Cell-associated nonesterified fatty acid levels and their alteration during lipolysis in the isolated mouse adipose cell

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Abstract A rapid and flexible method has been developed for measuring cell-associated, probably intracellular, nonesterified fatty acids (CAFA) in isolated mouse adipose cells. A variety of lipolytic agents as well as various concentrations of epinephrine elevate CAFA levels in rough proportion to their stimulation of glycerol and fatty acid release. Insulin reduces epinephrine-elevated CAFA levels. A detailed, quantitative study of the relationship among lipolytic activity, CAFA levels, and the extracellular molar ratio of fatty acids to albumin has been carried out. Epinephrine-elevated CAFA levels rise linearly with, while epinephrine-stimulated lipolytic activity is independent of, fatty acid to albumin ratios below 2–3. As the ratio increases from 3 to 5, CAFA levels continue to increase, whereas lipolytic activity decreases. Above ratios of 5, fatty acid release almost completely ceases; CAFA levels increase dramatically with residual glycerol release. A temperature-dependent efflux of epinephrine-elevated CAFA can be elicited through blockade of stimulated lipolysis with propranolol, but only in the presence of extracellular fatty acid to albumin ratios below 3. These observations suggest that during stimulated lipolysis, a fatty acid gradient exists between the cell and extracellular serum albumin and that CAFA represent the intracellular component of this gradient. In addition, these observations support the concept that intracellular fatty acids play a role in the feedback regulation of adipose cell function as extracellular fatty acids accumulate during the lipolytic response.

Supplementary key words lipolytic activity · intracellular fatty acids · fatty acid release · glycerol release · fatty acid to albumin ratio · epinephrine · insulin · glucose

The observation by Jungas and Ball (1) that insulin could enhance epinephrine-stimulated glycerol release from rat fat pads in the presence of glucose was among the first to suggest that elevated intracellular levels of nonesterified fatty acids inhibit lipolytic activity and, thereby, play a role in the regulation of adipose cell function during lipolysis. Subsequent studies by Bally et al. (2) and Hall and Ball (3), in which tissue fatty acid levels were actually measured, failed, however, to substantiate this suggestion. Interpretation of any of these experiments with intact adipose tissue is rendered difficult due to the multitude of compartments, both intra- and extracellular, in which nonesterified fatty acids might accumulate.

Recent studies of isolated adipose cells have yielded considerably more and consistent data supporting the concept that fluctuations in intracellular nonesterified fatty acid levels might be responsible for the modulation of cell function in response to a variety of stimuli (4). Due, however, to the technical problems of accurately measuring the very small amounts of fatty acid inside the isolated cell and of preventing the contamination of these intracellular fatty acids by the large amounts of fatty acid released from the cell into the extracellular medium during stimulated lipolysis, only three reports actually describe such measurements and, even then, only in limited detail and under a minimum of experimental conditions (5–7). Most of the available data, then, are indirect in nature; the effects of lipolytic agents on such parameters of adipose cell function as glucose metabolism (8–10), cell ATP levels (11–13), and α-aminoisobutyric acid and potassium uptake (14–17) have been observed to increase in response to decreasing extracellular serum albumin concentrations in spite of associated decreasing rates of lipolysis.

This communication reports the application of an extremely sensitive method for the assay of nonesterified fatty acids (18, 19) to the measurement of levels of intracellular fatty acids, more appropriately denoted cell-associated fatty acids (CAFA) since neither their metabolic or-

Abbreviations: CAFA, cell-associated nonesterified fatty acids.

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igin nor their intracellular location has been established, in isolated mouse adipose cells. A detailed, quantitative study of the relationship between CAFA levels and lipolytic activity has been carried out in an examination of the role that intracellular fatty acids might play in the regulation of adipose cell function.

METHODS

Isolated adipose cells were prepared from the epididymal fat pads of male Swiss albino mice (Ivanovas, Kisslegg im Allgäu, Germany), fed ad lib. with Altromin R laboratory chow (Kunath Co., Aarau, Switzerland), by the method described by Rodbell (20) and modified by Cushman (21). The concentration of collagenase (Worthington Biochemical Corp., Freehold, N.J.) was, however, reduced to 5 mg/6 ml of medium. Krebs-Ringer–bicarbonate buffer containing at least 2 g of dialyzed human serum albumin (Croix Rouge Suisse, Berne, Switzerland)/100 ml was used for the preparation of cells as well as for the subsequent experimental incubations. Tissue and cells were continuously kept at 37°C; glucose or pyruvate was present only where indicated.

Prior to the addition of cells, incubation tubes were prepared to contain pyruvate or glucose and one of several lipolytic agents (epinephrine [Adrenaline, 0.1%, Vifor Laboratories, Geneva, Switzerland], dibutyryl cyclic AMP [C. F. Boehringer und Sohne GmbH, Mannheim, Germany], caffeine [E. Merck A.G., Darmstadt, Germany], or ACTH [Synacthen, CIBA, Basel, Switzerland]), either alone or in combination with insulin (10 times crystallized monocomponent pork insulin, Novo Laboratories, Copenhagen, Denmark). The final cell concentration was 30–100 mg of cell lipid/ml in a final incubation volume of 1–3 ml. All incubations were carried out at least in triplicate, and all analyses were corrected for the appropriate blank values obtained from samples of cells incubated for 1 min in an ice–water mixture. The data are presented below as the results ± SD obtained in single exemplary experiments; each experiment has been carried out, however, at least three times.

At the end of the experimental incubation, a 0.5-ml, or larger, sample of cell-free medium was aspirated from each tube and chilled in an ice–water mixture. Glycerol was assayed directly in 0.1 ml of the cell-free medium, without deproteinization, by the enzymatic method described by Wieland (22). The results have been expressed relative to the weight of dried total cell lipid extracts obtained from samples of cells taken specifically for this purpose during the distribution of cells to the incubation tubes. Fatty acids were extracted from 0.2 ml of the cell-free medium by the method described by Dole (23); reagent volumes were adjusted to yield a final 2-ml volume of organic phase.

Fatty acids were assayed by the following modification of the radioactive method described by Ho and Meng (18) and Ho (19). A portion of the organic phase of each fatty acid extract was dried at room temperature in a small glass tube under a stream of nitrogen. The dried extracts were then redissolved in a chloroform–heptane solution (8:7, v/v), and 200 μl was assayed directly for fatty acids with 10 μl of the radioactive reagent. Palmitic acid standards were assayed in parallel during each analysis. Fatty acids released by the cells into the incubation medium have also been expressed relative to cell lipid weight.

