Studies on the cellular mechanism of free fatty acid uptake using an analog, hexadecanol

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Abstract Hexadecanol was employed as a fatty acid analog in an attempt to elucidate the role of the carboxyl group in free fatty acid uptake. Large quantities of albumin-bound [1-^{14}C]hexadecanol were taken up by Ehrlich ascites cells during in vitro incubation. More than 90% of the ^{14}C that was taken up remained as hexadecanol even after 1 hr of incubation at 37°C. Addition of unlabeled hexadecanol did not appreciably alter the rate of [U-^{14}C]glucose oxidation or incorporation into total lipids, suggesting that the slow rate of hexadecanol metabolism was not due to a toxic effect of this analog. However, more of the labeled glucose was incorporated into phospholipids and less into glycerides, indicating that hexadecanol did exert some metabolic effect on the cells. Uptake was temperature dependent but relatively unresponsive to the presence of glucose or fluoride and cyanide. Hexadecanol was incorporated into exchangeable and nonexchangeable cellular pools as determined by its availability for release to a medium containing albumin. These results indicate that a mammalian cell can rapidly take up large amounts of a long-chain hydrocarbon derivative that does not contain a carboxyl group. Furthermore, the data are compatible with the hypothesis that free fatty acids are taken up by a nonenzymatic process such as diffusion into the lipid phase of the cell membrane.

Supplementary key words membranes · transport · binding · serum albumin · Ehrlich ascites cells · tumor

The uptake of long-chain free fatty acid by mammalian cells occurs in two steps (1). One involves the reversible binding of the fatty acid to sites located on the plasma membrane (2–6). The other involves entry of some of the fatty acid into a nonexchangeable pool that is located either in the membrane or inside the cell (4, 5). There is general agreement that fatty acid binding to the superficial membrane sites occurs through a physical adsorption process (2–5, 7). However, the mechanism of entry into the nonexchangeable pool is the subject of some debate. Certain studies are compatible with a nonenzymatic diffusion process (4, 8), whereas others suggest an energy-dependent mechanism (5, 7) such as an acylcarnitine translocase (9) or a lecithin–lysolecithin cycle (10–12). These enzymatic mechanisms involve esterification reactions and, therefore, require the availability of the fatty acid carboxyl group.

In an attempt to learn whether esterification is essential for the rapid uptake of a long-chain hydrocarbon compound by a cell, we initially investigated the utilization of fatty acid methyl esters. This work was inconclusive because the methyl esters were hydrolyzed rapidly (13, 14). The possibility that hydrolysis occurred at the cell surface and that the released fatty acid rather than the intact methyl ester actually entered the cell could not be excluded. In order to circumvent this problem, we have examined the uptake of another fatty acid analog, [1-^{14}C]hexadecanol. These experiments were performed with Ehrlich ascites tumor cells, a model system that has been used extensively to investigate the mechanism of free fatty acid uptake (1, 15).

METHODS

[1-^{14}C]Hexadecanol was purchased from New England Nuclear, Boston, Mass. It was dissolved in 20 ml of hexane and purified by extracting the hexane solution three times with 20 ml of alkaline ethanol (4). Analysis of an aliquot of the hexane phase by thin-layer chromatography with a solvent system containing diethyl ether–heptane–glacial acetic acid 70:30:1 revealed that essentially all of the radioactivity migrated in the region of the hexadecanol standard. Fraction V bovine serum albumin, purchased from Miles Laboratories, Kankakee, Ill., was incubated with activated charcoal and dialyzed in order to remove adsorbed impurities (16, 17). Protein

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concentration was measured by the biuret method (18). Ehrlich ascites tumor cells were transplanted and prepared for incubation as described previously (4). Cell counts were made with a clinical hemocytometer and microscope.

