papers on methodology

Release of small amounts of free fatty acids from human adipocytes as determined by chemiluminescence

Birgitta Näslund,[1] Kerstin Bernström, Arne Lundin, and Peter Arner

Clinical Research Centre and Department of Medicine, Karolinska Institute, Huddinge University Hospital F-61, S-141 86 Huddinge, Sweden

Abstract A semiautomatic luminometric method for determination of small amounts of free fatty acids (FFA) released from human adipocytes in vitro is described. Bovine serum albumin (BSA) is used as acceptor of free fatty acids in the incubation medium of isolated fat cells. The assay involves pretreatment with the detergent sodium dodecyl sulfate (SDS) to liberate the free fatty acids from the bovine serum albumin before activation by acyl-CoA synthetase (ACS) (EC 6.2.1.3). This is followed by oxidation of the resulting thioesters by acyl-CoA oxidase (ACO). The H_2O_2 formed is subsequently measured in a horseradish peroxidase (HRP) (EC 1.11.1.7)-catalyzed luminol reaction. The assay is linear in the interval of 0.01–1 nmol in the cuvette corresponding to 2–200 μM in the sample, and 25 samples are automatically assayed in the luminometer within 75 min. FFA release could easily be studied in a small incubation volume (200 μl) of very diluted (10^4 cells/ml) human adipocyte suspensions. Samples (25 μl) containing 0.25% BSA from incubates of adipose tissue cells did not interfere with the standard curve. The analytical interference from different factors that could be used in studies of lipolysis was investigated. No interference was observed up to the following concentrations: 5 μM epinephrine, 5 μM norepinephrine, 80 μM isoproterenol, 1 mM insulin, 2.5 mM propranolol, 5 mM phentolamine, and 5 μM ascorbate. Results obtained with the present assay were highly correlated (r = 0.997) with those obtained by a 260-times less sensitive spectrophotometric kit method. The present assay can thus be used for serial studies of FFA release in diluted cell suspensions using small amounts of human adipose tissue obtained by needle biopsy. At least 250 separate determinations of FFA release can be made with 1 g of tissue.—**Näslund, B., K. Bernström, A. Lundin, and P. Arner.** Release of small amounts of free fatty acids from human adipocytes as determined by chemiluminescence. *J. Lipid Res.* 1993. **34:** 633–641.

Supplementary key words fat cell luminescence • acyl-CoA synthetase • acyl-Coa oxidase • ascorbate • peroxidase • luminol

The adipose tissue is an important organ for energy storage. The metabolic processes are hormone-regulated and are altered in diseases such as diabetes and obesity. In the fat cell the free fatty acids and glycerol are bound as esters in triacylglycerols and the FFA are released upon hydrolysis of the triacylglycerols by hormone-sensitive lipase. Some FFA are reesterified and the remainder are transported in serum as albumin-bound free fatty acids. FFA release has been investigated intensively in vitro using rat adipocytes. However, human investigations are rare mainly because of methodological problems. The amount of human adipose tissue that is available is usually limited and the rate of lipid mobilization from human fat cells is low compared to that of rat fat cells.

Both chemical and enzymatic methods for determination of free fatty acids in human blood and serum have been developed (1–10, and references in 11). However, several authors have reported an incomplete recovery of FFA from plasma (7) and that bovine serum albumin interferes with the FFA assays (3–6, 9) which results in an underestimation of FFA values in serum or plasma samples. To overcome this, the extraction method has been modified (7), BSA has been included in the standard curve (9), or *p*-toluenesulfonic acid has been used (3).

The methods discussed above for FFA determination are unsuitable for human in vitro studies because the detection limit is too high using small amounts of human adipose cells with usually low lipolytic activity. We have recently published a chemiluminometric method for the determination of low levels of FFA based on hydrogen peroxide determination in a peroxidase luminescence reaction (11). However, the method could not be used for fat cell studies because albumin (which must be added to the incubation buffer as FFA carrier) resulted in nonlinear FFA standard curves. In the present investigation we have

Abbreviations: FFA, free fatty acids; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; ACS, acyl-CoA synthetase; ACO, acyl-CoA oxidase; CoASH, reduced CoA; NEM, N-ethylmaleimide; HRP, horseradish peroxidase; DTPA, diethylenetriaminepentaacetic acid; KRP buffer, Krebs-Ringer phosphate buffer; CV%, coefficient of variation.

[1]To whom correspondence should be addressed.

developed a chemiluminometric assay of FFA that can be used in biological samples containing BSA.

Principles of the assay

In **step 1,** the sample is treated with the detergent (sodium dodecyl sulfate) to liberate FFA from BSA. In **step 2,** endpoint incubation with acyl-CoA synthetase is performed in the presence of pyrophosphatase to avoid a reverse reaction when excess reduced CoA (CoASH) is inactivated in the following step. In **step 3,** N-ethylmaleimide (NEM) is added to inactivate the excess of CoASH (which otherwise would interfere with the luminometric assay). In **step 4,** hydrogen peroxide is formed by endpoint incubation with acyl-CoA oxidase. Subsequently, in **step 5,** the resulting hydrogen peroxide is measured by a horseradish peroxidase-catalyzed luminol reaction in the presence of diethylenetriaminepentaacetic acid (DTPA). Steps 4 and 5 are automatically performed in the luminometer with 25 samples in a single run.

