Intracellular accumulation of free fatty acids in isolated white adipose cells

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ABSTRACT A simple, rapid, and accurate method was developed for measuring intracellular FFA levels in isolated white adipose cells using sucrose-^{14}C or inulin carboxyl-^{14}C as nontransportable, nonutilizable markers of the extracellular space. Following incubation, medium and cells were separated by centrifugation and the infranatant medium was removed by aspiration. The volume of medium trapped between cells was determined by measuring the amount of sucrose-^{14}C or inulin carboxyl-^{14}C retained in the floating packed adipose cells. In this way the FFA content of the adipose cells could be corrected for contamination by FFA bound to extracellular albumin.

With this technique the initial events in hormone-activated lipolysis were studied under conditions of maximal and constant rates of triglyceride hydrolysis. The FFA content of isolated adipocytes of fed rats was 0.5 μmole/g cell lipid. On addition of norepinephrine in the presence of medium albumin, the concentration of intracellular FFA rapidly increased and reached a plateau at a concentration of 2–2.5 μmoles/g cell lipid. In the presence of medium albumin an initial lag in glycerol release occurred and this was attributed to partial hydrolysis of triglyceride with retention of lower glycerides. After 5 min of incubation FFA and glycerol output was constant. In the absence of medium albumin norepinephrine-stimulated lipolysis was reduced more than 90% and extracellular FFA release was not detected. Nevertheless, intracellular FFA accumulation was identical to that seen in the presence of albumin. The data suggest that most of this intracellular pool of FFA is bound to cytoplasmic constituents.

SUPPLEMENTARY KEY WORDS lipolytic rates · hormone-stimulated lipolysis · intracellular FFA pools · fatty acid transport · fatty acid acceptors · sucrose space · fatty acid–glycerol balance

THE ABILITY of adipose cells to mobilize lipid in the form of FFA differentiates these cells from all other mammalian tissues. While a good deal is known about the various nutritional (1) and hormonal (2, 3) factors that regulate this event, and while the relationship of the adenyl-cyclase system to the lipolytic process has been examined in detail (4–6), very little is known about the physical transfer of fatty acids through the cytoplasmic compartment and across the plasma membrane.

In studies with slices of adipose tissue incubated in medium containing albumin, a significant proportion of the fatty acids produced as a result of treatment with lipolytic hormones is confined to the tissue fragments, probably because of interstitial diffusion barriers (7–9). This observation is particularly significant since it is commonly stated that many of the metabolic effects of lipolytic agents are secondary to intracellular accumulation of FFA (10–12). A number of reports have recently suggested that lipolytic hormones exhibit a direct effect on glucose metabolism in adipocytes by a mechanism unrelated to the lipolytic process (13, 14). Since intracellular FFA levels were not determined in any of these studies, it is not possible to reconcile the theories that differentiate primary and secondary effects of lipolytic hormones.

The present report describes a method for the simple, rapid, and accurate measurement of intracellular FFA in adipose cells without the need for repeated cell washings to remove medium FFA. This was accomplished by using sucrose-^{14}C as a nontransportable, nonutilizable marker of the extracellular space (8, 15). With this method, the initial events in triglyceride hydrolysis were studied in order to characterize the pattern of intracellular and extracellular FFA accumulation under optimal conditions of maximal and linear lipolytic rates.

Abbreviations: FFA, free fatty acid(s); KRB, Krebs-Ringer bicarbonate buffer; TLC, thin-layer chromatography.

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MATERIALS AND METHODS

Isolated adipose cells were prepared by the method of Rodbell (16) from epididymal fat of male Wistar rats raised on Purina Chow. The animals had continuous access to food and water and were killed by a sharp blow to the head. 3 or 12 animals (depending on whether albumin was present in the incubation medium) weighing 160–230 g each were used for each experiment. Cells were suspended in KRB pH 7.4 containing 5% bovine serum albumin (Cohn Fraction V, Armour Pharmaceutical Co., Chicago, Ill.) and they were then distributed in 2-ml volumes to a series of plastic vials (Nalgene) for incubation. In experiments with albumin-free medium the isolated cells were washed three times in KRB pH 7.4 prior to suspension in the final medium. Before distribution of cells into the incubation vials, 4.0 μCi of sucrose-U-^{14}C (or inulin carboxyl-^{14}C) was added in a small volume of distilled water. After 10 min preincubation, zero time samples were taken and norepinephrine (Sigma Chemical Co., St. Louis, Mo.) was added to the appropriate flasks in a final concentration of 10 μg/ml.

