papers and notes on methodology

Sensitive kinetic bioluminescent assay of glycerol release from human fat cells

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Abstract A sensitive and accurate assay of lipolysis has been developed, measuring the rate of glycerol release from fat cells with a bioluminescent assay. The rate of glycerol-dependent ATP-consumption in a system consisting of glycerokinase, ATP, luciferin, and luciferase was determined kinetically as a decrease of the ATP-induced luminescence and used for calculation of the concentration of glycerol. Under the conditions employed, it was possible to measure the concentration of glycerol down to a level of about 0.5 μmol/l. Under the same conditions, the detection limit of the usual fluorometric method was about 15 μmol/l. The coefficient of variation obtained with the bioluminescent assay was 11% at a level of 1.0 μmol of glycerol, 2–6% at a level of about 5 μmol/l, and 1–3% at a level of about 20 μmol/l. Satisfactory results were obtained in different recovery experiments. Using human fat cells, it was possible to determine the rate of glycerol release with a cell concentration in the medium of only 5,000–10,000 cells/ml. It is concluded that the bioluminescent assay of glycerol release should be preferred when there is a demand for sensitivity, e.g., when the rate of lipolysis is low and when only a small amount of biopsy material is available.—**Björkhem, I., P. Arner, A. Thore, and J. Östman.** Sensitive kinetic bioluminescent assay of glycerol release from human fat cells. *J. Lipid Res.* 1981. **22:** 1142–1147.

Supplementary key words glycerokinase · ATP · luciferin

The rate of glycerol release from adipose tissue is generally used as an index of the rate of lipolysis in vitro (1, 2). Such studies have been greatly facilitated by the development of enzymatic-fluorometric assays for glycerol (3). In human adipose tissue, however, the rate of glycerol release is low; values reported for tissue segments or isolated adipocytes in vitro are in the range 0.3–1.0 μmol/hr per g (basal) and 2–6 μmol/hr per g (maximally stimulated) (1, 2). In the enzymatic-fluorometric assay, relatively large amounts of tissue (50–100 mg/assay) must be used for accurate determination of the rate of glycerol release.

Thus, it is evident that there is a need for a more sensitive assay. The present report describes a simple glycerol assay based on bioluminescence, which is more than one order of magnitude more sensitive than the fluorometric method, and which should allow for assay of lipolytic activity in microsamples of human fat tissue.

METHODS

Materials

A crystalline suspension of glycerokinase (isolated from *Candida mycoderma* (sp act 89 U/mg protein) was obtained from Sigma (St Louis, MO). It was necessary to purify the preparation prior to use (cf. Results). The material was chromatographed on a column (50 × 1 cm) of Sephadex G-50, using Tris-Cl^- buffer (0.5 mol/l, pH 7.4) containing 3 mmol/l of dithiothreitol as eluant. Under the conditions employed, this chromatography essentially corresponded to a desalting, and the protein and the activity are eluted together in a single peak. The peak fractions were combined and immediately frozen in aliquots at −20°C. Aliquots of the purified preparation were thawed prior to use.

A bioluminescent ATP-monitoring kit (ATP Monitoring Reagent, 1259-120) containing firefly luciferase, luciferin, bovine serum albumin (50 mg), and magnesium acetate (0.5 mmol) was obtained from

LKB (LKB-Wallac Oy, Turku, Finland). The lyophilized material was reconstituted with 10 ml of redistilled water prior to use. According to the manufacturer, 0.2 ml of the reconstituted solution is suitable for assay of ATP in a total volume of 1 ml.

All other reagents and solvents were of analytical grade.

Human subcutaneous tissue was obtained from four different patients undergoing routine abdominal surgery. They fasted overnight and only saline was given intravenously prior to removal of the tissue samples, which was done at the start of the operation. Anesthesia was induced by a short-acting barbiturate and maintained by fentanyl. The study was approved by the Ethical Committee of the Karolinska Institutet. Informed consent was obtained from the patients.

