notes on methodology

pH as an indicator of free fatty acid release from adipocytes

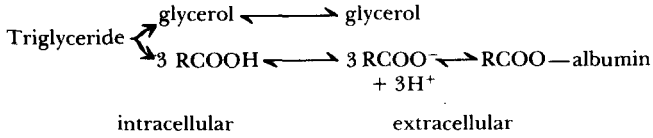
Herman Meisner and Karen Tenney

Department of Pharmacology, Case Western Reserve University School of Medicine, Cleveland, OH 44106

Summary A convenient method of measuring initial rates of free fatty acid efflux from isolated adipocytes during triglyceride breakdown by hormone-sensitive lipase is described. The procedure is based on the dissociation of protons from carboxyl groups of free fatty acids. A recording pH meter is used to monitor H^+ concentration in the medium continuously as an index of free fatty acid release. A stoichiometric relationship was demonstrated between proton release and extracellular free fatty acid concentration as determined by the ^{63}Ni radioassay method of Ho (1970. *Anal. Biochem.* **36:** 105–113). An acid pH (6.8) caused a reduction in proton release, which was immediately and completely reversed by raising the pH to 7.4.

Supplementary key words: lipolysis · proton · epinephrine

Stimulation of adipose tissue hormone-sensitive lipase by catecholamines and other lipolytic agents results in the breakdown of triglyceride to glycerol plus free fatty acids. As can be seen by the following scheme, the FFA thus released are in the protonated form intracellularly (1). Short and medium chain FFA (2), and presumably long chain FFA, diffuse passively across the cell membrane as the undissociated species into the extracellular fluid, provided that albumin is present (3). Rudman and Shank (1) have demonstrated that a proton is released into the extracellular medium from the carboxyl group of FFA, essentially stoichiometrically at a pH of 7.4 (1).



Provided that sufficient H^+ is released, it should be possible to continually monitor H^+ concentration in the medium as a convenient index of the appearance of extracellular FFA. The following report demonstrates that, during activated lipolysis, H^+ release from adipocytes can be determined continuously by using a recording pH meter, and is equated with FFA liberation.

Methods and Materials

Preparation of adipocytes. Fat cells from epididymal fat pads of Sprague-Dawley rats, 130–200 g, fed ad libitum were isolated by the method of Rodbell (4). Generally, coarsely minced fat pads from three rats were incubated in a 2% BSA (Fraction V, Sigma Chemical Co., St. Louis, MO) Krebs-Ringer phosphate solution, pH 7.4, containing 1 mg/ml collagenase (Worthington Biochemical Corp., Freehold, NJ) with moderate shaking for 1 hr at 37°C. After passing the crude digest through four layers of cheesecloth, the cells were washed four times by low speed centrifugation in 2% BSA–Krebs-Ringer phosphate.

Cells were tested for quality by measuring the extent of stimulation of [1-^{14}C]glucose into $^{14}CO_2$ by insulin (5), and preparations having ratios less than 3 (±insulin) were not used.

pH recording. The cells were added to a constantly stirred, water-jacketed chamber, maintained at 37°C, containing Krebs-Ringer phosphate buffer in a final volume of 2.0 ml. After a 10-min preincubation, the lipolytic drug was added and pH changes were measured with an Orion 701A pH meter (Orion Research, Inc., Cambridge, MA) connected to a Datel DPP-7 printer (Datel Systems, Inc., Canton, MA). Values were recorded at pre-set intervals of 15–90 sec. At the end of each experiment, an aliquot of NaOH (normality determined by titration) was added to convert pH into H^+.

In certain experiments, aliquots were removed and cell-free extracts, obtained by briefly centrifuging cells, were used to measure FFA release by the ^{63}Ni method of Ho (6), or to measure glycerol after deproteinization by the procedure of Wieland (7).

