

Radioisotopic method for the measurement of lipolysis in small samples of human adipose tissue

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Abstract To facilitate the study of adrenoreceptor response in small needle biopsy samples of human subcutaneous adipose tissue, we developed a dual radioisotopic technique for measuring lipolysis rate. Aliquots (20–75 mg) of adipose tissue fragments were incubated in a buffered albumin medium containing [³H]palmitate and [¹⁴C]glucose, each of high specific activity. In neutral glycerides synthesized in this system, [¹⁴C]glucose is incorporated exclusively into the glyceride-glycerol moiety and ³H appears solely in the esterified fatty acids. Alpha-2 and beta-1 adrenoreceptor activation of tissue incubated in this system does not alter rates of ¹⁴C-labeled glyceride accumulation, but does produce a respective increase or decrease in the specific activity of fatty acids esterified into newly synthesized glycerides. This alteration in esterified fatty acid specific activity is reflected in the ratio of ¹⁴C:³H in newly synthesized triglycerides extracted from the incubated adipose tissue. There is a high correlation ($r = 0.90$) between the ¹⁴C:³H ratio in triglycerides and the rate of lipolysis as reflected in glycerol release into the incubation medium. The degree of adrenoreceptor activation by various concentrations of lipolytic and anti-lipolytic substances can be assessed by comparing this ratio in stimulated tissue to that characterizing unstimulated tissue or the incubation medium. This technique permits the study of very small, unweighed tissue biopsy fragments, the only limitation on sensitivity being the specific activity of the medium glucose and palmitate. It is, therefore, useful for serial examinations of adipose tissue adrenoreceptor dose-response characteristics under a variety of clinical circumstances.—Leibel, R. L., J. Hirsch, E. M. Berry, and R. K. Gruen. Radioisotopic method for the measurement of lipolysis in small samples of human adipose tissue. *J. Lipid Res.* 1984. 25: 49–57.

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Human adipocyte plasma membranes contain both alpha and beta type adrenoreceptors which are important in regulating lipolysis. Activation of beta receptors increases lipolysis by stimulating the adenylate cyclase–cyclic AMP cascade, while alpha-2 receptor activation inhibits lipolysis by reducing levels of adenylate cyclase in the membrane (1). The physiologic balance of these opposing receptors is dependent upon anatomical site, endocrine, autonomic, and nutritional status (2). Functional studies of this receptor system in human adipose tissue have used measures of in vitro glycerol release, adenylate cyclase,

and cyclic AMP accumulation, and direct ligand binding as end points (3). However, cellular levels of cyclic AMP, or the number of various types of membrane adrenoreceptors, do not necessarily reflect changes in the actual rates of lipolysis (4–8). Additionally, these analytic techniques require relatively large amounts of tissue and/or the preparation of membrane fractions or cell suspensions from collagenase digests of adipose tissue. Requirement for large amounts of tissue limits the number of separate assays that can be performed on a single tissue sample, and often necessitates the use of tissue obtained during surgery rather than under more physiologic circumstances. In this paper, we describe a radioisotopic method for the in vitro determination of lipolysis rate in very small fragments of human adipose tissue obtained by needle aspiration. The method permits a quantitative functional assessment of adrenoreceptor status in such fragments which can be obtained from any subcutaneous site (5), repeatedly, during the course of study.

METHODS

A. Experimental subjects

Six obese (five females, one male) and six never-obese, normal weight (two females, four males) subjects had gluteal adipose tissue aspirations performed as described in section D. This tissue was incubated in the dual isotope medium (section D) to which various adrenoreceptor-active agents (section F) had been added. For an analysis of the effects of certain media types, two of the obese subjects were studied twice. There are, therefore, a total of eight studies on six subjects for these media. All subjects were studied when weight-stable on either an ad libitum

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solid food diet or a prescribed liquid formula diet (40% carbohydrate, 40% fat, 20% protein). In addition, three of the obese subjects were studied a total of eight times after fasting for 1 week or taking 600 kcal/day of a formula feeding for 1–2 weeks ("semi-fasted"). During weight maintenance and weight loss, all obese subjects were given 2 g of NaCl and 75 meq K⁺ daily. No subject was diabetic or taking any drug known to alter adipose tissue metabolism. Informed consent was obtained from all subjects and all procedures were approved by the Rockefeller University Institutional Review Board.

