Rates of tissue uptake of palmitic acid-1-^{14}C complexed with albumin by two different procedures

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ABSTRACT The effect was investigated of two different methods of preparing an albumin–palmitic acid complex on the tissue uptake of the palmitic acid, both in vivo and in vitro. Complex A was prepared by exposing monomolecular layers of palmitic acid-1-^{14}C deposited on a solid surface to albumin dissolved in buffer. Complex B was prepared by the interaction of albumin with a micellar solution of palmitate-1-^{14}C. The radioactivities and chemical compositions of the two complexes were almost identical.

Rat epididymal fat pads took up, during a 1 hr incubation, about 2.5 times as much palmitic acid from complex A as from complex B; the extent of esterification of the incorporated label was equal for the two complexes. The fractional turnover rate of palmitic acid of complex A, administered intravenously to dogs, was about twice that of palmitic acid from complex B. The label of the two complexes recirculated in the esterified fatty acid fraction of plasma to an equal extent.

It is proposed that differences in the orientation of the fatty acid molecules may affect their interaction with the binding sites of albumin and that the metabolic differences of the resulting complexes are related to differences in the ease of transfer of the fatty acid from the complex to receptor sites of tissues.

KEY WORDS metabolism · palmitic acid-1-^{14}C · fatty acid–albumin complexes · epididymal fat pads · dog · fatty acid monolayers · micelles · binding sites

THE METABOLIC FUNCTIONS of plasma free fatty acids (FFA) have been amply substantiated (1, 2). At physiological plasma concentrations, long-chain fatty acids (FA) are predominantly and very tightly bound to albumin (3). If such FA–albumin binding occurs in vivo, the very short half-life and rapid transfer of circulating FFA to the cells can be explained by postulating the presence of cellular receptor sites that have an affinity for FA and can compete effectively for FA with albumin. Another possibility is that a different type of association of FA with albumin exists in which FA are less tightly bound, and from which FA can be more easily transferred to cellular receptor sites.

In studies of the metabolism of long-chain FA so far reported, albumin–FA complexes were prepared from micellar solutions of soaps; the FA were added to albumin in a molar ratio close to or slightly exceeding the capacity of the albumin sites that bind FA most tightly (3). In the present study, albumin–FA complexes were prepared from monomolecular layers of FA, in which the molecules become aligned in a regular way (4). We hypothesized that differences in the interaction between albumin and FA molecules, aligned in a parallel order in these layers or oriented less evenly in micellar aggregates of FA soaps, might result in complexes with different properties; in particular, we investigated the rate of uptake by tissues of palmitic acid from albumin complexes prepared by the two different procedures.

MATERIALS AND METHODS

Bovine Serum Albumin

The same batch of albumin (bovine serum albumin, Fraction V, Armour Pharmaceutical Co., Kankakee, Ill.)

Abbreviations: FFA, free fatty acids; FA, fatty acids.

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was used in all the experiments. The albumin was treated according to the procedure of Goodman (5), in order to reduce the content of long-chain FA, and then was either vacuum-dried or lyophilized and stored at −10°C. Before the experiments the albumin was dissolved in Krebs-Ringer phosphate buffer (0.1 M, pH 7.4, half Ca^{++} strength) (6) and dialyzed in the cold for 72 hr against the buffer. By these procedures the FA content of the original albumin was reduced from 0.6 μmole to 0.2 μmole per μmole of albumin.

Palmitic Acid-1-^{14}C

The palmitic acid-1-^{14}C used here had a purity greater than 99% and a specific activity of 1.95 μc/μmole (New England Nuclear Corp., Boston, Mass.).

Preparation of Complex A (from Monomolecular Layers)

A monomolecular layer of palmitic acid-1-^{14}C was prepared by the dropwise addition of the acid, dissolved in petroleum ether (bp 38–40°C), onto the surface of a hypophase of 2×10^{-4} M $KHCO_3$ and 10^{-4} M barium acetate, pH 7.2 (4). After evaporation of the petroleum ether a monolayer of FA molecules, oriented with their hydrophilic carboxyl group toward the aqueous medium, is formed on the surface of the hypophase (Fig. 1). When a chromium slide is immersed vertically into the hypophase, a double layer of FA molecules covers the slide, one layer being deposited as the slide is immersed and one as it is withdrawn (7). As this operation is repeated, a known number of double layers of FA can be deposited on the slide.

