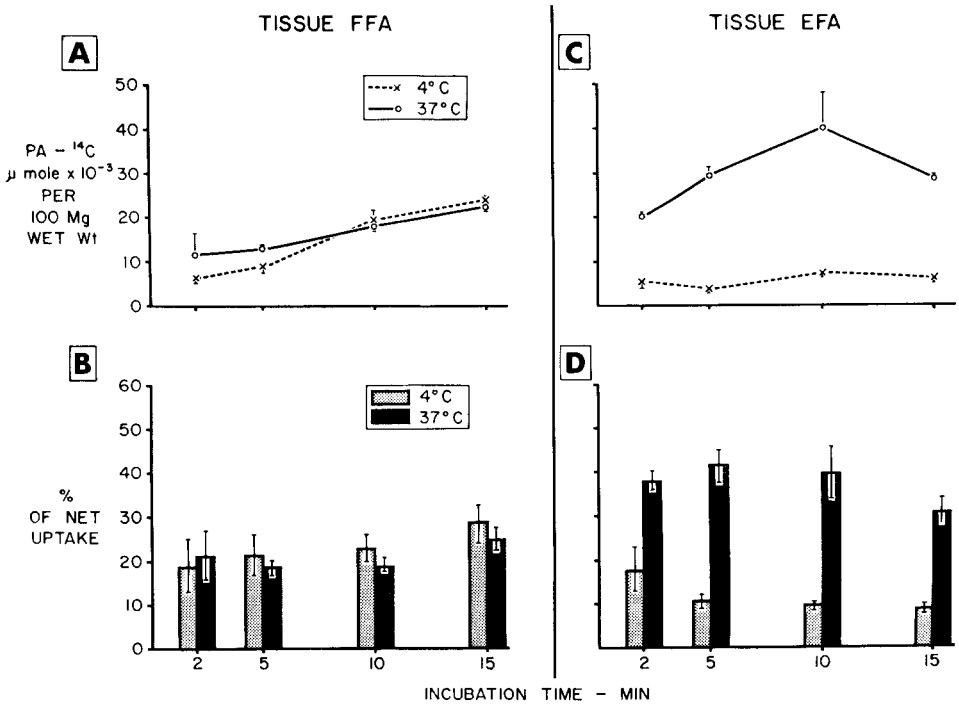

FIG. 6. Effect of duration and temperature of incubation on the distribution of [1-^{14}C]palmitic acid (PA-^{14}C) in the tissue free fatty acid (*FFA*) and tissue esterified fatty acid (*EFA*) compartments. *A*, absolute amount of [^{14}C]palmitic acid in the tissue FFA compartment; *B*, % of the net uptake of [^{14}C]palmitic acid in the tissue FFA compartment; *C*, absolute amount of [^{14}C]palmitic acid in the tissue EFA compartment; and *D*, % of the net uptake of [^{14}C]palmitic acid in the tissue EFA compartment.

incubation temperature (Fig. 6, *D*); a minor exception to this generalization occurred at 2 min of incubation (4°C).

The data presented in Figs. 5 and 6 indicate that for a given incubation temperature, the fraction of the net amount of labeled palmitic acid taken up in each compartment was relatively constant over the range of incubation times used. Although the factors which regulated the distribution of palmitic acid into these compartments could not be determined from the present experimental data, it was clear that an inverse relationship existed between the esterifying capacity of the tissue (Fig. 6, *D*) and the amount of [1-^{14}C]palmitic acid released by rinsing in albumin solution (Fig. 5, *D*). Mathematical analysis of the data indicated that two distinct regression lines could be derived for incubation at 4°C and 37°C, shown as P-4 and P-37, respectively, in Fig. 7. The slope of P-4 was clearly greater than that of P-37 ($P < 0.01$), and in addition, the individual values for radioactive palmitic acid released by rinsing in albumin solution after incubation at 4°C were always in excess of those which could have been predicted by extrapolation of the regression line for incubation at 37°C (P-37). This suggested that a process in addition to esterification had contributed to the amount of radioactive palmitic acid in the albumin-extractable compartment at 4°C. However, in view of the fact that P-37 was derived from values for esterified radioactive palmitic acid which never went below 13% of the net uptake, the theoretical possibility existed that for very low levels of esterified [1-^{14}C]palmitic acid, P-37 would not continue as a straight line but would instead curve upwards to merge with P-4. If this were the case, then the reduced esterifying capacity of the tissue would by itself account for the amount of labeled palmitic acid releasable by rinsing in albumin solution. In order to rule out this possibility, it was necessary to obtain data for the entire range of esterification under consideration using incubation at 37°C only. This was achieved by using gut sacs prepared from both the proximal and the distal intestine, as it was found that the terminal portions of the intestine possessed very low esterifying activity at 37°C. The results obtained yielded a regression line (PD-37, Fig. 7) which was very similar to P-37 and extended over a range of esterification from 1.5 to 50.0%, confirming that the inverse relationship between esterification and the amount of labeled palmitic acid releasable by rinsing in albumin solution was linear, even at very low levels of esterification. These results lent further support to the conclusion that an additional process was operative at 4°C. The possible significance of this finding will be discussed in a later section.

