# Glycerolipid synthesis in isolated adipocytes: substrate dependence and influence of norepinephrine

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**Abstract** Studies of fatty acid (FA) esterification by adipocytes have led to conflicting views with respect to how the process is regulated by norepinephrine (NE). It remains unclear whether NE directly modulates the pathway or whether its effects are indirect and reflect its well-known action to activate lipolysis. Changes in lipolysis can complicate estimation of esterification rates by altering both medium FA and the hydrolysis of newly formed FA esters. In this report, we describe an experimental approach that determined the effect of NE on FA esterification, amidst the complications introduced by activation of lipolysis. Esterification rates were estimated from the simultaneous incorporations (0.1–60 min) of [$^{14}$C]glucose and [$^{3}$H]oleate into diglyceride (DG), phospholipid (PL), and triglyceride (TG). Saturation kinetics of incorporation rates, with respect to FA, and more specifically to unbound or albumin-free FA (ubFA), were determined in both basal and NE-treated cells. To obtain true estimates of ester synthesis, incorporation rates were adjusted for label loss from breakdown of labeled esters. Our findings were: *1*) In basal versus NE-treated cells, [$^{3}$H]oleate, on its pathway to esterification, was diluted, respectively, by 2 and 50% of measured cell FA, and the diluting FA appeared derived from lipolysis. *2*) Syntheses of PL, DG, and TG, estimated from incorporation of [$^{14}$C]glucose, saturated at low ubFA. The $K_m$ for TG synthesis (0.06 $\mu$M) was within the physiological range of ubFA which meant that changes in plasma FA will modulate TG synthesis. PL synthesis, on the other hand ($K_m$ <0.01 $\mu$M), would be largely saturated under physiological conditions. *3)* NE treatment increased the molar ratio of FA to albumin in the medium an average 8-fold and ubFA about 87-fold. In addition, NE accelerated hydrolysis of labeled PL and DG. Adjusting incorporation rates for these changes indicated that NE does not directly regulate glyceride synthesis. The assays described should allow estimation of glycerolipid synthesis under various metabolic or disease states and will distinguish direct effects from those reflecting changes in FA concentration or in hydrolysis of labeled FA esters.—**Melki, S. A., and N. A. Abumrad.** Glycerolipid synthesis in isolated adipocytes: substrate dependence and influence of norepinephrine. *J. Lipid Res.* 1992. **33:** 669–678.

**Supplementary key words** phospholipid synthesis • diglyceride synthesis • kinetics

Turnover of glycerolipids is of great importance for adipocytes. Diglycerides (DG) and phospholipids (PL) are essential components of cell membranes and/or mediators of many hormone actions (1, 2), and stored triglycerides (TG) are central to the major role of the adipocyte in energy balance. The action of norepinephrine (NE) to accelerate hydrolysis of glycerolipids is well established, but its influence on glycerolipid synthesis is less clear. Both inhibitory (3, 4) and stimulatory effects (5, 6) of NE on synthesis have been reported.

Glycerolipid synthesis is generally estimated from lipid incorporation of labeled precursors, fatty acids (FA), or glucose. This sensitive method allows the study of pathway regulation in situ in the presence of native concentrations of cofactors and inhibitors. However, in the case of adipocytes treated with NE, interpretation of incorporation data can be very complicated. Acceleration, by NE, of glycerolipid hydrolysis changes *1*) the concentration of the FA precursor and *2*) the rate of breakdown of newly formed, labeled glycerides (7–9). The consequences of the changes on incorporation rates have not been investigated. For example, medium FA in adipocyte incubations treated with NE is increased and variable; however, the effect of FA concentration on precursor incorporation remains largely unknown, since few studies have addressed saturation kinetics of glycerolipid synthesis. Furthermore, no studies have related rates to albumin-free or unbound FA (ubFA) which is the FA fraction important for cellular

Abbreviations: NE, norepinephrine; FA, fatty acid; ubFA, unbound FA; TG, triglyceride; DG, diglyceride; PL, phospholipid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid; KRH, Krebs Ringers HEPES; BSA, bovine serum albumin; SA, specific activity.

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uptake (10–12). Finally, the effect of label loss, from NE-induced hydrolysis of new esters, on incorporation rates remains undetermined, although it could be quite significant. In this report, we have investigated whether NE directly regulates glycerolipid synthesis or whether its effects only reflect the complications introduced by activation of glycerolipid hydrolysis.

Our data indicated that glycerolipid synthesis saturates at low ubFA concentrations and that NE does not directly regulate synthetic activity. NE, however, significantly alters lipid incorporation of glucose and FA as a result of changes in ubFA concentration and in breakdown of labeled glycerides.

