Metabolism and Structure of Triacylglycerols in Rat Epididymal Fat Pad Adipocytes Determined by $^{13}$C Nuclear Magnetic Resonance*

(Received for publication, August 23, 1985)

Laurel O. Sillerud, Chung H. Han, Mark W. Bitensky, and Albert A. Francendese

From the Life Sciences Division, Los Alamos National Laboratory, University of California, Los Alamos, New Mexico 87545

Carbon-13 nuclear magnetic resonance (NMR) methods have been applied to a study of the structure and metabolism of the triacylglycerols from rat epididymal fat pad adipocytes. Complete NMR signal assignments are provided for adipocytes, the extracted triacylglycerols, and methyl esters of the derived fatty acids. $^{13}$C NMR provided nondestructive, quantitative analysis of the amounts of unsaturation of the fatty acyl chains; in cells from rats given ad libitum access to a standard laboratory diet the predominant fatty acids were found to be palmitate (29.9%), oleate (27.9%), and linoleate (34.1%). These results agreed with gas chromatographic separation of the derived methyl esters of the extracted lipids. Lipid dynamics were examined in situ and showed a substantial restriction of motion of glyceride-glycerol as compared with free glycerol; the nuclear magnetic spin-lattice relaxation times for free glycerol of $2.52 \pm 0.12$ (C1,3) and $4.37 \pm 0.21$ (C2) s decreased to $0.15 \pm 0.009$ and $0.21 \pm 0.013$ s, respectively, upon esterification. Segmental motion of the chains, monitored by relaxation time measurements, increased progressively from the $\alpha$-carbon ($T_1 = 0.70$ s) to the methyl ends of the chains ($T_1 = 9.63$ s). The incorporation of C-13-labeled substrates ([1-$^{13}$C]glucose and [3-$^{13}$C]lactate) into the glycerol moiety of triacylglycerols was monitored in real time, in the presence of insulin. Lactate (10 mM) inhibited the incorporation of glucose (5.5 mM) into glyceride-glycerol. Lipolysis at the natural abundance level of $^{13}$C was measured in the presence of 10 $\mu$M isoproterenol. Simultaneous lipogenesis and lipolysis were found to occur in situ and were measured with the aid of [1-$^{13}$C]glucose and isoproterenol; the labeling pattern of medium glycerol versus extracted triacylglycerols was significantly different from that found using natural abundance glucose. Our results indicate that $^{13}$C NMR is a useful new method for the real-time monitoring of lipid structure and metabolism in vivo.

Adipose tissue constitutes an important site for the synthesis, storage, and mobilization of triacylglycerols. Its involvement in the metabolism of lipid, carbohydrate, and other substrates has implicated major roles for this tissue in a variety of normal and abnormal physiological events, including the etiologies of cardiovascular disorders and adult onset diabetes mellitus (1). The metabolism of adipose tissue is regulated by hormones which alter both lipogenesis and lipolysis. Adipose tissue metabolism has been extensively studied in the past with the aid of radioactive tracers in cell suspensions, perfused fat pads, and in animals. The well-known dangers associated with radioactivity have limited the applications of radioisotope technology in man. Consequently, it would be desirable to find a method for the noninvasive examination of the hormonal regulation of adipose tissue metabolism in normal and pathological states in vivo.

Carbon-13 nuclear magnetic resonance (NMR) methods offer, for the first time, the means by which metabolic regulation may be examined in adipose tissue in vivo, in a noninvasive manner, without the use of radioactive materials. In principle, it should be possible to apply these NMR methods to the study of lipid metabolism in adipocyte suspensions, perfused fat pads, and eventually, in situ. We have chosen to initially determine the applicability of $^{13}$C NMR to the study of triacylglycerol metabolism in adipocytes from the epididymal fat pad of the rat as a model system. It has previously been shown that $^{13}$C NMR spectra from tissue lipids can be obtained in vivo from the rat head, abdomen, and hind leg, human arm (3), and the liver of the rat (4).

We report here the basic $^{13}$C NMR data needed to establish the biochemical utility of the triacylglycerol resonances from adipocyte suspensions. These data include the lipid chemical shift assignments, fatty acyl chain compositions, spin-lattice relaxation times, line widths, and nuclear Overhauser enhancements. In addition, we demonstrate that $^{13}$C NMR can be utilized to follow lipid metabolism in real time in situ within the adipocyte without the need for lengthy chemical extractions. The $^{13}$C NMR method is shown to provide unique information with respect to substrate cycling, the integrity of chemical bonds, and lipid composition and dynamics. Finally, we show how the use of $^{13}$C-labeled substrates can provide information on the hormonal regulation of metabolism in the intact adipocyte.

MATERIALS AND METHODS

Male Sprague-Dawley rats, CD strain (180-250 g), purchased from the Charles River Breeding Laboratories (Wilmington, MA) were given ad libitum access to a standard rat chow (Teklad feed diet, A. Harlam Industries, Madison, WI). Parker medium 199 was obtained from Gibco (Grand Island, NY). Fetal bovine serum was purchased from Armour. Collagenases were obtained from Sigma, Millipore Corp. (Bedford, MA), and Worthington. Hormones and fatty acid-free albumin were purchased from Sigma. Fatty acid methyl esters as standards for gas chromatography were obtained from Sigma. [U-$^{13}$C]Glucose was obtained from New England Nuclear. The reagents for the determination of triacylglycerols were purchased from Sigma. [1-$^{13}$C]-β-Glucose and [3-$^{13}$C]-L-lactate enriched to 90 atom % were purchased from Merck.

The abbreviations used are: NMR, nuclear magnetic resonance; TAG, triacylglycerol; GC, gas chromatography; $T_2$, NMR spin-lattice relaxation time; $T_\beta$, NMR spin-spin relaxation time; Me$_3$Si, tetramethylsilane.