Assay of glycerol by bioluminescence

Under standard conditions, 0.6 ml of Tris-Cl^- buffer (0.1 mol/l, pH 8.0), 0.2 ml of the reconstituted ATP-monitoring reagent, 10 μl of an aqueous solution containing 10^{-5} mol/l ATP, and 20 μl of a solution containing about 20 μg of purified glycerokinase were mixed carefully and the slightly declining level of bioluminescence was followed for about 90 sec using an LKB-Wallac Luminometer 1250 (Wallac Oy, Turku, Finland). A chart speed of 10 mm/min and a sensitivity of 20 mV was used in the recording. The analyte was then added in 200 μl of Tris-Cl buffer (0.1 mol/l, pH 8.0) or in 200 μl of incubation medium, and the ATP-signal was followed for an additional period of 90 sec. The slope of the tracing obtained in the presence of the analyte was measured manually with the use of a ruler, and was corrected for the lowering in intensity of the ATP-signal caused by the incubation medium containing the analyte (cf. Results). Under the conditions employed, the error due to the manual measurement of the slope was calculated to be below 2%. The slope obtained in the absence of the analyte was then subtracted from the corrected slope obtained in the presence of the analyte, and the resulting value was used for the calculation of the glycerol concentration with the use of a standard curve. In the analysis of incubations, standard mixtures of glycerol were always prepared in the same medium as that used in the incubations.

Assay of glycerol by fluorometry

Glycerol was assayed in 200 μl of the incubation medium by the method described by Wieland (4) and modified by Chernick (3), using an Aminco fluorometer (Silver Spring, MD).

Incubations with fat cells

Fat cells were isolated from adipose tissue according to the method of Rodbell (5) as modified by Smith, Sjöström, and Björntorp (6) and incubated for 2 hr in 1 ml of Krebs-Henseleit bicarbonate buffer (pH 7.4, 37°C), containing albumin (40 mg/ml) and glucose (1 mg/ml). The number of fat cells incubated was estimated by dividing the total lipid weight of the sample by the average cellular lipid weight. The latter was determined according to the method of Hirsch and Gallian (7). The incubations were run in quadruplicate. In the experiment shown in Table 1, air was used as the gas phase. In the experiments shown in Fig. 6, 95% O_2, 5% CO_2 was used as the gas phase. In separate experiments it was observed that, under these conditions, the rate of lipolysis is linear with time for at least 4 hr. After incubation, aliquots of the medium were removed, diluted 1:1 with distilled water, and treated at 100°C for 3 min prior to analysis by fluorometry and bioluminescence.

RESULTS

Fig. 1 demonstrates the principles of the assay. Luciferin, luciferase, and ATP gave a signal that decreased slightly in intensity with time. Addition of glycerokinase slightly increased, and further addition of glycerol markedly increased the rate of decrease of the bioluminescence intensity.

Additional ATP added at the end of the experiment resulted in the same response as obtained with the ATP initially present (Fig. 1). Therefore, the decrease

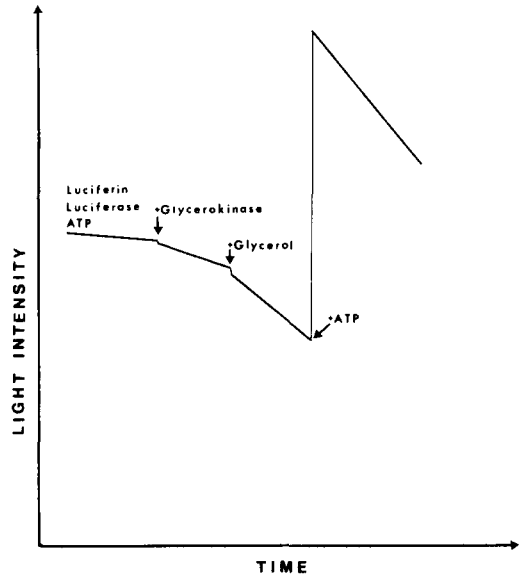

Fig. 1. Principle for assay of glycerol by bioluminescence. The amount of ATP added in the end of the experiment was the same as that initially present, (10^{-10} mol).

in signal was not due to loss of reactivity of luciferase towards ATP but to disappearance of ATP.