Steps performed outside the luminometer are:

$$\text{Step 1} \quad \text{BSA}-(\text{FFA})_n + n\ \text{SDS} \rightarrow \text{BSA}-(\text{SDS})_n + n\ \text{FFA}$$

$$\text{Step 2a} \quad \text{FFA} + \text{ATP} + \text{CoASH} \xrightarrow{\text{ACS}} \text{acyl-CoA} + \text{AMP} + \text{PP}_i$$

$$\text{Step 2b} \quad \text{PP}_i + \text{H}_2\text{O} \xrightarrow{\text{pyrophosphatase}} 2\text{P}_i$$

$$\text{Step 3} \quad \text{CoASH} + \text{NEM} \rightarrow \text{covalent binding of CoASH}$$

Steps performed in the luminometer are:

$$\text{Step 4} \quad \text{acyl-CoA} + \text{O}_2 \xrightarrow{\text{ACO}} \text{2,3-}\textit{trans-enoyl}\text{-CoA} + \text{H}_2\text{O}_2$$

$$\text{Step 5} \quad 2\ \text{H}_2\text{O}_2 + \text{luminol} \xrightarrow[\text{DTPA}]{\text{HRP}} \text{3-aminophthalic acid} + \text{N}_2 + 2\ \text{H}_2\text{O} + \text{light}$$

MATERIALS AND METHODS

Chemicals

The following chemicals were obtained from Sigma Chemical Company (St. Louis, MO): free fatty acids (except myristic acid), acyl-CoA synthetase (EC 6.2.1.3) from *Pseudomonas* spec., acyl-CoA oxidase from *Candida lipolytica,* ascorbate oxidase, adenosine deaminase, Triton X-100, ATP, reduced CoA (lithium salt), sodium dodecyl sulfate (lauryl sulfate), N-ethylmaleimide, bovine serum albumin (essentially fatty acid-free; order no. A-0281 was used for the development of the assay, and A-6003 was used for the fat cell incubations), dimethyl sulfoxide, epinephrine-bitartrate, norepinephrine-bitartrate, DL-propranolol (hydrochloride), (−)-isoproterenol (hydrochloride), and horseradish peroxidase (EC 1.11.1.7, Type VI; 250-330 U/mg, order no. P 8375). The latter (10 mg of protein/ml) was stored in water and propylene glycol (50% v/v) at −20°C. Phentolamine (Regitin) was obtained from Ciba-Geigy AG (Basel, Germany). Insulin (bovine, 1 mM, 150 U/ml) was purchased from Novo BioLabs (Bagsvaerd, Denmark). Hydrogen peroxide, Trizma base, myristic acid, diethylenetriaminepentaacetic acid, and L(+)-ascorbic acid were obtained from Merck (Darmstadt, Germany). Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) was obtained from BioThema AB (Dalarö, Sweden) and was stored as a 100 mM solution in dimethyl sulfoxide at 4°C. The hydrogen peroxide (1 mol/liter) was stored at 4°C in water acidified with phosphoric acid (0.5 mM), and the concentration of hydrogen peroxide was determined by titration against potassium permanganate in the presence of sulfuric acid according to Kolthoff and Sandell (12). Inorganic pyrophosphatase (EC 3.6.1.1) was purchased from Boehringer Mannheim (Mannheim, West Germany) and prepared as described earlier (11). The NEFA C test kit was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Ascorbate oxidase (100 U/ml), ACS (10 U/ml), and ACO (12.5 U/ml) were dissolved in 50 mM Tris-acetate buffer, pH 8, with 50% propylene glycol and stored at −20°C. The FFA stock solutions (0.5–1 mM) were prepared and diluted in 50 mM Tris-acetate buffer containing 0.25% (w/v) Triton X-100 as described earlier (11). The following stable buffers were prepared and stored in dark bottles at room temperature in a dark cupboard: 50 mM Tris-acetate containing 0.25% (w/v) Triton X-100, pH 8.0, and 50 mM Tris-phosphate containing 40 mM DTPA, pH 7.75. To minimize the background light emission the DTPA/Tris-phosphate buffer was prepared at least 1 week before use. The water used in this study was of reagent grade (which had not been exposed to UV light) and was obtained using Millipore equipment.

Fat cell incubations

The isolation and incubation technique has been described in detail (13 and references therein). In brief, subcutaneous adipose tissue was obtained during routine surgery. The study was approved by the local committee on ethics. Fat cells were isolated, and incubated in a shaking waterbath (37°C) in Krebs-Ringer phosphate (KRP) buffer, pH 7.4, in a total volume of 200 μl containing: 1% (v/v) fat cells corresponding to 10^4 cells/ml (dilute condition), 0.25% (w/v) BSA, 1 mg/ml glucose, 0.35 μg/ml ascorbic acid, and 1 U/ml adenosine deaminase. To some incubations insulin (50 μU/ml), and/or isoproterenol (10^{-15}–10^{-6} M) were added. Fat cells were separated by

flotation and cell-free samples were collected from the bottom of the tubes. To obtain large samples for the comparison of methods, 500-μl identical incubations in triplicate containing 10% (v/v) cells corresponding to 10^5 cells/ml (dense condition) were used. In this experiment 1% BSA was used, and the triplicates of cell-free samples were pooled. The samples were stored at −20°C.