*This work was performed under the auspices of the Department of Energy. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
**13C Nuclear Magnetic Resonance—**13C NMR spectra were obtained with the aid of Bruker WM300WB and AM400WB Fourier transform spectrometers, operating at 75.42 MHz (and 100.61 MHz) using proton noise decoupling. Spectra were accumulated in 16,000–32,000 data points in a spectral width of 16,129 Hz (22,727 Hz; 213.748 ppm) with an acquisition time of 0.508 s (0.721 s), for the two different instruments, respectively. Further details are given in the figure captions.

The pulse angle was optimized according to the spin-lattice relaxation times of the slowest relaxing signal of interest (5). This signal was usually from C1 of glucose (T1 = 1.28 s). Spin-lattice relaxation times for the triacylglycerols in both isolated adipocytes and cholesterol extracts were measured by means of an inversion recovery sequence (180°–90°). The relaxation times and standard deviations were determined from 3-parameter fits to the data using the spectrometer system software (DISNMRP, 830701) and a convergence limit of 1 × 10−3. The Relaxation times of the slowest relaxing signal of interest captions.

Natural abundance 13C NMR spectra of the isolated fat cells were obtained from suspensions containing 50–70% cells by volume in medium 199 supplemented with 10% fetal bovine serum and 5.5 mM glucose. Because adipocytes have a density (0.97 g/mL) substantially lower than that of water, they will rapidly rise to the top of the suspension. In order to ensure that the cells remained within the radiofrequency coil of the NMR probe throughout the experiment we used a simple volume (1.8 mL) just large enough to fill the observation coil. The 10-mm NMR tubes were siliconized prior to use to prevent cells from sticking to the glass walls.

A similar concentration of adipocytes was used in experiments designed to monitor the hormone stimulation of triacylglycerol synthesis and degradation. The T1-corrected (2) integrals of the C1 and C3,13C glucose and the ratio, r, of the corrected integrals of C1.3 to C2 for the triacylglycerols were determined to probe the hormone effect. The metabolic enrichment, ε = n (r − 2), where n is the natural abundance of 13C (1.1%), gives the per cent of 13C above natural background. When 13C glucose was used for the substrate, prior washings of the cells with medium containing no natural abundance glucose were performed.

The cells were oxygenated by blowing water-saturated O2:CO2 (5%:5%) on the surface of the NMR sample. Due to the ease of fat cell breakage and the resulting formation of a triacylglycerol layer on the surface of the cultures, stirring was reduced or discontinued prior to the initiation of the experiments reported here, and neither a field-frequency lock nor sample spinning were used.

**Preparation of Fat Cells—**Rats were placed in a CO2-saturated desiccator for 2–4 min. The epididymal fat pads were removed and rinsed in phosphate-buffered saline, pH 7.4, and placed into small pieces weighing about 10 mg. The pieces were transferred to a 125-mL polypropylene Erlenmeyer flask containing 30 mL of Parker’s medium 199 with 10% fetal bovine serum, 5.5 mM glucose, and collagenase (580 μg/mL, Millipore Corp., see "Results"). The suspension of tissue was incubated at 37°C for 1 h in a New Brunswick Gyrotory water bath/shaker (New Brunswick Scientific Co., Inc., Edison, NJ). Several collagenases were tested for the known adverse effects on hormone responsiveness of contaminating phospholipases and proteases (7–9). Suitability of collagenases was determined by insulin stimulation of adipocyte [13C]glucose incorporation into [13C]CO2 and triacylglycerols (Table 1) in the presence of standard radioisotopic procedures (24). The Millipore collagenase (lot 41108) gave the best insulin responses and was used throughout the experiments reported here. The isolated adipocytes and the remaining tissue fragments were poured through nylon mesh (Small Parts Inc., Miami, FL) with a pore size of 500 μm and the cells were isolated using a 125-mL graduated polypropylene centrifuge tube. The isolated adipocytes were allowed to float for 10 min, and the infranatant containing the medium and stromal vascular cells was removed by aspiration. The cell layer was washed 3 times with 20 mL of medium (37°C) without collagenase. Washed cells were resuspended in the desired volume of medium 199.

**Incubation of Fat Cells—**The cell suspension was gently swirled to ensure homogeneity, and 1.0-ml aliquots were transferred to 25-ml polypropylene Erlenmeyer flasks containing medium 199, hormones, and substrates to a total sample volume of 2.0–4.0 mL. The flasks were covered with Parafilm, placed in a Gyrotory water/shaker bath at 37°C, and swirled gently (80 rpm) and incubated for various times. The number of fat cells in each incubation flask was determined by the following method. The cell suspension (50 μL in a 5-ml polypropylene tube) was diluted with 300 μL of phosphate-buffered saline and 150 μL of a 10% scintidine orange solution. Immediately after staining, cell numbers were determined using a Zeiss fluorescence microscope and a standard hemocytometer (American Optics, Inc.). Cell suspensions contained approximately 2 × 10^6 cells/mL. Cell size was also estimated by measuring the diameters of 300 cells using a calibrated reticule. Mean volumes were calculated from the measured diameters and the formula for the volume of a sphere. Cells fell within the volume range 130–870 μL, with a mean cell volume of 452 μL.

**Determination of the Effect of Hormones on [U-13C]Glucose Metabolism and Lipolysis—**[U-13C]Glucose was added to the incubation flask containing 3.0 mL of the rat cell suspension to give a final specific activity of 0.1 μCi/μmol of glucose. The final insulin concentration in the incubation flask was 10−M (1.52 micromol/L). This concentration gave the maximum effect of insulin (13) on glucose incorporation into triacylglycerol by the isolated fat cells during a 3-h incubation at 37°C. At the end of this time, a 0.5-ml aliquot of the cell suspension was removed during gentle swirling of the flask. The reaction was terminated by mixing the cell aliquot with 1.0 mL of chloroform:methanol (2:1). The triacylglycerols were extracted (see below) by vigorous vortex mixing. The chloroform layer was washed 3 times with 0.5 mL of a 10% NaCl solution. A 0.5-ml aliquot of the chloroform layer was air dried, and the radioactivity determined using standard scintillation techniques. The quantity of [13C]glucose carbon converted to triacylglycerol was calculated from the initial specific activity of glucose in the medium and the quantity of radioactivity in the product.