To the cells left in each incubation tube was added directly 5 ml of ice-cold Krebs-Ringer–bicarbonate buffer containing a concentration of glutaraldehyde (Fisher Scientific Co., Fairlawn, N.J.) such that the final concentration would be 1 g/100 ml. The fixed cell suspension was then chilled in an ice–water mixture for at least 5 min, poured into a small plastic tube, and centrifuged for 10 sec in a desk-top centrifuge. The floating cells were transferred to a 25-mm, 7-μm Sartorius filter (Sartorius, Göttingen, West Germany) with an Eppendorf automatic pipette (Eppendorf, Hamburg, Germany), and the fatty acids were extracted and assayed by the methods described above for cell-free medium. Assay by titration yielded results comparable with those obtained with the radioactive method, although roughly 5–10 times as much cell extract was required. CAFA levels have been expressed relative to the dried cell lipid weight of each sample.

Two important potential sources of error in the quantitation of CAFA are (*1*) contamination of CAFA by fatty acids released from the cell into the incubation medium and (*2*) rapid alteration of CAFA levels by continued production, release, or metabolism during manipulation of cells between incubation and extraction. The combination of a simultaneous sixfold dilution of incubation medium and rapid fixation of cells with cold Krebs-Ringer–bicarbonate buffer containing glutaraldehyde, followed by complete separation of cells and medium through filtration, appears, however, to have minimized both of these errors. When adipose cells are collected and extracted immediately after dilution, fixation with glutaraldehyde is not required; concentrations of glutaraldehyde of 1 g/100 ml or greater, however, render the cells considerably more stable during the collection procedure than do lower concentrations or no glutaraldehyde at all, and permit a delay of up to 90 min between dilution and collection. Neither fixation followed by dilution nor washing the fixed cells with cold albumin-free Krebs-Ringer–bicarbonate buffer yields results significantly different from those obtained simply by simultaneous fixation and dilution. If the albumin of the incubation medium is preloaded with oleic acid up to a molar ratio of 5:1, only at the highest molar ratio is there a noticeable, but small, elevation of CAFA levels in cells exposed to medium before fixation and dilution compared with cells added to previously fixed and diluted me-

TABLE 1. Comparison of two methods for measurement of epinephrine-elevated cell-associated fatty acid levels [a]

Method [b]	Incubation Time (min)						
	0	5	10	20	40	60	90
	μmoles/g lipid [c]						
Glutaraldehyde	1.0 ±1.0	2.2 ±0.6	4.2 ±0.6	6.3 ±0.6	12.1 ±3.2	17.0 ±4.4	29.8 ±6.4
Medium marker	1.5 ±1.0	2.1 ±0.4	3.7 ±0.4	5.7 ±1.2	10.8 ±1.8	16.8 ±2.8	28.9 ±6.0

[a] Isolated mouse adipose cells (approximately 120 mg of cell lipid) were incubated in 2.0 ml of medium containing 3.5 g of human serum albumin/100 ml, 1 μg of epinephrine/ml, and 1 mM sodium pyruvate.
[b] Glutaraldehyde and medium marker methods for the measurement of cell-associated fatty acids are described in Methods.
[c] Results are means ± SD of quadruplicate samples.

dium. Serum albumin has been assumed to have a molecular weight of 68,000 (24). When the number of cells incubated in a constant volume of medium is varied, no significant difference in CAFA levels is observed.

A second method for quantitating CAFA has recently been reported (6) and now modified for use with the isolated mouse adipose cell preparation (25). In this method, cells and some contaminating medium are extracted together, and the results are corrected on the basis of a radioactive medium marker. Table 1 illustrates that comparable results are obtained with the two independent techniques.

RESULTS

Table 2 indicates that incubation of isolated mouse adipose cells with a variety of lipolytic agents produces an elevation of CAFA levels and a concomitant stimulation of glycerol and fatty acid release. Fig. 1, in turn, illustrates that increasing CAFA levels are associated with increasing rates of lipolysis when the concentration of epinephrine is varied. After 30 min of incubation, however, the elevation of CAFA levels is detectable only at higher concentrations of epinephrine than is the stimulation of glycerol and fatty acid release, a reflection, presumably, of the cumulative effect of incubation time on released glycerol and fatty acids and the absence of such an effect on CAFA levels.

TABLE 2. Effects of lipolytic agents on released glycerol and fatty acids and cell-associated fatty acid levels [a]

Addition	Released Glycerol	Released Fatty Acids	Cell-associated Fatty Acids
	μmoles/g lipid [b]		
None	0.8 ± 0.0	1.3 ± 0.0	1.1 ± 0.1
Epinephrine (1 μg/ml)	22.7 ± 1.5	40.6 ± 1.0	5.1 ± 0.2
Caffeine (5 mM)	20.1 ± 1.1	38.7 ± 0.7	6.7 ± 0.2
Dibutyryl cyclic AMP (1 mM)	20.8 ± 1.4	39.2 ± 2.2	5.9 ± 0.2
ACTH (1 μg/ml)	24.3 ± 0.9	38.9 ± 2.5	5.2 ± 0.2

[a] Isolated mouse adipose cells (approximately 50 mg of cell lipid) were incubated 20 min in 1.5 ml of medium containing 5 g of human serum albumin/100 ml but no substrate.
[b] Results are means ± SD of quadruplicate samples.

Furthermore, CAFA levels continue to increase with epinephrine concentrations higher than those necessary to maximally stimulate glycerol and fatty acid release and substantially raise the extracellular concentrations of these two products of lipolysis. In contrast, after only 10 min of incubation (Table 3), released fatty acids and glycerol accumulate in the incubation medium only to a small extent, even at very high epinephrine concentrations; under these conditions, parallel effects with increasing hormone concentrations and a near maximal effect with as little as 0.16 μg of epinephrine/ml are observed on all three parameters. The relationship between epinephrine-elevated CAFA levels and the accumulation of extracellular fatty acids is examined in detail below.

Neither insulin alone nor insulin plus glucose has a significant effect on basal CAFA levels or glycerol and fatty acid release. In the presence of 0.25 μg of epinephrine/ml, on the other hand, insulin alone is markedly antilipolytic on all three parameters (Fig. 2). The magnitude of the reduction by insulin of epinephrine-elevated CAFA levels and the inhibition by insulin of epinephrine-stimulated fatty acid and glycerol release reproducibly decrease, however, with increasing glucose concentration, at least in part due to the small effects on stimulated lipolysis of glucose alone. Insulin in the absence of glucose does not alter the molar ratio of released fatty acids to released glycerol observed in the presence of epinephrine; insulin plus increasing concentrations of glucose reduces this ratio.

