Release of free fatty acids from Ehrlich ascites tumor cells

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ABSTRACT Ehrlich ascites tumor cells release free fatty acids (FFA) during in vitro incubation in media that contain albumin. The released FFA are derived by lipolysis from endogenous lipid esters. Addition of glucose to the incubation medium greatly decreases the quantity of fatty acid released by the cells. Cyanide, which inhibits endogenous lipid oxidation but not lipolysis, increases the quantity of fatty acid released to media containing albumin and causes free fatty acid to accumulate in the cells in the absence of exogenous albumin. The release of fatty acid, either preformed or derived by lipolysis during prolonged incubations, occurs under conditions of net fatty acid uptake from the incubation medium. Net release of fatty acid from the cell occurs only when fatty acid-extracted albumin is present in the extracellular medium; extrapolation of the data suggests that net release will not occur under physiological conditions.

It is postulated that free fatty acid uptake and release are independent processes, the direction of net fatty acid movement being determined by the relationship between cellular free fatty acid concentration (regulating efflux) and the molar ratio of free fatty acid to albumin in the extracellular medium (regulating uptake).

KEY WORDS free fatty acid · transport · release · efflux · uptake · lipid utilization · oxidation · esters · palmitate · albumin · glucose · cyanide · Ehrlich ascites tumor

EVIDENCE FOR significant net release of free fatty acids (FFA) from mammalian cells to medium has so far been obtained only in the case of adipose tissue. Release of FFA radioactivity has been observed from intestine (1) and lung (2), but it is not clear to what extent this reflects isotopic exchange and to what extent, if any, a potential capacity for net release of FFA. The present communication demonstrates that a net release of FFA can occur from the Ehrlich ascites tumor cell during in vitro incubation, but only under particular conditions, and that even under conditions of net uptake there is a continuing release of cellular FFA to the medium. The origin of the FFA available for release, its composition, and aspects of the metabolic control of this process are described, and the possible physiologic significance of this release is discussed.

Abbreviations: FFA, free fatty acid(s); GLC, gas–liquid chromatography; $\bar{r}$, the approximate FFA:albumin molar ratio obtained by dividing total FFA concentration by total albumin concentration.

METHODS

The methods for preparation of the Ehrlich ascites tumor cells, fatty acids, and FFA-extracted albumin have been described in detail (3, 4). A buffer solution containing 0.116 M NaCl, 0.0049 M KCl, 0.0012 M $MgSO_4$, and 0.016 M sodium phosphate adjusted to pH 7.4 with 1 N HCl was used for all procedures, and all solutions were adjusted to pH 7.4 prior to use. Palmitate–albumin preparations were made by adding, drop by drop, a heated (60°C) solution of freshly prepared sodium palmitate in H_2O to a mechanically stirred solution of albumin in buffer.

Cells were labeled in vivo by injecting palmitate-1-^{14}C intraperitoneally into tumor-bearing mice. Mice were taken for study when the peritoneal cavity contained approximately 5 ml of ascitic fluid. They were injected three times at 30-min intervals with 0.5 ml of a solution containing 0.25 μeq of palmitate-1-^{14}C (3.75 μc) and 0.05 μmole of albumin. The mice were killed 30 min after the final injection and the ascitic contents pooled. Cells were separated and washed three times as before (3). The cells were then exposed for 3 min at 0°C to 15 ml of albumin made up to contain 1 μmole per 10^8 cells, centrifuged, washed in buffer, and suspended in fresh buffer to the cell concentration desired for subsequent in vitro

TABLE 1 DISTRIBUTION OF CELLULAR LIPID RADIOACTIVITY AFTER IN VIVO LABELING WITH PALMITATE-1-^{14}C

Fraction	Percentage of Total Radioactivity*		
	Expt. 1	Expt. 2	Expt. 3
Phospholipid	76.6	73.1	71.4
Monoglyceride, diglyceride, and cholesterol	1.0	6.6	6.5
Triglyceride	16.4	15.0	14.9
Cholesterol ester	5.7	5.0	7.2
FFA	0.3	0.3	0.2

* Mean of duplicate samples in each experiment.

incubations. In cells labeled in this way, more than 96% of the incorporated radioactivity was present in lipids, while 3% was present in unidentified water-soluble compounds. The distribution of radioactivity in the cellular lipid extract of three preparations is shown in Table 1. About 75% of the radioactivity was contained in phospholipids, 15% in triglycerides, 6% in cholesterol esters, 1–7% in lower glycerides, and 0.3% in FFA. By collecting fractions of the methyl esters of total cellular fatty acids as they were eluted from a GLC column and counting them in a scintillation counter, we could show that about 90% of the incorporated radioactivity remained as palmitic acid.