To measure the lipolytic effect of epinephrine (10−M) and other lipolytic hormones and compounds, incubations were performed as described above, but in the presence of added hormones. A 0.5-ml aliquot of the cell suspension was removed as described above and allowed to stand for 5–10 min at room temperature to allow the cells to float. The infranatant was gently aspirated, and glycerol in the medium was measured by means of the chromotrope acid assay (10). Data are expressed as a function of adipocyte number, and, in some cases, triacylglycerol concentration.

**Isolation of Triacylglycerols from the Isolated Fat Cells—**The triacylglycerols in 0.5 mL of a 50% cell suspension in a 15-ml centrifuge tube were extracted 4 times with 1.0-mL portions of chloroform by agitation on a vortex mixer. The first extract gave 85% of the total triacylglycerols, the second 12%, the third 3%, and the fourth a negligible amount of the total triacylglycerol. The combined chloroform extracts were washed with 0.1 M KCl (2 mL), dried, and evaporated under a stream of N2. Lipid analysis indicated that 0.5 mL of a 50% cell suspension gave 74 μg of triacylglycerol.

**Isolation of Fatty Acids from Fat Cell Triacylglycerols—**The fat cell triacylglycerols from above were dissolved in 10 μL of chloroform/toluene (2:8, v/v) and mixed with 20 μL of anhydrous methanolic HCl (5%) by the method of Eisen et al. (11) with the following modifications. Five mL of 2,5-dimethoxypropane was added, and the mixture was stirred in a capped flask overnight. Water (20 mL) was added, and the fatty acid methyl esters were extracted three times with 20-μL aliquots of petroleum ether. The combined extract was dried with anhydrous ammonium sulfate and sodium bicarbonate (4:1) and evaporated with a stream of N2.

The methyl esters of the fatty acids were separated by gas chromatography with a Hewlett-Packard model 3390A on an EGSS column (6 feet x 1/8 inch) at 200°C. Identification of each fatty acid was done by comparing the retention time to that of a known fatty acid methyl ester. The amount of each fatty acid was determined from an integration of the peaks.

**RESULTS**

**Chemical Shift Assignments**—Assignments for the 13C resonance of lactate (C3, 20.9; C2, 69.3; and C1, 183.1 ppm), glycerol (C1.3, 63.5; C2, 73.0 ppm), and glucose (12) are well known. The assignments for the triacylglycerol (Table I), free fatty acids, and fatty acid methyl esters (Table II) follow.

---

2. L. O. Sillerud, unpublished data.
### Table I

| Assignment | In situ | | In CDCl₃ | |
| --- | --- | --- | --- | --- |
| | δ ppm | T₁ s | α + δ | δ ppm | T₁ s |
| C1, C3 (glycerol) | 62.67 | 0.148 | 1.67 | 62.90 | 0.321 | 2.88 |
| C2' (glycerol) | 69.90 | 0.206 | 1.74 | 69.80 | 0.490 | 3.10 |
| C1=O (δ) | 172.46 | 2.49 | 1.00 | 173.86 | 5.90 | 1.88 |
| C2 (α, γ) | 34.54 | 0.351 | 1.44 | 34.83 | 0.761 | 1.94 |
| C2 (β) | 34.54 | 0.351 | 1.44 | 34.96 | 0.718 | 1.94 |
| C3 | 25.59 | 0.458 | 1.69 | 25.67 | 0.913 | 2.52 |
| C4 | — | — | — | 29.89 | — | — |
| C5, 6 | — | — | — | 30.12 | — | — |
| (−CH₂−)ₙ | 30.51 | 0.628 | 1.67 | 30.47 | 1.35 | 2.86 |
| C=O (C16, 18:2) | — | — | — | 130.96 | 2.49 | 2.49 |
| C=O (C10, 18:1, C9, 18:2) | — | — | — | 130.77 | 1.95 | 2.88 |
| C=O (C9, 18:1) | 130.45 | 0.923 | 1.66 | 130.42 | 1.80 | 2.37 |
| C=O (C10, 18:2; 18:3) | 128.79 | 1.26 | 1.61 | 128.91 | 2.60 | 2.36 |
| C=O (C12, 18:2) | — | — | — | 128.73 | 3.42 | 2.46 |
| C=O (C15, 18:3) | — | — | — | 127.87 | — | — |
| C=O (C16, 18:3) | — | — | — | 132.33 | — | — |
| C11 (18:1) and C8, C14 (18:2) | 27.92 | — | — | 38.01 | 1.67 | 2.60 |
| C11 (18:2, 18:3) | 26.35 | 1.05 | 1.96 | 26.46 | 1.59 | 2.34 |
| C (w−4) | 30.31 | — | — | 30.35 | — | — |
| C (w−3) | — | — | — | 30.05 | — | — |
| C (w−2) | 32.76 | 1.30 | 1.65 | 32.71 | 3.49 | 2.37 |
| C (w−1, 18:2) | — | — | — | 23.35 | — | — |
| C (w) | — | — | — | 14.83 | 5.22 | 3.01 |

* Estimated error ± 6%.
* Average error ± 9.6%.
* Set to δ = 23.45 ppm with respect to Me₄Si to coincide with our previous work (6).