## EXPERIMENTAL PROCEDURES

### Materials

D-[$^{14}$C-U]Glucose, [9, 10-$^{3}$H]oleate and nickel-63 were obtained from New England Nuclear (Boston, MA). [$\alpha$-$^{32}$P]CTP and aqueous scintillation fluid (ACS) were from Amersham (Arlington Heights, IL). Collagenase (type I) was from Worthington (Freehold, NJ). Fatty acids, di- and triolein, albumin (bovine serum albumin, fraction V, essentially fatty acid-free), and phospholipid standards for thin-layer chromatography were from Sigma (St. Louis, MO). Thin-layer plates were from Analtech (Newark, DE) for silica G and from Altech (Deerfield, IL) for silica 60. All organic chemicals were the highest reagent grade available and were from Fisher Scientific (Springfield, NJ).

### Cell preparation

Adipocytes were prepared from the epididymal fat of Sprague-Dawley rats (170–200 g, Harlan Industries Inc.). Cell isolation followed the methodology first described by Rodbell (13) and modified as detailed previously (11, 14). The washed cells were suspended in a Krebs-Ringer solution buffered (pH 7.5) with HEPES (KRH) containing 2% fatty acid-free bovine serum albumin and glucose (2 mM) except where indicated. Cell density was adjusted to 30 or 10% (v/v) using packed cell volume determined by centrifugation of a 7-$\mu$l aliquot in microcapillary tubes. Adipocytes, prepared as described above, were incubated without or with NE for 10 min at 37°C. NE was added in a few microliters from a concentrated stock to a final concentration of 0.1 $\mu$g/ml.

### Substrate uptake

The uptake values of [$^{3}$H]oleate and [$^{14}$C]glucose were measured simultaneously at 37°C in an Isolette incubator (Narco, Warminster, PA). The isotopic solution [[$^{3}$H]oleate (40–700 $\mu$M, about 3000 cpm/$\mu$l) complexed to FA-free bovine serum albumin (BSA) in a 4:1 ratio + [$^{14}$C]glucose (2 mM, about 8000 cpm/$\mu$l)], incubation tubes, and pipette tips were prewarmed to 37°C and kept at that temperature throughout the assay. Uptake was measured using previously validated methodology (11, 14). Briefly, an aliquot of 30–100 $\mu$l cell suspension, mixed by use of an automatic pipette, was placed in the bottom of polystyrene tubes. Label uptake was started by rapidly ejecting 40–100 $\mu$l isotope solution, and this was followed by gentle swirling. The volume of isotope added was sufficient to keep the 60-min cellular uptake below 25% of medium FA. At the desired time, uptake was stopped by the addition of 5 ml of ice-cold KRH buffer containing 200 $\mu$M phloretin. Cells were separated from medium by filtration at very low pressure (50 mm Hg) on glass-fiber filters (Gelman Sciences, MI) and washed twice with 1 ml of ice-cold stop solution. Under these conditions there was no gain or loss of FA by the cells and no damage to cell integrity (11). Filters with cells were added to 5 ml of aqueous scintillation fluid (ACS), and cell-associated radioactivity was measured in a three-channel counter (LS 3300, Beckman, Fullerton, CA) programmed for [$^{3}$H]/[$^{14}$C] discrimination. Radioactive standards were counted under the same quenching conditions. Label separation was done with a computer routine which also adjusted for counting efficiency.

In all uptake studies, background radioactivity, representing isotope trapped extracellularly and that bound nonspecifically by the cells, was determined from zero-time incubations to which stop solution was added before isotope (11, 14). Background radioactivity amounted to 20–40% of the total uptake values at 6 sec at FA:BSA ratios between 0.5 and 5.0 and was routinely subtracted from the total uptake values. Background values, at each FA:BSA ratio, were proportional to extracellular radioactivity (cpm/$\mu$l). As uptake proceeded, less radioactivity was present extracellularly so background values were decreased by the percent of radioactivity taken up by the cells. Uptake, measured under the above conditions, represented the total radioactivity ($^{3}$H and $^{14}$C) recovered in the cells prior to extraction.