The cells were incubated in a Dubnoff metabolic shaker at 37°C with 95% O_2, 5% CO_2 as the gas phase. After incubation the contents of each vial was transferred to a 15-ml glass centrifuge tube and centrifuged at top speed (about 300 *g*) for 15 sec in a clinical bench centrifuge (International Equipment Co., Needham Hgts., Mass.). The infranatant medium was aspirated into a 3-ml disposable syringe attached to a 6-inch-long, stainless steel, no. 22 needle. In this way, dispersion of the adipose cell "float" was kept to a minimum and 95% of the medium was easily removed. The time interval between termination of incubation and addition of solvents to the cells was less than 1.5 min. The adipose cell "float" containing a small amount of entrapped medium was extracted with 5 ml of Dole's extraction mixture (17) and 0.5 ml of the aspirated medium was similarly treated. Water was added to each extract (1 ml to the cell extract and 0.5 ml to the medium extract) to maintain the recommended ratio of extraction fluid to sample of 5:1. After further addition of water and heptane (17), 4 ml of the resultant upper phase was washed with 4 ml of 0.05% H_2SO_4, as described by Trout, Estes, and Friedberg (18), to remove extraneous titratable acidity, and 3 ml of the washed heptane phase was then titrated for fatty acids (17). 0.3-ml aliquots of the initial heptane phase were evaporated and weighed in tared flasks to determine the amount of cell lipid. Water blanks, medium blanks, and fatty acid standards were carried through each extraction and wash procedure. The FFA content of the KRB–albumin medium was 0.36 μEq/ml.

Measurement of intracellular FFA (Table 1) was accomplished by titrating the FFA of the adipose cell "float" and correcting for extracellular albumin-bound FFA in the small amount of medium trapped between cells. The extracellular volume of the adipose cell "float" was reflected by the amount of sucrose-^{14}C retained after gross separation of medium and cells. Its measurement was simplified by the finding in preliminary experiments that sucrose-U-^{14}C partitioned quantitatively into the lower (polar) phase of Dole's extraction system. Accordingly, the polar phase of the extract of the cell "float" was centrifuged to sediment denatured protein and 1 ml was assayed for sucrose-^{14}C. A 50-μl aliquot of the incubation medium was also counted to determine the concentration of isotope (cpm/ml medium). Knowing these two values, the volume of medium trapped in the adipose cell "float" was calculated from the ratio:

TABLE 1 MEASUREMENT OF INTRACELLULAR FFA IN WHITE ADIPOSE CELLS

		A	B	C	D	E	F	G	H
Time of Incubation	Norepinephrine	Medium Sucrose-^{14}C	Sucrose-^{14}C Content of Adipose "Float"	Volume of Trapped Medium in "Float" (B/A)	FFA in Medium	FFA Content of "Float"	Intracellular FFA E − (D × C)	Cell Mass	Adipocyte FFA
min	*10 µg/ml*	*cpm/ml*	*cpm*	*ml*	*µEq/ml*	*µEq*	*µEq*	*g of lipid*	*µEq/g lipid*
0	−	119,036*	5689	0.048	0.367	0.065	0.047	0.083	0.57
0	−	±1,334	6772	0.057	0.405	0.072	0.049	0.084	0.58
60	−	"	6735	0.057	0.416	0.080	0.056	0.078	0.72
60	−		6218	0.052	0.465	0.072	0.048	0.064	0.75
60	+	"	7648	0.064	2.841	0.389	0.207	0.073	2.84
60	+		8902	0.075	2.534	0.367	0.177	0.063	2.81

Isolated white adipose cells were incubated in KRB–5% albumin buffer with or without norepinephrine, 10 μg/ml. Each treatment was performed in duplicate. Sucrose-U-^{14}C was added to the medium–cell mixture as an extracellular marker. After 60 min each incubate was centrifuged briefly and the infranatant medium was separated from the adipose cell "float" by aspiration. The FFA and sucrose-^{14}C content of medium and "float" were determined (see Methods for details) and the intracellular FFA content was calculated as shown. Zero time values were taken after 10 min preincubation at 37°C in 95% O_2–5% CO_2 gas phase.