Results

Fig. 1A indicates that incubation of isolated rat adipocytes at pH 7.4 in 2% BSA (0.3 mM) with a variety of lipolytic agents produces a stimulation of proton release, as indicated by a progressive decline in pH of the medium. Addition of 2 mM dibutyryl cyclic AMP (Fig. 1*B*) consistently produced greater

Abbreviations: FFA, free fatty acids; BSA, bovine serum albumin.

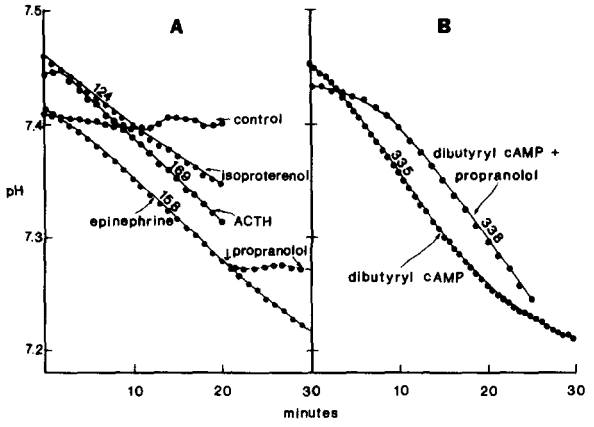


Fig. 1. Effect of lipolytic agents on H^+ release by isolated adipocytes. Fat cells (36.7 mg lipid) from 117-g rats were preincubated at 37°C in buffer containing 2% BSA for 10 min before addition of various agents at 0 min. Final concentrations were: *l*-epinephrine, 0.5 μg/ml; dibutyryl cyclic AMP, 2 mM; ACTH, 1 μg/ml; isoproterenol 1×10^{-5}M; propranolol 2×10^{-5}M. Numbers refer to maximal rates of release of H^+, in terms of nmol $H^+ \cdot$ mg lipid$^{-1} \cdot$ hr^{-1}. Filled circles are actual data points taken from the printer at 30 or 60 sec intervals.

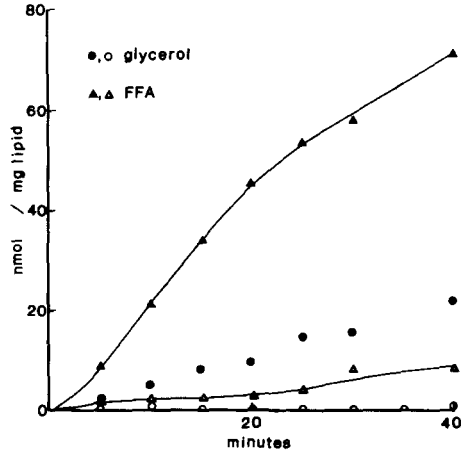


Fig. 3. Relationship between H^+, FFA, and glycerol release in adipocytes. Fat cells from 150-g rats were added to salts–albumin buffer, at 7.8 mg lipid/ml, in a total volume of 20 ml. After a 10-min preincubation period, *l*-epinephrine (0.25 μg/ml) was added, and duplicate samples removed at intervals for FFA and glycerol analyses of cell-free extracts. pH was continuously recorded. (——), H^+ release; solid symbols, plus epinephrine; open symbols, minus epinephrine.

maximal rates (355 nmol $H^+ \cdot$mg lipid$^{-1} \cdot$hr^{-1}) than were obtained with saturating levels of other drugs (124–169 nmol $H^+ \cdot$mg lipid$^{-1} \cdot$hr^{-1}). Propranolol, a β-adrenergic antagonist, completely blocked the epinephrine-stimulated H^+release but had no effect on that caused by 2 mM dibutyryl cyclic AMP, although the lag time before H^+ release approached a maximum was more prolonged. A decrease in the rate of dibutyryl cyclic AMP-stimulated H^+ release was evident at 30 min, and may have been associated with the high FFA/BSA molar ratio (8), which was estimated to be 6.1. In the absence of BSA (not shown), lipolytic agents caused no change in the pH of the medium.