In vitro incubations were performed with constant shaking under air in a 37°C water bath. After incubation, the flask contents were added to 30 ml of buffer and the cells separated at 0°C by centrifugation at 2000 × *g* for 2 min. The cells were washed in buffer, sedimented again, and dispersed in 20 ml of chloroform–methanol 2:1. The phases were separated by adding 4 ml of 0.004 N HCl, and the chloroform phase was isolated and evaporated to dryness under nitrogen (3). The residue was taken up in chloroform–methanol, and aliquots of the extract were analyzed as required. When the extracellular medium was to be analyzed, the flask contents were transferred without dilution to chilled plastic tubes and centrifuged at 10,000 × *g* at 0°C for 2 min. One milliliter of the supernatant fluid was added to 5 ml of isopropanol–isooctane–N H_2SO_4 40:10:1; the mixture was vigorously agitated and allowed to remain at room temperature for 30 min. After addition of 3 ml of isooctane and 2 ml of 0.1 N H_2SO_4, the isooctane phase was removed, evaporated to dryness under nitrogen, taken up in chloroform, and analyzed. When $^{14}CO_2$ production was determined, additional flasks were incubated for this purpose. CO_2 was collected in removable center wells with 0.2 ml of 1 N KOH. The reaction was stopped by injection of 0.25 ml of 10 N H_2SO_4, and shaking was continued for 1 hr in order that the $^{14}CO_2$ might be essentially completely trapped in the KOH (3). Lipid components were separated by thin-layer chromatography on Silica Gel G (E. Merck A. G., Darmstadt, Germany) with hexane–diethyl ether–methanol–acetic acid 90:20:2:3 as solvent (5). For routine analyses, the lipids were made visible by exposure to I_2 vapor, and the I_2 was then sublimed at room temperature. When GLC was to be performed, lipids were detected under ultraviolet light after being sprayed with Rhodamine 6G. For radioactivity determinations, the outlined segments of silica gel were scraped onto glassine paper, powdered, and dispersed in 15 ml of scintillator solution containing 3.47% Cab-O-Sil (Packard Instrument Co., La Grange, Ill.). For measurement of FFA content or composition, the segments of silica gel that contained FFA were similarly removed and then extracted twice by shaking with 5 ml of chloroform. The chloroform eluates were combined, dried under nitrogen, and taken up in the appropriate solvent.

The dried lipid residue was saponified by dissolving it in 19 ml of ethanol, adding 1 ml of aqueous 50% KOH, and heating for 1 hr at 65°C. The solution was diluted to 40 ml with distilled water and extracted twice with 20 ml of *n*-heptane in order to remove nonsaponifiable material. The aqueous phase was then acidified with HCl and extracted twice with 20 ml of isooctane.

The saponifiable fraction of the cellular lipid and the FFA eluate from the silica gel plates were methylated similarly. The dried residues were placed in tubes with Teflon-lined screw caps, dissolved in 1 ml of BF_3 in methanol (14% w/v) (Applied Science Laboratories Inc., State College, Pa.), and heated in a 60°C water bath for 2 min in an atmosphere of N_2. After addition of 1 ml of petroleum ether and 1 ml of water, the tubes were shaken vigorously and centrifuged at 2000 × *g* for 5 min. The ether phase was isolated and dried under N_2, and the methyl esters were dissolved in fresh petroleum ether.

GLC was performed on a 240 cm column (I.D., 4 mm) containing 11% EGS-X[1] on 80–100 mesh Gas-Chrom P (Applied Science). A Barber-Colman apparatus equipped with a hydrogen flame detector was used. The column was standardized at 180°C with a mixture of purified methyl esters (NIH mixture D). Results are given as uncorrected area percentages. FFA were determined by the method of Duncombe (6) with palmitic acid as the reference standard. Radioactivity was determined by liquid scintillation techniques. Samples subjected to thin-layer chromatography were counted in Cab-O-Sil as described above, while other lipid samples were dissolved directly in a toluene scintillator (3, 4). CO_2 radioactivity was counted by adding the 0.2 ml of 1 N KOH contained in the center well of the incubation flasks to 18 ml of a toluene–methanol scintillator (3). Appropriate quenching corrections with the use of internal standards were made in calculation of the results.

[1] Ethylene glycol succinate polyester.