Instruments and data collection

A 1251 luminometer (Bio-Orbit Oy, Turku, Finland) with a sample carousel for 25 samples, temperature control, and three connected dispensers was used. The sensitivity of the luminometer was reduced by a factor of 16 using the special Service program (instructions can be obtained from the manufacturer or from the authors). Cuvettes (polypropylene, order no. 2174-701) were obtained from Boehringer Mannheim Scandinavia AB (Bromma, Sweden).

The luminometer was connected to a Z-88 computer for data collection. Data were transferred to and calculated on a MacIntosh computer using MacTerminal and Microsoft Excel programs. The background was linearly increased with time in the luminometer. Therefore the reagent blank was determined in four cuvettes (containing 180 μl 40 mM DTPA–50 mM Tris-phosphate buffer, pH 7.75) evenly distributed in the sample carousel, and the position-dependent light emission was calculated using linear regression. The values (in mVs) were first corrected for instrument blanks (signal before addition of HRP reagent to each individual cuvette), then for the reagent blank (cf above) in the final reaction, and at last for the standard or the sample blank (cf below). Because the cuvette in the first position was not used, up to 20 FFA samples could be analyzed per carousel.

Standard procedure

Step 1: Samples were diluted with Tris-acetate/Triton buffer to contain 0.25% BSA. The following samples were assayed: samples from fat cell incubations and sample blanks (fat cell incubation buffer without fat cells treated as the sample) and from 2–200 μM palmitic acid standards and standard blanks (Tris-acetate/Triton buffer). The samples (25 μl) were treated with 25 μl 1.6 mM SDS in 50 mM Tris-acetate buffer with 0.25% (w/v) Triton X-100, pH 8.0, for 20 min at 37°C in Eppendorf tubes.

Step 2: Addition of ACS-reagent mixture (25 μl) containing: 1.5 mM CoASH, 6 mM ATP, 15 mM $MgCl_2$, 60 mM KCl, 0.3 U/ml ACS, 3 U/ml pyrophosphatase, 4.5 U/ml ascorbate oxidase in 50 mM Tris-acetate buffer with 0.25% (w/v) Triton X-100, pH 8.0. Endpoint incubation was performed for 10 min at 37°C.

Step 3: NEM-reagent (25 μl of freshly prepared 5 mM N-ethylmaleimide in 50 mM Tris-phosphate buffer, pH 7.75) was added and aliquots of 20 μl from the incubations were added to cuvettes containing 160 μl of 40 mM DTPA–50 mM Tris-phosphate buffer, pH 7.75.

Steps 4 and 5: In the luminometer, after 5 min of preincubation at 25°C, cuvette no. 1 entered the injection position and 20 μl of ACO-reagent (0.8 U/ml in 40 mM DTPA–50 mM Tris-phosphate buffer, pH 7.75) from the first dispenser was added. After 2 min, cuvette no. 2 received ACO-reagent and so on.

After the addition of ACO-reagent to cuvette no. 6, 10 min had passed since ACO-reagent was added to the first cuvette and this was analyzed by the addition of 790 μl luminol-reagent (200 μM luminol, 40 mM DTPA–50 mM Tris-phosphate buffer, pH 7.75, freshly prepared for every carousel) from the second dispenser. The light reaction was immediately started by the addition of 10 μl of HRP-reagent (0.9 mg/ml HRP in 40 mM DTPA–50 mM Tris-phosphate buffer, pH 7.75) from the third dispenser. Subsequently ACO-reagent was added to cuvette no. 7, and luminol- and HRP-reagents were added to cuvette no. 2. Repeating the procedure in this manner resulted in a 10-min time-span between the addition of oxidase and the luminol reaction. The total light emission was integrated for 106 s and 25 samples were analyzed within 75 min.

Effect of SDS on FFA samples containing BSA

Palmitic acid and oleic standards were prepared (see Chemicals) and diluted in 50 mM Tris-acetate containing 0.25% (w/v) Triton X-100, pH 8.0, with or without 0.25% BSA. Samples from 2–200 μM palmitic acid or oleic standard with or without SA were treated with SDS (5 nmol) or Tris-acetate/Triton buffer without SDS.

Recovery of different FFAs

The following free fatty acids were used for the recovery experiment: lauric, myristic, stearic, oleic, linoleic, and linolenic. The FFAs were dissolved (see Chemicals) and diluted in KRP buffer, pH 7.4, to the concentration of 200 μM FFA and 0.25% BSA.

Analytical interference

The effect of different compounds was studied by assaying single samples with 50 μM FFA to which the compound in increasing concentration had been added. The concentrations of these compounds in the samples were 0.3 μM to 10 mM epinephrine, norepinephrine, isoproterenol, propranolol, phentolamine, and 0.03 μM to 1 mM insulin. The concentrations of ascorbate (the ascorbic acid stock solution was adjusted to pH 8.0 with sodium hydroxide) in the samples were 0.5 μM to 90 mM (0.1 μg/ml to 16 mg/ml).

