notes on methodology

Continuous monitoring of free fatty acid release from adipocytes by pH-stat titration

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Summary A method for direct and continuous monitoring of free fatty acid release in adipocyte suspensions is described. Using a pH-stat apparatus the protons from the released free fatty acids are continuously titrated and the accumulated amount of OH^- added is monitored on a recorder against time, the slope thus indicating the rate of free fatty acid release. Since pH is kept constant, an incubation medium with a low buffering capacity can be used, which gives the method a high sensitivity. Under the conditions described, free fatty acid release from 5% of maximal norepinephrine stimulation of rat adipocytes can be accurately measured and the kinetics can be followed over extended periods of time.—**Nilsson, N. Ö., and P. Belfrage.** Continuous monitoring of free fatty acid release from adipocytes by pH-stat titration. 1979. *J. Lipid Res.* **20:** 557–560.

Supplementary key words lipolysis · buffer capacity · norepinephrine · insulin

Release of free fatty acids (FFA) from adipose tissue slices is associated with an equivalent production of protons leading to a decline of the pH of the incubation medium (1). With isolated rat adipocytes that have been stimulated with lipolytic hormones, the pH decrease can be monitored and the rate of FFA release calculated (2). However, the pH decrease in itself influences this rate (3). Furthermore, changes in the concentration of albumin, used as a FFA acceptor, or in K^+ concentration influence lipolysis differently at reduced and normal pH levels (3). In this note we describe a method for the determination of FFA release from adipocytes in suspension under such conditions that the medium pH is kept constant and the difficulties above are thereby avoided. The FFA release is monitored continuously by titration of the stoichiometrical amount of protons released, with the use of a recording pH-stat titration apparatus.

PROCEDURE

Preparation of acipocytes

Rat adipocytes were prepared according to Rodbell (4) as modified by Gliemann (5) from epididymal and perirenal fat from 120–150 g male Sprague–Dawley rats (Anticimex, Stockholm, Sweden) using 0.5 mg/ml of collagenase in Krebs–Ringer buffer with HEPES, 24 mM; albumin, 3.5% (w/v); and glucose, 0.55 mM. The rats were fed a standard pellet diet. The adipocyte concentration was determined (6) as packed cell volume (PCV) and the cell suspension was diluted to 50 μl PCV/ml in Krebs–Ringer buffer with HEPES, 24 mM; albumin, 1% (w/v); and glucose, 0.55 mM (storage medium).

The cell number in 1 μl of PCV was calculated from the cell diameter, measured with an ocular micrometer; the small volume of medium trapped between the cells, about 1% (6), was neglected in the calculation. Since the cell number in 1 μl of PCV is greatly affected by the cell diameter, and cell size increases with the weight of the rats, it is important to use rats of the same weight to minimize day to day variation. One μl of PCV contained $1.58 \cdot 10^4 \pm 0.23 \cdot 10^4$ cells from rats of weight 137 ± 12.6 g (mean ± SD, $n = 10$).

Viability was tested by microscopic appearance and by insulin stimulation of the conversion of medium [3-^{3}H]glucose into adipocyte ^{3}H-labeled lipids (7). Only cells that gave at least a 10-fold maximal increase of this parameter were used. Half maximal stimulation usually occurred at 7 μU/ml of insulin.

Human adipocytes were prepared from surgical biopsy specimens taken during general anesthesia. The biopsy specimens were minced in the collagenase solution immediately after removal and treated in the same way as the rat tissue. Viability was tested by microscopic appearance. The cell number per μl of PCV was $6.3 \cdot 10^2$ for the experiment described in Fig. 4.

Incubation conditions and pH-stat titration

Aliquots of 2 ml of cell suspension (50 μl PCV/ml) were stored for up to 4 hr in polyethylene vials at 37°C in a water bath with gentle shaking. Immediately before starting each pH-stat titration the storage medium was removed and replaced with (unless otherwise stated) a modified Krebs–Ringer buffer with low buffering capacity: NaCl, 131 mM; KCl, 4.95 mM; $CaCl_2$, 2.54 mM; KH_2PO_4, 1.19 mM; $MgSO_4$, 1.19 mM; bovine serum albumin, 1% (w/v); pH 7.40 (incubation medium). For this replacement the cell suspension (100 μl PCV of cells) was transferred to a centrifuge tube containing a thin polyethylene tubing.

Abbreviations: FFA, free fatty acids; HEPES, 2[4-(2-hydroxyethyl)-piperazinyl-(1)]-ethanesulfonic acid; PCV, packed cell volume; NE, norepinephrine; Ins, insulin.