RESULTS

Utilization of Endogenous Lipid by Cells Previously Labeled In Vivo with Palmitate-1-^{14}C

Table 2 shows the time course of radioactive CO_2 production by cells previously labeled in vivo with palmitate-1-^{14}C and then incubated in buffer, buffer plus FFA-free albumin, or buffer plus albumin containing added unlabeled palmitate. There was a rapid release of $^{14}CO_2$ during the first 15 min of incubation in each case; the rate then decreased but remained essentially linear for the next 105 min. About 13% of the initial cellular lipid radioactivity was oxidized to CO_2 in 2 hr by the cells incubated in buffer; addition of fatty acid-extracted albumin had no effect on the quantity of $^{14}CO_2$ evolved. However, addition to the medium of the same amount of albumin containing unlabeled palmitate decreased the amount of $^{14}CO_2$ evolved in 2 hr by 60%. The total radioactivity recovered as CO_2 after 2 hr of incubation far exceeded the total quantity present before incubation in cell fractions other than lipid esters (1300 cpm as FFA and 15,600 cpm as water-soluble material), which indicates that much of the radioactivity converted to CO_2 must have been derived from endogenous esterified lipid. However, the "sparing" of endogenous lipid oxidation when exogenous FFA was available was not accompanied by conservation of endogenous lipid radioactivity, as is shown in Table 3. Seven separate experiments are shown in which cells labeled in vivo with palmitate-1-^{14}C were incubated in buffer, albumin, or unlabeled palmitate–albumin for 90–180 min. The decrease or depletion of endogenous lipid radioactivity was essentially the same in the presence of palmitate–albumin as it was in the presence of buffer alone. The apparent discrepancy between the "sparing" effect of palmitate–albumin, shown by the $^{14}CO_2$ data (Table 2), and the absence of "sparing" effect in depletion of lipid ester radioactivity suggested that there was significant release of radioactive lipid from cells to medium in the presence of palmitate–albumin.

TABLE 2 RADIOACTIVE CO_2 PRODUCTION DURING IN VITRO INCUBATION OF CELLS PREVIOUSLY LABELED WITH PALMITATE-1-^{14}C*

Time of Incubation	Radioactivity Recovered as CO_2 during Incubation in:		
	Buffer	Albumin†	Palmitate–Albumin‡
min		*cpm*	
15	16,200	14,300	12,500
30	24,300	22,200	14,300
60	39,200	38,100	18,500
90	50,000	52,600	20,900
120	58,700	58,800	24,000

* Each flask contained 0.63 × 10^8 cells in a total volume of 3 ml. Before incubation, 0.63 × 10^8 cells contained 446,300 cpm in the total cellular lipid extract. Results are the mean of duplicate determinations.

† Incubation medium contained total of 0.88 μmole of albumin.

‡ In addition to 0.88 μmole of albumin, the incubation medium contained 4.0 μeq of unlabeled palmitate, $\bar{\nu}$ = 4.5 ($\bar{\nu}$ = number of moles of palmitate per mole of albumin).

TABLE 3 DEPLETION OF CELLULAR LIPID RADIOACTIVITY DURING IN VITRO INCUBATION OF CELLS PREVIOUSLY LABELED WITH PALMITATE-1-^{14}C

Expt. No.	Time of Incubation	Cellular Lipid Radioactivity before Incubation	Percentage Depletion in Cellular Lipid Radioactivity after Incubation in:*		
			Buffer	Albumin†	Palmitate–Albumin‡
	min	*cpm/10^8 cells*			
1	90	400,200	7.2	15.8	12.9
2	90	496,300	16.1	19.8	—
3	90	576,600	—	11.2	11.1
4	90	531,300	—	19.0	18.1
5	120	531,200	11.3	18.2	10.0
6	120	596,000	16.4	17.8	17.5
7	180	630,000	28.1	—	29.7

* Mean of duplicate determinations.

† Medium contained 1 μmole of albumin in a total volume of 3 ml.

‡ In addition to 1 μmole of albumin, the medium contained 5 μeq of unlabeled palmitate, $\bar{\nu}$ 5.

Release of FFA Radioactivity from Previously Labeled Cells

During incubation of cells previously labeled in vivo with palmitate-1-^{14}C there was significant release of labeled lipid to albumin-containing media. The radioactivity released was essentially all FFA (Table 4). The total amount of FFA radioactivity released far exceeded that present in the cells as FFA at the start of incubation. A very small quantity of labeled lipid was released to an albumin-free medium, about 5% of that released to an albumin-containing medium. Only 67% of the radioactivity released to the buffer medium was in the form of FFA, whereas 96–99% of that released to albumin-containing media was in FFA form. The ester radioactivity found in the buffer medium may reflect incomplete sedimentation of cells or cell fragments. This may occur also in the presence of albumin-containing media, but the much larger total FFA release would make the contribution of ester radioactivity insignificant.