The luminescence signal has been shown to be proportional to ATP concentration in the interval 10^{-12}–10^{-6} mol/l (8). Therefore, the rate of decrease of luminescence can be converted to the rate of hydrolysis of ATP, using for calibration the luminescent response to the ATP initially added.

In all following experiments, luciferin, luciferase, ATP, and glycerokinase were present at the beginning of the experiment. The glycerol concentration was calculated from the slope of the tracings obtained in the presence and absence of the analyte.

Effect of added buffer

Addition of buffer or incubation medium in volumes above 25 μl significantly decreased the intensity of the ATP signal (**Fig. 2**). Addition of 500 μl of the reaction mixture decreased the ATP signal to about 50%, presumably due to an inhibiting effect of substances in the medium on the luciferase reaction. Similar effects have been demonstrated with numerous substances, e.g., salts, chaotropic ions, organic solvents, protein (9). To compensate for the decreased luminescence-ATP ratio, all values were corrected by use of a factor corresponding to the ratio between the intensity of the ATP signal prior to and after addition of the buffer or sample (a/b in Fig. 2). Thus the initial ATP response could be used to calculate reaction rates.

The above compensation was used in all experiments. In all subsequent experiments, a volume of buffer or incubation medium of 200 μl was used.

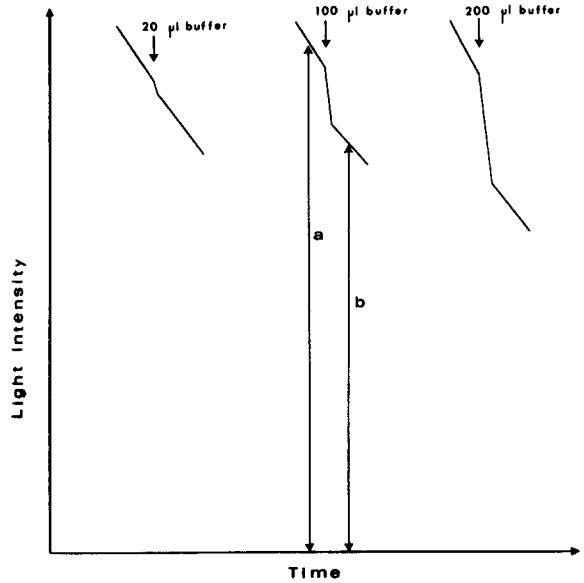

Fig. 2. Effect of buffer on ATP-signal. Standard conditions were used, with the exception that glycerol (corresponding to 1 nmol) was present in the reaction vessel prior to addition of buffer.

Effect of glycerokinase

The rate of hydrolysis of ATP was found to be linear with the amount of glycerokinase added (**Fig. 3 A**). The sensitivity of the assay increased with increasing amounts of added glycerokinase up to about 40 μg. Due to the presence of ATP-consuming components in the enzyme preparation, no further gain in sensitivity was achieved with more than 20 μg of glycerokinase. With this amount of enzyme, the rate of background ATP-hydrolysis was more than twice that obtained in the presence of luciferase reagent and ATP alone.

Attempts to increase the sensitivity of the assay by further purification of the glycerokinase failed. Direct use of commercial glycerokinase without any purification step (cf. Experimental) decreased the sensitivity by at least one order of magnitude due to the high background level of ATP hydrolysis.

In the following experiments, 10 or 20 μg of purified glycerokinase was used. The purified glycerokinase was stored for a maximum of 5 weeks in aliquots at −20°C; they were thawed immediately prior to use.

Effect of ATP

The rate of ATP hydrolysis was linear with the concentration of ATP in the investigated range of 10^{-8}–10^{-7} mol/l. In all subsequent experiments a concentration of 10^{-7} mol/l was used (Fig. 3B).

Effect of pH

As shown in **Fig. 4,** the rate of ATP hydrolysis had a broad pH optimum extending to about pH 8.5, whereas the bioluminescent ATP response decreased at pH values above pH 8.1. Occasionally, with aged preparations of glycerokinase, slightly aberrant pH activity profiles could be observed. A pH of 8.0 was used in all subsequent experiments.