B. Media preparation

Isotopic Medium. Labeled [9,10-³H(N)]palmitic acid (NET 043, New England Nuclear, Boston, MA) of specific activity approximately 17 Ci/mol was repurified in an aqueous ethanol-heptane system by alkalization, repeat extraction, acidification, and heptane extraction. A thin film of approximately 6 Ci/mol was prepared by evaporation of a twofold molar excess of ethanolic sodium hydroxide with labeled and unlabeled palmitic acid (Applied Science Laboratories Inc., State College, PA) in a rotary evaporator at 70°C. To this film, a solution (8%) of dialyzed, bovine fatty acid-poor albumin (Fraction V, Accurate Chemical and Scientific Corporation, Westbury, NY) at pH 7.4 was added to produce a final palmitate concentration of 1.0 mM. The molar ratio of fatty acid to albumin was approximately 1:1. The solution was filtered and then stored at -20°C in aliquots of convenient size. Incubation medium (pH 7.4) was prepared by mixing equal volumes of the albumin-palmitate solution with double strength Krebs-Henseleit original Ringer bicarbonate buffer (gassed with 95% O₂ and 5% CO₂) and glucose to produce glucose, palmitate, calcium, and albumin concentrations of 4.16 mM, 0.5 mM, 2.5 mM and 4%, respectively. Sufficient [¹⁴C]glucose (D-[¹⁴C(u)]-glucose, 1–5 Ci/mol; NEC 042, New England Nuclear) in water was added to give a glucose specific activity of approximately 2 Ci/mol. The addition of labeled glucose raised the final glucose concentration in the medium to 5–6 mM. The exact specific activities of palmitate and glucose in the media of each preparation were determined at the beginning and end of each tissue incubation, by methods described below.

Preincubation medium. The 8% solution of dialyzed bovine fatty acid-poor albumin was mixed with an equal volume of double strength Krebs-Henseleit original Ringer bicarbonate buffer and glucose added to give a final concentration of 4.16 mM at pH 7.4. No radioisotopes were present.

C. Adrenoreceptor agonists and antagonists

Epinephrine, a mixed adrenoreceptor agonist, activates both alpha and beta adrenoreceptors in human adipose

tissue. Maximal beta-1 (lipolytic) effect of epinephrine is seen when the alpha receptors are blocked with an alpha antagonist such as phentolamine. Conversely, alpha-2 receptor activation (resulting in anti-lipolysis) is best demonstrated when beta receptors are blocked with a beta antagonist such as propranolol. These, and other compounds as noted below, were added to the incubation medium to stimulate alpha or beta adrenoreceptor activity in incubated adipose tissue fragments: L-epinephrine d-bitartrate (Sigma Chemical, St. Louis, MO); propranolol HCl (gift of Ayerst Laboratories, New York, NY); phentolamine HCl (gift of Ciba Pharmaceutical Company, Summit, NJ); L-isoproterenol d-bitartrate (gift of Sterling Winthrop Research Institute, Rensselaer, NY); clonidine HCl (gift of Boehringer Ingelheim Ltd., Pearl River, NY). Solutions of these compounds in 0.9% saline were prepared fresh on the day of an experiment and added to incubation media to give final concentrations of 3.5×10^{-4} M (propranolol, phentolamine) or 3.5×10^{-5} M (epinephrine, isoproterenol) as described below. These concentrations are similar to those reported by Burns et al. (9) for optimal alpha and beta adrenoreceptor responses.

D. Tissue sampling and incubation procedure

With subjects under local anesthesia with 1% xylocaine, 100–1000 mg of subcutaneous adipose tissue was aspirated through a 14-gauge needle attached to a 50-ml syringe (10). The aspiration procedure took less than 5 min. The amount of tissue obtained varied depending upon experimental needs. No adverse effects other than minimal bruising were noted. The tissue was transported in a thermos containing 0.9% saline at 37°C and copiously washed with warm saline over a nylon filter within 5 min of being aspirated. The duration of tissue exposure to local anesthetic was kept below that shown to influence glyceride metabolism and lipolysis (11).

After rinsing with 0.9% saline at 37°C, the tissue was placed in 20 ml of preincubation medium (section B) in a 50-ml polypropylene vial (gas phase 95% O₂/5% CO₂). The vial was shaken gently in a water bath at 37°C for 30 min. Following this preincubation, the tissue was again gently washed with 0.9% saline at 37°C over a nylon mesh and 50–75 mg aliquots were then placed in separate 15-ml polypropylene vials containing 3 ml of isotopic incubation medium (section B) and any added adrenoreceptor agonists/antagonists. Final media volume was 3.10 ml, and the gas phase was 95% O₂/5% CO₂. Tissue samples were incubated in triplicate in separate flasks for each media preparation and results for each triplicate were averaged. The tissue was incubated for 2 hr in a water bath at 37°C with brisk shaking to ensure rapid and complete equilibration with the medium of any fatty acids or glycerol released by the tissue. After washing on

a nylon mesh with distilled water to remove any adherent medium, the tissue was immediately placed in 15 ml of chloroform-methanol 2:1 (v:v) (freshly redistilled solvents) for extraction of lipids (12). One-ml aliquots of media were frozen for later determination of glycerol content on a Centrifchem 400 Autoanalyzer by the enzymatic method of Eggstein and Kreutz as modified by Bucolo and David (13). One ml of media was immediately extracted for free fatty acids by the method of Dole and Meinertz (14) and used to monitor changes in media fatty acid specific activity due to the release of fatty acids by the incubated tissue. Fatty acid concentration was determined either by the titration method of Dole and Meinertz (14) or by a modification of the radioisotopic method of Ho (15) which included a ^{63}Ni -free control tube, allowing for subtraction from total heptane phase radioactivity of those counts contributed by ^3H . Because of the large volume of incubation medium relative to tissue aliquot size, the release of free fatty acids had negligible effects on the media specific activity of free fatty acids during the course of an incubation. Thus, in the comparison of glycerol release and dual isotope techniques reported here, no correction is made for small shifts in media specific activity over the 2-hr incubation period.