### Table II

| Carbon number | 12:0 | 14:0 | 14:1 | 16:0 | 16:1 | 18:0 | 18:1 | 18:2 | 18:3 |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| CH₃O | 52.04 | 52.11 | 51.99 | 52.08 | 52.09 | 52.20 | 52.19 | 52.02 | 52.02 |
| C (w−4) | 174.92 | 175.03 | 174.83 | 175.02 | 174.89 | 181.34 | 175.07 | 181.18 | 175.02 | 175.64 |
| C (w−3) | 34.86 | 34.88 | 34.91 | 34.88 | 34.91 | 34.91 | 34.88 | 34.88 | 34.88 | 34.88 |
| C (w−2, 18:2) | 25.87 | 25.74 | 25.69 | 25.75 | 25.79 | 25.45 | 25.72 | 25.45 | 25.45 | 25.87 | 25.73 |
| C (w−2, 18:3) | 35.76 | 25.95 | 25.86 | 25.90 | 25.95 | 25.77 | 25.45 | 25.72 | 25.45 | 25.45 | 25.87 | 25.73 |
| C (w−1) | 30.08 | 30.05 | 29.86 | 30.06 | 29.95 | 29.87 | 29.92 | 29.88 | 30.03 | 30.03 | 29.99 |
| C (w−1, 18:2) | 30.08 | 30.39 | 29.85 | 30.24 | 29.95 | 30.23 | 29.92 | 29.88 | 30.03 | 30.03 | 29.89 |
| C (w−1, 18:3) | 32.70 | 30.39 | 29.85 | 30.24 | 29.95 | 30.23 | 29.92 | 29.88 | 30.03 | 30.03 | 29.89 |
| C (w−2) | 30.37 | 30.39 | 29.85 | 30.24 | 29.95 | 30.23 | 29.92 | 29.88 | 30.03 | 30.03 | 29.89 |
| C (w−2, 18:3) | 32.70 | 30.39 | 29.85 | 30.24 | 29.95 | 30.23 | 29.92 | 29.88 | 30.03 | 30.03 | 29.89 |
| C (w−1) | 30.37 | 30.39 | 29.85 | 30.24 | 29.95 | 30.23 | 29.92 | 29.88 | 30.03 | 30.03 | 29.89 |
| C (w−1, 18:2) | 30.37 | 30.39 | 29.85 | 30.24 | 29.95 | 30.23 | 29.92 | 29.88 | 30.03 | 30.03 | 29.89 |
| C (w−1, 18:3) | 32.70 | 30.39 | 29.85 | 30.24 | 29.95 | 30.23 | 29.92 | 29.88 | 30.03 | 30.03 | 29.89 |
| C (w−2) | 30.37 | 30.39 | 29.85 | 30.24 | 29.95 | 30.23 | 29.92 | 29.88 | 30.03 | 30.03 | 29.89 |

* Set to δ = 23.45 ppm with respect to Me₄Si to coincide with our previous work (6). To convert to an internal Me₄Si scale subtract 0.74 ppm from the chemical shifts in this table.

---

the work of Batchelor et al. (13). Isolated intact epididymal adipocytes, in the incubation medium at pH 7.4, produced the natural abundance ¹³C NMR spectrum shown in Fig. 1A. The spectrum has four distinct chemical shift regions which are assigned to the following classes of carbons: methyl and methylene (14–35 ppm) (see also Fig. 2A), esterified glycerol (62–70 ppm), unsaturated carbons (127–131 ppm) (see also Fig. 3A), and carbonyls (172–174 ppm). The ¹³C NMR spec-
13C NMR of Triacylglycerols in Adipocytes

**Fig. 1.** Natural abundance 13C NMR spectrum of (A) a suspension of rat epi-
dydymal fat pad adipocytes. This proton-
decoupled spectrum was taken from about 3.5 x 10^6 cells using 32,000 data
points at a frequency of 100.625 MHz, at
a temperature of 310 K, with 1024 scans
and an acquisition time of 0.72 s and a
40° pulse. B, a spectrum of the extracted
triacylglycerols from the cells shown in
A dissolved in CDC13 with internal MeSi
(TMS) as a reference. This spectrum was
accumulated with the same parameters
as in A except for the use of a 20° pulse
and 128 scans.

**Fig. 2.** An expansion of the aliphatic region of the natural abundance 13C NMR spectrum of the (A)
adipocytes and (B) extracted triacyl-
glycerols as shown in Fig. 1.

Important information about the types and sites of unsat-
uration of the fatty acyl chains of the triacylglycerols can be
rapidly obtained from the NMR spectrum. Characteristic
signals from double bonded carbons appear in two places in
the spectrum, near 130 ppm (Fig. 3) and in the methylene
region (Fig. 2). The olefinic region of the spectrum is domi-
nated by two major groups of resonances separated by about
1.9 ppm (Fig. 3). This separation arises from the magnetic
inequivalence of monoenoic and polyenoic fatty acids. The
carbons in a single double bond in a long-chain fatty acid
such as oleate (18:1) and resonate at 130.51 and 130.76 ppm
(Table II) and the addition of another double bond, the form
linoleate, shifts the resonances to higher (128.73, 128.91 ppm)
and lower (130.96 ppm) fields. Distinct signals from the
carbons in linolenate (18:3) are visible at 128.73, 128.91 ppm;
signals from the other three olefinic carbon nuclei
in this fatty acid overlap with those of oleate and linoleate
(Table II).

The resonances from methylene carbons adjacent to olefinic
sites in the fatty acyl chains are shifted due to changes in
their shielding arising from their olefinic neighbors. The signal at 23.55 ppm arises from carbon nuclei in the penulti-
mate position (15) of the acyl chain of linoleate (Fig. 2A). The
peak at 26.35 ppm is indicative of an allylic carbon in a cis-
diene system; this could arise from either linoleate or linolen-
Corresponding to C5 and C6. These carbons are shifted 0.30 ppm upfield with respect to their field positions in the corresponding alkanes (13). The signal at 30.12 ppm was tentatively assigned to a cis-allylic carbon (18,29); this could arise from either oleate or palmitoleate. The resonance at 27.92 ppm is characteristic of double bonds, carbonyls, or terminal methyl groups.