### Determination of label incorporation into various lipid fractions

Cells were incubated with isotopic solution for various times and ice-cold stop solution was added to stop uptake and metabolism. The cells were then filtered as described above. Cells on filters were lysed in 1.3 ml methanol and the lysate was added to 2.6 ml chloroform. The chloroform–methanol mixtures were shaken for 10 min. Then two phases were separated by addition of 0.2 volume of water. The organic layer contained in all cases 99% of cell radioactivity from [$^{3}$H]oleate and 85–90% of cell radioactivity from [$^{14}$C]glucose. The aqueous layer, which would contain fatty acyl CoA, reproducibly had less than 1% of cell $^{3}$H. Extractions were quantitative since total radioactivity in the extract was within 2% of cell uptake measured in parallel incubations. An aliquot of the chloro-

form layer was concentrated by evaporation under nitrogen and subjected to thin-layer chromatography on silica gel G with petroleum ether–diethyl ether–acetic acid 80:20:1. The major lipid groups were identified by comparison with standards that were run simultaneously on one side of the plate and visualized by Rhodamine. For counting of radioactivity in each lipid fraction, the scraped silica was added directly to counting fluid (ACS) and the vials were shaken for 20 min before counting. To quantitate the total radioactivity in a fraction, the percent (of total radioactivity) recovered was multiplied by uptake by cells assayed in parallel but not extracted. Incorporation and uptake could be directly related to label specific activity in the medium, as the same quenching conditions were used to estimate medium and cell radioactivity. Radioactivity recovered in the FA fraction was corrected for extracellularly trapped radioactivity (background radioactivity) determined from zero-time incubations.

### Estimation of DG and PL levels

Phospholipids were estimated as total phosphorus in concentrated cell lipid extracts by a modification of the method of Harris and Popat (15). Diglycerides in lipid extracts were estimated by the kinase assay of Preiss et al. (16) as modified by Wright et al. (17).

### Measurement of medium and cell fatty acids

To determine medium FA, an aliquot of cell suspension was mixed well and centrifuged, then 300–500 $\mu$l medium was added to 3 ml Dole extraction mixture, isopropanol–heptane–1 N sulfuric acid 4:1:0.1 (v/v/v). The fatty acids recovered in heptane were assayed by the $^{63}$Ni technique of Ho and Meng (18). Blanks consisted of a similar aliquot of buffer. To determine cell FA during uptake experiments, aliquots of cell incubations were added to 5 ml ice-cold stop solution, filtered, and washed as before. Cells on filters were then lysed in isopropanol followed by extraction in heptane–isopropanol–sulfuric acid (19). The heptane layer containing FA standard or sample (10–100 nmol FA) was routinely concentrated 5-fold under nitrogen. Under these conditions, the Ho and Meng technique (18) reproducibly measured 10 nmol of FA. Recovery of FA was complete as tested in experiments where known amounts of oleate (20–100 nmol) were added to incubation buffer and processed alongside other samples.

## RESULTS

### Concentration of FA and of ubFA in the assay medium

Characteristics of cell incubations important for uptake and lipid incorporation of FA and glucose are shown in **Table 1**. The ratios of FA to BSA in the assay medium after mixing of cell suspension and isotope solution are shown. The isotopic solution (composition shown under Substrate Uptake) contained negligible concentrations of [$^3$H]oleate and albumin relative to those present in the cell suspension. This meant that the FA:BSA ratio and concentration of unbound FA (ubFA) were not changed significantly upon isotope addition. At the start of the uptake, assay media from basal cells contained about 0.19 nmol FA per $\mu$l medium. Treatment with 0.1 $\mu$g/ml NE increased medium FA and the ratio of FA:BSA by 8-fold on average, resulting in an average 87-fold increase in ubFA. This concentration of NE was shown to produce optimal effects on FA uptake (20, 21) and on lipolysis (S. A. Melki and N. A. Abumrad, unpublished observations).

The molar ratio of FA to BSA (not the total FA) and the concentration of the FA:BSA complex are the main factors controlling FA availability for uptake (10–12), as they determine the concentration of unbound FA in solution and its rate of supply as it is taken up by the cells. Under our conditions, on the basis of current estimates of dissociation of the FA:BSA complex (23, 24), 44 and 3813

TABLE 1. Characteristics of cell incubations important for oleate uptake

| Parameter | Basal | NE-Treated |
|---|---|---|
| Cell FA (nmol/$10^5$ cells) | 9.20 ± 2.00 | 18.0 ± 4.80 |
| Medium FA ($\mu$mol/ml medium) | 0.19 ± 0.07 | 1.60 ± 0.09 |
| Medium FA:BSA ($\mu$mol/ml medium) | 0.65 ± 0.10 | 5.00 ± 0.20 |
| Medium unbound FA (nmol/ml medium) | 0.06 ± 0.02 | 5.20 ± 0.60 |
| FA dissociation rate (nmol/min) | 44.0 ± 13.0 | 3813 ± 1100 |
| Initial uptake rate (nmol/min/$10^5$) | 1.85 ± 0.40 | 15.0 ± 2.30 |

Isolated adipocytes were preincubated without or with norepinephrine (NE) (0.1 $\mu$g/ml, 10 min), incubated as described in Substrate Uptake, and then either recovered by filtration (for measurement of total uptake or of cell FA) or centrifuged (for measurement of medium FA). Unbound FA in the medium was calculated as previously described (11), based on the dissociation constants of FA and BSA (35). FA dissociation under the conditions of the studies was determined from published values for FA:BSA dissociation rate constants (23, 24). Since these reported values were for low ratios of FA:BSA, those for higher ratios, in NE-treated cells, were increased in proportion to the increase in unbound FA. All values are given for incubations containing $10^5$ cells. Values are means ± SE for four to six experiments. Differences between values for basal and NE-treated cells were statistically significant ($P < 0.05$ for cell FA and $P < 0.001$ for all other parameters).

nmol of FA dissociated per min in media from basal and NE-treated cells, respectively, which greatly exceeded the fastest early uptake rates (Table 1).