In pulse-chase experiments in which adipose tissue acylglycerides were prelabeled with [^3H]palmitate and ^{14}C -labeled glyceride-glycerol, the beta adrenergic stimulation of lipolysis *a*) did not alter the $^{14}\text{C}/^3\text{H}$ ratio in triglycerides formed over the ensuing 2 hr and *b*) released free fatty acids of specific activity two orders of magnitude below that of [^3H]palmitate in the labeling medium. Thus, recycling of [^3H] palmitate within the adipocyte or in the relatively large volume of incubation medium was negligible.

Glyceride synthesis and glycerol release were linear over the 2 hr of incubation in dual isotopic medium.

E. Tissue extraction and chemical analysis

Tissue aliquots were extracted in 15 ml of chloroform-methanol 2:1 at 5°C for 48 hr. The solvent mixture was then layered gently with distilled-demineralized water to generate a 10-ml lower phase of chloroform containing tissue lipids. Methanolysis or saponification of newly synthesized glycerides (from both unstimulated and beta adrenoceptor-stimulated tissue) demonstrated that over 95% of ^3H counts and ^{14}C counts were in the fatty acid and glycerol moieties of the molecules, respectively. An aliquot (0.5 ml) of the chloroform phase of the tissue extract was dried. A standard mixture containing a total of 2.5 mg with equal weights of monoglyceride, free fatty acid, 1,2 diglyceride, 1,3 diglyceride standards was added (Applied Science Laboratories Inc.). The residue was redissolved in 0.5 ml of chloroform and duplicate 100- μl aliquots were placed 2 cm apart on aluminum-backed 200

$\times 20 \times 0.2$ mm precoated silica gel chromatography plates (E.M. Reagents. Cat. 5539 60F 254. MC/B Manufacturing Chemists, Inc., Cincinnati, OH). The plates were developed in chloroform-methanol-glacial acetic acid 100:2:1.5 (v:v:v). Tri-, di-, and mono-glycerides as well as free fatty acid regions were identified under ultraviolet light after spraying the plate with 0.125% Rhodamine B in ethanol (Sigma Chemical Co.). Specific regions, corresponding to each standard and the origin spots, were then separately removed with a suction funnel (16), eluted with diethyl ether and ^3H and ^{14}C dpm were determined by counting in 10 ml of Ultrafluor (National Diagnostics, Somerville, NJ) in a Packard Model 300 scintillation counter equipped to correct cpm and generate ^3H and ^{14}C dpm's from the degree of quenching of an external standard. The efficiencies of ^3H and ^{14}C counting were 45% and 67%, respectively. Scintillation counting was continued until at least 1000 counts of ^{14}C were accumulated. Approximately 95% of the radioactivity applied to the plates was recovered in the six areas scraped and assayed. Under most circumstances, 60–75% of the radioactivity was in triglycerides and 25–30% in diglycerides. The combined triglyceride regions contained, on average, 2500 cpm ^3H and 200 cpm ^{14}C . Cholesteryl esters, which were at the solvent front in this system, were not labeled to any detectable amount in these tissues. A cholesterol standard was found to co-migrate with diglycerides in this solvent system. However, saponification and heptane extraction of the isotopically labeled diglyceride regions failed to demonstrate the presence of any ^3H label in cholesterol in extracts of tissue incubated for up to three hours in the dual isotope medium described. Thus, virtually all of the tissue extract ^3H and ^{14}C were in the fatty acid and glyceride-glycerol moieties, respectively.

F. Incubations in medium containing $^3\text{H}_2\text{O}$ and [^{14}C]glucose

Cytoplasmic water provides protons for the synthesis of glycerolphosphate from glucose. If adipocyte cytoplasmic water has a specific activity equal to that of tritium-labeled medium water, each molecule of glycerol incorporated into triglyceride would be expected to contain 3.3 atoms of tritium (17). Reasoning that endogenous, unlabeled glucose would contribute to ^3H - but not ^{14}C -labeled triglyceride glycerol in tissue incubated with $^3\text{H}_2\text{O}$ and [^{14}C]glucose, we determined rates of triglyceride-glycerol formation from the accumulation of both isotopes in tissue under circumstances of basal and isoproterenol-stimulated lipolysis. Medium was prepared as described above (section B). $^3\text{H}_2\text{O}$ (1.8 Ci/mol NET-001D; New England Nuclear) and [^{14}C]glucose (D-[^{14}C (u)]glucose, 1–5 Ci/mole; NEC 042) were added to yield final respective specific activities of 0.00337 Ci/mol and 0.8616 Ci/mol.