TABLE 3. Lipolytic response to epinephrine in short (10 min) incubation time [a]

Epinephrine	Released Glycerol	Released Fatty Acids	Cell-associated Fatty Acids
μg/ml		*μmoles/g lipid* [b]	
0.00	0.8 ± 0.3	1.3 ± 0.8	1.16 ± 0.05
0.16	5.4 ± 0.2	13.3 ± 1.9	2.26 ± 0.20
1.25	6.7 ± 0.0	14.2 ± 0.9	2.60 ± 0.20
10.00	6.3 ± 0.2	14.0 ± 1.5	2.34 ± 0.19

[a] Isolated mouse adipose cells (approximately 80 mg of cell lipid) were incubated 10 min in 2.0 ml of medium containing 4 g of human serum albumin/100 ml but no substrate.
[b] Results are means ± SD of quadruplicate samples.

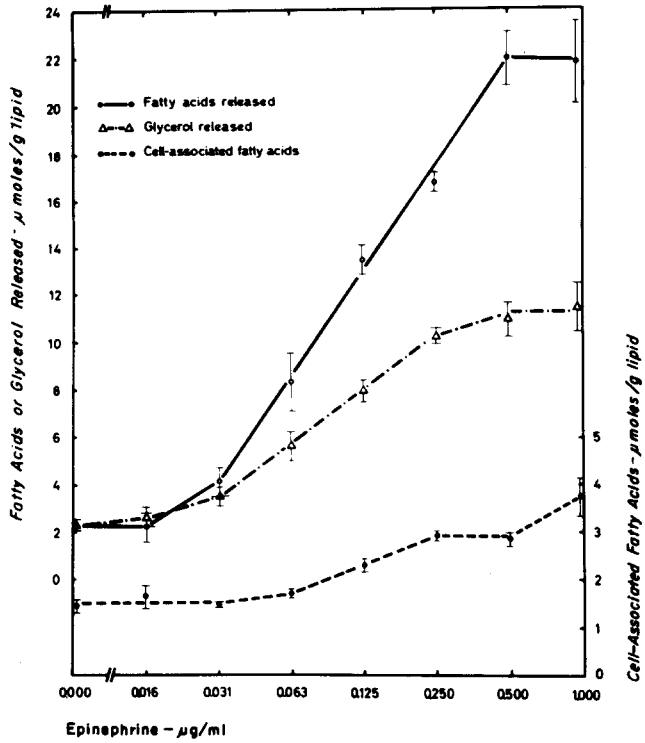

Fig. 1. Lipolytic response to epinephrine. Isolated mouse adipose cells (approximately 80 mg of cell lipid) were incubated 30 min in 2.0 ml of medium containing 4 g of human serum albumin/100 ml but no substrate. Results are means ± SD of triplicate samples.

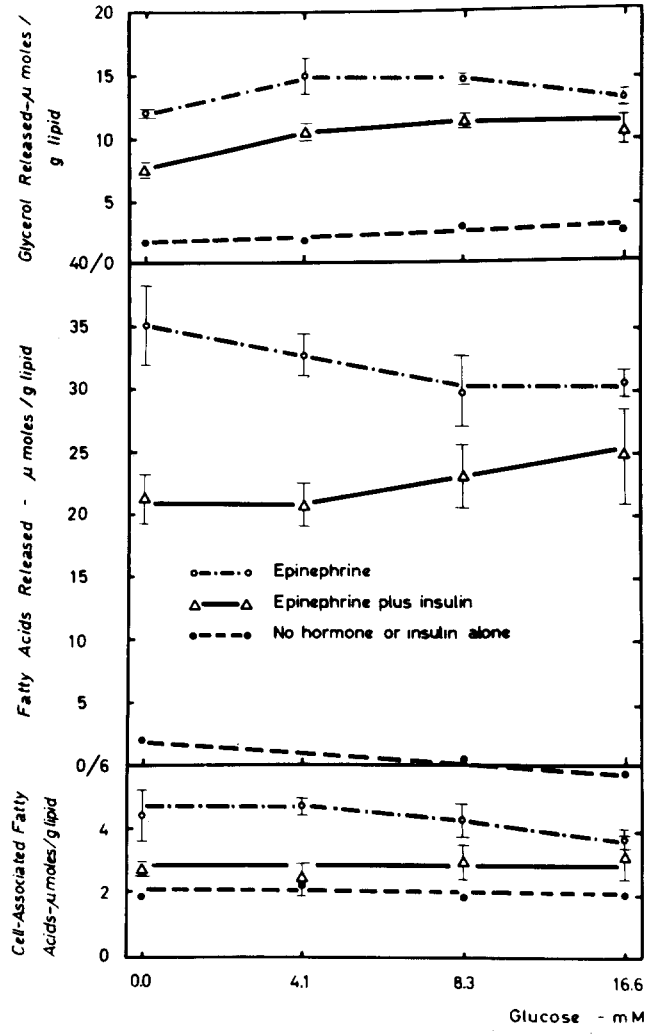

Fig. 2. Effects of glucose on lipolytic response to, and antilipolytic response to insulin against, epinephrine. Isolated mouse adipose cells (approximately 80 mg of cell lipid) were incubated 30 min in 1.5 ml of medium containing 4 g of human serum albumin/100 ml, 0 or 0.25 μg of epinephrine/ml, and 0 or 100 μU of insulin/ml. Results are means ± SD of triplicate samples.

Neither the elevation of CAFA levels nor the stimulation of glycerol and fatty acid release by a maximal concentration of epinephrine, on the other hand, is affected in any reproducible fashion by the presence of 0–22.2 mM glucose or 5.0 mM pyruvate either with 15 or 60 min of incubation (data not shown). A modest decrease in the molar ratio of released fatty acids to released glycerol is sufficient, however, to account for the reduction of this ratio observed in the presence of glucose plus insulin.

The elevation of CAFA levels by epinephrine in isolated mouse adipose cells is closely related to the extracellular molar ratio of fatty acids to serum albumin and contrasts with the relationship between epinephrine-stimulated glycerol and fatty acid release and this same ratio. These effects are reflected in the time-dependent evolution of the lipolytic response in three phases. The first phase commences with the initiation of the lipolytic response (Fig. 3) and evolves over a range of fatty acid to albumin ratios of nearly 0 to approximately 3 (Fig. 4, 0–10 min). During this phase, CAFA levels rise slowly after an initial small increase reproducibly detectable with a maximal concentration of epinephrine as early in time as measurements can be made. Glycerol release is almost immediate in onset, roughly linear with time, and occurs at a rate determined only by the concentration of hormone. Fatty acid release is also linear with time, but its onset always lags behind both the initial elevation of CAFA and stimulation of glycerol release.

The second phase of the lipolytic response evolves over a range of fatty acid to albumin ratios of approximately 3, to 5–6 (Fig. 4, 10–30 min). During the second phase, CAFA levels rise dramatically with time, the rate of glycerol release slows, returning toward that observed in the absence of hormone, and fatty acid release slows and nearly ceases. The third phase is that occurring after the almost complete cessation of fatty acid release at fatty acid to albumin ratios above 5–6; CAFA levels continue to rise only as glycerol continues to be released (Fig. 4, 30–60 min). This final phase can be observed only if cells are incubated in the presence of hormone for a period of time sufficiently long to permit a maximum accumulation of released fatty acids in the incubation medium.