The time course of FFA efflux from labeled cells and the effect of the concentration of exogenous unlabeled FFA are shown in Fig. 1. With an albumin-free medium, only trace quantities of radioactivity were released, and very little increment in released radioactivity occurred after 2 min of incubation. In the presence of albumin, increasing amounts of FFA-^{14}C were recovered during a 60 min incubation, but the rate of release was lower in the latter part of the incubation. When unlabeled pal-

TABLE 4 DISTRIBUTION OF RADIOACTIVITY RELEASED BY CELLS LABELED IN VIVO WITH PALMITATE-1-^{14}C

Expt. No.	Time of Incubation	Cellular FFA Radioactivity before Incubation	Incubation Medium	Radioactivity Release to Incubation Medium*		
				Total	FFA	Percentage as FFA
	min	*cpm*		*cpm*		
1	60	1200	Buffer	1,200	800	67
1	60	1200	Albumin†	14,000	13,500	96
1	60	1200	Palmitate‡ + albumin	30,300	29,900	99
2	90	800	Albumin + cyanide§	31,200	30,300	97

* Mean of duplicate determinations.
† 1 μmole of albumin in a total volume of 3 ml.
‡ 5 μeq of unlabeled palmitate plus 1 μmole of albumin.
§ 1 × 10^{-2} M NaCN.

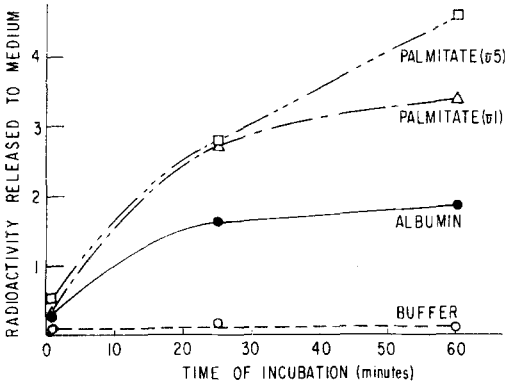

FIG. 1. Time course of FFA release from cellular lipid esters to medium. Radioactivity released is shown as cpm × 10^{-4} per 10^8 cells. The albumin concentration of the palmitate media was identical with that of the medium containing albumin alone (0.36 μmole/ml).

mitate was added to the same quantity of albumin so that the extracellular FFA:albumin molar ratio ($\bar{r}$) was 1, 80% more FFA radioactivity was recovered from the medium after 60 min of incubation, the decreasing rate of release again being noted. When the exogenous unlabeled palmitate concentration was raised further so that the initial medium $\bar{r}$ was 5, the release of FFA radioactivity remained more nearly linear over the 1 hr incubation, and the total release was greater. In each case, albumin concentration in the medium was the same. The values obtained with medium containing unlabeled palmitate–albumin of $\bar{r}$ 5 probably are a more accurate representation of total endogenous fatty acid efflux, since the radioactive FFA released are mixed into a large reservoir of unlabeled palmitate and thus a smaller fraction of the radioactive material is reincorporated by the cell. This apparently accounts for the continued linearity of radioactive FFA release seen only with the $\bar{r}$ 5 medium.

The release of appreciable amounts of endogenous radioactive lipid as FFA requires the presence of albumin acceptor throughout the time of incubation. Incubation in buffer followed by addition of buffer just before sedimentation of the cells led to the recovery of only 370 cpm, while incubation in albumin followed by addition of buffer caused a release of 12,000 cpm (Table 5). However, incubation in buffer followed by addition of the same amount of albumin prior to sedimentation of the cells led to recovery of only 940 cpm, which suggests that accumulation of cellular FFA occurs to only a limited extent during incubation in the absence of extracellular albumin.

Addition of glucose to the incubation medium decreased the net efflux of radioactive FFA from labeled cells (Table 6). In a medium containing albumin, 83% less FFA radioactivity was recovered after 90 min of incubation when glucose was present. In the presence of palmitate–albumin, addition of glucose decreased the net radioactive FFA release by 71% relative to that in glucose-free palmitate–albumin. The effect was independent of initial glucose concentration, provided glucose remained available throughout the incubation. Total glucose utilization in 1 hr varied from 16 to 22 μmoles/10^8 cells (7).

Addition of cyanide to the incubation medium containing labeled cells inhibited oxidation of endogenous lipid to CO_2. When cyanide was added to an albumin me-

TABLE 5 CONDITIONS AFFECTING FFA RELEASE FROM CELLS LABELED IN VIVO WITH PALMITATE-1-^{14}C

Time of Incubation	Contents of Incubation Medium	Additions after Incubation	FFA Radioactivity Recovered from Medium*
min			*cpm*
90	Buffer	Buffer	370
90	Buffer	Albumin†	940
90	Albumin†	Buffer	12,000

* Mean of duplicate determinations.
† 2 μmoles of albumin in a total volume of 3 ml.