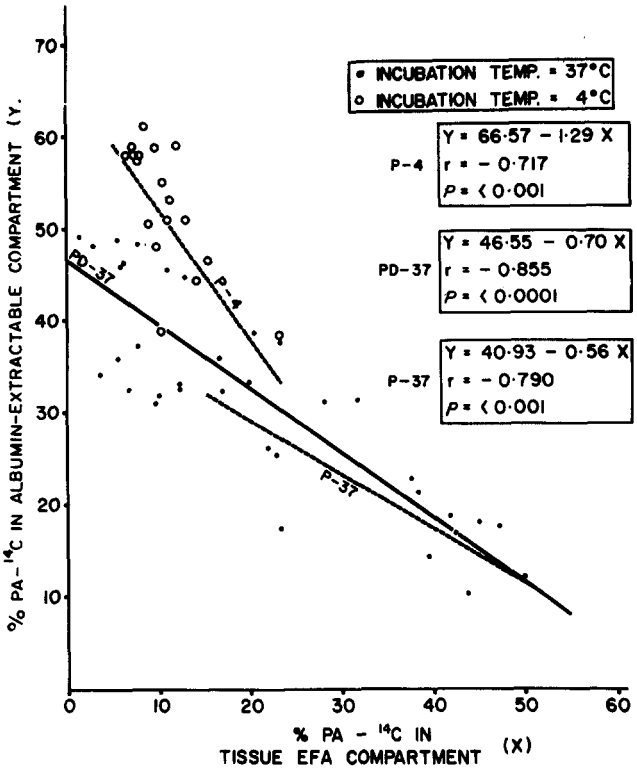

FIG. 7. Relationship between the percentage of the net uptake of [1-^{14}C]palmitic acid (PA-^{14}C) in the tissue esterified fatty acid (EFA) compartment and the albumin-extractable compartment for proximal intestine incubated at 4°C (*P-4*) and 37°C (*P-37*), and for proximal and distal intestine incubated at 37°C (*PD-37*).

Uptake and release of micellar [1-^{14}C]palmitic acid by a brush border preparation of hamster intestine

In order to characterize further the site of binding of palmitic acid by the intestinal sacs and to determine the relationship between the binding properties and the intracellular concentration and esterification of radioactive palmitic acid, experiments with a brush border preparation were undertaken. The results, summarized in Table 1, indicate that in contrast to the results with everted sacs, the uptake of micellar palmitic acid was significantly greater during incubation at 4°C. Furthermore, at least 98% of the radioactivity taken up at either temperature could be removed by subsequent washing in KRP buffer and albumin. It was found that after three rinses with KRP buffer very little additional radioactive palmitic acid could be removed with continued rinsing. Exposure of the brush border to albumin at this time resulted in the release of almost all of the remaining radioactive palmitic acid. No radioactivity in the esterified fatty acid fraction could be detected in any of the rinsing solutions or in the brush border pellets, irrespective of the incubation temperature used. The possibility that the greater uptake of palmitic acid at 4°C by the brush border preparation may have been due to alterations in the physical properties of the micellar incubation medium at 4°C, such as crystallization and aggregation of unsolubilized palmitic acid or sodium palmitate, or both, was reasonably excluded by previous experiments in which ultracentrifugation (100,000 *g* for 6 hr) at 4°C and 37°C of an identical micellar solution, containing ^{14}C-labeled palmitic acid, [^{3}H]taurodeoxycholate, and ^{14}C-labeled carboxyl inulin (33), showed an analogous distribution of the amounts and ratios of radioactivities along the length of the centrifuge tube. In addition, no changes in the amounts or in the ratios of the radioactivities were observed after passage of the micellar solution at 37°C and 4°C through a 50-nm Millipore filter (Millipore Filter Corp., Bedford, Mass.). The experiments with everted sacs at 4°C also do not support the possibility that temperature-induced alterations of the physical properties of the incubation medium may have accounted for the results with brush borders at 4°C, unless it is assumed that such alterations may occur only in the presence of brush borders and not of everted sacs. Although the experiments with brush borders do not exclude the above possibility, they do indicate that the reversible binding of palmitic acid is accomplished by the superficial components of the intestinal epithelium, and they substantiate further the finding that the binding of greater amounts of palmitic acid at 4°C is independent of fatty acid esterification.