Complexes with albumin were formed from palmitic acid-1-^{14}C monolayers as follows. 5 mg of albumin was dissolved in 30 ml of phosphate buffer (0.016 M, pH 7.2) in a square cuvette, and four slides, each covered with 30 double layers of palmitic acid-1-^{14}C, were placed with their backs flush against its walls. The solution was maintained at 30°C and stirred for 1 hr. The slides were then replaced with four new slides, each covered with 15 double layers, and the solution was stirred for an additional 30 min.

Previous experiments (8–10) have shown that under these conditions monolayers of FA become attached to the albumin (by a process of "ripping off") in a maximal ratio of 4.5–4.7 μmoles of FA per μmole of albumin. The exact number of moles of FA complexed to albumin can be determined from changes in the thickness of the monolayers on the slide, measured by monochromatic interferometry (4) or by ellipsometry (11), before and after the interaction with albumin. The amount of labeled FA complexes to albumin can also be calculated from the specific activity of the acid and the radioactivity of the albumin–FA complex (12).

To obtain a solution of albumin that was "half-saturated" with labeled palmitic acid (molar ratio of 1:2.3) we added an additional 5 mg of albumin; the final solution was dialyzed against distilled water (for 72 hr at 4°C) and lyophilized. The resulting powder was dissolved in Krebs-Ringer phosphate buffer (0.1 M, pH 7.4, half Ca^{++} strength) to a final albumin concentration of 5%. The final solution was stable and optically clear.

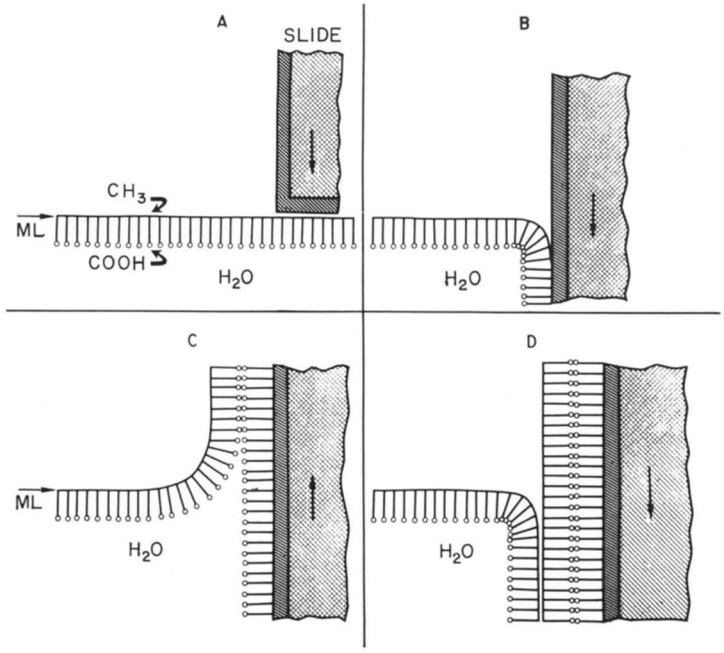

FIG. 1. Formation of monomolecular layers of FA and their molecular orientation after deposition on a glass or metal slide [after Langmuir (7)]. *ML*, monomolecular layer of FA.

Preparation of Complex B (from Soap Solution)

The procedure employed was essentially that of Fillerup, Migliore, and Mead (13). In a 10 ml volumetric flask 7.7 mg of palmitic acid-1-^{14}C was added to 6 ml of the Krebs-Ringer buffer made alkaline with a few drops of 1 N NaOH. The solution was warmed until optically clear and diluted to the mark with buffer. The mixture was adjusted to pH 7.4 with dilute HCl and added while warm (37–40°C) with vigorous stirring to 700 mg of albumin dissolved in 4 ml buffer. The final solution remained clear and no precipitation of albumin occurred.