Certain features of these assignments have particular relevance with regard to the usage of $^{13}$C NMR for metabolic studies. Upon esterification, the signals from glycerol shift upfield sufficiently (C1,3, -0.8; C2, -3.1 ppm) so that free and bound glycerol can be easily resolved. This implies that the integrity of the ester bond in triacylglycerols can be monitored by means of $^{13}$C NMR. The methyl carbon of lactate resonates between the two signals from the ultimate and penultimate carbons of the fatty acyl chains (Table I). The carboxyl carbons of fatty acids shift upfield by 6.3 ppm upon methyl ester formation (Table II), and an additional 1.3–2.6 ppm upon esterification with glycerol (Table I). This shift of the carboxyl resonances offers another way to monitor the integrity of the ester bond by $^{13}$C NMR.

Nuclear resonances from carbons near the glycerol moiety are influenced by the dielectric constant of the medium (Table I). For example, there is a 1.2–1.4 ppm shift of the carboxyl carbon resonances of the triacylglycerols when they are extracted from the adipocyte and dissolved in chloroform. A similar effect of the solvent on the resonance frequencies of the carboxyl carbons of phospholipids has been interpreted as the result of solvent competition for intermolecular hydrogen bonds (21).

We are able to identify resonances from the 9 major fatty acyl chain lengths in the $^{13}$C NMR spectrum (Fig. 1B) of a chloroform extract of the fat pads from rats given ad libitum access to a standard laboratory diet. The amounts and saturation patterns of the various fatty acyl chain lengths were measured from the $^{13}$C NMR spectra, and independently, by means of gas chromatography (Tables II and III). The amount of saturation was estimated from the difference between the integrals of C-3 and the total integrals of the unsaturated carbons. For the four fatty acyl chain types which give rise to unique $^{13}$C nuclear resonances (16:0, 18:1, 18:2, and 18:3) the mol % determined by NMR (Table III) agrees well with that found using GC analysis. The NMR determination is much faster (10 min) than the GC method which relies on the quantitative hydrolysis of the esters and their subsequent quantitative methylation (2–3 days). The average difference between the mol % determined by the two methods is 2 mol %. In the case of linoleic acid (18:2) we could determine its amount in the triacylglycerol mixture in three independent ways from the $^{13}$C NMR spectrum (Figs. 2 and 3, and Table IV). Integrals of the signals at 23.35 ppm (ω-1), 26.35 ppm (C11), and 32.29 ppm (ω-2) (Fig. 2B) from linoleic acid carbons were used (Table IV) to calculate a mol percentage of 34.1 ± 1.8%. This determination agrees within the error limits quoted with that found by GC analysis.

The results of both the GC and the NMR unsaturation analyses are in agreement that the major fatty acids found in these adipocytes are palmitic, oleic, and linoleic (Table III). An examination of the integrals of the signals from the olefinic sites in the fatty acyl chains indicates that 45.0 ± 3.0% of the unsaturated side chains consists of oleic acid, based on the integrals of the signals at 130.42 and 130.77 ppm. Integrals of the 4 signals at 128.73, 128.91, 130.77, and 130.96 ppm give an average amount of linoleate of 53.0 ± 6.3%. The minor resolved signals from linolenate (n = 5) constitute an average amount of 2.7 ± 1.7% of the total unsaturated sites.

Table III

Comparison of the determination of the fatty acid composition of adipocyte triacylglycerols by $^{13}$C NMR and gas chromatography

The NMR analysis was done on CDC13 solutions of extracted triacylglycerols, while the GC analysis was done on derived fatty acid methyl esters.

| Fatty acid | % by $^{13}$C NMR | % by GC |
|-----------|------------------|--------|
| 16:0      | 29.9             | 35.3   |
| 18:1      | 27.9             | 29.7   |
| 18:2      | 34.1 ± 1.8%      | 33.2   |
| 18:3      | 1.9 ± 0.8%       | 0.5    |
| 12:0      |                  | 0.3    |
| 14:0      |                  | 1.5    |
| 14:1      |                  | 0.3    |
| 16:1      |                  | 2.4    |
| 18:0      |                  | 0.6    |

*Based on the integrals of the ω-2 (32.71 ppm) carbon signals and signals from monoenic and saturated fatty acids (23.35 ppm) (28).

Based on the integral of the ω-1 carbon of 18:2 and the total integrals of the unsaturated carbons.

See Table IV.
The resolution of the triacylglycerol $^{13}$C NMR spectrum is greater when these molecules are dissolved in CDCl$_3$ as compared with the in situ spectrum. The widths of the resonances were measured in both states and at two different magnetic field strengths (2.0 and 7.05 tesla); the protonated carbons gave signal widths of 3.0 ± 0.2 and 6.8 ± 1.4 Hz, respectively, in CDCl$_3$ and 3.8 ± 0.5 and 48–77 Hz, respectively, in situ. The larger widths found at the higher field are due in part to inhomogeneous broadening by the particulate nature of the cellular sample and partly due to chemical shift anisotropy in these long bulky molecules, particularly for the triacylglycerols in the viscous lipid droplet within the adipocyte. At the lower field (2 tesla) the carboxyls give resonance widths of 1.7 ± 0.5 Hz in either state; we expect that these molecules would have resonance widths of around 1 Hz based on their mass of about 1000 daltons. At 7 tesla, on the other hand, the width of the carboxyl resonances changes from 6.9 ± 0.5 to almost 25 Hz when one compares the extracted molecules in CDCl$_3$ to those in situ within the adipocyte. These widths in situ at 7 tesla scale approximately as the square of the field, lending support to the proposal of chemical shift anisotropy as an important line-broadening mechanism for the lipid signals from within the adipocyte.