TABLE 6 EFFECT OF GLUCOSE ON THE RELEASE OF RADIOACTIVE FFA FROM CELLS LABELED IN VIVO WITH PALMITATE-1-^{14}C

Contents of Medium	Radioactivity Released as FFA after 90 Min Incubation*
	cpm
Albumin†	26,300
Albumin + glucose‡	4,500
Albumin + palmitate§	47,500
Albumin + palmitate + glucose	13,600

* Mean of duplicate determinations.
† 0.6 μmole of albumin in a total volume of 3 ml.
‡ 1.4×10^{-2} M glucose.
§ 3.0 μeq of unlabeled palmitate + 0.6 μmole of albumin. Prior to incubation, an aliquot of cells contained 382,800 cpm.

dium, $^{14}CO_2$ production was only one-tenth that in the control incubation, but more than twice the amount of radioactive FFA was released to the medium (Table 7). Moreover, cyanide did not appear to inhibit transport of FFA from cell to medium (Table 4). As shown in Table 8, cells incubated with cyanide in the absence of exogenous albumin accumulated FFA radioactivity (24,300 cpm). Total cellular lipid radioactivity decreased only slightly during incubation in the albumin-free medium containing 1×10^{-2} M NaCN, but the accumulation of a large amount of FFA radioactivity in the cell was accompanied by a corresponding decrease in esterified lipid radioactivity. When albumin was added to the medium containing cyanide, FFA radioactivity did not accumulate in the cell but was released to the medium. As can be seen from Table 7, the net loss of cellular lipid radioactivity in the presence of a medium containing albumin ($^{14}CO_2$ plus FFA-^{14}C released) was only 15% lower when cyanide was added. These results suggest that cyanide itself did not importantly influence lipolysis, although the conclusion must remain tentative until possible changes in reincorporation of radioactivity can be evaluated. Even in the presence of both 5×10^{-2} M NaF and cyanide, the efflux of cellular FFA radioactivity to

TABLE 7 UTILIZATION OF ENDOGENOUS LIPID RADIOACTIVITY BY CELLS LABELED IN VIVO WITH PALMITATE-1-^{14}C DURING A 90 MIN INCUBATION WITH AND WITHOUT CYANIDE

Contents of Medium	Radioactivity Recovered after Incubation*		
	CO_2	Incubation Medium (FFA)	Total
		cpm	
Albumin†	62,600	28,500	91,100
Albumin† + cyanide‡	6,500	72,500	79,000

* Mean of duplicate determinations. Before incubation, the cellular lipid contained 301,700 cpm, 1700 cpm being in the FFA fraction.
† 0.6 μmole of albumin in a total volume of 3 ml.
‡ 1×10^{-2} M sodium cyanide.

TABLE 8 CHANGES IN CELLULAR ENDOGENOUS LIPID RADIOACTIVITY FOLLOWING 90 MIN INCUBATION IN MEDIA CONTAINING 1×10^{-2} M CYANIDE

Cellular Lipid Fraction	Total Lipid Radioactivity in Cells*		
	Before Incubation	After 90 Min Incubation in:	
		NaCN†	NaCN† + Albumin‡
		cpm	
Esters	265,200	237,500	210,100
FFA	1,500	24,300	1,500
Total lipids	266,700	261,800	211,600

* Mean of duplicate determinations.
† 1×10^{-2} M NaCN.
‡ 0.6 μmole of albumin in a total volume of 3 ml.

albumin was twice that seen in the presence of albumin alone. There was 25% less FFA radioactivity released from the cells when both fluoride and cyanide were present, as compared to that released in albumin media containing only cyanide. However, addition of fluoride alone to media containing albumin did not alter the quantity of FFA radioactivity released.

Net Release of FFA from Cell to Medium

Since albumin preparations cannot be freed entirely from FFA (8), the observed release of radioactive FFA might reflect only an isotopic exchange process. However, direct evidence for net release was obtained by incubating unlabeled Ehrlich ascites tumor cells in FFA-poor albumin and demonstrating a net FFA efflux (Table 9). The albumin used in this and the following experiment had been repeatedly extracted by the method of Goodman (8) in order to reduce its FFA content as much as possible (to <0.01 μeq/μmole of protein). Before incubation, an aliquot of cells corresponding to the number used in each incubation flask contained less than 0.01 μeq of FFA. A progressive increase in FFA concentration in the medium occurred during the 1st hour of incubation, and at 1 hr the FFA present in the medium was about 100 times greater than the amount present in the cells at zero time. Some of the FFA originally released by the cells probably was reincorporated during the incubation, and the observed increments in FFA in the medium thus represent minimum values for the total FFA efflux.

A similar experiment was carried out in which the composition of the total cellular fatty acids at zero time and of the FFA in the medium after 45 and 90 min of incubation was determined by GLC (Table 10). It is evident that the composition of the fatty acid released by the cells differed from that of the total cellular fatty acids. Relatively more saturated than unsaturated fatty acids were released.