Portions (40–50 mg) of abdominal subcutaneous adipose tissue were incubated for 2 hr in 2.10 ml of this medium, with and without added isoproterenol (10^{-6} M). Similar incubations were also performed in the dual radioisotope medium containing [^3H]palmitate and [^{14}C]glucose (section B) in order to quantify the lipolytic response to isoproterenol. Following incubation, the tissue was treated as in section E and the ratio of ^3H to ^{14}C dpm in triglyceride was determined.

G. Statistical analysis

All statistical analyses were performed on a PDP-11 computer using BMDP statistical software (University of California, 1981). The specific types of analyses used are cited in the relevant portions of the text.

RESULTS

^{14}C Accumulation in the tissue lipid extract is a minimum estimate of the net rate of glyceride formation. This is so because incoming [^{14}C]glucose is, to some extent, diluted by interstitial and intracellular glucose (glycogen) and by glucose metabolites already in the glycolytic pathway at the beginning of the incubation period. Thus, the specific activity of the glucose pool used as substrate for glyceride synthesis is somewhat below that of the incubation medium. This pool of glucose (or glycogen) is evidently quite small and does not affect the calculations. Neither preincubation of tissue in glucose prior to exposure to the dual isotope medium, nor intense beta adrenoceptor stimulation of the tissue (which would be expected to cause glycogenolysis) significantly changed the rate of ^{14}C -labeled glyceride accumulation in the tissue. In addition, the newly synthesized triglycerides of adipose tissue incubated in medium containing both $^3\text{H}_2\text{O}$ and [^{14}C]glucose (section F), had a ^3H atom: ^{14}C glyceride-glycerol molecule ratio of $3.40 \pm .05$ (SEM) in unstimulated tissue and $3.34 \pm .083$ in tissue exposed to 1×10^{-6} M isoproterenol (lipolysis rate = 221% basal). This ratio, determined in human adipose tissue, is identical to the value of 3.3:1 reported by Jungas (17) in rat adipose tissue. The fact that the ratio is unaltered by a twofold increase in lipolysis rate implies that, under the circumstances of our experiments, the endogenous pool of glucose or intracellular glycogenolytic and glyceroneogenic metabolites that might contribute to the synthesis of glycerolphosphate is unmeasurable and unaffected by large changes in the rate of lipolysis. Hence, the accumulation of ^{14}C in adipose tissue lipids is an accurate measure of glyceride synthesis.

In tissue incubated in the [^3H]palmitate-[^{14}C]glucose medium, ^3H accumulation in any glyceride species is due to esterification of medium-derived fatty acids to newly

synthesized ^{14}C -labeled L- α -glycerolphosphate. Since the specific activity of medium free fatty acids is known, the molar ratio of newly synthesized ^{14}C -labeled glyceride to ^3H -labeled palmitate which has been esterified can be calculated. In practice, there are always fewer moles of esterified [^3H]palmitate than would be expected if every newly synthesized glyceride molecule contained only fatty acid of specific activity equal to that of the medium. This dilution is due to the mixture of medium fatty acids with fatty acids released by intracellular lipolysis of triglyceride. In fact, the $^{14}\text{C}/^3\text{H}$ ratio, which measures the relative rate of dilution of ^3H label being esterified to ^{14}C -containing α -glycerolphosphate, is a precise and accurate measure of the rate of lipolysis. The extent of the decrease in the specific activity of esterified fatty acids is exactly proportional to the rate of lipolysis in the tissue during the period of incubation. Thus, changes in the amount of [^3H]palmitate accumulated relative to ^{14}C -labeled glyceride synthesis can be used as a measure of lipolysis rate. In time course studies not shown here, both the glycerol release rate and the $^{14}\text{C}/^3\text{H}$ ratio in triglyceride were constant from 30 min (earliest time point) to 2 hr of incubation.

Effects of increased rates of lipolysis on ^{14}C -labeled glyceride synthesis and [^3H]palmitate esterification

As demonstrated in Fig. 1, the main effect of beta adrenoceptor-mediated stimulation of lipolysis with epinephrine in the presence of phentolamine is to alter the amount of [^3H]palmitate esterified per mole of newly synthesized ^{14}C -labeled glyceride. Changes in the rate of ^{14}C -labeled glyceride formation are small and cannot account for the reduced accumulation of esterified