### Oleate uptake

We have previously characterized membrane transport of oleate in rat adipocytes under conditions where oleate metabolism was prevented (11, 14). The present study, conducted at 37°C and in the presence of glucose, deals with oleate uptake which represents the sum of transport and metabolism. Uptake of [$^3$H]oleate, as nmol per $10^5$ cells at a given time point, was obtained based on the average specific activity of medium FA up to that time point. The average was estimated from the arithmetic mean between specific activity at the time point and that at zero time. This was sufficiently accurate since label specific activity decreased (by about 25% at 60 min) mainly as a result of nearly linear label uptake by the cells. Medium FA, measured at different time points during the assay, did not change in most experiments. In some experiments a small (20–30%) increase was observed in medium FA at the end of the 60-min incubation. Under these conditions it was determined that the arithmetic mean was still adequate as it overestimated the true average specific activity, obtained by integral calculation, by less than 3% for all time points.

Oleate uptake representing total intracellular [$^3$H]oleate (free + esterified) estimated from medium specific activity is shown in **Fig. 1** (top panel). At least two phases could be distinguished in the uptake time course consistent with previous observations (11). An early phase represented FA transfer across the membrane, approach of intracellular FA to isotopic steady state (shown in the middle panel), and accumulation of fast turnover metabolites such as diglycerides (shown later in Fig. 2). The short (0.2 min) half-time of the early phase indicated that only a small fraction of cell FA in basal cells equilibrated with exogenous FA. If all cell FA could exchange with exogenous FA, isotopic equilibration would have required about 5 min (turnover time (5 min) = cell FA (9.2 nmol/$10^5$ cells) divided by uptake rate (1.8 nmol/min per $10^5$ cells). The late phase of the 60-min time course (Fig. 1, top panel, insert) was linear and, as shown later (Fig. 2), represented incorporation of oleate into slow-turnover lipids, mainly TG. Entry of extracellular FA was 8 times faster in cells treated with 0.1 $\mu$g/ml NE as compared to basal cells.

### Oleate incorporation into cellular lipids

Oleate incorporation (nmol oleate per $10^5$ cells) into various lipid fractions estimated on the basis of specific activity (SA) of medium FA is shown in Figs. 1 and 2.

Free intracellular [$^3$H]oleate increased in the first 0.5 min and then stabilized for the rest of the assay period (Fig. 1, middle and lower panels). Intracellular accumulation of labeled FA was very low in basal cells but generally increased in parallel with ubFA in the medium. It ranged between 0.004 nmol/$10^5$ cells at an FA:BSA ratio of 0.02 to 0.17 nmol/$10^5$ cells at an FA:BSA ratio of 1.7. All cell preparations had reached a steady state with respect to FA release before the start of the uptake assay. In basal preparations with low FA:BSA ratios, addition of the isotope solution, which had a ratio of 4, was associated with a transient peak (the first minute) of intracellular [$^3$H]oleate. This reflected the rapid establishment of a new steady state of ubFA in the medium. Basal preparations with relatively high FA:BSA ratios (higher than 0.75) did not exhibit a transient peak of intracellular [$^3$H]FA. Little change in medium FA occurred during the 60-min assay period.