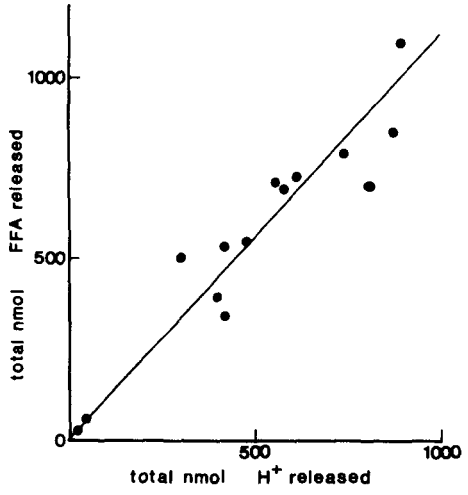


Fig. 2. Stoichiometry between H^+ release and FFA accumulation by isolated adipocytes. Cells were incubated as described in Fig. 1, with different lipolytic drugs. After measuring H^+ release, aliquots were immediately removed, centrifuged at low speed for 1–2 min, and the cell-free medium was analyzed for FFA. Medium FFA were also determined in the absence of lipolytic drug and subtracted from experimental values.

In order to show that H^+ and FFA release are stoichiometrically related, samples were removed at various times under different conditions and analyzed for FFA content by the ^{63}Ni method (6). As illustrated in **Fig. 2**, the resulting values (total nmol FFA/sample) closely correlated with the FFA content of these samples as calculated by proton release. The solid line indicates a linear regression slope of 1.027, which is statistically not significantly different from the expected slope of unity. **Fig. 3** illustrates an experiment in which epinephrine-stimulated release of H^+, FFA, and glycerol were simultaneously monitored. In this example, H^+ production correlated exactly with the values of medium FFA obtained by the ^{63}Ni assay. Glycerol release, another indicator of lipolysis, paralleled the accumulation of FFA in the medium. In other experiments, we found that dibutyryl cyclic AMP-stimulated lipolysis was also associated with a H^+:FFA ratio of 1.0, which ruled out the butyrate ion as a contributing factor in the greater rate of H^+ efflux.

Acidosis has been found to inhibit catecholamine-stimulated lipolysis in vivo (9) and in vitro (10, 11). In **Fig. 4**, we have confirmed these results by following pH changes in the extracellular fluid. Furthermore, the 50% reduction in FFA release at pH 6.8 is immediately reversible, with no detectable time lag, upon increase of the pH to 7.4. Thus, a shift from pH 6.75 to 7.39 raised the rate of FFA

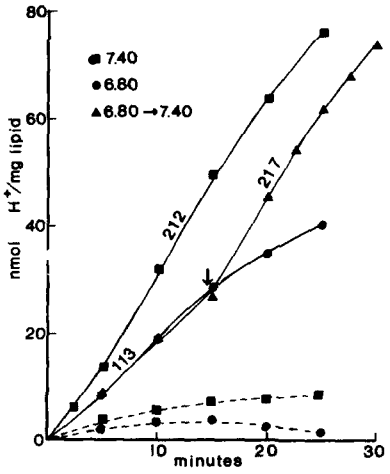


Fig. 4. Effect of pH on H^+ release in adipocytes. Cells (39.2 mg lipid) from 130-g rats were added to Krebs-Ringer phosphate medium, pH 7.40 or 6.80. After 10 min, 1 μg of epinephrine was added. At the arrow, the pH of the medium was raised from 6.754 to 7.387 by addition of NaOH. Solid lines are cells incubated with epinephrine; dotted lines are controls.

release from 113 to 217 nmol $H^+ \cdot$mg lipid$^{-1} \cdot$ hr^{-1}, which is identical to the rate of H^+ release in cells maintained at pH 7.4.