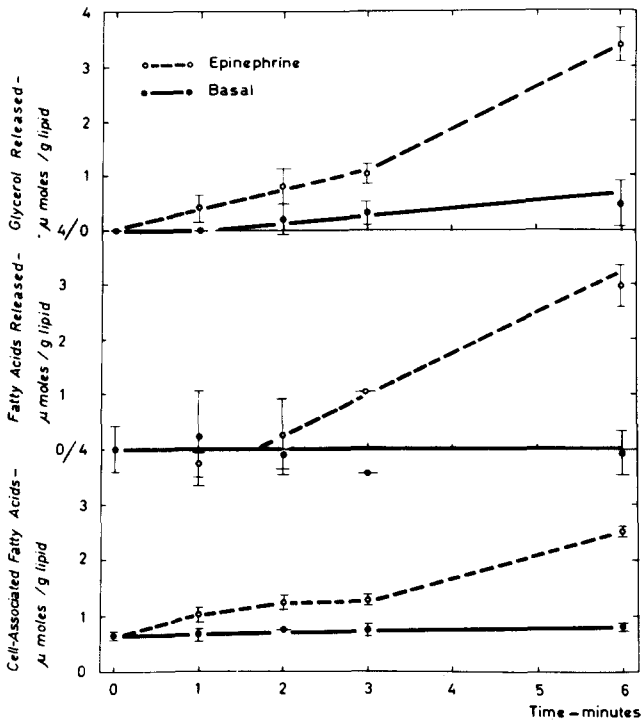

Fig. 3. Short time course of lipolytic response to epinephrine. Isolated mouse adipose cells (approximately 100 mg of cell lipid) were incubated in 1.5 ml of medium containing 4 g of human serum albumin/100 ml and 0 or 1 μg of epinephrine/ml, but no substrate. Results are means ± SD of quadruplicate samples.

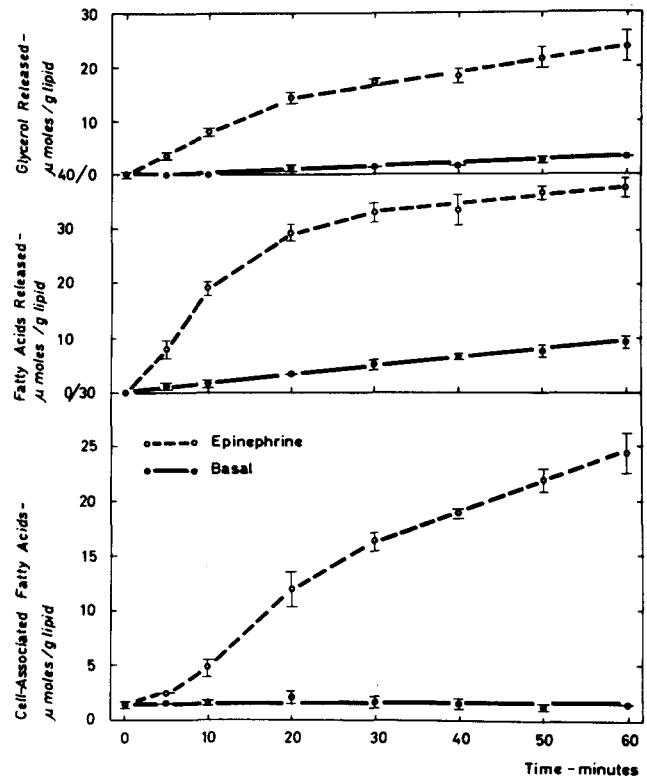

Fig. 4. Time course of lipolytic response to epinephrine. Isolated mouse adipose cells (approximately 110 mg of cell lipid) were incubated in 1.5 ml of medium containing 3.5 g of human serum albumin/100 ml and 0 or 1 μg of epinephrine/ml, but no substrate. Results are means ± SD of quadruplicate samples. The molar ratios of medium fatty acids to albumin present in the incubation medium in the presence of epinephrine are 3.6 ± 0.1, 5.0 ± 0.2, 5.4 ± 0.2, and 6.1 ± 0.2 at 10, 20, 30, and 60 min, respectively.

The length of incubation time over which this triphasic response to epinephrine can be observed experimentally is readily manipulated by controlling the rate of fatty acid release relative to the extracellular albumin concentration. An increase in the albumin concentration, a decrease in the cell concentration, a decrease in the epinephrine concentration, or simultaneous exposure of cells to antilipolytic agents such as insulin lengthens the time required for the evolution of qualitatively and quantitatively identical epinephrine responses. During each phase and for each set of incubation conditions, as long as glucose is not present, the sum of CAFA plus released fatty acids expressed relative to released glycerol approaches the theoretical molar ratio of 3:1.

TABLE 4. Effect of human serum albumin on lipolytic response to epinephrine[a]

Incubation Time[b]	Human Serum Albumin	Released Glycerol	Released Fatty Acids	Cell-associated Fatty Acids[c]	Medium Fatty Acids/Albumin
min	*g/100 ml*		*μmoles/g lipid*[d]		*molar ratio*[d]
15	2	5.0 ± 0.0	10.0 ± 0.4	4.08 ± 0.18	2.93 ± 0.09
	3	6.2 ± 0.0	12.8 ± 0.7	2.60 ± 0.19	2.65 ± 0.12
	4	5.9 ± 0.2	12.8 ± 0.8	2.08 ± 0.10	2.19 ± 0.09
	5	6.4 ± 0.2	13.5 ± 0.5	1.67 ± 0.05	1.96 ± 0.05
60	2	20.5 ± 0.5	30.5 ± 2.0	15.80 ± 1.80	4.42 ± 0.27
	3	30.0 ± 0.4	40.9 ± 5.1	16.60 ± 1.20	3.96 ± 0.45
	4	37.0 ± 1.3	67.3 ± 7.3	14.30 ± 1.30	4.67 ± 0.46
	5	42.6 ± 1.2	75.5 ± 6.9	12.40 ± 1.00	4.31 ± 0.35

[a] Isolated mouse adipose cells (approximately 80 or 140 mg of cell lipid) were incubated in 2.0 ml of medium containing 1 μg of epinephrine/ml but no substrate.

[b] Each incubation time represents a separate experiment.

[c] Cell-associated fatty acid levels in the absence of epinephrine range from 0.69 ± 0.09 to 0.98 ± 0.07 μmoles/g lipid.

[d] Results are means ± SD of quadruplicate samples.