Effect of glycerol

Under optimal conditions, at pH 8.0, the rate of hydrolysis of ATP was linear with the concentration of glycerol (concentration of glycerol in added solution) in the range 0.6–5 μmol/l (**Fig. 5**). The sensitivity of the assay did not increase with higher pH. When working with glycerol concentrations in the range 5–50 μmol/l, it was practical either to dilute the solution ten-fold prior to assay, or decrease the added volume to 20 μl instead of 200 μl.

Precision of the assay

The coefficient of variation as determined by five

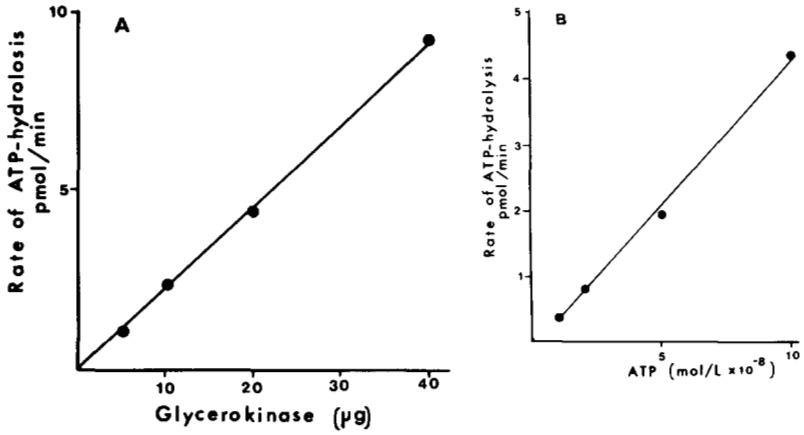

Fig. 3. Effect of glycerokinase (A), ATP (B). Except for the varied parameter, standard conditions were used in each experiment.

replicate measurements was 11% at a level of 1 µmol/l of glycerol, 2–6% at a level of about 5 µmol/l, and 1–3% at a level of about 20 µmol/l.

Recovery experiments

The possibility that compounds transferred from the fat cells into the medium during the incubation could interfere with the assay was excluded by addition of known amounts of glycerol to the incubation medium after the incubations and subsequent assay of glycerol.

In one such experiment, the glycerol concentration of the incubated medium was estimated to be 4.9 ± 0.1 µmol/l (mean ± S.E.M., n = 4). After addition of glycerol in an amount corresponding to 17 µmol/l, the glycerol concentration of the medium was found to be 22.7 ± 0.2 µmol/l. The difference between expected and found value was only 4%.

In another experiment, the glycerol concentration of the incubated medium was estimated to be 6.3 ± 0.1 µmol/l. After addition of glycerol in an amount corresponding to 2.5 µmol/l, the glycerol concentration of the medium was found to be 9.1 ± 0.1 µmol/l. The difference between expected and found values was only 3%.

Comparison with fluorometric assay

Human fat cells in different concentrations (0.5–20% in incubation medium, corresponding to 6–224 $\times 10^3$ cells/ml) were incubated under the conditions described in Methods. The incubation medium was removed after the incubations, diluted with water, boiled, and assayed for glycerol with the bioluminescent technique as well as with the fluorometric technique. The results are summarized in **Table 1.**

It should be mentioned that the variation in the

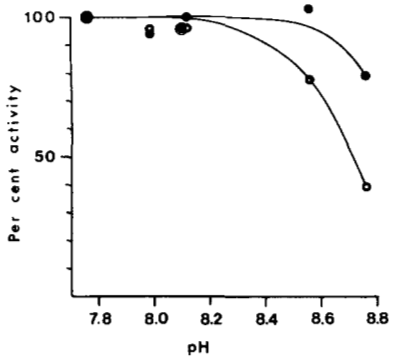

Fig. 4. Effect of pH on glycerokinase (●) and luciferase (○) activity. Standard conditions were used, except that pH of the 0.1 mol/l Tris-Cl-buffer was varied. Luciferase activity was defined as the luminescent response to 10^{-7} mol/l of ATP. Activities at pH 7.75 were arbitrarily set to 100%.