The measurement of the relaxation parameters for the triacylglycerols has a value beyond the study of segmental motion in lipids. These parameters must be known if peak integrals are to be converted to amounts of $^{13}$C so that NMR can be used as a quantitative detector for carbon-13 isotopic enrichment. In situations where there is no $^{13}$C recycling or scrambling into all molecular sites, certain sites will remain at the natural abundance level of 1.1% $^{13}$C. These latter sites can be used, once corrected for saturation and Overhauser effects, as internal standards for resonance signals from those sites in the biomolecules which do incorporate $^{13}$C as a result of metabolic flow. We can use $^{13}$C NMR in this way to measure the synthesis of chemical amounts of metabolites.

**Inulin Stimulation of $[1-^{13}$C]Glucose Incorporation into Triacylglycerols**—A key advantage of the use of $^{13}$C NMR in the study of the metabolism of adipose tissue is the fact that the natural abundance spectrum of the tissue contains resonances from many distinct carbon nuclei (Fig. 1, Table I). In particular, the resonances from the glycerol moiety of the triacylglycerols appear in a window removed from those of the other carbons and nuclei (Fig. 3). In the glycolytic pathway responsible for the formation of glycerol 3-phosphate, the $^{13}$C label added as C1 of glucose appears as C3 of the glycerol, while no $^{13}$C flows directly to the C2 position of the glycerol. Since these two glycerol positions give rise to distinct NMR signals, one can separately determine the time course of the intensities of these signals and, therefore, use the C2 signal as a constant internal control during the extended NMR time courses. Furthermore, the C2 signal has an integral equal to that of a single carbon nucleus in a triacylglycerol at natural abundance so that it acts as a standard for determining the absolute incorporation of $^{13}$C into the glycerol carbon 3. The chemical shifts of free glycerol and the glycerol moiety of triacylglycerols are also distinct so that one can monitor the formation and breakdown of triacylglycerols during lipogenesis and lipolysis from an examination of the time dependence of the $^{13}$C NMR spectra of adipocytes.

One of the classical effects of insulin on adipose tissue is to promote the incorporation of glucose into the glycerol moiety of triacylglycerols. In our incubations with [U-$^{14}$C]glucose, insulin at a concentration of 10 nm caused a 5.5-fold increase in the rate of $^{13}$C glucose incorporation into triacylglycerols; the rate in the presence of insulin was found to be 450 nmol/10$^6$ cells/h. These results indicated that it would be feasible.
to monitor with $^{13}$C NMR the flow of $^{13}$C-labeled glucose into the triacylglycerol pool of adipocytes, since an average adipocyte contains from 1 to 10 nmol of triacylglycerol and this rate would then correspond to a change in the NMR signal of from 0.3 to 3.0%/h. Changes of this magnitude (~1 µmol/sample/h) can be measured given the very high signal to noise ratio (~100:1 for glycerol C2 in 1 min) obtained from the adipocyte suspensions.

In the presence of a physiological concentration of insulin (10 nM) the incorporation of glucose into the glycerol moiety of adipocyte triacylglycerols is stimulated. In order to determine the efficacy of $^{13}$C NMR as applied to a study of this process we incubated adipocytes with insulin (10 nM) and [1-$^{13}$C (90%)]-D-glucose. The $^{13}$C NMR spectrum of the adipocyte system (1.8 ml of a 50% cell suspension) was taken and stored on the disk in 10–20-min blocks. The results (Fig. 4) show that there is a significant decrease in the $^{13}$C NMR signal from the added [1-$^{13}$C]glucose over the course of this 3-h experiment. At the same time, we observed no change in the integral of the signal from carbon 2 of the triacylglycerol. The constancy of this internal control signal indicates that our adipocyte suspension was fully contained within the NMR coils and that sample flotation out of the sensitive volume was not a problem.

In order to demonstrate that the NMR method is capable of detecting chemical amounts of triacylglycerol synthesis against a large natural abundance $^{13}$C background, we incubated adipocytes for 18 h with insulin (10 nM) and [1-$^{13}$C]glucose (10 mM), with natural abundance glucose plus insulin and with [1-$^{13}$C]glucose in the absence of insulin. Following extraction with CHCl$_3$, the natural abundance control spectrum exhibited a ratio of the glycereide-glycerol C1,3 to C2 resonance integrals of 2.0; the labeled glucose without insulin gave a ratio of $r = 2.0256$, corresponding to an enrichment above natural abundance of $e = 0.0252\%$ $^{13}$C, and the [1-$^{13}$C]glucose plus insulin gave a ratio of $r = 2.294$, corresponding to an excess enrichment of $e = 0.323\%$ $^{13}$C. These data show a marked effect of insulin on the glycolytic flux in these adipocytes. The control ratio is 2 because $^{13}$C is randomly distributed in nature and there are 2 carbons contributing to the C1,3 signal as opposed to only 1 for the C2 resonance. These data also serve as a good illustration of the use of one of the triacylglycerol signals as internal controls so that very accurate measurements of the amount of $^{13}$C at a labeled site can be made.