* Mean ± SEM of six replicates.

$$\frac{\text{total cpm in lower phase of Dole's extract of adipose cell "float"}}{\text{cpm/ml medium}}$$

Since the medium FFA concentration was determined by titration, and the volume of medium trapped in the adipose cell "float" was known, the extracellular FFA in the adipose cell "float" could be calculated and the intracellular FFA was determined as follows:

$$\text{Intracellular FFA} = \text{FFA in adipose cell "float"} - \text{FFA in medium trapped between cells}$$

In the experiments to be described the volume of trapped medium in the adipose cell "float" was 40–100 μl. This volume varied according to the number of adipocytes in a given incubation flask and the completeness of the initial medium aspiration. Inulin carboxyl-^{14}C could be substituted for sucrose-^{14}C with identical results.

The percentage retention of marker in washed adipocytes was calculated as follows:

$$\frac{\text{cpm in washed cells}}{\text{cpm in incubation medium} + \text{cpm in wash fluid} + \text{cpm in washed cells}} \times 100$$

Glycerol was measured enzymatically by the spectrophotometric procedure of Garland and Randle (19). Since facilitated diffusion of glycerol is extremely rapid (20, 21), and since the ratio of volume of intracellular water to volume of extracellular water is approximately 1:400,[1] the rate of glycerol release into the incubation medium is taken to represent the rate of glycerol production.

Use of Palmitic Acid-1-^{14}C as an Extracellular Marker

Palmitic acid-1-^{14}C was purified twice by TLC. It was converted to its Na^+ salt by the addition of $^1/_3$ excess NaOH while being stirred in a heated water bath. A portion was taken up in a warmed pipette and added drop by drop to a small volume of warmed (40°C) KRB–5% albumin medium. This solution was stirred for 15 min and then passed through a 0.22-μ Millipore filter to remove noncomplexed fatty acid crystals; the filtered solution was gassed with 95% O_2–5% CO_2 to pH 7.4 before use. Each flask contained 40 μl of this stock solution (ca. 6.0 × 10^5 cpm) in 2 ml of cell–medium mixture. After 10 min equilibration at 37°C, zero time samples were taken. The distribution of radioactivity in the different lipid classes of the adipose cell "float" was determined by TLC of portions of both the organic and polar phases of Dole extracts and radioassay of silica gel scrapings (23). The proportion of lipid radioactivity present in esterified form could thus be determined and subtracted from the total radioactivity in the "float." Apart from this correction, the mathematical treatment was identical to that outlined in Table 1 for sucrose-U-^{14}C.

Sucrose-U-^{14}C, 98% pure, specific activity 10.4 mCi/mmole was purchased from Amersham/Searle Corp., Arlington Hgts., Ill.; inulin carboxyl-^{14}C, greater than 99% pure according to manufacturer's specification, specific activity 2 μCi/mg, mol wt 5000–5500, and palmitic acid-1-^{14}C, 97% pure, specific activity 10 mCi/mmole, were purchased from New England Nuclear Corp., Boston, Mass. Radioactivity was assayed at 15°C in a Beckman Liquid Scintillation Spectrometer, model LS-250, equipped with an external standard. The fluids were counted directly in 15 ml of Bray's liquid scintillation solution (24); with the volumes used corrections for quenching were not necessary.