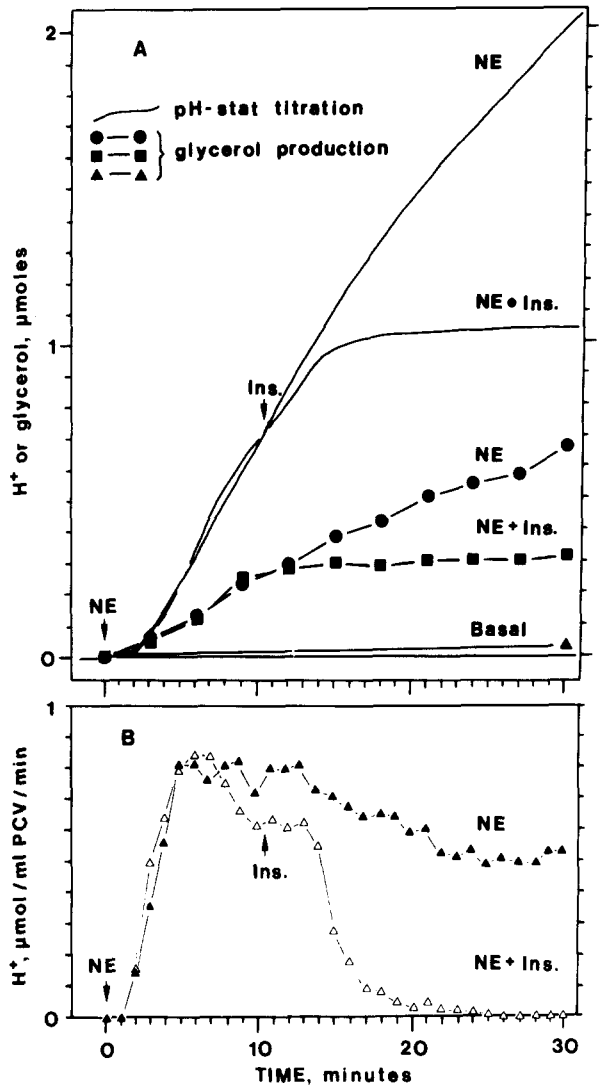

Fig. 1. *A.* Recorder charts from pH-stat titration experiments. Proton release from suspended rat adipocytes (10 μl PCV/ml) was measured by continuous titration under basal conditions, after the addition of norepinephrine (final concentration 5 ng/ml), or norepinephrine followed by insulin (10 μU/ml). Glycerol concentration was measured enzymatically fluorimetrically in single samples (150 μl) from the incubation mixture and plotted in the same scale. Each set of data are from one representative experiment. No correction has been made for the volume change during the incubation. *B.* Rates of proton release calculated over 1-min intervals from the pH-stat data of the experiments in Fig. 1*A*. The data have been corrected for the volume change caused by the glycerol sampling.

After centrifugation (100 *g* for 30 sec at room temperature) the storage medium under the flotated cells was removed through the thin tubing and the cells were resuspended in fresh incubation medium, usually 10 ml (37°C). The low cell concentration (10 μl PCV/ml) was chosen to keep the hormonal degradation low (8). The pH-stat incubation vial (polystyrene, 25 mm i.d.) was thermostated to 37 ± 0.1°C by a water jacket and supplied with a stream of water-saturated O_2 above the cell suspension. The latter was mixed with a magnetic stirrer set at a speed which just prevented flotation of cells.

The pH-stat titrator was a combination of DK 10, electrode potential amplifier; DK 11, rate and endpoint control; DV 11, burette drive; DV 201, 1-ml interchangeable burette; and DV 13/131 automatic valve control, from Mettler Instrumente AG, Greifensee-Zürich, Switzerland. To the pH-stat was fitted a combined glass electrode (EA 120, Metrohm AG, Herisau, Switzerland). Volumes of 0.2 μl could be delivered, i.e., 2 nmol of OH^- with the 10 mM NaOH usually employed. The pH-stat was adjusted to the pH of the incubation medium. After 5–10 min of preincubation (from the change to incubation medium), steady-state conditions were achieved. Then, zero time was usually defined by the addition of norepinephrine. The titrated volume was recorded on a Vitatron 2001 recorder equipped with a Multirange Module B for variation of *y*-scale amplification. This module was set to 100 mV, and paper movement (*x* scale) was set to 10 mm/min. The electrode was treated with pepsin (5% in 0.1 M HCl) after each experimental day to maintain its sensitivity.