**Natural Abundance $^{13}$C NMR Monitoring of Lipolysis—**

Hormonal regulation of metabolic pathways often takes the form of opposing effectors. It is well known that insulin promotes lipogenesis while the adrenergic effectors such as epinephrine and isoproterenol stimulate lipolysis. We have already shown that $^{13}$C NMR can monitor lipogenesis. Our results show that during incubations of adipocytes with 10 µM isoproterenol we could also monitor lipolysis in real time (Figs. 5 and 6). Noteworthy is the decrease with time of the glycereide-glycerol C1,3 signal over the course of the experiment, while essentially no change occurs in the control penultimate carbon resonance. Similar control results (data not shown) were obtained when the time dependence of the glycerol C2 signal was examined. These data provide values for

---

**Fig. 4.** Time course of the [1-$^{13}$C]glucose and adipocyte triacylglycerol $^{13}$C NMR signals of a suspension of rat epididymal fat pad cells during insulin-stimulated lipogenesis from glucose. The line labeled Glc 1 is the sum of the [1-$^{13}$C]glucose and $^{13}$C C1 resonances, TAG(1,3) is the signal from the glycerol C2 carbon of the triacylglycerols, and $^{13}$C TAG(1,3) is the signal from the glycerol carbon C(1,3) of the triacylglycerols. P is the peak height in centimeters. The lines are least square fits to the data and have slopes of $9.315 \times 10^{-4}$ for TAG(1,3), $6.189 \times 10^{-4}$ for TAG(2), and $-1.659 \times 10^{-2}$ cm/min for Glc 1, respectively.

**Fig. 5.** Time dependence of the natural abundance $^{13}$C NMR signals from rat adipocytes during isoproterenol-stimulated lipolysis. The line labeled $^{13}$C TAG(1,3) is the signal from carbons 1,3 from the glycerol moiety of the triacylglycerols, $^{13}$C TAG(1,3) is the control signal from the penultimate carbon in the fatty acyl chain of the triacylglycerols, and $^{13}$C TAG(2) is the signal from carbon 2 of the glycerol moiety of the triacylglycerols. $^{13}$C P is the peak height in centimeters. The lines are least square fits to the data and have slopes of $-2.75 \times 10^{-3}$ for TAG(1,3), $1.75 \times 10^{-4}$ for TAG(2), and $-1.28 \times 10^{-2}$ cm/min, respectively. The slope of the $^{13}$C TAG(1,3) line is about twice that of the $^{13}$C TAG(2) line since two carbon nuclei contribute intensity to it as opposed to only one for the latter line.

**Fig. 6.** Carboxyl region of the proton-decoupled natural abundance $^{13}$C NMR spectrum of the extracted triacylglycerols from a 192-h incubation of rat adipocytes with 10 µM isoproterenol. For this spectrum the lipids were dissolved in CDCl$_3$. Note the substantial signal from free fatty acids which were cleaved as the result of lipolysis during this period.
the rate of lipolysis of 300 nmol of glycerol/10^6 cells/min. The success of this method depends on the fact that there are substantial shifts of several of the carbon resonances when the fatty ester bond is broken. As the triacylglycerol is degraded, the glyceride-glycerol signal decreases and, if enough is formed, a signal from free glycerol appears, either in the cells or from the medium. Adipocytes lack glycerol kinase so that the free glycerol formed during lipolysis cannot be reesterified and is given up to the medium.

The free carboxyls from the cleaved fatty acids will also resonate in a different and distinct position of the 13C NMR spectrum from those ester bound into triacylglycerols (Tables I and II). Exploitation of these properties was accomplished by monitoring isoproterenol-stimulated lipolysis at natural abundance in adipocytes incubated with 10 µM hormone for 192 h. This extensive time was chosen in order to provide for substantial chemical-scale hydrolysis of the triacylglycerols in the adipocytes. The results (Fig. 6) show that the bound and free fatty acids are easily resolved in the 13C NMR spectrum of the extracted triacylglycerols and that 37.4 ± 1.4% of the lipids were cleaved over this time span. Chemical analyses of the incubation media and extracted triacylglycerols indicated that 38.6% of the pre-existing lipids were cleaved over this time period; the agreement between these two different methods of measuring lipolysis is excellent.

Lactate Inhibition of [1-13C]Glucose Metabolism—In the present set of experiments in which adipocytes were incubated simultaneously with [3-13C]-l-lactate and [1-13C]-d-glucose, we monitored both of these labeled sites simultaneously in the medium. The lactate methyl carbon resonates at 20.9 ppm and is resolved from the ω-1 (23.45 ppm) and ω (14.72 ppm) carbons of the triacylglycerols, while the glucose C1 resonances are found around 95 ppm, far away from any competing resonances from the lipids (Fig. 1, Table I).

Substrate interactions are indeed of some interest in adipose tissue with respect to metabolic disorders, such as diabetes and obesity. Using radio-labeled procedures, lactate, in particular, has been shown by us (24) to exert a potential glucose-sparing effect on adipocyte metabolism by virtue of its preferential utilization over glucose for glycerogenesis, when both are present in the medium. Given the excellent resolution available from the 13C NMR spectrum of adipocytes, we sought to measure the rates of glucose and lactate utilization in the presence of insulin (10 nM). The results (Fig. 7) show that lactate is a good substrate for the glycerol portion of triacylglycerols. The lactate was consumed at a rate of about 4.2%/h or 220 nmol/10^6 cells/h, while 13C from lactate appeared in the glycercide-glycerol at a rate of 2.3%/h or 121 nmol/10^6 cells/h. The consumption of glucose was significantly decreased in this experiment by the presence of the lactate: glucose only accounts for approximately one-quarter of the amount of 13C incorporated into lipids. We observed a slight upward trend to the glycerol C2 signal; the rate was about 6-fold smaller than that found for carbons C1 and C3. Lactate and glucose consumption was about 60% greater than the rate of incorporation of 13C into glycercide-glycerol; the excess lactate- and glucose-derived 13C was lost to the atmosphere as 13CO2. Subsequent experiments using lysine as a carbon dioxide trap and 13C NMR have demonstrated significant 13C labeling of the evolved CO2 (data not shown).