Comparison of the present method with a kit method

The present method was compared with the spectrophotometric NEFA C test kit method based on the studies of Duncombe (1) and Itaya and Ui (2). To make this possible we increased the sample volume for the kit from 25 μl, which is recommended for serum samples, to 200 μl. The concentration of FFA was determined in samples from incubations of fat cells (10^5 cells/ml, with 1% BSA, with and without isoproterenol 0.1 μM, incubated 1–2 h) of human subcutaneous adipose tissue. For the NEFA C kit, a single sample volume of 200 μl was used in a final assay volume of 1.7 ml. The same volume was used as an individual sample blank. The same incubates were also analyzed by incubating 25 μl of sample (diluted 1:4) in duplicates using the present method (corresponding to 1.25 μl of undiluted sample used in the luminometer).

Precision study

Samples were obtained from three different fat tissue sources and the cells were incubated in KRP-buffer containing 0.25% BSA with or without isoproterenol (0.1 μM). The six separate pools of fat cell incubations containing 11.8–85.7 μM FFA were frozen as 25-μl samples and analyzed in duplicate for 3–5 days.

Statistical methods

Linear regression, analysis of correlation, determination of coefficient of variation (CV%), and Student's *t*-test were performed according to Snedecor and Cochran (14). The linear regression analysis was performed after calculating logarithms of light emission and concentration values.

RESULTS

Effect of SDS on FFA samples containing BSA

A preincubation with SDS before the ACS reaction resulted in increased recovery of two different common fatty acids, palmitic and oleic acids, from a buffer containing 0.25% BSA. These FFA curves were significantly different from the curves without SDS as illustrated by a palmitic acid standard curve in **Fig. 1.** The light emission was significantly increased at FFA concentrations up to 3 10^{-8} M (in cuvette), when the effect of SDS at individual concentrations of three palmitic acid standard curves containing 0.25% BSA was tested. When SDS was included in the assay, identical standard curves of palmitic acid with and without BSA were obtained. No interference with the assay of at least up to 80 nmol of SDS was observed (data not shown). The ACS-incubation was completed within 2 min (**Fig. 2A**) at pH 8.0. The ACO-reaction was completed within 1 min at pH 7.75 and at 25°C. Using 1/10 of the usual concentration of ACO, this reaction was completed within 6 min (Fig. 2B).

Recovery of different FFAs

The following recoveries, compared to palmitic acid (100% recovery compared to hydrogen peroxide standard), of different FFAs was obtained: 102% lauric, 101% myristic, 102% stearic, 103% oleic, 99% linoleic, and 106% linolenic acid (one expt.).

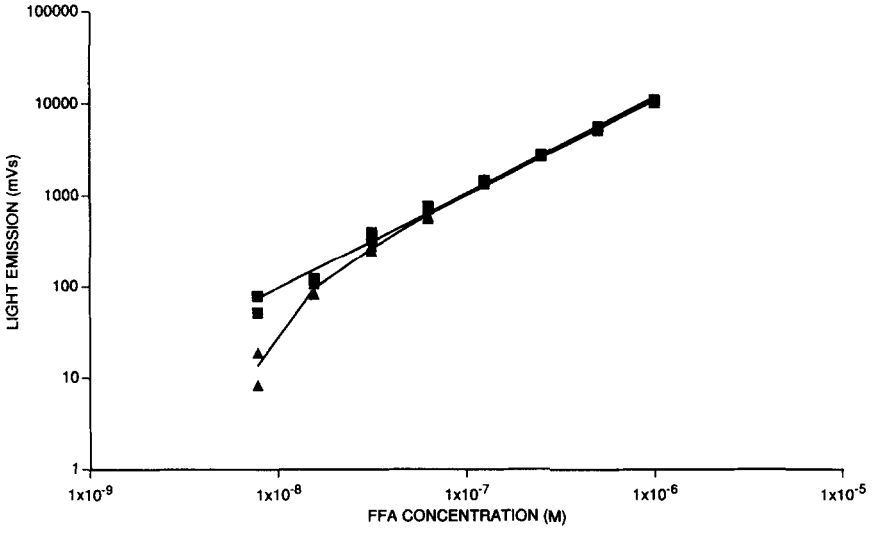

Fig. 1. The effect of SDS on FFA standard curve containing 0.25% BSA. Double logarithmic plots of palmitic acid standard curves (final concentrations in cuvette) are shown. For each concentration of standard in duplicate (the logarithmic plot hides the duplicates at high concentrations) the sample was treated with 25 μl 50 mM Tris-acetate–0.25% Triton buffer, pH 7.75, containing 5 nmol SDS (■) or the corresponding volume of buffer (▲).