Hexadecanol–albumin solutions were prepared by the "Celite method" (13, 19). A weighed amount of unlabeled hexadecanol obtained from Applied Science Laboratories, State College, Pa., was dissolved in the hexane solution containing the purified radioactive alcohol. Three aliquots of this solution were analyzed, using a Packard Tri-Carb liquid scintillation spectrometer, in order to determine the hexadecanol specific radioactivity. A toluene–methanol 70:30 (v/v) scintillator solution was employed, and quenching was monitored with the external standard (13). The remainder of the hexane solution was added to washed Celite, and the solvent was evaporated under nitrogen. Most preparations contained 20 μmoles of hexadecanol/g of Celite, and the specific activity of the [1-^{14}C]hexadecanol usually was 0.2 Ci/mole. Hexadecanol-coated Celite was incubated for 30 min at 24°C with 0.5 μmole/ml albumin containing 116 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, and 16 mM Na_2HPO_4 adjusted to pH 7.4 with HCl (19). The Celite was removed by centrifugation at 10,000 *g* for 10 min at 0°C. This supernatant solution was passed through a 1.2-μm Millipore filter and was readjusted to pH 7.4, and aliquots of the filtrate were assayed for radioactivity. The hexadecanol concentration of these solutions ranged from 0.1 to 1.0 mM, depending upon the quantity of Celite incubated with the albumin solution. Penicillin and streptomycin were added to the solution in a final concentration of 25 μg/ml of each.

Suspensions of washed Ehrlich cells were incubated with these [1-^{14}C]hexadecanol–albumin solutions in a water bath incubator with shaking. Air served as the gas phase. The incubation was terminated by pouring the contents of each flask into chilled polypropylene centrifuge tubes containing 25 ml of the buffered salt solution described above. Following centrifugation at 2000 *g* for 3 min at 0°C, the supernatant solution was siphoned off and the cells were redispersed in 25 ml of fresh buffered salt solution. Sedimentation and washing were repeated twice. The resulting cell pellet was extracted with 20 ml of chloroform–methanol 2:1 (v/v), and the chloroform phase was isolated and dried under nitrogen (13). The lipid residue was dissolved in 2 ml of chloroform, and an aliquot of this solution was dried under nitrogen and assayed for total lipid radioactivity (13). The lipids contained in another aliquot of the chloroform solution were separated by thin-layer chromatography in the ether–hexane–acetic acid system. Lipid standards purchased from Applied Science Laboratories were added to each chromatogram. The radioactivity present in the segments of the silica gel containing (*a*) fatty alcohols, (*b*) free fatty acids, (*c*) phospholipids, and (*d*) neutral lipid esters was measured in the liquid scintillation spectrometer using a dioxane–water scintillator solution (13, 20).

In one series of experiments, $^{14}CO_2$ collections were made using incubation flasks with removable center wells containing 0.2 ml of 1 N KOH (13). In other experiments, the cells were incubated briefly with albumin-bound [1-^{14}C]hexadecanol, washed, and suspended in fresh phosphate-buffered salt solution. Aliquots of the labeled cell suspension were incubated for various periods of time in media containing albumin, and either the medium or the cells were assayed for radioactivity. Other aliquots of the cells were extracted with chloroform and methanol in order to determine the content and distribution of radioactivity prior to exposure to albumin (13).

RESULTS

Addition of [1-^{14}C]hexadecanol to albumin

The transfer of [1-^{14}C]hexadecanol from Celite to media containing bovine serum albumin occurred rapidly, as is illustrated in Fig. 1. Similar kinetics of incorporation were noted over the entire range of Celite concentrations tested. The molar ratio of hexadecanol to albumin in the resulting solution was dependent upon the amount of hexadecanol-coated Celite that was added. Thin-layer chromatographic analysis of the lipid extracted from these media indicated that at least 97–98% of the radioactivity was present as fatty alcohol.

Uptake of [1-^{14}C]hexadecanol

The time course of [1-^{14}C]hexadecanol uptake and utilization by Ehrlich ascites cells is shown in Table 1. Similar results were obtained when the molar ratio of

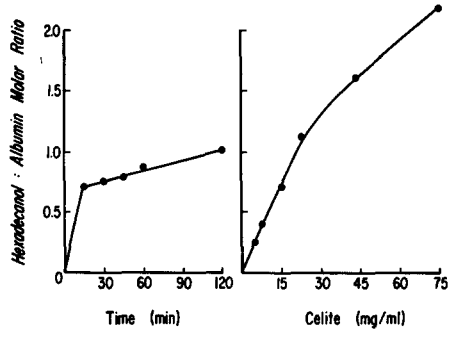

FIG. 1. Addition of [1-^{14}C]hexadecanol to bovine serum albumin. In the experiment shown on the left side, each flask contained 2 ml of albumin and 60 mg of Celite. Each flask also contained 2 ml of albumin in the experiment illustrated on the right side, and the time of incubation was 30 min.