Comparison of Complex A with Complex B

Aliquots of complex A and complex B solutions were added to 15 ml of Bray's scintillation mixture (14) and the radioactivity was assayed in a liquid scintillation spectrometer (Packard Tri-Carb) with a counting efficiency of 65–68% for ^{14}C. The FA content of each complex was determined by the method of Dole (15) as modified by Trout, Estes, and Friedberg (16). Paper electrophoresis (17) of the two complexes was performed and the radioactivity on the paper strip was detected with a 4 pi Actigraph Scanner (Nuclear-Chicago Corporation, Des Plaines, Ill.

Uptake of Fatty Acid from Complexes by Rat Epididymal Fat Pads

Male rats of the Sprague-Dawley strain, weighing 200–250 g, were fasted overnight and killed by cervical dislocation. The epididymal fat pads were removed, washed in ice-cold saline, and placed separately in 25-ml Erlenmeyer flasks containing 4 ml of complex A or complex B solution, to which glycose had been added to a final concentration of 0.05%. One pad of each rat was incubated with complex A and the contralateral pad with complex B. After 1 hr of incubation in air at 37°C in a Dubnoff metabolic shaker the pads were washed in ice-cold saline, blotted, weighed, and homogenized in 14 ml of Dole's extraction mixture (15). The homogenate was decanted and the rod and tube of the homogenizer were washed with 6 ml of extraction mixture followed by 10 ml of *n*-heptane. Water was added to break the phases. Esterified FA and FFA in the heptane phase were separated by the method of Borgström (18) and aliquots were taken for radioactivity and FFA determinations. The radioactivity of the incubation media at zero time was determined by extracting portions of unincubated complex A and complex B solutions and assaying the radioactivity in the esterified FA and FFA fractions. The FFA fraction contained all the radioactivity.

The results were expressed as the percentage of total radioactivity in the incubation media at zero time taken up by 1 g of epididymal adipose tissue during 1 hr of incubation (% cpm/g per hr).

Metabolism of Complexes A and B Administered Intravenously to Dogs

Six healthy mongrel dogs weighing 20–25 kg were used. After an overnight fast the dogs were anesthetized with sodium pentobarbital (25–30 mg/kg of body weight) and a polyethylene catheter was placed in a femoral vein. 4 ml of complex A or B solution was injected into a forepaw vein and 5-ml samples were drawn through the femoral catheter at 2, 3, 4, 5, 8, and 10 min. During the interval between collections the catheter was kept open by drawing and reinjecting 2–3 ml of blood. In the same manner samples were collected at various intervals up to 60 min, and in two experiments, up to 120 min. Each dog was used twice—once for studies with complex A and after 4–6 weeks for studies with complex B, or vice versa.

The samples were drawn in ice-chilled syringes and tubes containing a few drops of heparin, and centrifuged in a refrigerated centrifuge. The plasma was kept at 4°C and analyzed within 1–2 hr. Plasma lipids were extracted and separated into esterified FA and FFA as described above and aliquots were taken for radioactivity and FFA determinations. Portions of complex A and complex B solutions were extracted and counted similarly, and the injected total radioactivity was calculated. All the results were normalized to a constant dose of 10^6 cpm/kg of body weight. The plasma FFA radioactivity was plotted semilogarithmically, and half times of disappearance ($t_{1/2}$) were calculated from the portion of the graph between the 2nd and 4th min after the injection. As indicated in the studies of Fredrickson and Gordon (19) and of Friedberg, Harlan, Trout, and Estes (20), this region represents the first truly straight segment of the graph after an interval of time when mixing of the labeled FA in the plasma pool has become reasonably complete. Fractional turnover rates ($0.693/t_{1/2}$) and turnover rates (fractional turnover rate × concentration of FFA per liter × plasma volume in liters) were also calculated. Plasma volume was estimated as 5% of body weight.