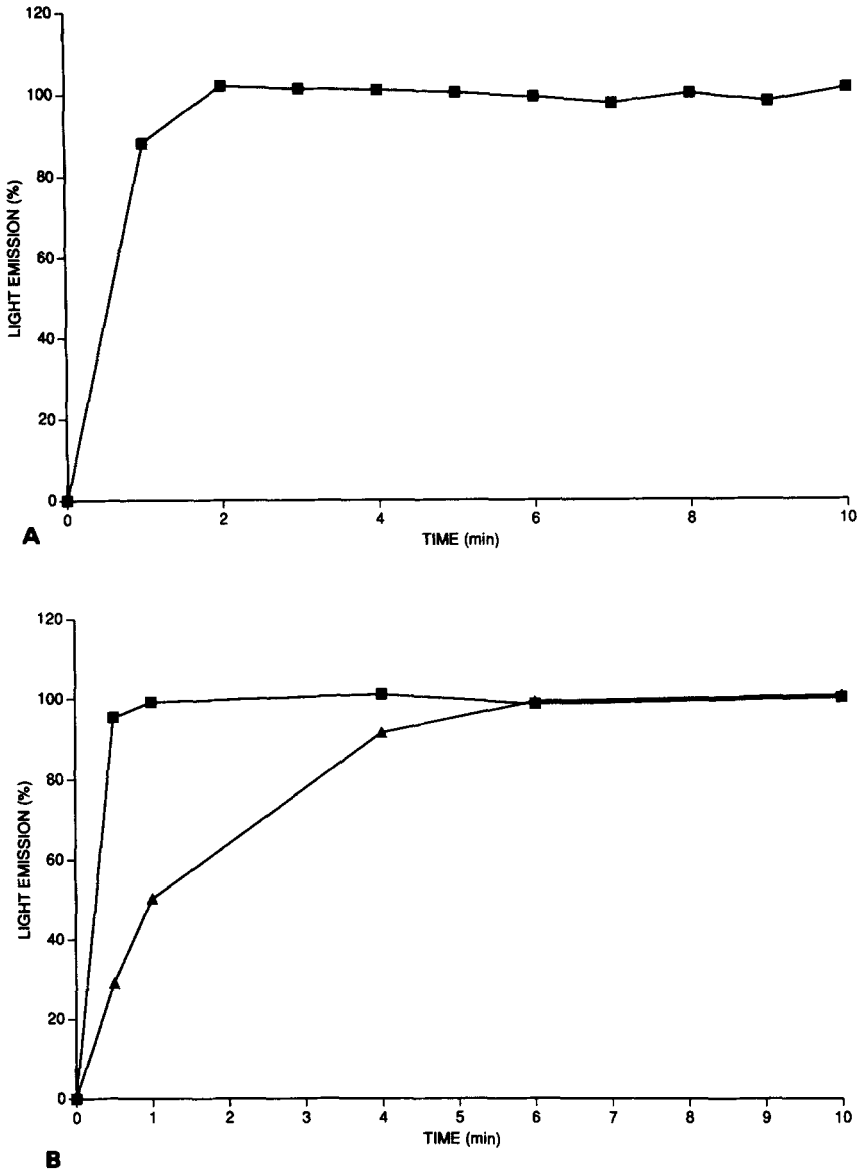

Fig. 2. A: Time-curve of the ACS-reaction. Samples (25 μl) from 200 μM palmitic acid standard containing 0.25% BSA were incubated. The ACS-reaction was stopped by the addition of NEM-reagent. B: Time-curve of the ACO-reaction with the normal (■) and with 1/10 (▲) of the normal enzyme level using 200 μM palmitic standard containing 0.25% BSA. After different times, the light reaction was started by the addition of luminol-reagent and of HRP-reagent. The luminometer program was changed for this experiment.

FFA standard curves and effects of fat cell sample and KRP buffer

A linear relation between analyte and signal requires that the slope is unity in double logarithmic plots after subtraction of blanks. The slope of the double logarithmic plots for three standard curves was 1.00 ± 0.04 (mean ± SD). Thus the assay was linear in the range 0.01 to 1 nmol of FFA in cuvette. The detection limit defined as three standard deviations of the blank was 5 pmol in the final assay mixture.

The possible interference from fat cell samples and from KRP buffer on the standard curve was studied. The curves with fat cell sample or KRP buffer included were not different from the normal FFA standard curve. It was possible to use a 40-μl aliquot rather than 20 μl of the ACS incubation in the luminometric step without affecting the standard curve (results not shown). Thus the detection limit may be improved by a factor of 2.

TABLE 1. Effect of different factors on the assay

Factor	No Interference Up to Conc.	10% Stimulation Up to Conc.	50% Inhibition Up to Conc.
Ascorbate (– oxidase)	5 μM	13 μM	1.7 mM
Ascorbate (+ oxidase)	5 μM	20 μM	17 mM
Epinephrine	5 μM	15 μM	940 μM
Norepinephrine	5 μM	ne	60 μM
Isoproterenol	80 μM	ne	470 μM
Propranolol	2.5 mM	ne	ne
Phentolamine	5.0 mM	ne	ne
Insulin	1.0 mM	ne	ne

The effect of different factors was studied by assaying single samples with 50 μM FFA to which increasing factor concentration had been added. The concentrations of factors in the samples were 0.3 μM to 10 mM epinephrine, norepinephrine, propranolol, phentolamine, and 0.03 μM to 1 mM insulin. The concentrations of ascorbate (the ascorbic acid stock solution was adjusted to pH 8.0 with sodium hydroxide) in the samples were 0.5 μM to 90 mM (0.1 μg/ml to 16 mg/ml); ne, no effect.

Analytical interference from factors used in fat cell studies

The influence of different factors that might interfere with the assay was investigated (**Table 1**). At concentrations of catecholamines and insulin that were at least 10 times higher than the concentrations used for in vitro experiments with human fat cells (15) or more than 500 times the in vivo concentrations of catecholamines (16) and of insulin (17), there was no effect on the assay. As for epinephrine, addition of ascorbate resulted in an increase of emitted light. Ascorbate oxidase did not abolish this increment but the inhibition by ascorbate was displaced 10-times upward.