Glycerol and fatty acid determination

Samples (150 μl) were taken from 10-ml incubations at various time intervals. The incubation was interrupted by a rapid centrifugation (Beckman Micro-

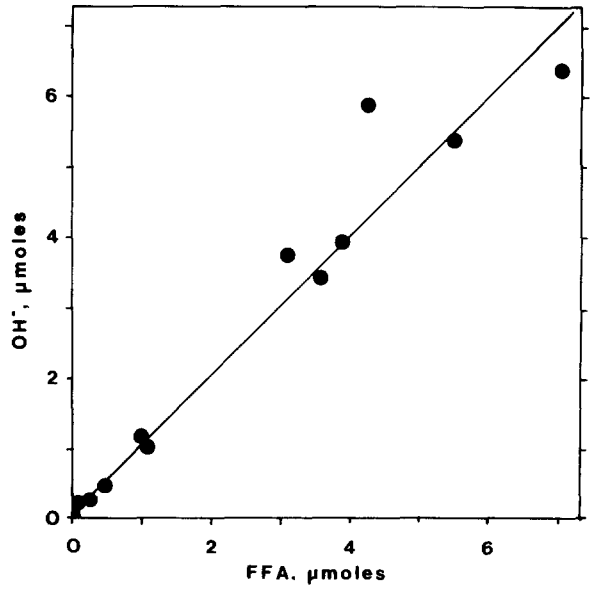

Fig. 2. Relationship between proton and FFA release. Adipocytes were incubated as in Fig. 1 but were stimulated with 100 ng/ml of norepinephrine. Samples of the incubation medium were taken at various times and analyzed for fatty acids by a colorimetric method. Values corrected for medium FFA before hormonal stimulation. Curve calculated by linear regression analysis. $r = 0.977$.

fuge B, 1 min, 10,000 *g*) through a silicone oil layer (6), leaving the medium separated from the cells for analysis. Glycerol was determined after deproteinization with the enzymatic, fluorometric method (9) modified for higher sensitivity (10). FFA was determined as Cu-complexes with a colorimetric method (11).

Materials

Norepinephrine bitartrate was from Sigma Chemical Co., St. Louis, MO; monocomponent porcine insulin was a gift from Novo, Copenhagen, Denmark; [3-^{3}H]glucose was from the Radiochemical Centre, Amersham, England; and HEPES (2-[4-2-hydroxyethyl)-piperazinyl-(1)]-ethanesulfonic acid) was from Schwarz–Mann, NY. Bovine serum albumin (Cohn Fraction V) from Sigma Chemical Co. was extensively dialyzed and filtered (0.8 μm). Collagenase type CLS (Batch No. 45K137) was from Worthington Biochemical Corp., Freehold, NJ. All other chemicals were of analytical grade.

RESULTS

FFA release from rat adipocytes continuously monitored by pH-stat titration

The time course of norepinephrine stimulation of FFA production, measured as proton release, and the inhibition of this stimulation by insulin could be conveniently followed by continuous pH-stat titration (**Fig. 1***A*). The effects were even more evident if rates

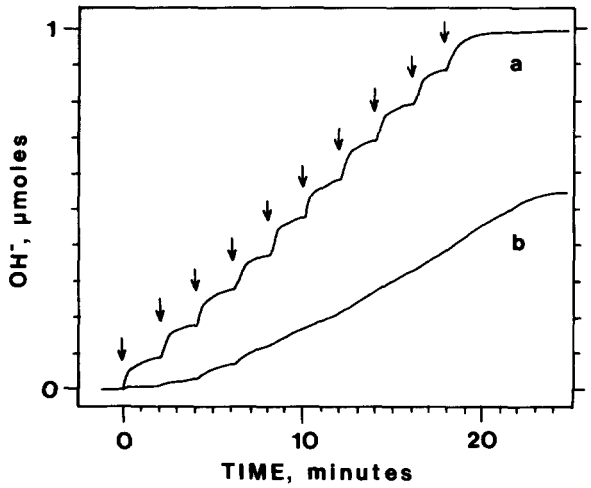

Fig. 3. Effect of buffering capacity of incubation medium on titrations of added fatty acid. One hundred nanomoles of palmitic acid (in 10 μl ethanol) was added to 10 ml of incubation medium *a* or *b* at arrows. *a*, Incubation medium with low buffering capacity used in all cell incubations (see Procedure); *b*, Krebs–Ringer solution buffered with 24 mM HEPES and with 1% (w/v) bovine serum albumin.