TABLE 1. Uptake and metabolism of [1-^{14}C]hexadecanol[a]

Incubation Time	[1-^{14}C]Hexadecanol Utilization					
	$\bar{v}$ = 0.3[b]			$\bar{v}$ = 0.7[b]		
	Total Incorporation	Oxidation to $^{14}CO_2$	^{14}C Remaining as Fatty Alcohol	Total Incorporation	Oxidation to $^{14}CO_2$	^{14}C Remaining as Fatty Alcohol
min	*nmoles/10^8 cells* ± SE[c]		%	*nmoles/10^8 cells* ± SE[c]		%
5	94 ± 5	0	99.4	220 ± 6	0	99.2
10	113 ± 7	0	98.3	266 ± 6	0	98.8
30	128 ± 2	0.5 ± 0.1	96.6	295 ± 5	1.2 ± 0.1	96.1
60	144 ± 5	1.3 ± 0.2	94.5	308 ± 8	2.8 ± 0.3	92.6

[a] Incubation was at 37°C with air as the gas phase.
[b] Molar ratio of hexadecanol to albumin present in the incubation medium.
[c] Means of six determinations.

hexadecanol to albumin was either 0.3 or 0.7, except that the uptake was considerably larger at the higher ratio. In both cases, a large fraction of the total uptake occurred during the first 5–10 min of incubation. Only very small amounts of hexadecanol were oxidized to $^{14}CO_2$ even after 60 min of incubation. Most of the radioactivity that was taken up by the cells remained as fatty alcohol. Of the small quantity of radioactivity incorporated into the other lipid fractions, 60% was recovered in neutral lipids and 40% in phospholipids. Results similar to these were noted in one additional experiment in which the hexadecanol–albumin molar ratio was 1.2.

In order to determine whether the failure to note greater metabolism of hexadecanol might be due to a toxic effect of this analog, we examined the utilization of [U-^{14}C]glucose by the Ehrlich cells in the presence of unlabeled hexadecanol. As shown in Table 2, glucose oxidation decreased only slightly when hexadecanol was contained in the incubation medium. Likewise, the amount of glucose radioactivity incorporated into cell lipids was essentially unchanged. The main difference noted was that a larger fraction of the glucose radioactivity was recovered in phospholipids when the medium contained hexadecanol.

TABLE 2. Effect of hexadecanol on the metabolism of [U-^{14}C]glucose[a]

Hexadecanol	[U-^{14}C]Glucose Utilization[b]		
	Oxidation to $^{14}CO_2$	Incorporation into Cell Lipids	Percentage of Lipid Radioactivity Present in Phospholipids
mM	*nmoles/10^8 cells* × *30 min*		%
0	215	65	56
0.5	213	59	85
1.0	201	57	84
1.5	195	64	93

[a] Incubation for 30 min at 37°C with air as the gas phase.
[b] Each value is the mean of two determinations. These media contained 11 mM [U-^{14}C]glucose.

The effect on uptake of the molar ratio of hexadecanol to albumin is illustrated in Fig. 2. Since the time of incubation in this experiment was 30 min, these hexadecanol uptakes are equilibrium values at each molar ratio. Uptake increased markedly as the molar ratio was raised, a finding similar to that observed with palmitate and other long-chain free fatty acids (15). In other experiments we noted that the uptake of hexadecanol was 36–42% greater at a given molar ratio when the medium contained human albumin instead of bovine albumin. More palmitic acid also was taken up when the medium contained human albumin in place of bovine albumin (15).

Experiments with [1-^{14}C]hexadecanol bound to ^{131}I-labeled albumin were done in order to determine whether uptake was due to transfer of the alcohol from albumin to the cell or to incorporation of the intact albumin complex. Under conditions where 57% of the labeled hexa-

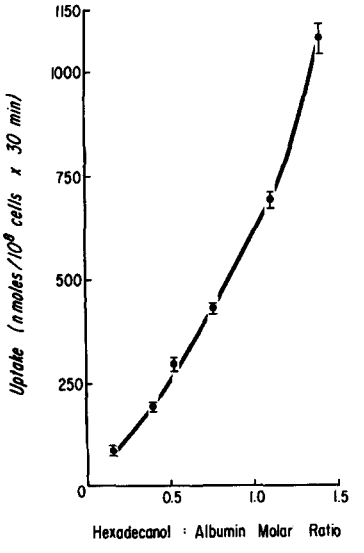

FIG. 2. Uptake of [1-^{14}C]hexadecanol relative to the molar ratio of hexadecanol to albumin in the incubation medium. Incubation was for 30 min at 37°C with air as the gas phase. The molar ratio that is plotted on the abscissa is that present at the end of the incubation with the cells.

decanol was taken up, the ^{131}I radioactivity present in the cells was not significantly different from background. The ^{14}C–^{131}I ratio in the medium decreased from 113 to 51 after incubation with the cells for 30 min. Therefore, almost all of the hexadecanol uptake was due to transfer from albumin to the cells, not to incorporation of the intact albumin complex.