Comparison of the present assay with a routine method

The results of FFA determination of samples from incubations that included the lipolytic agent isoproterenol (using 6.25 μl out of which 1/5 was used in the luminometer) were compared to values obtained using the spectrophotometric NEFA C kit method that assays 200 μl of undiluted samples. The limit of detection defined as three standard deviations of the reagent blank was 1.3 nmol with the NEFA C kit method. The correlation coefficient between the two methods was 0.997 (**Fig. 3**).

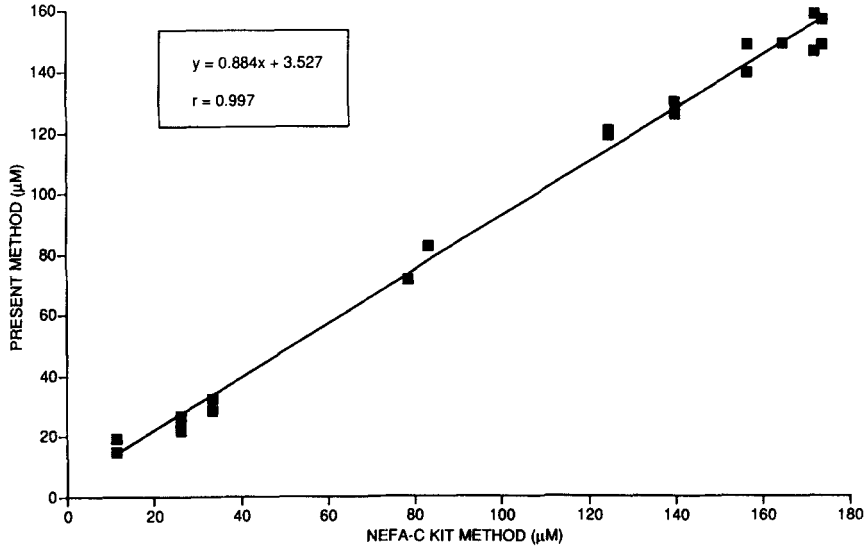

Fig. 3. Correlation between luminometric and spectrophotometric methods. The data are expressed as the concentration of FFA in the fat cell incubation medium.

Precision of the method

Repeated assays for 3–5 days of samples obtained from different fat cell incubations containing 11.8–85.7 μM FFA resulted in a within-run variation of 2.6–7.9% and a between-day variation of 3.8–13.2% (**Table 2**).

TABLE 2. Precision of the luminometric method

FFA Concentration Mean	Within Run CV	Between Day CV	Days No.
μM	%	%	
11.8	6.3	13.2	4
18.0	7.9	4.9	4
32.7	2.9	8.9	3
47.3	2.8	4.3	4
49.1	3.9	6.0	4
85.7	2.6	3.8	5

Samples were obtained from three different fat tissue sources and the cells were incubated in KRP-buffer, pH 7.4, containing 0.25% BSA with or without isoproterenol (0.1 μM). The six separate pools of fat cell incubations containing 11.8–85.7 μM FFA were frozen as 25-μl samples and analyzed in duplicate over a period of 3–5 days.

Rate of FFA release from fat cells

The concentrations of FFA in samples obtained from fat cells incubated under dense conditions, 10% (v/v) fat cells, were determined after different incubation times. A small but time-dependent increase in the levels of FFA was observed in the absence of lipolytic agent. In the presence of the lipolytic agent isoproterenol, the rate of FFA release was markedly increased (data not shown). Dose-response experiments were performed with diluted (1%, v/v) fat cell incubations (data not shown).

Isoproterenol caused a marked and concentration-dependent stimulation of FFA release. Addition of physiological concentration of insulin (50 μU/ml) resulted in a marked suppression of isoproterenol-induced FFA release.

DISCUSSION

In this investigation we have developed an ultrasensitive and partly automatic method for the determination of free fatty acid release in human adipocyte incubations. All major FFAs in human adipose tissue are completely recovered in this assay. The method has been developed by adaptation of a chemiluminometric assay of FFA (11) to a biological system.

When incubating adipocytes in vitro for lipolysis studies, to avoid intracellular accumulation of FFA, BSA has to be added as acceptor for the fatty acids that are released from the fat cells (16). BSA is known to bind five to seven fatty acids with high affinity (18, 19). The anionic detergent SDS is known to hydrophobically bind to and unfold the bovine serum albumin molecule (20). In the initial stage of the development of the assay, 5 nmol of SDS was used per sample containing 0.95 nmol of BSA (which is the amount deriving from the sample).

Pretreatment of samples containing 0.25% BSA with 5 nmol of the detergent SDS resulted in linear standard curves for FFA. Routinely, 40 nmol of SDS was used. Both BSA preparations used in this study contained less than 0.005% FFA (stated by the manufacturer) which corresponds to an FFA concentration below 0.4 μM in a sample containing 0.25% BSA, and was, as expected, not detectable. The effect of SDS was most pronounced at low FFA concentrations, while the effect at the upper part of the standard curve was probably hidden by the increased standard deviation.