RESULTS

Radioactivity and FFA Content of Complex A and Complex B

The content of labeled palmitic acid in complex A, calculated from the specific activity of palmitic acid-1-^{14}C and the amount of albumin, was 2.5 μmoles/μmole of albumin, which is in good agreement with the ratio of 2.3:1 determined from changes in the thickness or radioactivity of the palmitic acid monolayers on the slides. Complex B contained 2.8 μmoles of labeled palmitate per μmole of albumin, which is equal to the molar

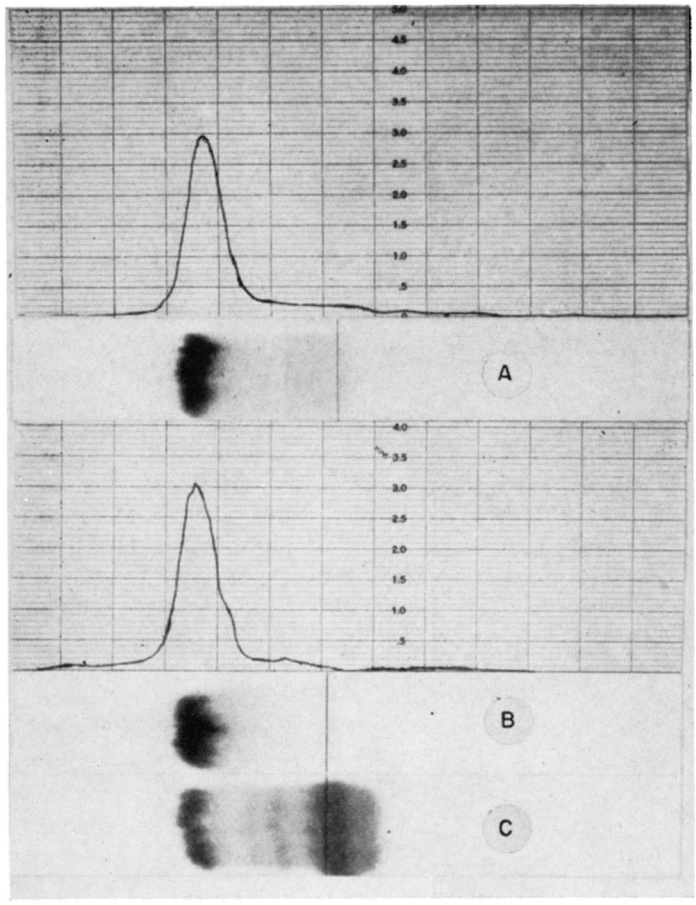

FIG. 2. Electrophoretic mobilities of complexes A and B and location of palmitic acid-1-^{14}C radioactivity in relation to the electrophoretic mobility of plasma proteins (*C*).

ratio of 2.8:1 calculated from the amounts of palmitic acid and albumin used in the preparation of the complex. The total FFA (stable and radioactive) contents determined for complex A and complex B were 2.6 and 3.0 μmoles/μmole of albumin, respectively. The differences between the total FA content and the content of labeled palmitic acid can be accounted for by FA remaining on the albumin after the extraction procedures (see above). Paper electrophoresis and radioactivity scanning of the paper strips (Fig. 2) of complexes A and B showed that the peak radioactivity of each complex corresponded to the electrophoretic mobility of serum albumin.

Uptake by Fat Pads

In preliminary experiments it was shown that the incorporation of labeled palmitic acid by epididymal pads was nearly linear during the 1st hr. In subsequent experiments the pads were routinely incubated for a period of 1 hr.

The extent of incorporation of palmitic acid from complex A was much greater than that from complex B (Table 1). This difference cannot be accounted for by the negligible difference in the amount of labeled palmitic acid in complexes A and B (2.5 μmoles vs. 2.8 μmoles). On the contrary, the lesser amount of label in complex A would be expected to lead to a smaller uptake from this complex.

The extent of esterification of the incorporated label was the same in the two groups of fat pads (Table 1).

TABLE 1 INCORPORATION OF PALMITIC ACID-1-^{14}C FROM COMPLEXES A AND B BY RAT EPIDIDYMAL FAT PADS

Complex*	No. of Experiments	Palmitic acid-1-^{14}C Incorporated†		Esterification‡
		% cpm/g per hr	*μeq/g per hr*	*%*
A	12	29.3 ± 5.6§	2.1 ± 0.4	85.7 ± 5.4
B	12	12.4 ± 1.7	1.0 ± 0.1	88.9 ± 6.1

* Complex A derived from monolayers; B from soap solution.