The effects of serum albumin concentration on the lipolytic response of isolated cells to epinephrine as a function of time demonstrates directly the specific relationship between elevated CAFA levels and the extracellular molar ratio of fatty acids to albumin (Table 4). Epinephrine-elevated CAFA levels closely parallel the fatty acid to albumin ratio and are unrelated to the fatty acid concentration either with 15 min of incubation, where glycerol and fatty acid release are independent of albumin concentrations above 2 g/100 ml, or with 60 min of incubation, where these two parameters vary directly with the albumin concentration.

The same quantitative relationship among the three lipolytic parameters can be observed when cells are exposed

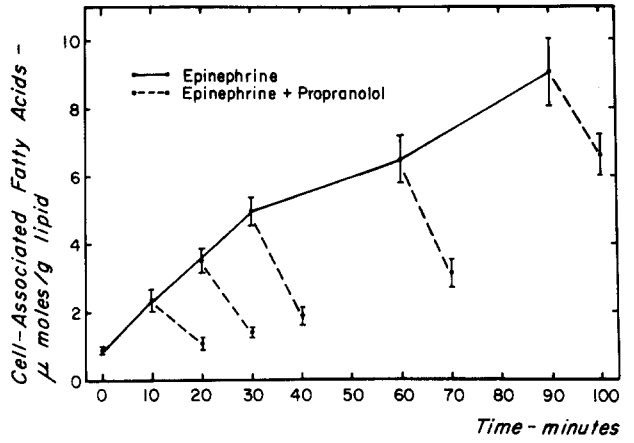

Fig. 5. Reduction of epinephrine-elevated cell-associated fatty acid levels by propranolol. Isolated mouse adipose cells (approximately 35 mg of cell lipid) were incubated in 1.5 ml of medium containing 7 g of human serum albumin/100 ml and 1 μg of epinephrine/ml, but no substrate. The effect of propranolol was measured 10 min after the addition of 50 μl of a solution of 1000 μg of propranolol/ml in water. Results are means ± SD of quadruplicate samples. The molar ratios of medium fatty acids to albumin present in the incubation medium prior to the addition of propranolol are 0.4 ± 0.1, 1.0 ± 0.1, 1.6 ± 0.1, 3.0 ± 0.1, and 4.4 ± 0.1 at 10, 20, 30, 60, and 90 min, respectively.

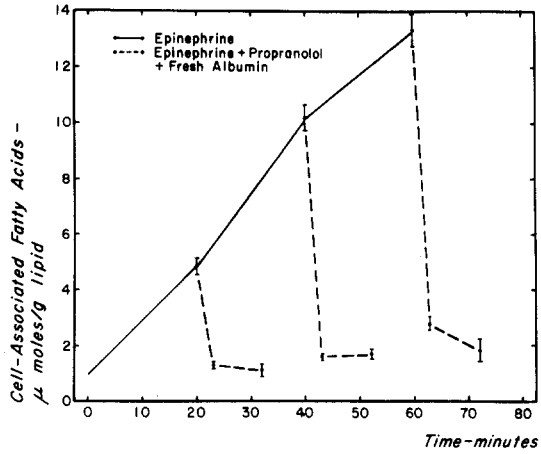

Fig. 6. Reduction of epinephrine-elevated cell-associated fatty acid levels by propranolol and fresh albumin. Isolated mouse adipose cells (approximately 50 mg of cell lipid) were incubated in 1.0 ml of medium containing 2 g of human serum albumin/100 ml and 1 μg of epinephrine/ml, but no substrate. The effect of propranolol and fresh albumin was measured 3 or 12 min after the addition of 1.0 ml of a solution of 10 μg of propranolol/ml in fresh medium containing 8 g of human serum albumin/100 ml but no substrate. Results are means ± SD of quadruplicate samples. The molar ratios of medium fatty acids to albumin present in the incubation medium prior to the addition of propranolol are 3.6 ± 0.2, 5.5 ± 0.1, and 5.6 ± 0.2 at 20, 40, and 60 min, respectively.

for a fixed incubation time and in the presence of a constant epinephrine concentration to a medium in which a fixed amount of albumin is variably preloaded with oleic acid (Table 5). With increasing fatty acid to albumin ratios, net CAFA levels rise, accounting for an increased proportion of the total fatty acids produced, released fatty acids plus CAFA, per unit released glycerol. At the same time, epinephrine-stimulated glycerol release decreases, and net fatty acid release decreases, then ceases completely.

An attempt has been made to determine more specifically the functional significance of CAFA levels and of the relationship between CAFA and the extracellular fatty acid to albumin ratio in the fatty acid releasing process. Fig. 5 illustrates that the addition of an epinephrine antagonist, propranolol (Inderal, 0.1%, Vifor Laboratories), at various incubation times (26) results in a precipitous fall in epinephrine-elevated CAFA levels except at 90 min, when the extracellular fatty acid to albumin ratio is well above 3:1. The addition of propranolol plus fresh albumin, on the other hand, elicits a rapid efflux of CAFA independent of the fatty acid to albumin ratio to which cells have previously been exposed (Fig. 6).

Fig. 7, in turn, indicates in detail not only the kinetics but also the temperature dependency of CAFA efflux. Within 3 min after the addition of propranolol plus fresh albumin at 37°C, a 50% reduction in CAFA and a corresponding increase in released fatty acids (data not shown) are observed. If the addition of propranolol plus fresh albumin is carried out at 10°C, CAFA efflux is almost entirely prevented and recovery of fatty acids in the incubation medium is decreased. Temperatures between 10 and 37°C produce graded rates of CAFA efflux. Addition of fresh albumin in amounts insufficient to lower the fatty acid to albumin ratio during efflux below 3:1 prevents CAFA efflux at all temperatures.

Fig. 8 summarizes the specific quantitative relationship between the net elevation of CAFA by epinephrine and the molar ratio of medium fatty acids to albumin observed over several experiments. Two relatively linear functions correspond to (*1*) the initial and second phases and (*2*) the final phase, respectively, of the time-dependent lipolytic

TABLE 5. Effects of exogenous oleic acid on lipolytic response to epinephrine[a]

Medium Fatty Acids/Albumin		Epinephrine Concentration	Released Glycerol	Released Fatty Acids	Cell-associated Fatty Acids
Initial[b]	Final				
molar ratio[c]		*μg/ml*		*μmoles/g lipid*[c]	
0.51 ± 0.03	0.70 ± 0.07	0.0	0.5 ± 0.0	3.1 ± 1.0	1.32 ± 0.15
	1.79 ± 0.05	1.0	8.2 ± 0.3	20.0 ± 0.8	3.59 ± 0.42
2.30 ± 0.04	2.41 ± 0.14	0.0	0.5 ± 0.3	1.8 ± 2.2	1.54 ± 0.04
	3.21 ± 0.06	1.0	6.6 ± 0.7	14.6 ± 1.0	4.74 ± 0.53
3.44 ± 0.09	3.56 ± 0.07	0.0	0.5 ± 0.3	1.9 ± 1.1	2.05 ± 0.17
	3.95 ± 0.05	1.0	4.6 ± 0.0	8.0 ± 0.8	5.77 ± 0.46
4.93 ± 0.10	5.02 ± 0.12	0.0	0.0 ± 0.0	1.4 ± 1.9	2.52 ± 0.33
	5.11 ± 0.09	1.0	3.0 ± 0.4	2.8 ± 1.5	7.15 ± 0.39

[a] Isolated mouse adipose cells (approximately 80 mg of cell lipid) were incubated 15 min in 2.0 ml of medium containing 4 g of human serum albumin/100 ml but no substrate. Oleic acid was added as its sodium salt to all incubations except that series exhibiting the lowest initial ratio of medium fatty acids to albumin.