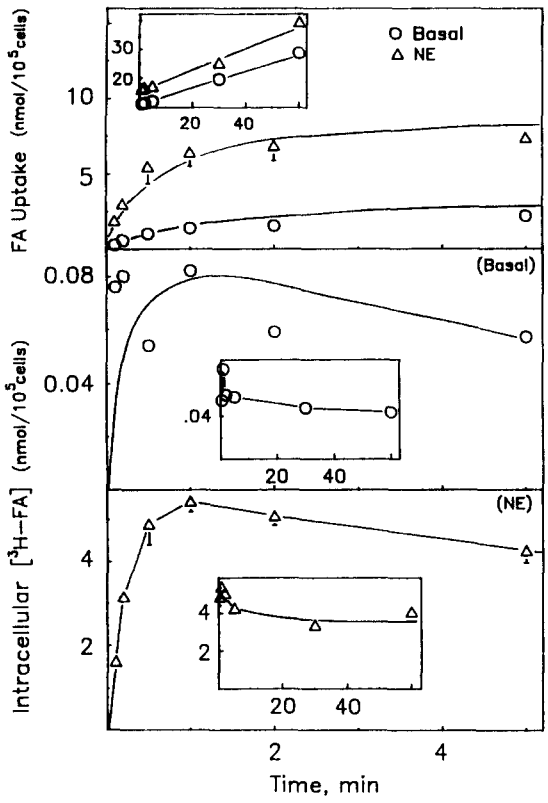


**Fig. 1.** Time course of [$^3$H]oleate uptake. Fat cells were preincubated without (○) or with (△) NE (0.1 $\mu$g/ml, 10 min) and then assayed for [$^3$H]oleate uptake. Uptake values (free + esterified cellular [$^3$H]oleate) are shown in the upper panel. They were calculated based on average medium specific activity up to each time point, estimated as described in the text. The values shown are means from five experiments. Standard errors are indicated by the bars which in some cases are hidden by the symbols. Differences between basal and NE-treated cells were statistically significant. Middle and lower panels show cellular free [$^3$H]oleate in basal and NE-treated cells, respectively. Owing to the great variability of medium unbound FA and consequently of intracellular [$^3$H]FA in basal incubations (see text for more detail), only a representative result from two experiments (FA:BSA = 0.5–0.7) is shown in the middle panel. ubFA was less variable in NE-treated cell preparations, so data were pooled from five experiments (lower panel).

NE treatment increased labeled cellular FA (Fig. 1, lower panel) by 50- to 500-fold. The variability reflected the different medium FA:BSA ratio present prior to addition of NE. As for basal cells, no significant changes in FA release were observed during the assay.

Incorporation of [$^3$H]oleate into the major lipid fractions was biphasic. A small amount of incorporation occurred rapidly (over the first minute), followed by a slower phase of linear incorporation for the remainder of the 60-min time course **(Fig. 2)**. Early incorporation rates (estimated from the linear phase, inserts) were 1.7, 0.22, and 0.95 nmol/$10^5$ cells per min for DG, PL, and TG, respectively. NE treatment produced an enhancement (40–70%) of oleate incorporation into all three fractions at 30–60 min.

Incorporation rates were also estimated based on intracellular SA of oleate. This was calculated from the cpm recovered as free oleate divided by cell free FA and averaged between zero time and each time point (area under SA time course divided by the time interval). The incorporation rates obtained exceeded those calculated on the basis of medium SA and gave estimates of DG levels that greatly exceeded chemical measurements. For example, oleate incorporation into DG (based on SA of cell FA) was about 600 nmol/$10^5$ cells at 60 min, as compared with measured DG levels of 135 nmol/$10^5$ cells. Similarly, incorporation into PL at 60 min was 641 nmol/$10^5$ cells which was about 60 times the amount present in the cell (8 nmol/$10^5$ cells). These results indicated that exogenous oleate was not mixing significantly with cell FA, in line with our earlier analysis of the uptake time course (Fig. 1).

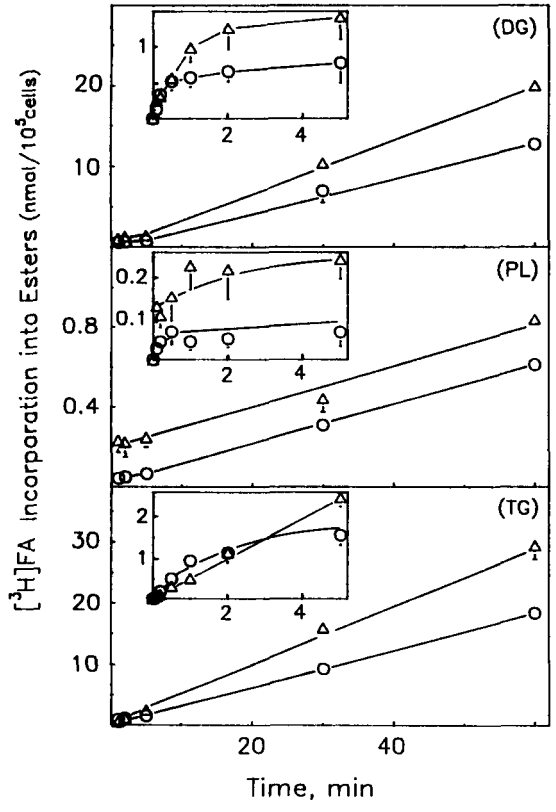


**Fig. 2.** Incorporation of [$^3$H]oleate into diglycerides (DG), phospholipids (PL), and triglycerides (TG). Basal (○) and NE-treated (△) cells were processed as described in Experimental Procedures and in the legend to Fig. 1. Incorporation into each lipid fraction was obtained by multiplying % radioactivity in the fraction by total uptake based on label specific activity in the medium. Data are means from three-five assays and are shown with their standard errors (bars, often hidden by the symbols). Differences in $^3$H incorporation between basal and NE-treated cells were significant for DG and TG at 30 and 60 min and for PL at all points except 0.5 and 30 min.