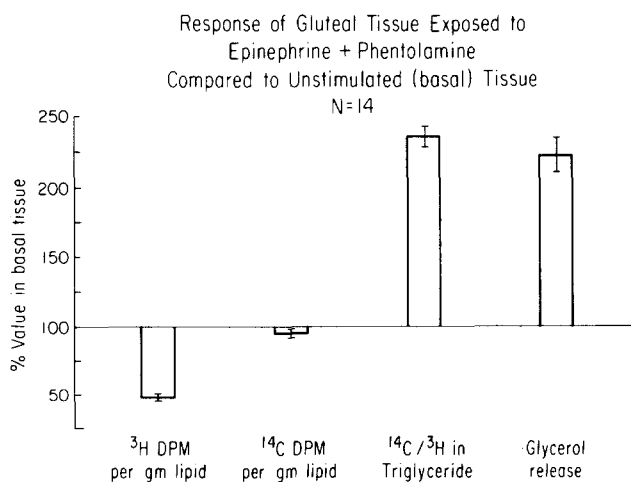


Fig. 1. Response of adipose tissue from obese and nonobese subjects. Medium containing both epinephrine (3.5×10^{-5} M) and phentolamine (3.5×10^{-4} M) is compared to basal medium. $N = 14$ studies on 12 subjects; bars = SEM. $^{14}\text{C}/^3\text{H}$ is triglyceride is the ratio of dpm in triglyceride formed during a 2-hr incubation of gluteal adipose tissue in medium containing [^3H]palmitate and [^{14}C]glucose.

[³H]palmitate. The reduction in [³H]palmitate esterification is due mainly to decreased [³H]palmitate uptake. The fact that ¹⁴C-labeled glyceride synthetic rates are not substantially lowered by a maximal lipolytic stimulus, further supports the conclusion that the dilution of incoming [¹⁴C]glucose by glucose released from intracellular glycogen must be small.

Use of relative rates of ¹⁴C and ³H accumulation in glyceride species as a measure of the rate of lipolysis

¹⁴C-Labeled glyceride accumulation can be used as a measure of new glyceride synthesis under a wide range of rates of lipolysis. [³H]palmitate accumulation, on the other hand, is inversely related to the rate of flux of unlabeled free fatty acids out of the tissue during the period of incubation. For this reason, the relative proportions of ³H and ¹⁴C in newly formed glycerides reflect the rate of hydrolysis of the large tissue pool of unlabeled triglyceride during the course of the tissue incubation in medium containing [³H]palmitate and [¹⁴C]glucose. As the lipolysis rate increases, the ratio of ¹⁴C/³H in newly synthesized glycerides should increase due to a progressive fall in the specific activity of the free fatty acids being esterified to the ¹⁴C-labeled glyceride-glycerol. **Table 1** and **Table 2** demonstrate the close relationship of the ratio of ¹⁴C/³H in triglyceride to glycerol release for the three types of media adrenoreceptor agonist/antagonist mixtures employed in this study. Both ¹⁴C/³H in triglyceride and glycerol release measures are expressed in relationship to basal circumstances in order to allow comparison between experiments with somewhat different starting specific activities of media [¹⁴C]glucose and

[³H]palmitate. Comparison of Tables 1 and 2 indicates that the expected antilipolytic effect of alpha adrenergic stimulation (epinephrine plus propranolol) is most readily seen in the tissue of fasted and semi-fasted subjects. This is due to the fact that caloric restriction both raises the basal rate of lipolysis and increases the alpha-2 receptor responsiveness of human adipose tissue (18–20). However, the greater sensitivity of the ¹⁴C/³H in triglyceride measure allows the detection of the subtle antilipolytic effect of alpha adrenergic stimulation on tissue from a nonfasted subject (Table 1). **Fig. 2** demonstrates the close correlation between the rate of lipolysis as reflected by glycerol release and the ¹⁴C/³H ratio in triglyceride formed during the incubation period. An equivalent relationship is obtained if the ratio in diglyceride is used, but triglyceride is more convenient since it is the quantitatively predominant glyceride species formed in this incubation system.

The mean coefficient of variation for duplicate or triplicate tissue samples exposed to identical media conditions is 6.6% for the isotope method compared to 11.5% for glycerol release per cell. The greater precision of the dual isotope method clearly enhances its sensitivity to small changes in the rate of lipolysis.

The dual radioisotope method is substantially simplified and shortened by the omission of the thin-layer chromatography step. However, scintillation counting of the unchromatographed tissue extract gives a less sensitive measure of changes in lipolysis rate due to the presence of varying amounts of tritium in the form of free fatty acids. Partial correction of this problem may be achieved by shaking the tissue extract with a small amount (e.g., 200 mg) of magnesium oxide to absorb some of the free fatty acids.

TABLE 1. Obese and nonobese (mean ± SEM)

| Method | Epinephrine (n = 14) | Epinephrine + Phentolamine (n = 12) | Epinephrine + Propranolol (n = 14) |
|--|-------------------------|--|---------------------------------------|
| Glycerol release (% basal) | 132 ± 10 | 227 ± 13 | 109 ± 6 |
| ¹⁴ C/ ³ H in triglyceride (% basal) | 122 ± 5 | 246 ± 13 | 93 ± 3 |