† Calculated from the specific activity of palmitic acid and the radioactivity incorporated into each pad.

‡ Calculated from the radioactivity in the esterified FA fraction and the total lipid fraction.

§ Mean ± SD.

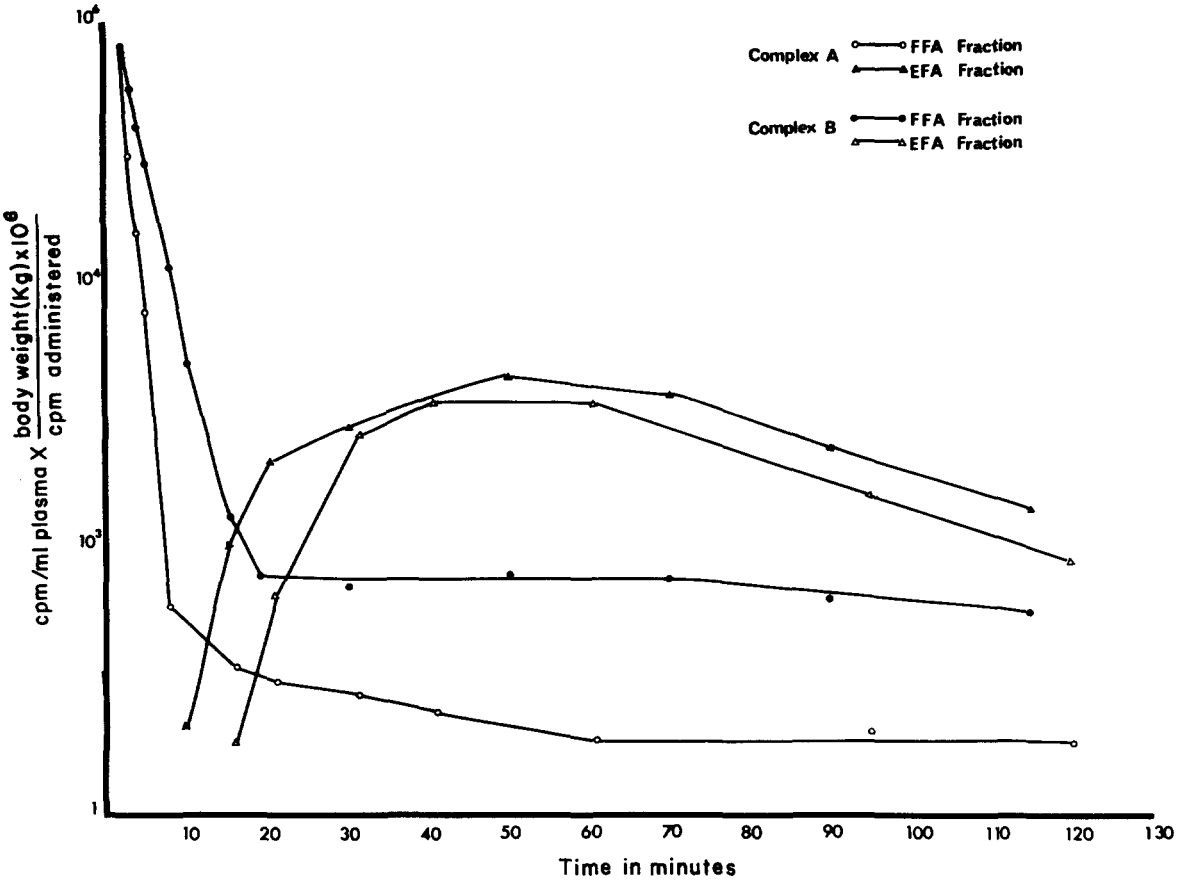

FIG. 3. Disappearance from the plasma, and recirculation in the esterified FA fraction, of palmitic acid-1-^{14}C from complexes A and B. *EFA*, esterified FA.