RESULTS

Time course experiments on the release of products of lipolysis are shown in Figs. 1–3. Since reutilization of glycerol by adipose cells is negligible (25), glycerol output is taken as a measure of lipolytic rate. In Fig. 1 the pattern of glycerol output is shown. An initial lag in the release of glycerol was noted but after 5 min incubation, glycerol output was linear at a rate of 16 μmoles/g of cell lipid per hr. The initial lag in glycerol output is

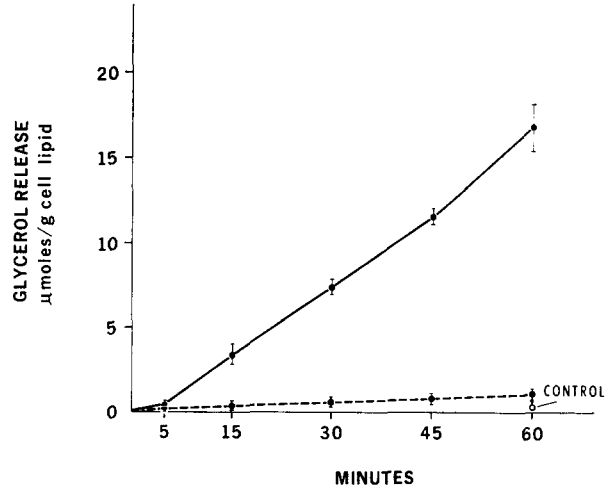

FIG. 1. A time course study on the initial rates of lipolysis in norepinephrine (10 μg/ml)-stimulated adipocytes. White adipose cells were incubated in KRB buffer with (———) or without (- - - -) 5% albumin. Hormone was added to the appropriate flasks after 10 min preincubation at 37°C. In calculating glycerol output at each interval, zero time values were subtracted. Each point and bracket represents the mean ± SEM (n = 6) of three experiments, each performed in duplicate. In the experiments with albumin the flasks contained (mean ± SEM, n = 12) 74 ± 0.5, 71 ± 1.8, and 77 ± 1.9 mg of cell lipid/2 ml, respectively. In experiments without albumin the incubates contained 212 ± 6.6, 190 ± 2.6, and 188 ± 4.9 mg of cell lipid/2 ml. Fewer cells were used in the former system to avoid saturation of medium albumin. More cells were employed in the latter situation to enhance the accuracy of measuring small increments in glycerol output.

[1] Calculated on the assumption that adipose cell water equals 5% wet weight (22) and that each incubation flask contained 100 mg of adipose cells in 1.9 ml of medium.

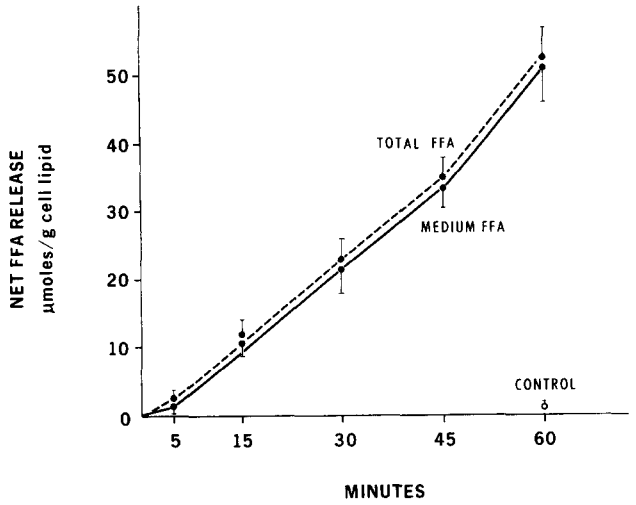

FIG. 2. The pattern of FFA release by isolated white adipose cells incubated with norepinephrine (10 μg/ml) in KRB–5% albumin buffer. Total FFA production (upper curve) was calculated by adding intracellular FFA levels (Fig. 3 solid curve corrected for zero time FFA content) to the medium content shown above. The final FFA–albumin molar ratio was 2.83 ± 0.53 (mean ± SEM, n = 6). Each point is the mean ± SEM of six observations from three experiments as described in the legend to Fig. 1.