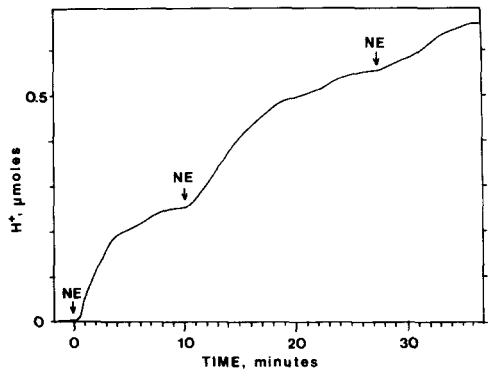

Fig. 4. FFA release from human adipocytes measured by continuous pH-stat titration. Norepinephrine, 100 ng/ml, added at arrows. Incubation conditions as in Fig. 1. The data are from one representative experiment.

were plotted (Fig. 1*B*) instead of the cumulative values obtained on the recorder. Under the incubation conditions used, with no glucose in the medium, the FFA release closely paralleled glycerol release, at a molar ratio of 3:1 (Fig. 1*A*). Thus, in these experiments, the rate of FFA release was also a measure of the rate of lipolysis, and the hormonal effects reflect changes in the activity of the hormone-sensitive lipase of the adipocytes.

The FFA release in Fig. 1 was approximately linear over 15 min. However, the concentration of norepinephrine used (5 ng/ml) gave less than half-maximal stimulation. At maximal stimulation (100 ng/ml) linearity was obtained for 30 min with a lipolytic rate of 2.25 μmol fatty acids released/ml PCV per min from 5 min after norepinephrine addition. Then the FFA/albumin ratio in the incubation medium exceeded 3 and thus was a limiting factor (12). This could be overcome by increasing the albumin concentration. The increase in buffer capacity caused by the increased albumin concentration was of no importance at these high lipolytic rates.

The equivalence between proton and FFA release is demonstrated in **Fig. 2.** Under the conditions used the FFA release obtained at 5% of maximal norepinephrine stimulation (2 ng/ml) represented the practical lower limit of the method. To obtain this sensitivity it was essential to use an incubation medium with a low buffering capacity (cf. **Fig. 3**). With the low cell concentration used (10 μl PCV/ml) FFA release in the absence of norepinephrine could not be measured (Fig. 1*A*).

Adipocytes could be used with the same hormonal sensitivity up to at least 4 hr after preparation if stored at 37°C with gentle shaking. The reproducibility of consecutive titrations was tested using cells from one large cell batch. After stimulation with 5 ng/ml of norepinephrine, the initial rate (calculated from 5–

10 min after norepinephrine addition) was 0.91 μmol/ml PCV per min with a variation coefficient of 5.6% (n = 7). The entire experiment lasted for 4 hr. There was no significant difference between the first three and the last three titrations.

The variation coefficient for the lipolytic rate obtained after norepinephrine (5 ng/ml) stimulation between different cell batches was 12.7% (0.89 ± 0.11 ($\bar{X}$ ± SD) μmol FFA/ml PCV per min for seven separate cell batches; the value for each batch was the mean of three titrations).

FFA release from human adipocytes

FFA release from human adipocytes could be monitored by continuous pH-stat titration (**Fig. 4**). However, with human cells the increased FFA release after norepinephrine was linear for only a few minutes, even with amounts of hormone (100 ng/ml) that gave maximal response in rat adipocytes. Furthermore, the responses after repeated additions of norepinephrine decreased successively (Fig. 4).

The lipolytic rate during the first 5 min was 0.4 μmol FFA/ml PCV per min. It can be calculated to correspond to 0.5 μmol FFA/g triacylglycerols per min (80 nmol/10^5 cells per min). As a comparison, although not directly comparable because of other incubation conditions, lipolytic rates of 0.2–0.3 μmol FFA/g triacylglycerols per min after incubation of human cells with 10^{-5} M isoproterenol were obtained by others (13).

DISCUSSION

Continuous pH-stat titration was found to be a convenient and rapid method to monitor FFA efflux from adipocytes, especially when it was desired to follow the time course of effects after perturbation with hormones and other agents. The method can also be used to follow the rate of lipolysis, i.e., the rate-limiting activity of the hormone-sensitive lipase (14), under such conditions that little reesterification of liberated fatty acids take place. Metabolites and enzymatic activities can easily be determined in samples of cells and medium taken at selected time points during the continuous monitoring of hormonal stimulation. The method should thus be of general value in studies of hormonal effects.

FFA release from adipocytes can also be followed as a decline of the pH of the incubation medium (2). However, the pH decrease in itself rapidly changes the conditions for the FFA release (cf. 3), especially when a high sensitivity is required and incubation medium with a low buffering capacity therefore is used. In contrast, in the present method the protons produced are continuously titrated and the pH is maintained constant regardless of the buffering capacity of the incubation medium. In our work the pH-stat titration technique has proven to be a more versatile instrument, particularly when the kinetics for the FFA release at low concentrations of hormones are studied.

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