Comparison of relative rates of lipolysis as determined by both glycerol release and the radioisotopic technique (¹⁴C/³H in TG) described in the text. The responses of gluteal adipose tissue samples from 12 subjects (6 obese; 6 nonobese) were examined. Because two obese subjects were studied twice for certain media combinations, n = 14 for some media types. Four types of media were employed: basal; epinephrine (3.5×10^{-5} M) = mixed alpha and beta adrenoreceptor activation; epinephrine (3.5×10^{-5} M) plus the alpha adrenoreceptor blocking agent phentolamine (3.5×10^{-4} M) = preferential beta adrenoreceptor activation; epinephrine (3.5×10^{-5} M) plus the beta adrenoreceptor blocking agent propranolol (3.5×10^{-4} M) = preferential alpha adrenoreceptor activation. ¹⁴C/³H in TG is the ratio of dpm in the triglyceride formed during a 2-hr incubation of adipose tissue in medium containing [³H]palmitate and [¹⁴C]glucose. Both isotope ratio and glycerol release were assayed for tissue in the same incubation flasks. Each media type was assayed in triplicate and the results were averaged. Two-way analysis of variance indicates a large effect of media type ($F = 15.02$; $P < 0.01$), within each of the two assay systems, but no significant difference between assay systems ($F = 0.463$; $P > 0.1$) across all media types. However, the difference between responses in the two assay systems for the epinephrine + propranolol media is significant ($t = 2.97$; $P < 0.02$, paired t test, two-tail). This discrepancy is apparently due to the ability of the isotopic method to detect a subtle suppression below basal levels of activity by alpha adrenergic stimulation which the glycerol release technique does not detect.

TABLE 2. Fasted and semi-fasted (mean \pm SEM)

| | Epinephrine (n = 8) | Epinephrine + Phentolamine (n = 8) | Epinephrine + Propranolol (n = 8) |
|---|------------------------|---------------------------------------|--------------------------------------|
| Glycerol release (% basal) | 102 \pm 10 | 215 \pm 19 | 62 \pm 6 |
| $^{14}\text{C}/^3\text{H}$ in triglyceride (% basal) | 112 \pm 9 | 198 \pm 23 | 67 \pm 6 |

Comparison of relative rates of lipolysis as determined by both glycerol release and radioisotopic ($^{14}\text{C}/^3\text{H}$ in TG) techniques. The responses of gluteal adipose tissue samples from three obese subjects were studied a total of eight times after either fasting for 1 week or taking 600 kcal/day of liquid formula diet for 1–2 weeks ("semi-fasted"). See legend to Table 1 for specifics of media composition. Analysis of variance indicates a large effect of media type ($F = 22.84$; $P < 0.01$) within each of the two assay systems, but no significant difference between assay systems across all three media types ($F = 0.021$; $P > 0.1$).

Example of the use of the dual radioisotope method in the preparation of dose response curves for unweighed fragments of human adipose tissue

Adipose tissue was obtained from the buttocks region of a never-obese female at stable weight. Unweighed aliquots of approximately 20 mg were incubated in 2.1 ml of dual isotope medium containing varying concentrations of isoproterenol (Fig. 3) or clonidine and theophylline (Fig. 4). Quadruplicate specimens were assayed at each concentration of pharmacologic agent. At a concentration of 3.5×10^{-4} M, theophylline increases the rate of lipolysis

to 200–250% of basal values. Clonidine, an alpha-2 agonist, inhibits lipolysis through membrane receptor-mediated inhibition of adenylate cyclase activity (20, 21). Because of the small size of the tissue samples, only 5 ml of Folch reagent was used to extract the samples. As only the triglyceride fraction was needed, chromatography was simplified by running the tissue extracts on 100-micron thick plastic-backed silica gel plates (Eastman Kodak, Rochester, NY) in hexane–diethyl ether 9:1 (v:v). The triglyceride spots were cut out and placed into scintillation vials for counting with Ultrafluor as described. The plastic backing of this plate does not significantly interfere with counting efficiency.

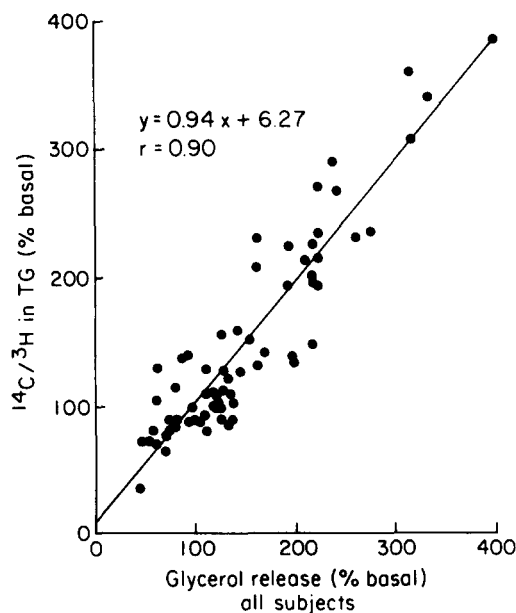


Fig. 2. Relationship of glycerol release to $^{14}\text{C}/^3\text{H}$ ratio in newly synthesized triglyceride in gluteal adipose tissue fragments exposed to epinephrine (3.5×10^{-5} M); epinephrine (3.5×10^{-5} M) plus phentolamine (3.5×10^{-4} M); and epinephrine (3.5×10^{-5} M) plus propranolol (3.5×10^{-4} M). Subjects include obese and nonobese described in the legend to Table 1 and fasted-semifasted obese described in Table 2. Separate correlation analyses for obese, nonobese, and fasted-semifasted gave similar regression equations with r values of 0.88–0.93; $P < 0.001$ for all correlations.