In the FFA assay, pyrophophatase was included to hydrolyze PP_i thereby avoiding a reverse reaction. N-ethylmaleimide, which was easier to handle and less expensive than Affi-Gel 501 used in the previous paper (11), was used to block unreacted CoASH which otherwise would interfere with the peroxidase reaction (5, 6). This concentration of NEM did not affect the peroxidase-catalyzed luminol reaction. Ascorbate oxidase was included in the ACS incubation to degrade ascorbate to avoid inhibition of the luminometric assay. The results obtained with the luminometric assay were compared to results obtained with the NEFA C test. The detection limit was 5 pmol for the luminometric method and 1.3 nmol for the NEFA C method. This is a difference of 260-fold. For the luminometric method 1.25 μl was used compared to 200 μl for the NEFA method in the analytic step. Thus the difference in volume used for the assays was 160-fold. If a sample blank has to be included for the NEFA C assay, 400 μl of sample is needed for a single determination. The small amount of FFA needed for the luminometric assay affords the possibility of reducing the amount of fat cells in the incubations and thereby reducing the amount of fat tissue needed.

Assuming a yield of 500 mg of collagenase-isolated fat cells per g of intact adipose tissue and using 1% (v/v) fat cell incubations, 250 assays could be performed on 0.5 ml packed isolated fat cells. This means that 1 g of inact human adipose tissue, which can easily be obtained with a needle biopsy, enables detailed investigations of FFA release under clinical conditions. Another advantage with the present method is that it can detect FFA in dilute fat cell suspensions. The latter is of methodological importance because when dense fat cell suspensions are used (to overcome low sensitivity of the FFA assay) endogenous metabolites such as adenosine or lactate may accumulate in the incubation medium and artificially inhibit FFA release. To increase the sensitivity, it was possible to double the sample volume used in the luminometric step.

It might also be possible to further increase the sensitivity by using a larger sample volume for the initial step in the assay. Because 40 nmol of SDS is in excess, it is possible that samples containing more than 0.25% BSA can be used.

Compared to earlier published spectrophotometric methods, with a lower limit normally at 1–10 nmol FFA in the sample (see references in 11), this method is much more sensitive in the analytical range between 0.05 and 5 nmol in the sample corresponding to 0.01 and 1 nmol in cuvette. A bioluminometric method reported earlier (10) has sensitivity similar to the present method, but involves seven enzymatic steps and has a rather limited linear range. The present assay involves only three coupled enzymatic steps, and the reagent cost is approximately 50 cents per assay as compared to 2.5 dollars for the NEFA assay.

Some improvements have been made as compared to the previous method in addition to the introduction of the SDS step of the actual FFA assay. The ACS-reaction, which earlier had been optimized, was unchanged (11). The luminol-peroxidase reaction was the same as in a glucose assay (21) where the pH was reduced from 8.0 to 7.75. The pH choice is a compromise between light emission, specificity of the reaction with hydrogen peroxide, and the activity of the oxidase enzymes.

The light emission is proportional to the amount of hydrogen peroxide, which is stoichiometric to the amount of fatty acid and is independent of chain length and degree of saturation. Furthermore, to simplify the laboratory procedure using one luminometer program, 10 min of oxidase incubation is used in common with the glucose assay (21). Because the oxidase reaction is performed in the luminometer before the luminol reaction, it was convenient to use the same buffer for these two reactions. Moreover, we now use the DTPA/Tris-phosphate buffer also for the oxidase step.

The ACO-concentration was doubled compared to that used at pH 8.0 (11). With this concentration the reaction was essentially completed within 1 min for palmitic acid. In the actual assay a 10-min reaction time was used as a safety margin. With this procedure a yield of around 100% for several of the FFAs with 12–18 carbons was obtained. This confirmed that in the present assay system an adequate excess of ACO was used not only for palmitic acid but for other FFAs as well. We chose the stable saturated palmitic acid, one of the major FFAs present in adipose tissue, as the standard.

The luminometer program used analyzes hydrogen peroxide in one cuvette while the oxidase reactions are running in the next five cuvettes with a 2-min delay between the cuvettes. A shorter oxidase incubation time will therefore decrease the total assay time only slightly. Because the present method is based on three end-point reactions and the amount of the ACS, ACO, and HRP enzymes are in large excess, variation between different enzyme batches is not critical as it would be in kinetic methods. Other enzyme sources are therefore probably suitable as well for this assay.

Because addition of samples taken from adipocyte incubations did not affect the standard curve, it was possible to analyze samples using an ordinary standard curve. The effects of some common metabolites and hormones on the assay were investigated. In general, interference with the assay was observed only at concentrations above those that would be used in adipocyte incubations. However, because a preceding and not FFA-dependent increase of the light emission was observed in the presence of ascorbate, which was not abolished in the presence of oxidase, it is recommended not to exceed 5 μM ascorbate in the sample which corresponds to 0.025 μM in the luminol-peroxidase reaction. It is advisable to use a sample from the fat cell incubation medium as blank which is treated identically as the sample. We have shown earlier that ascorbate at concentrations below 0.02 μM in the final step in cuvette did not interfere with the chemiluminometric glucose assay (21).

In summary we have developed a sensitive, simple, and inexpensive luminometric assay for free fatty acid release. The method is many hundred-fold more sensitive than standard spectrophotometric methods and can be used for serial studies of lipid metabolism in small amounts of human adipose tissue or for investigations of FFA release from very diluted human fat cell samples.