Removal from the Circulation

Two curves representative of the disappearance of palmitic acid-1-^{14}C from the plasma, obtained by semilogarithmic plotting of plasma FFA radioactivity against time, are shown in Fig. 3. These curves are qualitatively similar to those previously reported (19, 20). The half-times, fractional turnover rates, and turnover rates, presented in Table 2, were calculated from the initial, nearly linear portion of the curves (19, 20). It can be seen that palmitic acid from complex A was metabolized much faster than that from complex B.

TABLE 2 RATE OF DISAPPEARANCE FROM PLASMA OF PALMITIC ACID-1-^{14}C IN COMPLEXES A AND B

Complex	No. of Dogs	Half-Time	Fractional Turnover Rate*	Turnover Rate†
		min	*min*$^{-1}$	*μeq/min*
A	6	0.86 ± 0.08‡	0.816 ± 0.07	763 ± 88
B	6	1.67 ± 0.22	0.423 ± 0.06	398 ± 78

* $0.693/t_{1/2}$.

† Fractional turnover rate × concentration of plasma FFA × estimated plasma volume.

‡ Mean ± SD.

The observed differences in the metabolism of palmitic acid from the two complexes could have been due to different levels of plasma FFA, since it has been shown (19) that the fractional turnover rate of FFA varies inversely with the concentration of FFA at levels below 0.6–0.8 meq/liter. Injection of complex A or complex B, however, produced no changes in the level of plasma FFA, which remained fairly constant (0.75–0.85 meq/liter) throughout the experiment.

Fig. 3 shows also that a significant fraction of the label was recycled in the esterified FA fraction. Radioactivity appeared in this fraction about 10–15 min after the injection of either complex A or complex B, and reached a peak in 50–60 min. Although during the early phase of recycling the radioactivity in the esterified FA fraction was somewhat higher in the dogs injected with complex A, probably as a result of the faster removal of the label in FFA, the curves subsequently became similar for the two complexes.

DISCUSSION

This study indicates that the metabolism of palmitic acid can be significantly influenced by the method

whereby the fatty acid has been complexed with albumin. The rate of incorporation by rat epididymal fat pads in vitro, and the rate of disappearance from the circulation of the dog in vivo were both greater for palmitic acid from the complex prepared from monolayers than from that prepared from a micellar soap solution. This evidence suggests the possibility that palmitic acid from monolayers assumes a position on the albumin surface from which its transfer to cellular receptor sites is relatively easy. The same extent of esterification of the incorporated label by epididymal fat (Table 1), and the similar rates of recycling in plasma esterified FA of palmitic acid from the two complexes (Fig. 3) demonstrate that once the palmitic acid had been taken up from either complex its subsequent metabolism was the same.

From this study no definite conclusions can be drawn as to the nature and the number of FA binding sites on the surface of albumin, or whether complex formation of albumin with palmitic acid from monolayers or from micellar aggregates is accomplished through the same sites of binding. However, it is conceivable that the orientation of the FA molecules before binding could significantly affect the relationship between the binding sites of albumin and the nonpolar side chain of the FA, which participates in the binding process (3), and thus lead to differences in the strength of the association between albumin and FA. In support of this is the finding that in the formation of albumin complexes of palmitic acid from monolayers it is virtually impossible to exceed a molar ratio of albumin to palmitic acid of 1:4.7 (8–10), which is substantially lower than the values obtained by Goodman (3). Although differences in the conditions (pH, ionic strength, and temperature) employed by us and by Goodman may account for some of the differences in results, other possibilities should also be considered. For example, it may be that only a few "loose" binding sites on the albumin are accessible to the palmitic acid in monolayers; or that when complex A is formed, dimers or trimers of palmitic acid become attached to a single binding site, and the FA molecules, not attached to a binding site but only to other FA molecules, might be more easily taken up from the complex.

Further work is necessary to elucidate the nature of the FA–albumin binding in the two complexes, to determine the chemical structure of the binding sites, and finally, to establish the relationship of these complexes to the binding between FA and albumin that occurs in vivo.

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