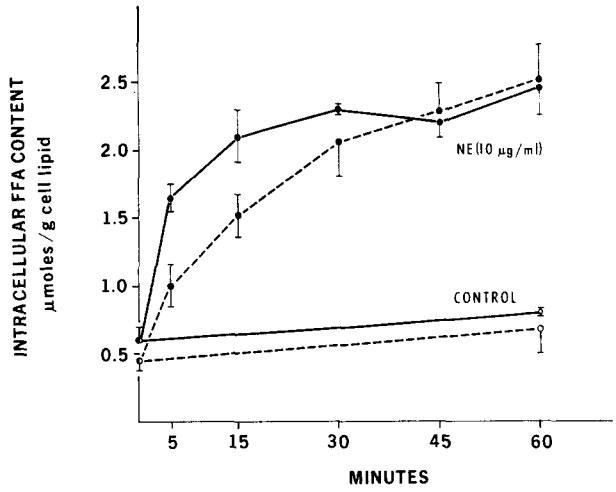

FIG. 3. Intracellular FFA accumulation in isolated white adipose cells during 60 min incubation with norepinephrine (*NE*) was determined as described in Table 1. Incubations were with (———) or without (– – – –) albumin. Each point is the mean ± SEM of six observations from three experiments as described in the legend to Fig. 1.

attributed to retention of partial glycerides. In the absence of medium albumin, glycerol output was markedly reduced to a rate of 1 μmole/g of cell lipid per hour. Unstimulated cells, whether in the presence or absence of albumin, released less than 0.2 μmole of glycerol/g of cell lipid per hr.

The pattern of FFA mobilization is shown in Fig. 2. As with glycerol output, a lag in fatty acid release into the medium was evident between zero and 5 min incubation, but subsequently the output of FFA was linear. In control flasks less than 1 μmole of fatty acid was released per g of cell lipid per hr. In the absence of medium albumin, fatty acid output was not detected whether or not catecholamine was present, and was not plotted. It is of interest that the initial lag in FFA release was less apparent in a plot of total FFA production (Fig. 2, upper curve), indicating that initiation of glyceride hydrolysis occurred very soon after addition of lipolytic hormone. Another point which is apparent in Fig. 2 is the distribution of FFA between medium and cells during the initial phase of the lipolytic reaction. Up to 50% of the total FFA produced was still within the cell after 5 min incubation, and at 15 min this had reduced to about 15% of the total. With time, the proportion within the cell decreased as medium fatty acid continued to accumulate, while the amount of intracellular FFA was constant. Determination of medium–cell partition of FFA would of course be obligatory in studies designed to relate initial rates of lipolysis to other metabolic events.

The pattern of intracellular fatty acid accumulation (Fig. 3) during 60 min incubation with norepinephrine differed appreciably from the pattern of FFA appearance in the medium (Fig. 2). At zero time the FFA content of isolated adipose cells was between 0.4 and 0.6 μmoles/g of cell lipid. Within 5 min exposure to catecholamine a rapid increase in intracellular titratable acidity occurred. This increase continued at a somewhat slower rate for about 30 min, after which the concentration was constant, between 2 and 2.5 μmoles/g of cell lipid. It is apparent that the pattern of fatty acid accumulation within the cell was similar whether albumin was present or absent from the incubation medium. In the absence of hormone, intracellular FFA increased about 0.25 μmole/g but this was not statistically significant.

Analysis of the FFA–glycerol balance (calculated using total FFA release [Fig. 2, upper curve] and glycerol output [Fig. 1, upper curve]) revealed that during the initial phases of lipolysis proportionately more FFA was released relative to glycerol compared with later times, since at 5 min the FFA–glycerol ratio was 5.5 and after 30 min of incubation the FFA–glycerol ratio approached 3, the theoretical value for complete hydrolysis of triglyceride. A ratio of 3.1 was maintained for the next 30 min, indicating the absence of significant FFA re-esterification.

It cannot be assumed that FFA associated with the adipose cells following incubation with lipolytic hormones are situated within the cytoplasmic compartment, since fatty acid adsorbed to the cell surface or bound to adsorbed albumin (26) would give identical results. It is also possible that a portion of FFA associated with the adipose cells could represent secondary incorporation from the medium by pinocytosis (27). Accordingly, ex-

TABLE 2 INTRACELLULAR FFA CONTENT IN ADIPOCYTES COMPARING SUCROSE-U-^{14}C AND INULIN CARBOXYL-^{14}C AS EXTRACELLULAR MARKERS