TABLE 9 TIME COURSE OF FFA RELEASE FROM CELLS TO MEDIUM CONTAINING ALBUMIN

Time of Incubation	FFA Released to Medium
min	*μeq*
0	0.00
15	0.04
30	0.06
60	0.11
90	0.10

Each flask contained 0.96×10^8 cells and 1.23 μmoles of albumin in a total volume of 2 ml. This albumin preparation was free from FFA as determined by the method of Duncombe (6). Each aliquot of cells (0.96×10^8) contained less than 0.01 μeq of FFA before incubation. After incubation, the cells were sedimented by centrifugation at $10,000 \times g$ for 2 min at 0°C. One milliliter of the supernatant solution was added to 5 ml of isooctane–isopropanol–acetic acid 40:10:1. After addition of 3 ml of isooctane and 2 ml of 0.1 N H_2SO_4, the lipids in the isooctane phase were isolated and chromatographed as described under Methods. The FFA band was eluted with chloroform, and the FFA content of the eluate determined by the method of Duncombe (6). Each value is the mean of duplicate determinations.

Cellular FFA Release During Net FFA Uptake

The data contained in Table 11 indicate that FFA continue to be released from the cells under conditions of net FFA uptake. Unlabeled cells were incubated for 1 hr in a medium containing palmitate-9,10-^{3}H–albumin. A large number of cells relative to the quantity of palmitate in the medium was used in order that any change in specific radioactivity of the medium that might occur, because of release of unlabeled FFA from the cells, would be magnified. A large immediate decrease in both FFA radioactivity and FFA mass in the medium was noted after 2 min of incubation, but the specific radioactivity of the FFA remained essentially unchanged. As the incubation progressed, the total FFA radioactivity in the medium continued to decrease, but the total FFA mass did not drop further. Thus, there was a progressive decline in specific radioactivity of FFA in the medium, which showed that unlabeled FFA were leaving the cell even in the course of uptake of labeled FFA from the incubation medium. This was confirmed by GLC analysis of aliquots of the FFA in the medium in the above experiment (Table 12). Before incubation, palmitate was the only fatty acid present in appreciable amounts. During the first 2 min of incubation, 0.34 μeq of palmitate was taken up and traces of laurate, myristate, and palmitoleate appeared in the medium. As the incubation continued, an additional 0.1 μeq of palmitate was taken up by the cells and increasing amounts of stearate, oleate, and linoleate were released to the medium. No further net uptake occurred after the first 2 min, the further uptake of palmitate being balanced by release of other fatty acids from the cells. Because some of the palmitate originally taken up probably was released again and some of each fatty acid released probably was reincorporated, these figures represent minimum values for both FFA uptake and release.

TABLE 10 FATTY ACID COMPOSITION OF CELLS AND MEDIUM BEFORE INCUBATION AND OF THE MEDIUM AFTER INCUBATION

Fatty Acid	Percentage of Total Fatty Acid Mass*		
	Before Incubation	Medium FFA after Incubation of:	
	Cells' Total Fatty Acid	45 min	90 min
12:0†	0.5	0.1	0.2
14:0	1.1	1.8	2.5
16:0	22.0	30.4	30.7
16:1	1.5	0.6	0.0
18:0	20.6	38.7	31.7
18:1	22.6	18.2	19.4
18:2	19.6	7.5	15.6
18:3	2.3	1.3	0.0
20:4	6.8	1.2	0.0
Unidentified	3.1	0.4	0.0

* Mean of duplicate determinations. Procedure identical to that of Table 9, except that the fatty acids contained in the chloroform eluate of the FFA bands were methylated with boron trifluoride and analyzed by GLC.

† Carbon chain length: degree of unsaturation.

That release of cellular FFA can occur even during the initial rapid uptake of exogenous FFA from the incubation medium was shown by the following experiment (Table 13). Cells previously labeled with palmitate-1-^{14}C were incubated in buffer containing cyanide for 50 min, which led to the accumulation of intracellular FFA described above (Table 8). These cells were then isolated and washed, and aliquots were reincubated for 20 sec in albumin or in albumin–palmitate-9,10-^{3}H of $\bar{\nu}$ 0.95 or $\bar{\nu}$ 4.5. The total albumin content was the same in all flasks. Approximately the same quantity of cellular FFA radioactivity was released in each case, corresponding to about 90% of the total cellular FFA-^{14}C content at zero time (Table 13). The cells incubated with albumin alone, of

TABLE 11 SPECIFIC RADIOACTIVITY OF FFA IN THE MEDIUM DURING INCUBATION OF CELLS WITH PALMITATE-9,10-^{3}H

Time of Incubation	Incubation Medium		
	Radioactivity*	FFA*	Specific Radioactivity
min	*μc*	*μeq*	*μc/μeq*
0	3.97	1.12	3.56
2	3.09	0.80	3.89
20	2.58	0.80	3.21
40	2.35	0.86	2.73
60	2.28	0.81	2.82