Thanks are due to Mrs. Eva Åkersten, and to Mrs. Kerstin Wåhlén for excellent technical assistance. We also thank Dr. Erik Söderman for a fruitful statistical discussion. This investigation was supported by grants from the Swedish Medical Research Council (No. MRF 19X-01034), the Swedish Diabetes Association, the Nordic Insulin Foundation, the Karolinska Institute, the Osterman and Stohne Foundations, the Swedish Athletics Confederation.

Manuscript received 9 January 1992 and in revised form 15 June 1992.

REFERENCES

1. Duncombe, W. G. 1964. The colorimetric microdetermination of nonesterified fatty acids in plasma. *Clin. Chim. Acta.* **9:** 122–125.
2. Itaya, K., and M. Ui. 1965. Colorimetric determination of free fatty acids in biological fluids. *J. Lipid Res.* **6:** 16–20.
3. Matsubara, C., Y. Nishikawa, Y. Yoshida, and K. Takamura. 1983. A spectrophotometric method for the determination of free fatty acid in serum using acyl-coenzyme A synthetase and acyl-coenzyme A oxidase. *Anal. Biochem.* **130:** 128–133.
4. Hosaka, K., T. Kikuchi, N. Mitsuhida, and A. Kawaguchi. 1981. A new colorimetric method for the determination of free fatty acids with acyl-CoA synthetase and acyl-CoA oxidase. *J. Biochem.* **89:** 1799–1803.

5. Shimizu, S., Y. Tani, H. Yamada, M. Tabata, and T. Murachi. 1980. Enzymatic determination of serum-free fatty acids: a colorimetric method. *Anal. Biochem.* **107:** 193–198.
6. Okabe, H., Y. Uji, K. Nagashima, and A. Noma. 1980. Enzymic determination of free fatty acids in serum. *Clin. Chem.* **26:** 1540–1543.
7. Duncombe, W. G., and T. J. Rising. 1973. Quantitative extraction and determination of nonesterified fatty acids in plasma. *J. Lipid Res.* **14:** 258–261.
8. Shimizu, S., and H. Yamada. 1985. Free fatty acids: colorimetric method. *In* Methods of Enzymatic Analysis. H. U. Bergmeyer, J. Bergmeyer, and M. Graβl, editors. Verlag Chemie, Weinheim. 19–25.
9. Miles, J., R. Glasscock, J. Aikens, J. Gerich, and M. Haymond. 1983. A microfluorometric method for the determination of free fatty acids in plasma. *J. Lipid Res.* **24:** 96–100.
10. Kather, H., and E. Wieland. 1985. Free fatty acids: luminometric method. *In* Methods of Enzymatic Analysis, H. U. Bergmeyer, J. Bergmeyer, and M. Graβl, editors. Verlag Chemie, Weinheim. 25–34.
11. Näslund, B. M. A., K. Bernström, A. Lundin, and P. Arner. 1989. Free fatty acid determination by peroxidase-catalysed luminol chemiluminescence. *J. Biolumin. Chemilumin.* **3:** 115–124.
12. Kolthoff, I. M., and E. B. Sandell. 1963. Textbook of Quantitative Inorganic Analysis. 3rd ed. Macmillan, New York. 564–578.
13. Hellmér, J., P. Arner, and A. Lundin. 1989. Automatic luminometric kinetic assay of glycerol for lipolysis studies. *Anal. Biochem.* **177:** 132–137.
14. Snedecor, G. W., and W. G. Cochran. 1980. Statistical Methods. 7th ed. Iowa State University Press, Ames, IA.
15. Engfeldt, P., J. Hellmér, H. Wahrenberg, and P. Arner. 1988. Effects of insulin on adrenoreceptor binding and the rate of catecholamine-induced lipolysis in isolated human fat cells. *J. Biol. Chem.* **263:** 15553–15560.
16. Lönnquist, F., B. Nyberg, H. Wahrenberg, and P. Arner. 1990. Catecholamine-induced lipolysis in adipose tissue of the elderly. *J. Clin. Invest.* **85:** 1614–1621.
17. Bolinder, J., H. Lithell, E. Skarfors, and P. Arner. 1986. Effects of obesity, hyperinsulinemia, and glucose intolerance on insulin action in adipose tissue of sixty-year-old men. *Diabetes.* **35:** 282–290.
18. Spector, A. A., and J. E. Fletcher. 1978. Transport of fatty acid in the circulation. *In* Disturbances in Lipid and Lipoprotein Metabolism. J. M. Dietschy, editor. Williams and Wilkins, Baltimore. 229–249.
19. Brown, J. R., and P. Shockley. 1982. Serum albumin: structure and characterization of its ligand binding sites. *In* Lipid-Protein Interactions P. C. Jost, and O. H. Griffith, editors. Wiley, New York. 25–68.
20. Reynolds, J. A., J. P. Gallagher, and J. Steinhardt. 1970. Effect of pH on the binding of N-alkyl sulfates to bovine serum albumin. *Biochemistry.* **9:** 1232–1238.
21. Näslund, B., P. Arner, J. Bolinder, L. Hallander, and A. Lundin. 1991. Glucose determination in samples taken by microdialysis by peroxidase-catalyzed luminol chemiluminescence. *Anal. Biochem.* **192:** 237–242.