[b] Initial ratios of medium fatty acids to albumin are those measured after 1 min of incubation at 4°C.

[c] Results are means ± SD of quadruplicate samples.

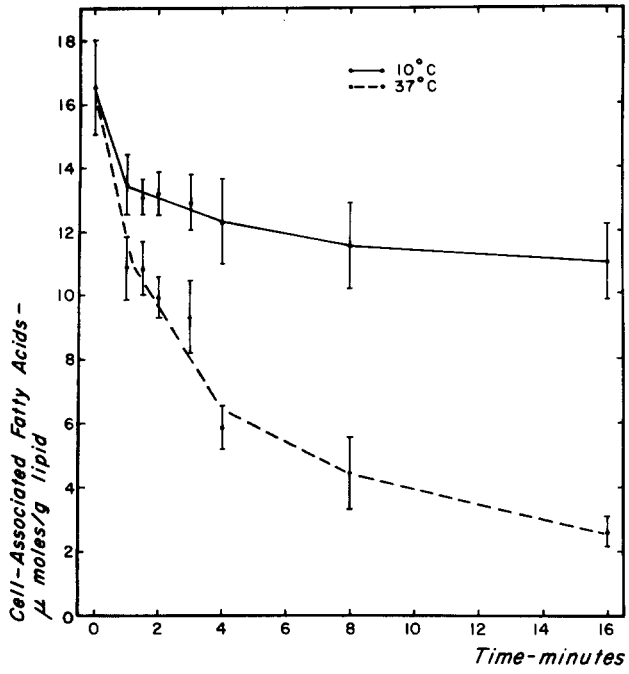

Fig. 7. Time course of, and effect of temperature on, efflux of epinephrine-elevated cell-associated fatty acids. Isolated mouse adipose cells (approximately 80 mg of cell lipid) were incubated 60 min in 1.0 ml of medium containing 2 g of human serum albumin/100 ml and 1 μg of epinephrine/ml, but no substrate. Efflux of cell-associated fatty acids was measured at 10 and 37°C after the addition of 1.0 ml of a solution of 10 μg of propranolol/ml in fresh medium containing 8 g of human serum albumin/100 ml but no substrate. Results are means ± SD of quadruplicate samples. After 16 min of efflux at 37°C, the molar ratio of medium fatty acids to albumin present in the incubation medium is 2.6 ± 0.2; 8.0 μmoles more cell-associated fatty acids/g of lipid are recovered in the incubation medium at 37°C than at 10°C.

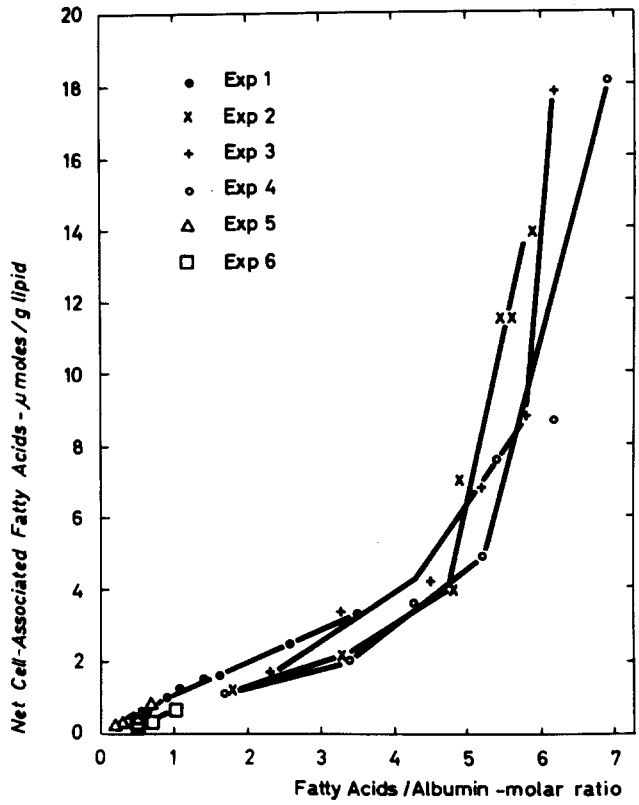

Fig. 8. Relationship between level of cell-associated fatty acids and ratio of medium fatty acids to albumin during lipolytic response to epinephrine. Isolated mouse adipose cells (approximately 40 mg of cell lipid/ml) were incubated 1–120 min in 2–3 ml of medium containing 4 g of human serum albumin/100 ml and 1 μg of epinephrine/ml, but no substrate. Results are means of quadruplicate samples; each set of points represents one of six separate experiments. Cell-associated fatty acid levels are expressed as the net increase in the presence of epinephrine relative to the basal level observed at the same incubation time.

response. During the final phase, commencing at a fatty acid to albumin ratio of approximately 5, large increments in CAFA level are associated with small increments in the ratio. A potential functional relationship between CAFA levels and fatty acid release is, in turn, suggested when net released fatty acids are expressed relative to net CAFA level and plotted against the fatty acid to albumin ratio (Fig. 9). While CAFA levels per se remain linearly related to fatty acid to albumin ratios up to 5, alterations in the relationship between fatty acid release and CAFA level become apparent at ratios as low as 2 and correspond to the second, or transition, phase of the lipolytic response. Over the range of fatty acid to albumin ratios of 2–5, declining rates of fatty acid release are associated with increasing CAFA levels. Above ratios of 5, fatty acids accumulate only very slowly in the incubation medium while CAFA account for nearly all those fatty acids produced by the residual rate of lipolysis.

DISCUSSION

The present studies (*1*) describe a rapid and flexible method for the detection and quantitation of cell-associated, probably intracellular, nonesterified fatty acids in a small sample of isolated adipose cells, (*2*) demonstrate that an elevation of CAFA levels is an early and intimate part of the response to a variety of lipolytic agents, and (*3*) support the predictions of many investigators that relatively high CAFA levels would be associated with the alteration of adipose cell function by lipolytic agents (1, 2, 4–17). In addition, this series of experiments elucidates a quantitative relationship between CAFA levels and the extracellular molar ratio of fatty acids to albumin that offers potential insight into the mechanism of fatty acid release and the control of lipolysis relative to the extracellular environment. Thus, the observations made by Rodbell (5) and by Angel, Desai, and Halperin (6, 7) on CAFA levels in isolated rat adipose cells are not only confirmed but are quantitated in detail and extended over the wide range of experimental conditions under which the lipolytic response of isolated adipose cells has been studied by others.