Hexadecanol uptake was temperature dependent. In an experiment in which the [1-^{14}C]hexadecanol–albumin molar ratio was 1.0, the uptake after 30 min of incubation was 108 ± 2 nmoles/10^8 cells at 0°C, 340 ± 3 nmoles/10^8 cells at 24°C, and 519 ± 5 nmoles/10^8 cells at 37°C (mean ± SE, six determinations). As shown in Fig. 3, the temperature dependence of hexadecanol uptake was evident throughout the course of a 1-hr incubation. In contrast, addition of metabolic inhibitors to the incubation medium had little effect on the amount of [1-^{14}C]hexadecanol taken up during a 2- or a 30-min incubation at 37°C (Table 3). The cells were exposed to the inhibitors for 15 min prior to addition of hexadecanol in order to allow for depletion of high energy metabolites, and the inhibitors remained in the medium during subsequent incubation with hexadecanol. Even when relatively high concentrations of both cyanide and fluoride were present, the uptake after 30 min was only 15% less than that in the control medium. In other experiments, we observed that addition of 11 mM glucose to the incubation medium did not appreciably increase [1-^{14}C]hexadecanol uptake. For example, when the hexadecanol–albumin molar ratio was 0.8 and the incubation was carried out for 30 min at 37°C, the uptake was 343 ± 6 nmoles/10^8 cells in the control medium and 352 ± 9 nmoles/10^8 cells when glucose was present.

Release of [1-^{14}C]hexadecanol from the cells

When cells were incubated with albumin-bound [1-^{14}C]hexadecanol, washed, resuspended in buffer, and

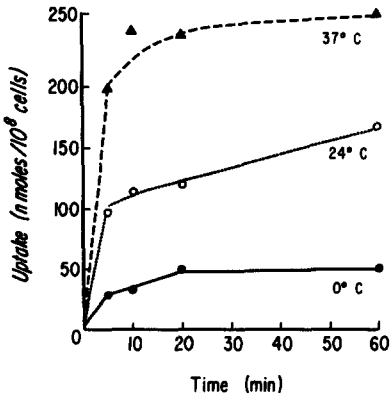

FIG. 3. Time course of [1-^{14}C]hexadecanol uptake relative to the temperature of incubation. The hexadecanol–albumin molar ratio was 0.5.

TABLE 3. Effect of metabolic inhibitors on [1-^{14}C]hexadecanol uptake[a]

Inhibitor	[1-^{14}C]Hexadecanol Uptake[b]	
	2 min[c]	30 min[c]
	nmoles/10^8 cells	
None	150 ± 12	420 ± 11
Cyanide (5 mM)	155 ± 14	375 ± 18
Fluoride (50 mM)	134 ± 11	401 ± 13
Cyanide + fluoride	160 ± 15	362 ± 14

[a] Cells were incubated initially for 15 min at 37°C with either buffer or buffer containing inhibitor. [1-^{14}C]Hexadecanol–albumin solution was added after this preliminary incubation. Air served as gas phase.

[b] Means of six determinations ± SE.

[c] Time of incubation at 37°C with the [1-^{14}C]hexadecanol–albumin solution of molar ratio 0.8. The inhibitors remained in the medium during this incubation period.

then incubated in a solution containing albumin, lipid soluble radioactivity was released from the cells. Analysis of an extract of the albumin-containing medium by thin-layer chromatography revealed that more than 99% of the released radioactivity was recovered as fatty alcohol. Approximately 85% of the radioactivity remaining in the cells after exposure to albumin also was present as fatty alcohol. As seen on the left side of Fig. 4, most of the efflux occurred during the first 5–10 min of incubation. Even after 1 hr at 37°C, only 65% of the [1-^{14}C]hexadecanol initially associated with the cells was released into the medium. Like uptake, efflux of hexadecanol from the cells was temperature dependent. Only 40% of the cellular hexadecanol content was released in 1 hr at 0°C. As seen on the right side of Fig. 4, the amount of [1-^{14}C]hexadecanol released from the cells was dependent upon the albumin content of the medium. Only trace quantities of hexadecanol were released in the absence of albumin. However, even when 2 μmoles of albumin were