## [$^{14}$C]Glucose uptake and incorporation into cellular lipids

**Figure 3** shows the time course of [$^{14}$C]glucose uptake by cells processed as described for "substrate uptake" and not extracted (Cell $^{14}$C in Fig. 3) and its incorporation into DG, PL, and TG. Incorporation into free FA was negligible over the 60-min experimental period and was undetectable in most experiments. Glucose uptake rates estimated from the early phase of cell $^{14}$C accumulation (prior to significant release of [$^{14}$C]glucose, [$^{14}$C]lactate, or $^{14}CO_2$) were about 0.3 nmol/min per $10^5$ basal cells. There was no significant delay or induction period for incorporation of glucose into DG and PL, indicating that glycolytic intermediates and glycerophosphate achieved steady-state labeling rapidly.

The [$^3$H]FA/[$^{14}$C]glycerol labeling ratio was computed for the different lipid fractions based on extracellular specific activities. This was done for time points between 5 and 60 min since label equilibration had occurred in the intracellular FA precursor pool while label incorporation into DG, PL, and TG continued at a steady rate. There were no trends with time, so ratios for 5- to 60-min time points were pooled. The [$^3$H]/[$^{14}$C] ratios in basal cells were 0.8, 0.7, and 1.5 for DG, PL, and TG, respectively **(Fig. 4)**. Comparison of these ratios to the expected chemical ratios for DG, PL, and TG (2, 2, and 3, respectively) suggested an approximate 2-fold dilution of labeled FA after cell entry and before incorporation into esters.

NE treatment slightly stimulated glucose uptake (Fig. 3, upper left panel), did not alter [$^{14}$C]glucose incorporation into DG or PL, and slightly enhanced its incorporation into TG. The [$^3$H]/[$^{14}$C] ratios for all three lipid fractions indicated a general 2-fold dilution of exogenous oleate (Fig. 4). Thus the 8-fold increase in FA entry was matched by a comparable increase in the diluting endogenous FA.

## Effect of increasing unbound FA on [$^{14}$C]glucose incorporation

Incorporation of [$^{14}$C]glucose was measured at various medium concentrations of total FA (200–600 $\mu$M) and with a constant concentration of BSA (294 $\mu$M, FA:BSA =