The presence of nonesterified fatty acids in the adipose cell isolated from either rats (6) or mice has now been established by two independent methods (Table 1). Each

method minimizes the potential error in such measurements due to contamination from extracellular fatty acids or continued metabolism between experimental incubation and analysis. The method described in this report, however, consisting of a rapid dilution and simultaneous fixation of adipose cells with cold glutaraldehyde followed by separation of cells and medium through filtration, maximizes the capability of arresting the cell's activity quickly at a desired time while permitting flexibility in the time at which analysis of the fixed cells is carried out. Nevertheless, neither method defines the location of these fatty acids within the cell nor distinguishes fatty acids that are directly of lipolytic origin from those that may have been taken up by the cell subsequent to their release. Cell-associated fatty acids, or CAFA, would appear, therefore, to be the most appropriate designation for the nonesterified fatty acids measured by either method. CAFA may very well be, in part, physiologically adsorbed to the cell surface, bound to membranous structures within the cell, including the inner surface of the cell membrane, or compartmentalized within some membrane-bound subcellular component of the cell, perhaps the vesicular fatty acid transport system postulated in an earlier series of reports (8, 9, 21). While lack of information with respect to the chemical state of CAFA renders a precise definition of their chemical role in adipose cell function virtually impossible, the characteristics of CAFA metabolism and of the specific relationship between CAFA levels and other metabolic parameters of the cell, described below, strongly imply their physiological significance.

A rise in cell-associated fatty acid levels appears to be as much a part of the lipolytic response of the isolated adipose cell as are glycerol and fatty acid release. Lipolytic agents as diverse in their mechanisms of action as epinephrine and ACTH, which operate at the adenyl cyclase level (27), caffeine, which operates by inhibiting cyclic AMP phosphodiesterase (28), and dibutyryl cyclic AMP, which may act in the same fashion as does cyclic AMP itself (29), produce elevations in CAFA levels that parallel their effects on glycerol and fatty acid release (Table 2). In addition, the graded lipolytic response to increasing concentrations of epinephrine[3] is roughly paralleled by a graded elevation of CAFA levels (Fig. 1 and Table 3). Finally, the inhibition by insulin of epinephrine-stimulated fatty acid and glycerol release, while varying with the concentration of glucose in the incubation medium, is accompanied by corresponding reductions in epinephrine-elevated CAFA levels (Fig. 2).

A role for cell-associated fatty acids in the lipolytic response of the isolated adipose cell as intermediates between the lipolytic reaction, producing fatty acids inside the cell, and the available acceptor, usually serum albumin, binding these fatty acids in the extracellular medium,

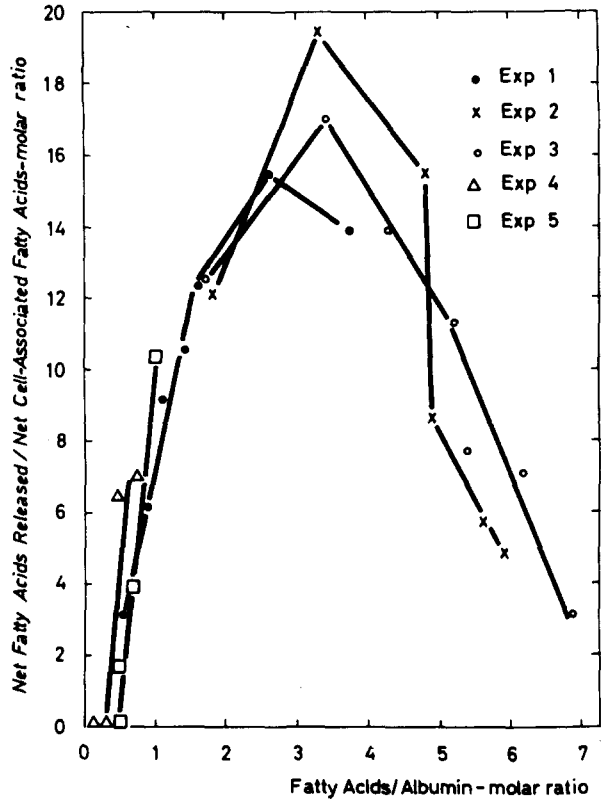

Fig. 9. Relationship between ratio of released fatty acids to level of cell-associated fatty acids and ratio of medium fatty acids to albumin during lipolytic response to epinephrine. Results are derived from experiments 1–4 and 6 described in Fig. 8. Ratios of released fatty acids to level of cell-associated fatty acids are expressed as the molar ratio of net increases in the presence of epinephrine relative to the basal quantity or level, respectively, observed at the same incubation time.

is suggested by short time-course studies. During the initiation of the lipolytic response to a maximal concentration of epinephrine, a very small, but highly reproducible, elevation of CAFA levels accompanies the stimulation of glycerol release and precedes by at least 1 min any demonstrable effect of the hormone on fatty acid release (Fig. 3). The small magnitude of the initial CAFA response to a large hormonal stimulus suggests that the lack of a detectable elevation of CAFA levels at low epinephrine concentrations (Fig. 1) reflects what must be very minute responses to lesser lipolytic stimuli. The time-dependent accumulation of released glycerol and fatty acids in the incubation medium, on the other hand, facilitates analysis of these two parameters even with the smallest lipolytic response.

The elevation of cell-associated fatty acid levels by epinephrine prior to the appearance of released fatty acids in the extracellular medium is strongly suggestive of a precursor–product relationship. CAFA efflux studies substantiate this suggestion. The maintenance of elevated CAFA levels by epinephrine, at least in the presence of low extracellular molar ratios of fatty acids to albumin, appears to strictly depend upon a continuous stimulation of

[3] Or dibutyryl cyclic AMP.

lipolysis by the hormone (Fig. 5); in spite of a progressive increase in CAFA levels as the fatty acid to albumin ratio increases, the cessation of stimulated lipolysis in response to propranolol is accompanied by an immediate and rapid efflux of CAFA. In the presence of higher fatty acid to albumin ratios, the rate of CAFA efflux decreases (Fig. 5); rapid efflux is restored, however, upon exposure of propranolol-treated cells to fresh albumin (Fig. 6).The exponential nature of CAFA efflux and the quantitative recovery of fatty acids in the incubation medium as CAFA levels decrease provide direct evidence that CAFA are an intermediate in the fatty acid releasing mechanism (Fig. 7). The temperature dependence of CAFA efflux supports the suggestion by other investigators that fatty acid release may be an energy-requiring process (30–32).