Extracellular Marker	Experiment A: Time of Incubation	Intracellular FFA	Medium FFA	Glycerol Release	Experiment B: % of Marker Retained in Washed Adipocytes
	min	*μmoles/g cell lipid*			
Sucrose-U-^{14}C	0	0.48 ± 0.14	0	0	0.014 ± 0.002
	30	0.63 ± 0.15	<0.5	<0.2	0.017 ± 0.004
+ Norepinephrine (*10 μg/ml*)	30	2.30 ± 0.08	32.29 ± 1.53	8.97 ± 0.25	0.029 ± 0.001
Inulin carboxyl-^{14}C	0	0.50 ± 0.02	0	0	0.006 ± 0.002
	30	0.53 ± 0.16	<0.5	<0.2	0.008 ± 0.002
+ Norepinephrine (*10 μg/ml*)	30	2.56 ± 0.11	32.99 ± 0.92	10.14 ± 0.92	0.007 ± 0.001

In Experiment A, medium and cells were separated by centrifugation and analyzed for intracellular FFA as described in Table 1 and the Method section. Medium FFA and glycerol content at zero time was subtracted from the 30-min values. Each flask contained 58–69 mg of adipose cells and each value is the mean ± SEM of three observations. In Experiment B, medium and cells were separated and then the adipose cells were washed four times with 2 ml of buffer–albumin and analyzed for radioactivity. Each flask contained 51–71 mg of adipose cells and each value is the mean ± SEM of triplicate determinations.

TABLE 3 RETENTION OF PALMITIC ACID-1-^{14}C IN WASHED ADIPOCYTES

Time of Incubation	Norepinephrine	% of Palmitic Acid-1-^{14}C Retained: Total Radioactivity	% of Palmitic Acid-1-^{14}C Retained: As FFA	Distribution of Lipid Radioactivity in Adipocytes: TG	FFA	DG	Origin (PL)	Intracellular FFA: Total	Medium-Derived	Endogenous
min	*10 μg/ml*			%				*μmoles/g of cell lipid*		
0	–	6.6	0.098	89.3	1.5	6.3	3.0	0.29	<0.01	0.29
0	–	6.8	0.096	89.2	1.4	7.3	2.1	0.39	<0.01	0.39
30	–	12.4	0.120	90.9	1.0	5.4	2.7	0.28	0.02	0.26
30	–	9.8	0.219	88.9	2.2	7.3	1.6	0.39	0.03	0.36
30	+	9.6	0.191	89.5	1.9	6.8	1.8	3.41	0.06	3.35
30	+	10.6	0.201	91.4	1.9	6.1	0.6	3.16	0.06	3.10

Tracer amounts of palmitic acid-1-^{14}C (6×10^5 cpm, approximately 0.04 μmole) were added to each incubation flask in a small volume of buffer–albumin. After 10 min preincubation, zero time samples were taken and norepinephrine was added. Each flask contained 59–83 mg of adipose cells and each incubation was carried out in duplicate. TG, triglyceride; DG, diglyceride; PL, phospholipid.

periments were undertaken in an effort to establish the relevance of these considerations.

Table 2 shows results of studies in which intracellular FFA was determined using sucrose-U-^{14}C and inulin carboxyl-^{14}C as extracellular markers. Similar results were obtained with both isotopic markers. It is apparent that less than 0.05% of labeled sucrose or labeled inulin was retained by adipocytes. If retention of these markers parallels the uptake and retention of extracellular FFA, it may be concluded that this extraneous source of fatty acid would be trivial, since 0.05% of medium FFA would amount to less than 1% of the FFA actually localized in the adipocyte after lipolytic stimulation.

To assess the significance of fatty acid adsorption as a possible source of error, a tracer amount of palmitic acid-^{14}C was added to adipose cells incubated in the presence or absence of norepinephrine (Table 3). After incubation cells were reisolated and washed four times with KRB and extracted by Dole's solvents (17). Up to 12.4% of the fatty acid label was retained by adipocytes and over one-half of this uptake occurred during the preincubation period. However, over 97% of this activity was in esterified lipid rather than in FFA. If the amount of cell radioactivity present as FFA (less than 0.22% of the added isotope) was assumed to represent adsorbed fatty acid and this value was multiplied by the final specific activity of medium FFA, a maximal value for the mass of medium-derived fatty acid associated with the adipocytes could be calculated. Incorporating this correction into the calculation resulted in a 2% adjustment in the values obtained for intracellular FFA concentration following 30 min incubation with lipolytic hormones (Table 3).