Discussion

The stoichiometric release of protons and FFA during activated triglyceride breakdown in adipocytes provides a convenient method to measure lipid mobilization by monitoring changes in medium pH. In addition, the system can be perturbed multiple times by addition of α- and β-adrenergic agonists, antagonists, or by antilipolytic drugs, and initial changes in the rate of appearance of protons (FFA) can be determined. The well-known variation in the FFA/glycerol ratio, which is dependent on the extent of FFA re-esterification, limits the method to providing an index of FFA release and not of total lipolytic activity. Nonetheless, under the conditions described here, as well as in the presence of insulin and glucose[1], H^+ and FFA appearance remain stoichiometric. The proposed role of FFA as a feedback regulator of lipolysis (12) lends importance to the determination of this parameter.

To the extent that pH can be used to measure FFA efflux, the data confirm the experiment of Rudman and Shank (3), who followed pH changes in the extracellular medium of perirenal and epididymal adipose tissue slices at half-hour intervals over a 3 hr period. By using suspensions of adipocytes, we have been able to measure rates of FFA release that are 30–60 times greater than were obtained in tissue slices, and to do so in a shorter time period.

The mechanism by which acidosis depresses lipolysis has been discussed (11) and is thought to be related to cyclic AMP and adenosine levels, although as yet no causal relationship has been established. Any theory will have to take into consideration the observation reported here that FFA release is immediately affected by pH, presumably before changes in these cellular metabolites can occur. Certainly, large pH changes in the circulation do not occur in vivo, under physiological conditions, although it remains possible that significant H^+ is released into the microcirculation of the fat organ to affect lipolysis.

This research was supported by grants from the Cleveland Diabetes Foundation, N.E. Ohio Heart Association, and N.I.H. HL 18887.

Manuscript received 27 January 1977 and accepted 21 April 1977.

[1] Meisner, H. Unpublished data.

REFERENCES

1. Rudman, D., and P. W. Shank. 1966. Observations on the production of hydrogen ions during mobilization of fatty acids from adipose tissue. *Endocrinology.* **79:** 1100–1108.
2. Sallee, V., and J. Dietschy. 1973. Determinants of intestinal mucosal uptake of short and medium chain fatty acids and alcohols. *J. Lipid Res.* **14:** 475–484.
3. Campbell, J., A. Martucci, and G. Green. 1964. Plasma albumin as an acceptor of free fatty acids. *Biochem. J.* **93:** 183–189.
4. Rodbell, M. 1964. Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. *J. Biol. Chem.* **239:** 375–380.
5. Gliemann, J. 1965. Insulin-like activity of dilute human serum assayed by an isolated adipose cell method. *Diabetes.* **14:** 643–649.
6. Ho, R. J. 1970. Radiochemical assay of long-chain fatty acids using ^{63}Ni as tracer. *Anal. Biochem.* **36:** 105–113.
7. Wieland, O. 1957. Eine Enzymatische Methode zur Bestimmung von Glycerin. *Biochem. Z.* **329:** 313–319.
8. Rodbell, M. 1965. Modulation of lipolysis in adipose tissue by fatty acid concentration in fat cell. *Ann. N.Y. Acad. Sci.* **131:** 302–314.
9. Hjemdahl, B., and P. Fredholm. 1974. Comparison of the lipolytic activity of circulating and locally released noradrenaline during acidosis. *Acta Physiol. Scand.* **92:** 1–11.
10. Poyart, C., and G. Nahas. 1968. Inhibition of activated lipolysis by acidosis. *Mol. Pharmacol.* **4:** 389–410.
11. Hjemdahl, P., and B. Fredholm. 1976. Cyclic AMP-dependent and independent inhibition of lipolysis by adenosine and decreased pH. *Acta Physiol. Scand.* **96:** 170–179.
12. Fain, J., and R. Shepherd. 1975. Free fatty acids as feedback regulators of adenylate cyclase and cyclic 3′:5′-AMP accumulation in rat fat cells. *J. Biol. Chem.* **250:** 6586–6592.