Superimposed on this precursor–product relationship between cell-associated fatty acids and released fatty acids, however, is a second relationship between CAFA levels and the extracellular molar ratio of fatty acids to serum albumin; this ratio perhaps best reflects the chemical state of extracellular fatty acids as they affect adipose cell metabolic activity (Figs. 4, 8, and 9 and Tables 4 and 5). As intermediates in the fatty acid releasing process, CAFA may represent the intracellular component of a fatty acid gradient between the cell and the fatty acid to albumin ratio. The parallelism among epinephrine-elevated CAFA levels, the fatty acid to albumin ratio, and previously demonstrated epinephrine-stimulated pinocytic activity in isolated rat adipose cells support the concept of compartmentalized fatty acid transport (8, 9, 21).

Quantitatively (Figs. 4 and 8 and Tables 4 and 5), at extracellular fatty acid to albumin ratios below 2, the net elevation of cell-associated fatty acid levels by epinephrine is roughly proportional to the ratio, and the rate of fatty acid release is constant and determined only by the rate of lipolysis. For fatty acid to albumin ratios increasing from 2 to approximately 5, increasing CAFA levels are associated with decreasing rates not only of fatty acid release but of lipolysis. At fatty acid to albumin ratios above 5–6, CAFA accumulate in proportion to the residual rate of lipolysis, fatty acid release having ceased completely. Thus, slowly increasing CAFA levels, rapid fatty acid release, and a constant rate of lipolysis accompany the filling of the two to three high-affinity fatty acid binding sites of extracellular albumin. Further increasing CAFA levels and decreasing rates of fatty acid release and lipolysis parallel the filling of roughly three to four additional low-affinity binding sites. The cessation of fatty acid release, the return of lipolysis to its basal rate, and the continued accumulation of additional fatty acids produced by lipolysis as CAFA mark the termination of the lipolytic response after saturation of the extracellular fatty acid acceptor.

This specific quantitative relationship among epinephrine-elevated cell-associated fatty acid levels, the rate of lipolysis, and the extracellular molar ratio of fatty acids to albumin appear to account for the time-dependent elevation of CAFA levels by graded concentrations of epinephrine (Fig. 1 and Table 3). Because the initial CAFA response even to a maximal lipolytic stimulus is very small, the higher CAFA levels observed in the presence of submaximal epinephrine concentrations with longer incubation times must reflect primarily the extracellular accumulation of released fatty acids. The magnitude of the elevation of CAFA levels depends very specifically upon the fatty acid to albumin ratio achieved with a given concentration of hormone and incubation time.

The rate of cell-associated fatty acid efflux in the absence of a lipolytic stimulus is either very rapid, in the presence of extracellular fatty acid to albumin ratios below 3, or very slow, in the presence of ratios above 3 (Figs. 5, 6, and 7). This observation provides an explanation for the changing relationship between epinephrine-elevated CAFA levels and the fatty acid to albumin ratio near ratios of 3 (Fig. 8). In contrast, in the presence of epinephrine, CAFA levels rise as a continuous, linear function of the fatty acid to albumin ratio even over the range below 3 (Fig. 8), where CAFA efflux is independent of the ratio. This latter observation suggests that fatty acid transport across the cell membrane may be the rate-limiting step in fatty acid release during stimulated lipolysis at all fatty acid to albumin ratios. How CAFA move through the cytoplasm from the intracellular site of lipolysis and ultimately traverse the cell membrane remains to be elucidated.

The existence of such a quantitative relationship among cell-associated fatty acid levels, the availability of extracellular fatty acid acceptor sites, and the rate of lipolysis provide evidence supporting the hypothesis that CAFA, through an as yet unidentified mechanism, may play a significant role in the feedback regulation of the lipolytic response of the adipose cell as fatty acids accumulate in the extracellular space. In addition, the reproducibility of this relationship under a wide variety of experimental conditions indirectly strengthens the possibility that CAFA, or some derivative thereof, may play a role in the mediation of several of the effects of lipolytic agents on adipose cell functions other than lipolysis, such as the depression of cell ATP levels, suggested by many other in vitro investigations (1, 2, 4–17).

High and quantitatively specific CAFA levels are reproducibly associated with the inhibition of lipolysis in response to fatty acid to albumin ratios above 3 (Fig. 4 and Tables 4 and 5). Furthermore, this association can be mathematically demonstrated when the relationship between fatty acid release and the CAFA level, expressed as a ratio, is examined relative to the fatty acid to albumin ratio. Even if the ratio of released fatty acids to CAFA level is not the appropriate description of a fatty acid gradient, postulated earlier, as it relates to the fatty acid releasing process, Fig. 9 clearly illustrates that an initially linear relationship is first perturbed then reversed as the

fatty acid to albumin ratio rises above 2. Below fatty acid to albumin ratios of 2, no inhibition of lipolysis is observed; if sufficient albumin were present such that ratios above 2 could not be attained, lipolysis would continue indefinitely with slightly elevated CAFA levels. Above ratios of 2, CAFA accumulate more rapidly relative to the rate of fatty acid release; such higher CAFA levels are associated, however, with an inhibition of lipolysis. The highest CAFA levels are associated with a return to the unstimulated rate of lipolysis and cessation of fatty acid release.

While it is anticipated that cell-associated fatty acids will prove to play a physiological role in vivo in the fatty acid releasing mechanism of the adipose cell, the importance of their levels as a regulator of lipolysis and other adipose cell functions cannot be determined without more knowledge of the specific extracellular environment of the adipose cell, the mechanisms by which fatty acids are transported across the capillary endothelium, and the relationship between these mechanisms and blood flow through adipose tissue. If the transport of fatty acids out of the extracellular space into the circulation were a rate-limiting step in the mobilization of fatty acids and/or if the albumin concentration in the extracellular fluid were lower than those observed in plasma, then fatty acid to albumin ratios in the cell's immediate environment could be substantially higher than those measured in serum; under these circumstances, the large changes in CAFA level in response to relatively small changes in the fatty acid to albumin ratio would represent a very sensitive mechanism for the regulation of adipose cell function.

The authors wish to thank Mrs. Thérese Cuche, Miss Françoise Roulet, and Mrs. Elizabeth Fischer-Hümmerich for their expert technical assistance.

This investigation was supported by grant no. 4848.3 of the Fonds National Suisse de la Recherche Scientifique, Berne, Switzerland, and by a grant-in-aid of Nestlé-Alimentana, Vevey, Switzerland. Preliminary reports of this work were presented at the Sixth Annual Meeting of the European Association for the Study of Diabetes, September 1970, and at the Annual Meeting of the Federation of American Societies for Experimental Biology, April 1972.

Manuscript received 8 February 1973; accepted 29 June 1973.

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