DISCUSSION

The results of these experiments have enabled us to conclude that the radioactive palmitic acid taken up by hamster intestinal mucosa during a short incubation at 4°C and 37°C was located in four functionally distinct compartments. A significant proportion of the labeled fatty acid was reversibly bound to the tissue and a se-

TABLE 1. Uptake and release of [1-^{14}C]palmitic acid by intestinal brush border

Incubation Temperature	Total Uptake	Distribution of Total Uptake		
		% Released into:		% Remaining after Washing
		KRP	Albumin	
	μmoles × 10^{-3}/*mg of protein*			
4°C (5)[a]	223.4 ± 49.3	11.0 ± 2.0	87.1 ± 1.5	1.8 ± 0.8
37°C (5)[a]	34.2 ± 4.9	2.3 ± 0.7	97.2 ± 1.1	0.5 ± 0.4

[a]Number of experiments.

quence of rinsing of the incubated sacs in KRP buffer (KRP-extractable compartment) and albumin (albumin-extractable compartment) resulted in a highly reproducible pattern of release of radioactive palmitic acid. After the rinsing sequence, an appreciable amount of radioactivity remained in the tissue in the form of free (free fatty acid compartment) and esterified (esterified fatty acid compartment) palmitic acid.

The amount of radioactive palmitic acid that could be removed by rinsing in KRP buffer was clearly independent of the duration and the temperature of incubation and contained a rather constant proportion of the radioactivity taken up by the tissue (Fig. 5, *A* and *B*). The very rapid release of this fraction into KRP buffer suggested that it may have been loosely adherent to the tissue and conceivably could have represented palmitic acid contained within the extracellular water compartment on and among the microvilli as well as the water entrapped in the fibrillar mesh of the glycocalyx. It is now recognized that the intestinal epithelium is coated by the glycocalyx, the integrity of which is maintained during digestion (34, 35). The role of the glycocalyx in fat absorption is unknown, although it is clear that fatty acid molecules must pass through this region before reaching the microvillous membrane, where actual absorption takes place. It is conceivable, however, that fatty acids alone or in micellar aggregates may be associated with the glycocalyx and thus may represent a part of the KRP-extractable compartment of our experiments. If this proves to be so, the glycocalyx can then be considered as a trapping device for the products of digestion. Such a mechanism would maintain a favorable concentration gradient for these substances at the microvillous membrane, thereby ensuring their efficient absorption into the cell interior.

The second fraction of releasable palmitic acid was held rather tightly by the tissue, and in contrast to the fraction released in KRP, it was markedly temperature-dependent and significantly larger (Fig. 5, *C* and *D*). The fact that a powerful fatty acid binding agent, namely albumin, was required to remove these molecules suggested that they were held to binding sites by forces analogous to those which mediate the linkage of fatty acid to albumin. There is ample evidence that the binding of palmitic acid to albumin is accomplished through low energy hydrophobic or electrostatic forces, or both (25, 30). On the basis of this evidence, it is not unreasonable to assume that the stronger binding of labeled palmitic acid to the tissue may have been mediated by similar forces and that one of the earliest steps in the uptake of fatty acid may involve the reversible binding of these molecules to binding sites, possibly located on the microvillous membrane or on the glycocalyx itself. At pH 6.3, the pH of our incubation medium as well as that measured in the proximal intestine of man and experimental animals, a significant proportion of palmitic acid would be in ionized form (2). This would certainly allow for electrostatic interaction with suitable receptor sites. The concept that the reversible binding of fatty acid may be a property of the outermost portions of the epithelial cell is strengthened by the finding that over 98% of the radioactive palmitic acid originally taken up by a brush border preparation of proximal intestine was available for release into KRP buffer and albumin (Table 1). Although we do not have a satisfactory explanation for the significantly greater uptake of palmitic acid by the brush borders at 4°C, it is possible that it may have been due to an increase in the affinity of the binding sites for palmitic acid in the cold, a characteristic of the noncovalent type of binding (36). An analogous alteration of fatty acid binding may have also resulted in the increased release of radioactivity in albumin at 4°C, which was in excess of that accountable for solely by the inhibition of esterification at this temperature (Fig. 7). The possibility that the observed differences in the release of labeled palmitic acid in KRP buffer or albumin could be attributed to differences in the solubility and rates of diffusion of palmitic acid into the rinsing solutions, rather than to differences in the binding properties of the tissue, is very pertinent to our conclusions. However, the amounts of palmitic acid available for release in each sequential rinse in KRP buffer or albumin (Figs. 2 and 3) were always well within the solubilizing capacities of the rinsing solutions and were in concentrations sufficient to maintain a favorable gradient for diffusion from tissue to rinsing medium. In addition, when the binding properties of proximal and distal sacs were compared (Fig. 7 and Ref. 33), differences were observed despite the fact that the tissues were subjected to the same rinsing solutions and the same rinsing sequence. The differences in the behavior of silk strings and boiled everted sacs also indicate that the properties of the intact tissue and not the fatty acid-solubilizing capacity of the rinsing solutions accounted for the differences in the amounts of labeled palmitic acid released in KRP or albumin.