Finally, the effect of norepinephrine on intracellular FFA levels was determined in experiments in which sucrose-U-^{14}C and palmitate-1-^{14}C were compared as ex-

TABLE 4 ADIPOCYTE FFA LEVELS USING SUCROSE-U-^{14}C OR PALMITATE-1-^{14}C AS AN EXTRACELLULAR MARKER

Marker	Intracellular FFA		Medium FFA		Glycerol Release		Medium FFA–Albumin Molar Ratio	
	NE	Control	NE	Control	NE	Control	NE	Control
	μmoles/g of cell lipid/30 min							
Sucrose-U-^{14}C	2.81 ± 0.19	0.49 ± 0.04	27.63 ± 1.51	0.89 ± 0.32	8.69 ± 0.40	<0.2	2.77 ± 0.57	0.64 ± 0.05
Palmitic acid-1-^{14}C	2.90 ± 0.19	0.43 ± 0.06	26.30 ± 2.11	0.49 ± 0.18	7.50 ± 0.48	<0.2	2.67 ± 0.58	0.68 ± 0.03

Intracellular FFA levels were determined in isolated cells after incubation with or without norepinephrine (NE), 10 μg/ml, to compare sucrose-U-^{14}C and palmitic acid-1-^{14}C as extracellular markers. After centrifugation the adipose cell "float" and medium were analyzed as described in Methods. TLC of palmitic acid-1-^{14}C-containing adipose cell "floats" was carried out to determine the correction for the amount of radioactivity present in lipids other than FFA. Medium glycerol and FFA content at zero time was subtracted from the 30-min values to give net release of lipolytic products. Each value is the mean ± SEM of nine observations from three experiments. Each flask contained 69–78 mg of adipose cells.

tracellular markers. For the palmitate studies there was an appropriate correction made for the amount of radioactivity in the adipose cell "float" present in esterified lipid rather than FFA. As can be seen in Table 4 intracellular FFA levels were similar whether labeled sucrose or labeled palmitic acid was used to identify the extracellular space. This indicates that under the conditions employed sucrose and exogenous FFA displayed essentially identical volumes of distribution. This was taken as strong evidence in favor of the conclusion that adsorption of medium FFA on adipocytes was negligible compared to the intra- and extracellular FFA concentrations encountered, and that FFA associated with adipocytes following catecholamine treatment is located within the cell rather than on the cell.

DISCUSSION

A simple, rapid, and reliable method for measurement of intracellular FFA levels in isolated adipose cells has been developed using sucrose-^{14}C as an extracellular marker. With this approach the initial events in the hormone-activated lipolytic reaction have been studied in detail. The principle employed is based on the assumption that no unusual, extracellularly derived, FFA pool is concentrated at the surface of the adipose cell in significant amounts and that if pinocytosis of medium FFA by isolated adipocytes occurs, uptake of labeled sucrose would be a concomitant event and hence systematically excluded by the mathematical treatment. This latter point warrants some elaboration. It should be apparent from the calculations shown in Table 1 that radioactivity in the adipose cell "float" (Column B) is assumed to represent marker in the extracellular space and is used to calculate the volume of medium trapped between cells (Column C). Since any labeled marker sequestered within pinocytotic vesicles is treated as part of the total "float" radioactivity, calculation of intracellular FFA (Column D) automatically excludes pinocytosed material. The validity of these assumptions was established in the experiments showing that retention of the extracellular marker by adipocytes was trivial and that over 98% of labeled palmitate marker which had been taken up by adipocytes was in the ester rather than free form. The fact that all three extracellular markers (sucrose-U-^{14}C, inulin carboxyl-^{14}C, and palmitate-1-^{14}C) gave essentially identical results further attests to the accuracy of the methodology. It should be emphasized that while some retention of markers by adipocytes was apparent the amounts were so small that corrections would not significantly alter the results. Whether the small amount of palmitate-1-^{14}C retained as the free acid or the retention of sucrose or inulin after washing represents either adsorption of these molecules on the cell surface or internalization by pinocytosis cannot be discerned from the available data.