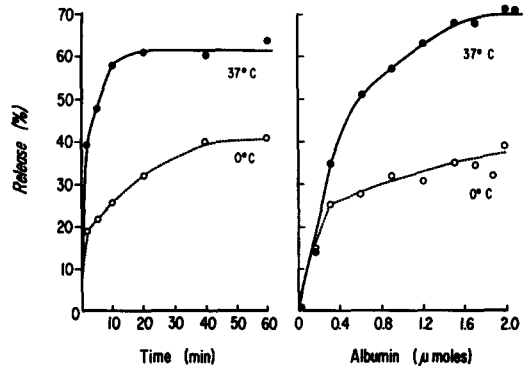

FIG. 4. Release of [1-^{14}C]hexadecanol from cells into media containing albumin. The medium contained 1 μmole of albumin in the experiment shown on the left side. The time of incubation was 20 min in the experiment shown on the right side. Each point is the mean of two determinations. A different preparation of cells was employed for each experiment.

TABLE 4. Relationship between the cellular [1-^{14}C]hexadecanol content and the amount released to albumin[a]

Experiment No.	Time of Incubation with [1-^{14}C]-Hexadecanol	[1-^{14}C]Hexadecanol Content of the Cells[b]	[1-^{14}C]Hexadecanol Released to Albumin	
			Amount	Percentage of Cell Content[c]
	min	*nmoles*	*nmoles*	%
1	10	45	32 ± 0.3[d]	70
	10	96	72 ± 0.5	75
	10	270	186 ± 1.6	69
2	10	329	214[e]	65
3	10	129	90[f]	70
	30	149	100	67

[a] Cells were incubated initially at 37°C with [1-^{14}C]hexadecanol–albumin solutions. The second incubation was for 20 min at 37°C in a medium containing 1 μmole of albumin, and the radioactivity released into this medium was measured.

[b] [1-^{14}C]Hexadecanol contained in the cells prior to incubation with albumin.

[c] [1-^{14}C]Hexadecanol released into the medium relative to the cellular hexadecanol content prior to exposure to albumin.

[d] Means of six determinations ± SE.

[e] Mean of two determinations.

[f] Means of three determinations.

present, only 70% of the cellular [1-^{14}C]hexadecanol content was released at 37°C and only 39% at 0°C. The maximum molar ratio of released hexadecanol to albumin in the medium was only 0.15.

As shown in Table 4, the fraction of the cellular [1-^{14}C]hexadecanol content that was available for rapid release to media containing albumin was similar over a wide range of hexadecanol uptakes. Differences in cellular [1-^{14}C]hexadecanol content were produced by varying the molar ratio of hexadecanol to albumin in the loading solution. Between 65 and 75% of the cellular [1-^{14}C]-hexadecanol content was released even though the initial hexadecanol content of the cells varied from 45 to 329 nmoles/10^8 cells (experiments 1 and 2). These cells were loaded with hexadecanol for 10 min. From 65 to 75% of the cellular [1-^{14}C]hexadecanol content also was released when the time of loading was extended to 30 min (experiment 3).

DISCUSSION

Fatty acid structural analogs have been employed to examine certain aspects of the mechanism of free fatty acid uptake in mammalian tissues. Previous studies with 3,3-dimethyl-14-phenylmyristate and 3,3,12,12-tetramethylmyristate provided some insight into the effect of hydrocarbon chain structure on uptake (4, 21). The purpose of the present work with hexadecanol was to examine the role of the fatty acid carboxyl group in the cellular uptake process. We recognized the possibility that the absence of the ionized carboxyl group might impart sufficiently different properties to the hydrocarbon chain that results with the alcohol would have little or no bearing on fatty acid uptake. However, every aspect of hexadecanol uptake that we investigated with the Ehrlich cells was similar to that observed with palmitic acid (15). These similarities lead us to suggest that the data with this analog probably are applicable to certain aspects of the fatty acid transport mechanism in Ehrlich cells. Competitive uptake studies between palmitate and hexadecanol might have demonstrated this point conclusively, but they could not be performed adequately for technical reasons. Palmitate and hexadecanol are poorly soluble in aqueous solutions, and a carrier was required in order to introduce sufficient amounts of these compounds into the incubation media. Both palmitate and hexadecanol bind to the currently available carriers, serum albumin and β-lactoglobulin (17, 22). Preliminary experiments revealed that the binding to these carriers was competitive, and we could not clearly distinguish between competition at the level of binding to the carrier as opposed to that at the level of cellular transport. Hence, this approach was not helpful and was discontinued.