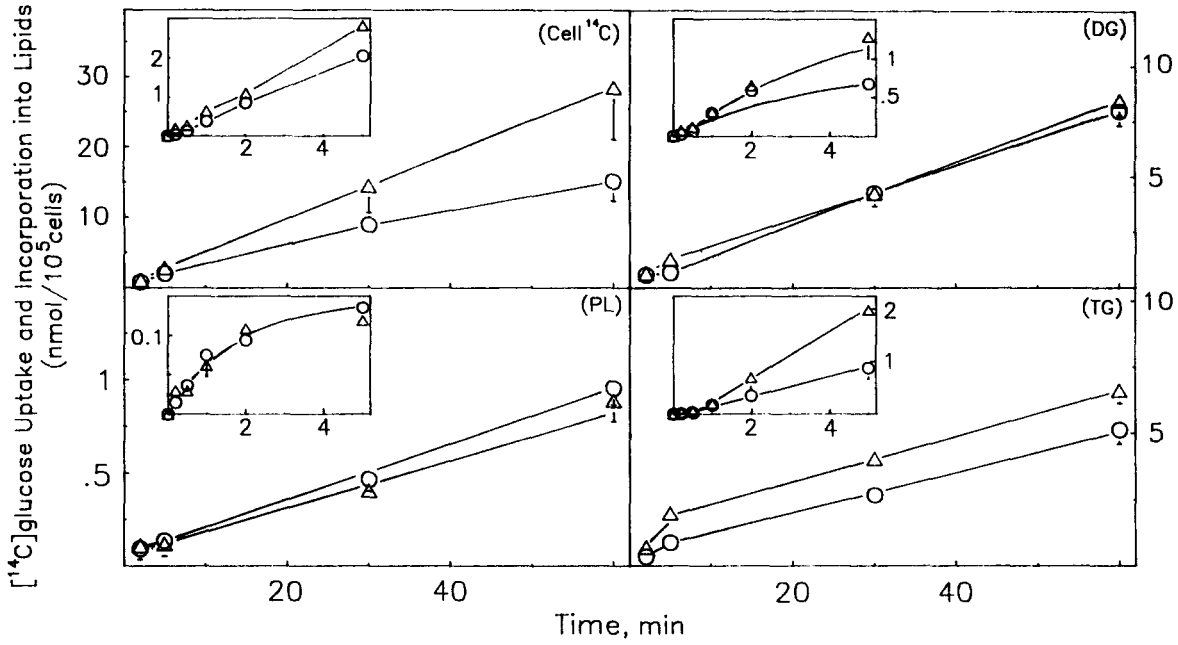


**Fig. 3.** [$^{14}$C]Glucose uptake and incorporation into diglycerides (DG), phospholipids (PL), and triglycerides (TG). Nanomoles of glucose taken up (Cell $^{14}$C) or incorporated into lipids (DG, PL, TG) were calculated based on medium [$^{14}$C]glucose specific activity. To obtain the nmoles of lipid synthesized, incorporation values shown should be multiplied by 2 (one mole of glucose yields two moles of lipid-glycerol so the SA of glycerol-phosphate is half that of glucose). Data are means from three-five experiments shown with their standard errors (bars, often hidden by the symbols). Effect of NE on incorporation into TG was significant at 5, 30, and 60 min, $P < 0.02$).

0.7-2). In these experiments, the cells were used at either a density of 10 or 30% (v/v) with similar results. Adipocytes (100 $\mu$l of cell suspension) were preequilibrated in buffer containing 2 mM glucose and the indicated concentration of FA and BSA for 20 min at room temperature and then for 4 min at 37°C. Uptake was started by addition of medium (10 $\mu$l) that contained tracer [$^{14}$C]glucose (300,000 cpm/$\mu$l) and no FA or albumin. As shown in

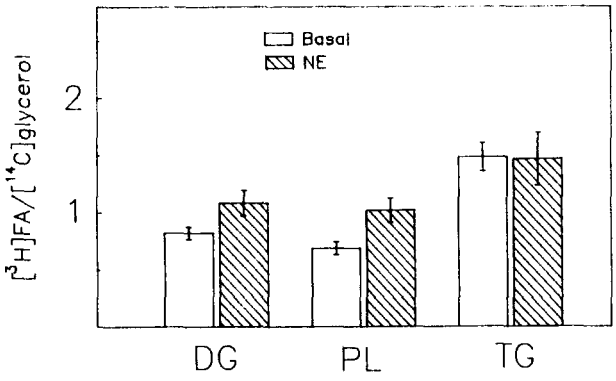


**Fig. 4.** [$^{3}$H]Oleate/[$^{14}$C]glycerol incorporation ratios. Ratios for incorporation of [$^{3}$H]oleate/oleate/[$^{14}$C]glycerol into DG, PL, and TG were calculated from data points 5, 30, and 60 min of Fig. 2 and those of Fig. 3 (after multiplying rates by 2). If there were no dilution of exogenous FA by cell FA and no recycling of lysolipids, the ratios for DG, PL, and TG would be 2, 2, and 3, respectively. Data are means shown ± SE from three-five experiments. Effects of NE on the ratios did not reach statistical significance.

**Fig. 5**, increasing external ubFA from 0.01 to 0.064 $\mu$M increased glucose uptake and its incorporation as glycerol into PL, DG, and TG. No additional increases in incorporation were observed when unbound FA was raised further to 0.260 $\mu$M (or to 3.4 $\mu$M with NE treatment).

Treatment with NE increased glucose incorporation into glycerides significantly above that in basal cells without added FA. The effect was larger in magnitude than that observed in Fig. 3 as a result of lower FA:BSA ratios (and lower unbound FA) in the basal cell preparations of Fig. 5. By comparison with basal cells in presence of added FA, NE-treated cells exhibited reduced glucose incorporation into DG and PL.

The data were analyzed in a Hanes plot (S/v versus S) to obtain kinetic parameters for glyceride synthesis. Incorporation of glucose into DG, PL, and TG exhibited half-saturation at low unbound FA concentrations (0.01-0.06 $\mu$M) **(Fig. 6)**. NE appeared to inhibit synthesis of DG (upper panel) and PL (middle panel) but not that of TG (lower panel).