* Mean of duplicate determinations. Each flask contained 1.55×10^8 cells, 1.12 μeq of palmitate and 0.3 μmole of albumin ($\bar{\nu}$ = 3.7). Prior to incubation, 1.55×10^8 cells contained 0.01 μeq of FFA. The procedure was identical to that of Table 10, except that the chloroform eluate of the FFA band was divided into 3 parts. One fraction was analyzed for radioactivity, the second for total fatty acid, and the third for fatty acid composition (see Table 12).

course, took up essentially no FFA; those incubated with palmitate of $\bar{\nu}$ 0.95 took up 0.03 μeq, and those incubated with palmitate of $\bar{\nu}$ 4.5 took up 0.14 μeq in 20 sec. Thus, most of the FFA radioactivity contained in the cells prior to incubation was released to the medium independent of the degree of concomitant FFA uptake from the medium.

DISCUSSION

These data demonstrate that under appropriate conditions a net release of fatty acid in the form of FFA can occur from the Ehrlich ascites tumor cell to media containing albumin. Since much more fatty acid was released from the cells than was present as cellular FFA at the start of incubation, the released fatty acids must be produced endogenously. Studies with cells previously labeled with palmitate-1-^{14}C in vivo support this conclusion and suggest that the released FFA are derived from lipolysis of endogenous lipid esters. However, the present data, considered in relation to previously reported data on FFA uptake by these cells (3, 4), make it seem unlikely that net release of fatty acid would occur under physiological conditions. A maximum net FFA release of about 0.1 μeq/10^8 cells in 1 hr was noted when albumin essentially free from FFA was present in the incubation medium. The isotopic data indicate that in the presence of glucose this would be reduced by 75%, i.e., to about 0.03 μeq/10^8 cells. Additional studies have shown that when glucose and palmitate–albumin of $\bar{\nu}$ 0.7 are contained in the incubation medium, a total palmitate *uptake* of 0.15 μeq/10^8 cells occurred in 1 hr (7). Moreover, as the $\bar{\nu}$ of the exogenous palmitate–albumin medium was raised, total uptake of exogenous palmitate increased further, especially in the presence of glucose (4, 7). While the isotopic data presented here indicate a greater efflux of endogenous fatty acid when the FFA concentration in the medium is increased, it appears that the increase in uptake with increasing $\bar{\nu}$ more than compensates for it, and the net change would be in the direction of FFA uptake. Therefore, under conditions likely to be encountered in vivo, net release of fatty acid from the Ehrlich ascites tumor cell would not be expected.

TABLE 12 CHANGES IN FFA COMPOSITION OF MEDIUM DURING INCUBATION OF CELLS WITH PALMITATE-9,10-^{3}H

Time of Incubation	Amount of FFA in Medium*						
	12:0	14:0	16:0	18:0	18:1	18:2	Total
min				*μeq*			
0	0	0	1.115	0	0	0	1.115
2	0.004	0.008	0.769	0.016	0	0	0.797
20	0.003	0.008	0.701	0.036	0.025	0.022	0.795
40	0.003	0.011	0.705	0.050	0.049	0.033	0.851
60	0.002	0.007	0.656	0.056	0.038	0.035	0.794

* Mean of duplicate determinations. Calculated from percentage distribution of GLC peak areas, taken to be proportional to mass, corrected for differences in molecular weight, and from the total FFA in the medium, determined by the method of Duncombe (6).

TABLE 13 EFFECT OF CELLULAR FFA UPTAKE ON THE RELEASE OF ENDOGENOUS FFA

Before Incubation		After Incubation	
Palmitate-9,10-^{3}H in Medium	Cellular FFA-^{14}C	Cellular Palmitate-9,10-^{3}H Uptake*	FFA-^{14}C in Medium
μeq	*cpm*	*μeq*	*cpm*
0.0	23,000	0.0	21,600
0.48	23,000	0.034	20,300
2.20	23,000	0.143	20,600

* Calculated from the decrease in palmitate-9,10-^{3}H content in the medium. Cells were labeled with palmitate-1-^{14}C in vivo, harvested, and incubated at 37°C in buffered salt solution containing 1×10^{-2} M NaCN for 50 min. After two washings at 0°C, the cells were suspended in buffer, and aliquots were added to 1 ml of medium, at 37°C, containing either 0.5 μmole of albumin alone, 0.5 μmole of albumin plus 0.48 μeq of palmitate-9,10-^{3}H, or 0.5 μmole of albumin plus 2.2 μeq of palmitate-9,10-^{3}H. After a 20 sec incubation, the plastic tubes were transferred to a centrifuge at 0°C, and the cells were sedimented at 10,000 × *g* for 2 min. One milliliter of the supernatant solution was analyzed for both ^{3}H- and ^{14}C-FFA content. Before incubation in the media containing albumin, 1 ml of cells contained 23,000 cpm as FFA-^{14}C.