Much of the free fatty acid taken up by Ehrlich cells is available for rapid release to a medium containing albumin (1, 15). This reversibly bound fatty acid appears to be associated with the cell membrane (2–6). A considerable fraction of the hexadecanol uptake also was bound reversibly to the cells, suggesting that the presence of the anionic form is not an absolute requirement for the reversible binding of a long-chain hydrocarbon to the cell surface. Sufficient energy for binding apparently is available from nonpolar interactions between the hydrocarbon chain and hydrophobic regions of the plasma membrane.

Another component of the free fatty acid uptake process involves accumulation of some of the fatty acid in a nonexchangeable pool (4, 5). This pool may be located at strong binding sites on the surface of the cell (6), deeper within the plasma membrane (5), or inside the cell (15). The fact that hexadecanol also entered a nonexchangeable pool suggests that this step, rather than being totally dependent upon an esterification reaction (9–12), can also occur through a nonenzymatic process. A mechanism such as diffusion is suggested by the observations that hexadecanol uptake was temperature dependent but was not appreciably altered by the presence of either cyanide and fluoride or glucose. The magnitude of the temperature effect (Fig. 3) is much larger than might be expected simply from the temperature dependence of the diffusion coefficient. However, other factors also would contribute to the temperature dependence of a diffusion process. These include hexadecanol binding to albumin, which determines the unbound hexadecanol concentration and, hence, the concentration of the diffusing substance, and

the viscosity of the membrane lipid phase, which determines resistance to hexadecanol uptake. At present, there is no information concerning temperature effects on these processes. However, as the temperature rises, both of these parameters should change in a direction that would increase diffusion.

The largest amounts of hexadecanol that were taken up by the Ehrlich cells were of the same magnitude as the largest palmitic acid uptakes that we have observed (15). Therefore, the absence of the carboxyl group or anionic form also did not appreciably alter the rate of uptake. At molar ratios between 0.2 and 1.5, the uptake of hexadecanol was much larger than that of palmitate (15). However, the binding of hexadecanol to albumin is weaker than that of palmitate (17). Hence, these quantitative differences in uptake of the alcohol and acid appear to result at least in part from differences in albumin binding, and they probably do not represent major differences in the rates of cellular uptake of these substances.

The rates of oxidation and esterification of hexadecanol were much smaller than those noted previously for palmitic acid (15). In fact, most of the hexadecanol taken up by the Ehrlich ascites cells was not metabolized further. Yet, fatty alcohols are known to be precursors of alkyl ethers and plasmalogens in mammalian tissues (23–26). Indeed, the incorporation of hexadecanol into these compounds has been demonstrated in Ehrlich cells (25, 27). These observations and the present findings actually are not contradictory. Ehrlich cells were incubated with tracer amounts of labeled hexadecanol for 6–24 hr in those studies in which appreciable incorporation into lipid ethers occurred (25, 27). In contrast, we used substrate amounts of hexadecanol and incubation times of only 1 hr or less. From 7 to 22 nmoles of hexadecanol were incorporated into Ehrlich cell lipids in our incubations (Table 1). Analysis of the distribution of this hexadecanol radioactivity between ether and ester bonds was beyond the scope of the present studies. However, the work of others suggests that at least some of it probably was present in ether linkage (24, 25, 27).

When hexadecanol was present, glucose oxidation was reduced only slightly and its total incorporation into cell lipids was essentially unchanged (Table 2). This indicates that the slow rate of hexadecanol metabolism was not due to a toxic effect of this substance. On the other hand, hexadecanol did affect lipid metabolism in these cells. Glucose incorporation into phospholipids was enhanced, and this was accompanied by a corresponding decrease in glucose incorporation into triglycerides. The mechanism of these metabolic effects is unknown. However, the observation is potentially important, for it suggests that metabolic alteration may occur when cells are exposed to an exogenous lipid that cannot be readily metabolized.

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