The concept that transport of lipids across membranes may be mediated by specific membrane receptors is not new (13). Glover and Green (37) have shown that the transport of dietary cholesterol across the intestinal epithelium of guinea pigs is accomplished through a reversible binding to membrane proteins. Also, Reshef and Shapiro (38) have shown that the uptake of albumin-bound fatty acids by adipose tissue involves binding to membrane proteins. More recently, a membrane-associated sterol carrier protein was demonstrated and implicated in the intracellular transport of cholesterol and its water-insoluble precursors (39). These results

seriously challenge the concept that cellular membranes behave as simple lipid interfaces and that the transport of lipid across cellular membranes is accomplished by passive molecular diffusion.

The tissue free fatty acid compartment contained radioactive palmitic acid which could not be removed by our washing techniques. In view of the fact that the labeled palmitic acid taken up by brush borders was almost entirely reversibly bound (Table 1), we presumed that the "irreversible" type of binding which characterized the tissue free fatty acid compartment may have occurred in a deeper region of the cell, possibly on the surface of intracellular organelles. This postulate is supported by the work of Sjöstrand and Borgström (40) in which subcellular fractions of rat intestine were isolated at various times after the oral administration of labeled triolein and oleic acid. During the earlier stages of fat absorption, a significant amount of free fatty acid radioactivity was located in the membrane fraction derived mainly from the smooth endoplasmic reticulum; 1 hr later, however, over 70% of the radioactivity in the smooth endoplasmic reticulum had become esterified. These findings suggest that the cellular free fatty acid was "irreversibly" bound to the membranes of the smooth endoplasmic reticulum preceding their esterification by the enzymes located in this structure (41).

The esterifying capacity of the tissue (tissue esterified fatty acid compartment) was markedly temperature-dependent and was significantly inhibited on incubation at 4°C (Fig. 6, *C* and *D*). The finding that no esterified [1-^{14}C]palmitic acid was available for release from the tissue is consistent with the observation that esterified fatty acids are confined to the membranes and to the interior of the smooth endoplasmic reticulum and Golgi apparatus (42).

The conclusion which can be drawn from these in vitro experiments with regard to the physiology of fatty acid absorption must of course remain speculative. It is conceivable that the distribution of fatty acids in the compartments described might be very different in the presence of intact blood supply and physiological temperatures. Furthermore, it is quite possible that the observed reversible binding of micellar fatty acid might have been a property of the in vitro system, and therefore no conclusions can be drawn as to whether and to what extent fatty acids taken up in vivo can be removed by the employed rinsing sequence. Alterations of the permeability of the tissue maintained for 25 min in unoxygenated solutions and after exposure to a relatively high concentration of NaTDC should be also considered. Preliminary in vivo experiments[1] in which the same parameters were investigated in isolated intestinal segments with intact circulation support our in vitro results and the conclusion that the absorption of fatty acid is a dynamic process involving the simultaneous uptake and release (influx and efflux) of these molecules, which conceivably may be accomplished through the reversible binding of fatty acid to superficially located binding sites. We believe that the subsequent transfer from these sites to the cell interior is dependent upon the rate of esterification, which by continuously removing intracellular free fatty acid would maintain an effective concentration gradient for fatty acid influx. In this respect, the inverse relationship between the content of radioactive palmitic acid in the tissue esterified fatty acid compartment and the amount of radioactivity released in albumin is of interest (Fig. 7). It is now well substantiated that esterification of fatty acids precedes their incorporation into lipoproteins and transport to the lymphatics (1). In addition, an inverse relationship between the esterifying capacity of the intestinal mucosa and the uptake of luminal fatty acids has been recently shown (43). This evidence would therefore suggest that the tissue esterified fatty acid compartment may determine the amount of palmitic acid releasable by albumin and that the latter may control the uptake of luminal fatty acids by the intestine. Inhibition of the esterification or block in formation and release of lipoprotein would then result in the saturation of the albumin-extractable compartment with fatty acids and finally in the impaired uptake of luminal fatty acids. This sequence of events would explain the steatorrhea in patients with abetalipoproteinemia (44) and in animals treated with ethionine and other inhibitors of protein synthesis (45).

It will be of interest to determine whether under physiological conditions different fatty acids will compete for attachment to these presumed binding sites. Such a mechanism may explain the difference in the rates of absorption of fatty acids with different chain lengths and degrees of unsaturation (46). Experiments designed to answer these questions are currently in progress.

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