Substrate Cycling during [1-13C]Glucose Metabolism in Adipocytes—We have shown that 13C NMR can monitor both major directions of flow in the metabolism of triacylglycerols: lipolysis and lipogenesis. In this regard, we sought to deter-
half only saline control additions. At the end of the incubations, after all the glucose had been consumed, the medium and the cells were separated and the triacylglycerols were extracted from the cells. In the absence of labeled glucose, the C-13 NMR spectrum of the medium and triacylglycerols (Fig. 8) shows a ratio of glycerol signal intensities of $n = 2.018 \pm 0.056$ ($n = 18$), while in the presence of labeled glucose this ratio increased to $3.02 \pm 0.06$ for both the glycerol and the triacylglycerols; isoproterenol had no effect on this ratio. The presence of the hormone did result in a 2-fold stimulation of the amount of glycerol formed during the incubations and in the appearance of labeled lactate in the medium (Fig. 8). These results indicate that the carbons of glycerol in the medium originated from medium glucose, rather than from the intrinsic triacylglycerols. The glucose in the medium was taken up, cleaved in glycolysis, formed into glyceride-glycerol, and cleaved in lipolysis to form glycerol. The $^{13}$C enrichment at glycerol carbons 1,3 was found to be $e = 1.122 \pm 0.055\%$.

**DISCUSSION**

Of primary interest in this study was the establishment of the efficacy and accuracy of $^{13}$C NMR as applied to the quantitative determination of the metabolic parameters of isolated rat epididymal adipocytes. From the results it is clear that nuclear resonance methods can be applied in many unique ways in the study of metabolic molecular transformations in adipocytes. The resolution, sensitivity, and specificity of the carbon NMR spectrum are well suited to the examination of the various factors which regulate the flow of metabolites through the glycolytic, lipogenic, and lipolytic pathways in adipocytes in vitro. Advances in surface coil NMR technology (3) will eventually enable the examination of these properties in vivo. Strengths of this approach include the large $^{13}$C chemical shift range (coupled with the assignments of the $^{13}$C NMR spectrum), the sensitivity of the shifts to the formation and degradation of covalent bonds, and the ability of the method to give structural information with respect to the naturally occurring fatty acyl chain composition and to the dynamic aspects of lipids in situ. The accuracy and precision of $^{13}$C NMR are sufficient to permit quantitative metabolic measurements to be made in cell suspensions. Extrapolation of this technique to the in vivo situation as has been done with the liver (3, 25, 26) and heart (27) will undoubtedly lead to important new insights.

**REFERENCES**

1. Lynch, M., Wong, S. L., and Blackburn, G. L. (1984) *Bariatric Surg.* 2, 1
2. Sillerud, L. O., and Shulman, R. G. (1983) *Biochemistry* 22, 1087
3. Alper, J. R., Sillerud, L. O., Behar, K. L., Gillies, R. J., Shulman, R. G., Gordon, R. E., and Hanley, P. E. (1981) *Science* 214, 660-662
4. Canioni, P. C., Alper, J. R., and Shulman R. G. (1983) *Biochemistry* 22, 4974
5. Christensen, K. A., Grant, D. M., Schultzman, E. M., and Wailing, C. (1974) *J. Phys. Chem.* 78, 1971
6. Sillerud, L. O., Prestgard, J. H., Yu, R. K., Schafar, D. E., and Koningsberg, W. H. (1976) *Biochemistry* 17, 2619
7. Kuo, J. F., Dill, I. K., and Holmlund, C. E. (1967) *J. Biol. Chem.* 242, 3659-3664
8. Rodbell, M., and Jones, A. B. (1966) *J. Biol. Chem.* 241, 140-142
9. Rodbell, M. (1966) *J. Biol. Chem.* 241, 130-139
10. Korn, E. D. (1955) *J. Biol. Chem.* 215, 1-14
11. Eisen, E. J., Cartwright, A. L., Weller, K. M., and Smith, K. J. (1982) *Lipids* 17, 136
12. Walker, T. E., London, R. E., Whaley, T. W., Barker, R., and Matwiyoff, N. A. (1976) *J. Am. Chem. Soc.* 98, 5807
13. Batchelor, J. G., Chuske, R. J., and Prestgard, J. H. (1974) *J. Org. Chem.* 39, 1698
14. Hay, C. E., and Helmer, G. (1981) *Lipids* 16, 102
15. Pfeffer, P. E., Sampugna, J., Schwartz, D. P., and Shoolery, J. N. (1977) *Lipids* 12, 869
16. Tulloch, A. P., and Bergter, L. (1979) *Lipids* 14, 996
17. Tulloch, A. P. (1985) *Lipids* 17, 544
18. Gunstone, F. D., Pollard, M. E., Serringeour, C. M., and Vedanaayagam, H. S. (1977) *Chem. Phys. Lipids* 18, 115
19. Bus, J., Sies, L., and Lie Ken Jie, M. S. (1976) *Chem. Phys. Lipids* 17, 501
20. Johns, S. R., Leslie, D. R., Willing, R. I., and Bishop, D. G. (1977) *Aust. J. Chem.* 30, 823
21. Schmidt, C. F., Barenholz, Y., Huang, C., and Thompson, T. E. (1977) *Biochemistry* 16, 3948
22. Ford, W. T. (1976) *J. Am. Chem. Soc.* 98, 2227
23. Gent, M. N., and Prestgard, J. H. (1977) *J. Magn. Reson.* 25, 243
24. Francendese, A. A., and DiGirolamo, M. (1981) *Biochem. J.* 194, 377
25. Alper, J. R., Behar, K. L., Rothman, D. L., and Shulman, R. G. (1984) *J. Magn. Reson.* 56, 334
26. Reo, N. V., Ewy, C. S., Siegfried, B. A., and Ackerman, J. J. H. (1984) *J. Magn. Reson.* 59, 76
27. Neurohr, K. J. (1984) *J. Magn. Reson.* 59, 511
28. Pfeffer, P. E., Luddy, P. E., and Unrath, J. (1977) *J. Am. Oil Chem. Soc.* 54, 380