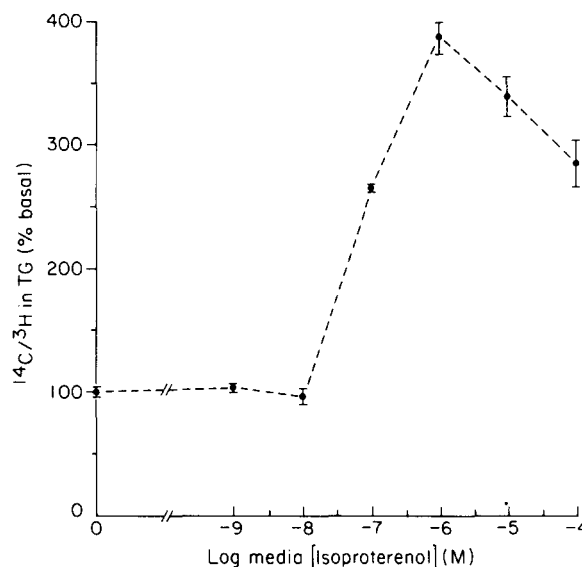


Fig. 3. Response of small (approximately 20 mg) fragments of gluteal adipose tissue from a never-obese subject to serial concentrations of the beta adrenoreceptor agonist, isoproterenol, in incubation medium containing [^{14}C]glucose and [^3H]palmitate. Response is expressed in relationship to the ratio of $^{14}\text{C}/^3\text{H}$ dpm in the triglyceride of unstimulated (basal) tissue. Quadruplicate tissue incubations were performed at each isoproterenol concentration and for basal medium. Partial inhibition of lipolysis at very high medium concentrations of isoproterenol is believed to reflect activation of alpha adrenoreceptors by isoproterenol (39). Bars = SEM.

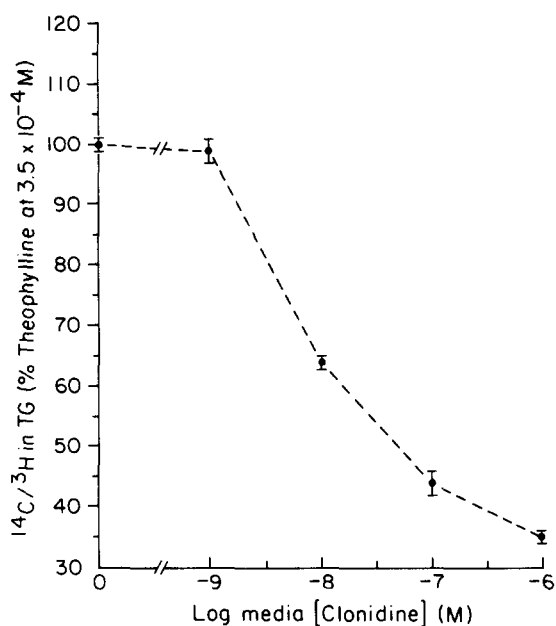


Fig. 4. Response of small adipose tissue fragments from the same subject as in Fig. 3. In order to demonstrate the response of the alpha-2 adrenoreceptor, the rate of lipolysis was increased to approximately 200% above basal by the addition of theophylline (medium concentration = 3.5×10^{-4} M). Serial doses of the alpha-2 adrenoreceptor agonist, clonidine, were added to the media. Quadruplicate tissue incubations were performed at each clonidine concentration and for the basal theophylline medium. Bars = SEM.

DISCUSSION

In studies aimed at examining rates of uptake of free fatty acids and glucose into human adipocytes, Waldius (22) first described the use of a dual radioisotope system similar to that employed in the experiments described above. He concluded that there was an inverse relationship between lipolysis rate and labeled free fatty acid uptake, and that the decline in esterification of labeled fatty acids accompanying increased rates of lipolysis was due both to reduced uptake of labeled free fatty acids and to dilution of these exogenous substrates in an enlarged intracellular free fatty acid pool. These results are in agreement with ours, and support our interpretation of the reasons that the simultaneous monitoring of the incorporation of [¹⁴C]glucose and [³H]palmitate into adipose tissue glycerides provides a sensitive and accurate means of examining rates of lipolysis in very small tissue fragments. The methodology described by us relies upon the observations that: 1) the cytoplasmic pool of glucose (as glycogen), glycogenolytic and glyceroneogenic precursors is small relative to the amount of isotopically labeled glucose entering the cell in the incubation system; 2) the rate of ¹⁴C-labeled glyceride formation is not significantly altered by acute, pharmacologically induced changes in the rate of lipolysis; 3) there is little or no synthesis of fatty acid carbon from [¹⁴C]glucose in human adipose