Certain aspects of the FFA release mechanism from the Ehrlich ascites tumor cell are evident from these data. As expected, the presence of exogenous albumin acceptor is required for appreciable FFA release. The composition of the FFA efflux differs from that of the total cellular fatty acid, a relatively higher percentage of saturated fatty acids being released. This may be due either to preferential release of saturated fatty acids once delivered into the cellular FFA pool or to a preferential input of saturated fatty acids from esterified lipids into the FFA pool. Release, like uptake (3), is a very rapid process, most of the cellular FFA content being released within 20 sec. The presence of concomitant FFA uptake appears not to interfere with cellular FFA release. The studies in the presence of cyanide and fluoride suggest that neither respiratory nor glycolytic energy is an absolute requirement, in agreement with findings in adipose tissue (9). However, since the very high FFA efflux occurring in the presence of cyanide is somewhat reduced when fluoride is added, an energy requirement for some part of the process (lipolysis or release) cannot be excluded.

The amount of FFA released in prolonged incubations appears to depend on the cellular FFA content which, in turn, is determined by the relative rates of lipolysis and utilization. Thus, glucose, as in the case of adipose tissue, probably decreases FFA release by stimulating reesterification of fatty acids, which reduces the FFA concentration in the cellular pool (4, 10). From previous work, it is

known that the amount of exogenous FFA taken up by the cell is related to the extracellular FFA : albumin molar ratio (3, 4). Therefore, the direction of net FFA movement at a given time is determined both by the cellular FFA content and the FFA content of the exogenous albumin. These factors also appear to be relevant to fatty acid transport in adipose tissue as discussed by Steinberg and Vaughan (9). However, adipose tissue releases FFA in net amounts into media of relatively high FFA concentration (high $\bar{v}$) whereas this tumor cell (and probably other tissues) exhibit net FFA uptake under similar conditions.

This does not necessarily mean that FFA release from adipose tissue is a qualitatively different process. The concentrations of FFA reached in adipose tissue are extremely high relative to those seen in other tissues, presumably because of the uniquely high lipolytic activity in adipose tissue. Certainly, if all or much of the FFA is in the narrow cytoplasmic ring of the adipose tissue cell, the FFA "activity" there far exceeds that in other cells. All our studies in the Ehrlich ascites tumor cell thus far are compatible with a passive transfer process in which uptake is controlled by the effective concentration of FFA in the medium (related to FFA:albumin molar ratio), on the one hand, and the availability of binding sites on the cell surface and their affinity for FFA on the other. As shown in the present studies, net release can be demonstrated from ascites tumor cells provided the "activity" of FFA in the surrounding medium is made sufficiently low. It is also shown that FFA generated by endogenous lipolysis are available for release under such conditions. Exactly how the intracellular FFA leave the cell is not clear, but it is reasonable to propose that they first are transferred to cell surface sites, perhaps the same set of sites involved in uptake.

Evidence that other tissues can release labeled FFA has been presented. Intestinal segments have been shown to release previously incorporated radioactive palmitate (1), and lung was shown to release radioactive FFA synthesized from acetate into a medium containing albumin (2). During perfusions of isolated hearts, changing FFA composition (11) and decreasing FFA specific radioactivity in the perfusate have been noted (12). This could be explained by release of endogenous FFA from the myocardium accompanying uptake of FFA from the perfusing fluid, the process shown to occur in the present incubations with tumor cells. In this connection, it is of interest to note the ability of the intestinal mucosa under appropriate conditions to effect net transfer of FFA into the portal blood. When bile is diverted from the intestine, long-chain fatty acids, which are normally transported as triglycerides in chylomicrons, are now transported via the portal vein, like short-chain fatty acids (13). In the absence of bile, FFA are poorly esterified by slices of intestine (14), and it seems likely, therefore, that under these conditions FFA build up to levels that permit transfer into the plasma. Normally, esterification probably keeps pace with mucosal cell uptake, keeping cellular FFA levels low and thus preventing appreciable transfer into plasma. We suggest, then, that there is no need to postulate a qualitatively unique transfer mechanism in the case of adipose tissue. The basic processes of FFA uptake and release may not be different from those in other cells but the ability to release FFA may reflect primarily the ability to generate intracellular (more correctly, cell surface) FFA of high activity.

Manuscript received 15 February 1966; accepted 18 May 1966.

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