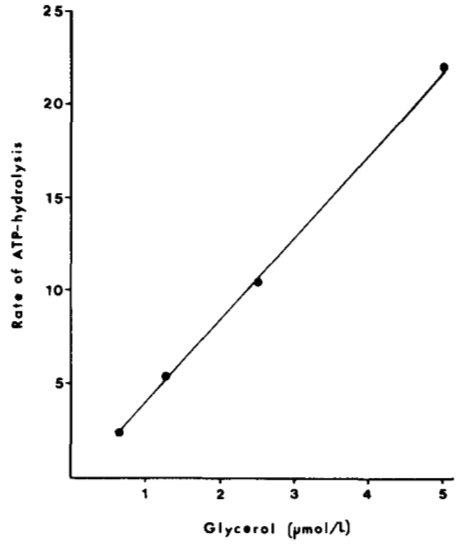

Fig. 5. Effect of glycerol concentration on ATP-hydrolysis. Standard conditions were used.

TABLE 1. Comparison of fluorometric and bioluminescent methods for glycerol determinations

Concentration of Cells in Incubation Medium	Fluorometric Assay	Bioluminescent Assay
	μmol/l	*μmol/l*
20%, Basal	15 ± 0.5	17 ± 0.2
20% + Noradrenaline	53 ± 2	49 ± 2
10%, Basal	BD[a]	8.0 ± 0.2
10% + Noradrenaline	45 ± 3	31 ± 2
5%, Basal	BD	3.5 ± 0.5
5% + Noradrenaline	20 ± 2	16 ± 1
2.5%, Basal	BD	2.0 ± 0.4
2.5% + Noradrenaline	BD	8.1 ± 0.0
1%, Basal	BD	0.8 ± 0.0
1% + Noradrenaline	BD	2.2 ± 0.0
0.5%, Basal	BD	0.3 ± 0.1
0.5% + Noradrenaline	BD	0.7 ± 0.1

[a] BD, below detection unit.

Human fat cells were isolated and incubated according to the procedure described in Methods. The preparation containing 1% cells in the medium corresponded to 11×10^3 cells/ml. The incubated preparations were diluted 1:1 with water, boiled, and assayed for glycerol with the fluorometric and bioluminescent techniques, respectively. The values given represent the mean ± SEM of four independent replicates. When added, 10^{-5} mol/l of noradrenaline was used. In the case of fluorometric measurements, only values above the detection limit (15 μmol/l) are shown.

fluorometric assay is due mainly to variations obtained in the blank reading, prior to addition of the glycerokinase. From the variations in the blank reading, it was calculated that the detection limit of the method, within a 95% confidence interval, should be 15 μmol/l. From this point of view, reliable results with the fluorometric technique would only be expected in experiments with >20% fat cells. In Table 1, only values above 15 μmol/l obtained with the fluorometric method are shown. Similar values were obtained by the two methods.

With the bioluminescent assay, the basal release of glycerol was approximately linear with the concentration of fat cells in the whole range studied (Table 1, **Fig. 6**). Addition of noradrenaline or isoprenaline increased the release of glycerol two- to four-fold.

DISCUSSION

In a recent study, a bioluminescent assay of glycerol in plasma triglycerides was presented (10). In that study, remaining ATP was assayed by bioluminescence after completion of the glycerokinase reaction. Such an endpoint analysis should be considerably less sensitive than the rate-analysis used here, which is made possible by the low rate of spontaneous decline of bioluminescence obtained with purified luciferase preparations (8). The concentration of glycerol in plasma triglycerides is about three orders of magnitude higher than the concentration of glycerol assayed in the present work.

Under conditions employed, the sensitivity of the present bioluminescent assay was limited by the presence of small amounts of ATP-consuming components in the purified glycerokinase fraction (possibly glycerol or ATP-hydrolyzing enzymes). If it had been possible to remove this interference, a somewhat higher sensitivity might have been achieved by a further increase in the amount of glycerokinase. It should be pointed out however, that the lowest concentration of glycerol possible to assay with the bioluminescent technique, about 0.5 μmol/l, is more than two orders of magnitude lower than the K_m of the glycerokinase, which was found to be about 90 μmol/l under the specific conditions employed. It cannot be excluded that much lower levels of substrate would lead to analytical problems, related to removal of substrate by adsorption, activation of enzyme, etc.