In the present study isolated adipocytes obtained from normal fed rats contained about 0.5 μmole of FFA/g of cell lipid and this level increased slightly after 60 min incubation in the absence of added substrate. This is of interest since the fatty acid content of intact adipose tissue is 2–3 μmoles/g wet wt (28–30), suggesting that most of the FFA titrated in intact adipose tissue is associated with stromal or vascular constituents rather than the adipocyte itself.

Following the addition of catecholamine to the incubation system, a lag in the release of glycerol was clearly seen (Fig. 1). Since FFA release exceeded that of glycerol (as indicated by a molar ratio greater than 3), it is likely that the lag in glycerol release was due to partial hydrolysis of glyceride with retention of lower glycerides. After 5 min incubation, however, both FFA and glycerol production were linear and approached the theoretical value for complete hydrolysis of triglyceride with no fatty acid reesterification. This implies that progressive accumulation of lower glycerides do not occur to any significant extent in this system. The constant FFA–glycerol molar ratios confirm an earlier observation (31) that reesterification of FFA in isolated adipocytes exposed to lipolytic hormone does not occur in the absence of glucose substrate.

The pattern of intracellular FFA accumulation differed appreciably from that of the medium after exposure to lipolytic hormones. Within 5 min, intracellular FFA levels rapidly increased, and within 30 min reached a constant concentration four times that of the unstimulated cell (Fig. 3). Since an identical pattern occurred in the absence of medium albumin it is concluded that activation of glyceride hydrolysis does not require the presence of an extracellular fatty acid acceptor and that the accumulation of intracellular FFA is not a function of lipolytic rate per se. The similarity in intracellular FFA levels, whether or not medium albumin was present, further validates the methodology for determining the volume of extraneous medium in the adipose cell "float" since FFA accumulation in the absence of acceptor cannot be attributed to surface adsorption of fatty acid or any other unidentified extracellular pool.

The present results are not in agreement with earlier claims (31) that significant lipolysis does not occur in the absence of medium albumin. While the lipolytic rate was 15 times greater in the presence than in the absence of medium albumin (Fig. 1), fatty acids produced in the latter situation were totally confined to the adipose cell, thus accounting for the similar intracellular FFA levels. If metabolic effects of lipolytic agents are secondary to fatty acid accumulation it is likely that intracellular rather than extracellular FFA are responsible (31, 32). Thus, the findings reported here also cast doubt on the notion that lipolytic agents augment glucose uptake and metabolism directly since that conclusion is based on the assumption that adipose cells incubated in albumin-free medium are insensitive to the lipolytic effects of catecholamines (13, 33).

The significance of the plateau in the intracellular FFA at a concentration of 2.5 μmoles/g of cell lipid during brisk and linear FFA mobilization deserves some comment. This is in close agreement with previous observations in adipocytes exposed to ACTH for 60 min (31). Since the water content of isolated adipocytes is approximately 5% of wet weight (15, 22) the FFA levels observed correspond to a theoretical concentration of 50 mM which far exceeds the limit of solubility; this suggests that most of the intracellular FFA must exist in bound form. If the large lipid droplet does not act as a sink into which FFA dissolve, it is tempting to speculate that the plateau reflects the binding capacity of cytoplasmic FFA acceptors. In this connection Reshef and Shapiro (34) showed that adipocyte mitochondria and microsomes have an affinity for FFA exceeding that of kidney and liver mitochondria as well as serum albumin. Alternatively, intracellular accumulation of fatty acids might be necessary to establish a concentration gradient to facilitate fatty acid transport out of the cell. Whether one or both of these possibilities apply cannot be established from the available data.

If intracellular FFA binding to organelle membranes is similar to the dissociable interaction of FFA with red blood cell plasma membrane (35), a dynamic equilibrium between fatty acid acceptor(s) and unbound FFA pools both within and outside the cell is envisaged. Now that methods are available for quantifying intracellular FFA levels the validity of this proposal is amenable to analysis.

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