tissue under these experimental circumstances; and 4) glycerol or partial glycerides released during lipolysis are not re-esterified with free fatty acids to any significant extent. The small size of the cytoplasmic pool of glucose or other glycerolphosphate precursors is indicated by the linearity of ¹⁴C-labeled glyceride accumulation over a 2–3 hr period, and by the failure of increased lipolysis to influence the ratio of ³H:¹⁴C in triglyceride formed during adipose tissue incubation in medium labeled with ³H₂O and [¹⁴C]glucose. In rat adipose tissue, the in vitro stimulation of lipolysis with beta adrenergic agonists has been shown by both isotopic (23) and nonisotopic (24) techniques to increase the in vitro rate of triglyceride synthesis. In human tissue, however, in vitro findings have been variable. Neither we, nor Björntorp, Karlsson, and Hovden (25), noted any significant change in the rate of glyceride synthesis from [¹⁴C]glucose when lipolysis was pharmacologically stimulated in subcutaneous adipose tissue. Others, however, have found catecholamine-induced increases in ¹⁴C-labeled glyceride synthesis from [¹⁴C]glucose in omental adipose tissue slices (26) and strips or fragments of subcutaneous adipose tissue (22, 27). The reasons for these discrepancies are not clear, but may relate to differences in methods for obtaining and handling the tissue. However, the dual isotope method may be used in circumstances where the rate of glyceride synthesis is influenced by lipolysis rate (or other factors). In such instances, tissue ¹⁴C accumulation is corrected for a relevant aspect of tissue mass (e.g., total lipid weight, cells per unit weight, etc.). ³H activity is then related to this corrected numerator to obtain the ¹⁴C/³H ratio in triglyceride.

Various levels of de novo fatty acid synthesis from glucose in human adipose tissue have been reported. An unmeasurable rate of fatty acid synthesis from glucose was demonstrated in the experiments described here, and similar results have been reported by others (28–30). Some investigators, however, have described high rates of fatty acid synthesis in vitro in human adipose tissue under both basal circumstances (31, 32) and following the stimulation of lipolysis with epinephrine (26, 33). Again, the precise reasons for these discordant results are not clear.

Finally, glycerol kinase activity is negligible in human adipose tissue (34), explaining the virtual absence of glycerol reutilization by the tissue. Arner et al. (35, 36) have reported evidence for the utilization of glycerol for neutral glyceride synthesis in human adipose tissue. Metabolism of substantial amounts of glycerol by such a pathway could introduce an artifactual reduction in ¹⁴C/³H ratio in triglyceride, particularly in circumstances of rapid lipolysis. However, the quantities of glyceride reported by Arner et al. to be formed via this pathway are less than 1% of that formed from glucose in our system. Therefore,

despite its considerable theoretical interest, the "monoacylglycerol pathway" cannot produce a quantitatively significant effect in the system described here.

The recently described, highly sensitive chemiluminescent assay for glycerol (37) offers sensitivity in a range comparable to that provided by the dual radioisotope assay. However, any measure of medium glycerol release requires the determination of tissue aliquot cell number or weight in order to normalize results. This, in turn, requires either the use of cell suspensions prepared by collagenase digestion of tissue samples or the assaying of small incubated tissue samples for lipid content. The former procedure may alter the metabolic status of the cells freed by collagenase digestion (38) and, in any event, delays their arrival in incubation medium. Finally, glycerol release is not an ideal technique for the investigation of the role of plasma factors in the regulation of lipolysis. Because plasma glycerol content is so large relative to the small amounts released even by maximally stimulated human adipose tissue, and because hydrolysis of plasma glycerides may contribute additional glycerol to the medium, the sensitivity of glycerol measures to true lipolysis rate is compromised in these circumstances. The dual radioisotope technique is not influenced by these factors.

If the specific activities of media glucose and free fatty acid are known, their ratio can be used to normalize the $^{14}\text{C}:$ ^3H ratio in tissue triglycerides. This permits the expression of lipolysis rate in absolute terms, and allows the comparison of experiments without resort to expression of data in a percentage-of-basal format.

Because this assay can be performed repeatedly on small samples obtained by needle aspiration from unanesthetized subjects, multiple dose response curves can be obtained under circumstances of rapidly changing metabolic status. Assay of plasma lipolytic factors is facilitated by the fact that levels of medium glycerol or triglyceride do not affect the assay system. In addition, a preliminary study has indicated that this system can detect variable effects of insulin on isoproterenol-induced lipolysis at a concentration of $1\ \mu\text{u}/\text{ml}$ incubation medium (lowest concentration tested). This concentration of insulin is below that influencing the rate of glyceride synthesis and the numerator of the $^{14}\text{C}:$ ^3H ratio in triglyceride is not increased. Thus, the dual isotope assay may also prove useful as a bioassay for the effects of insulin on lipolysis. ■■

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