Furthermore, the low rate of hydrolysis of ATP in the absence of either glycerokinase or glycerol is comparable to the increase of rate obtained with glycerokinase and 5 μmol/l of glycerol. Statistical variation of this background slope would not allow accurate determinations of glycerol concentrations below 0.5 μmol/l even with more highly purified glycerokinase unless higher levels of this enzyme were used.

The bioluminescent assay was found to be more than one magnitude more sensitive than the fluorometric assay. With the present volume of 1 ml of incubation medium, the number of fat cells required for assay of lipolysis can thus be reduced at least by a factor of 10–20. However, since the volume of the incubation medium can be reduced simultaneously

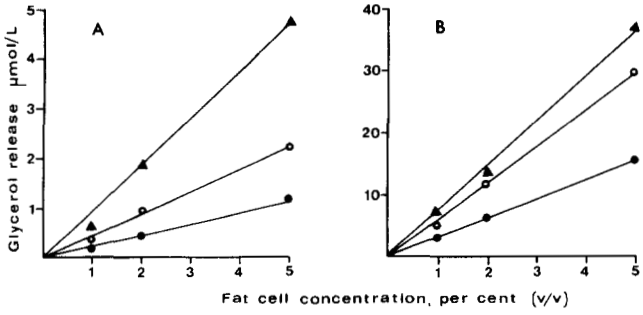

Fig. 6. Effect of fat cell concentration on glycerol release in different samples of fat. Human fat cells were isolated from three different subjects and incubated in the absence (A) or presence (B) of isoprenaline. With the exception that 95% O_2, 5% CO_2 was used as gas phase, the same conditions were used as in the experiment shown in Table 1.

with reduction in the number of cells, it should be possible to decrease the amount of cells even further by analyzing a larger fraction of the sample. At the lowest concentration possible to assay, about 0.5 μmol/l, the amount of glycerol present in the reaction vessel is in fact only 100 pmol. Since the total release of glycerol from 1 mg of human adipose tissue should be about 1,000 pmol during 2 hr of incubation (basal release (1, 2)), it should in fact be possible to assay lipolysis in samples corresponding to less than 1 mg.

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REFERENCES

1. Björntorp, P., and J. Östman. 1971. Human adipose tissue dynamics and regulation. *Adv. Metab. Disord.* **5:** 277–327.
2. Hales, C. N., H. P. Luzio, and K. Siddle. 1979. Hormonal control of adipose-tissue lipolysis. *Biochem. Soc. Symp.* **43:** 97–135.
3. Chernick, S. S. 1969. Determination of glycerol in acyl glycerols. *Methods Enzymol.* **14:** 627–360.
4. Wieland, O. 1957. Eine enzymatische Metode zur Bestimmung von Glycerin. *Biochem. Z.* **329:** 313.
5. Rodbell, M. 1964. Metabolism in isolated fat cells. 1. Effects of hormones on glucose metabolism and lipolysis. *J. Biol. Chem.* **239:** 375–380.
6. Smith, U., L. Sjöström, and P. Björntorp. 1972. Comparison of two methods for determining human adipose cell sizes. *J. Lipid Res.* **13:** 822–824.
7. Hirsch, J., and E. Gallian. 1968. Methods for the determination of adipose cell size and cell number in man and animals. *J. Lipid Res.* **9:** 110–119.
8. Lundin, A., A. Rickardsson, and A. Thore. 1976. Continuous monitoring of ATP-converting reactions by purified firefly luciferase. *Anal. Biochem.* **75:** 611–629.
9. Thore, A. 1979. Technical aspects of the bioluminescence firefly luciferase assay of ATP. *Sci. Tools.* **26:** 30–34.
10. Hercules, D. M., and T. L. Sheehan. 1978. Chemiluminescent determination of serum glycerol and triglycerides. *Anal. Chem.* **50:** 22–25.