Unlike labeled TG that enter a large intracellular pool, newly formed labeled DG and PL can be degraded in presence of $\beta$-agonists (7-9). We investigated whether this might explain the apparent inhibitory effect of the hormone on glucose incorporation into these fractions. In separate experiments (not shown) we followed degradation of newly formed lipids (labeled during a 5-min preincubation) in basal and NE-treated cells. Significant decreases of DG radioactivity (49% by 30 min) and of PL

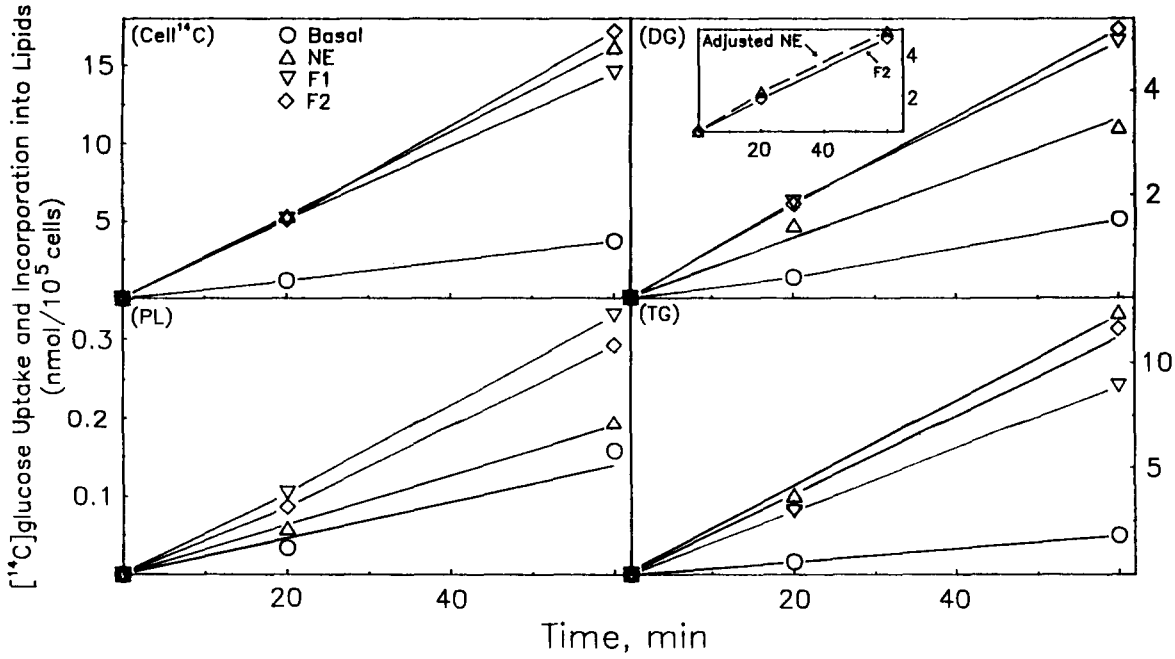


**Fig. 5.** Effects of medium FA concentration on [$^{14}$C]glucose uptake and incorporation into diglycerides (DG), phospholipids (PL), and triglycerides (TG). Cells were pre-equilibrated in buffer containing 2 mM glucose and without or with added oleate. Concentrations of unbound FA were: Basal, B = 0.01 $\mu$M, F1 = 0.064 $\mu$M, F2 = 0.260 $\mu$M, NE = 3.40 $\mu$M. Uptake was started by addition of medium containing tracer [$^{14}$C]glucose. Nanomoles glucose taken up or incorporated were calculated as explained under Fig. 3. Insert in DG panel shows incorporation into NE-treated cells adjusted for label loss from breakdown of labeled DG. Differences between basal and NE were significant for uptake (cell $^{14}$C) and for TG ($P < 0.005$). Differences between NE and F2 were significant for DG and PL at 60 min ($P < 0.05$).

radioactivity (40% by 30 min) were observed after exposure to NE. Radioactivity in TG remained stable. Incorporation of [$^{14}$C]glucose into DG in the presence of NE was then corrected for the amount of DG breakdown. This was obtained by multiplying incorporation in basal cells with 600 $\mu$M added FA (F2 in Fig. 5) by the average percent degradation of labeled DG up to each time point. When estimates for label loss from degraded DG were added to the amount of DG labeled in the presence of NE, incorporation rates were indistinguishable from those observed in basal cells with 600 $\mu$M FA (Fig. 5, upper right panel, insert). Similar results were obtained when rates of glucose incorporation into PL were adjusted for PL breakdown (data not shown).

## DISCUSSION

The present study helped clarify aspects related to glycerolipid synthesis and its regulation by norepinephrine. *1*) The fraction of cell FA, which exchanges with ubFA in the medium and which is accessible to esterification enzymes, was determined in basal and NE-treated cells. *2*) Glyceride formation was estimated as a function of the physiological substrate, ubFA, and its saturation kinetics were determined. *3*) The effect of label loss, from hydrolysis of newly formed esters, on incorporation rates was evaluated. And *4*), based on the above information, direct effects of NE on glyceride synthesis were distinguished from those reflecting complications introduced by its lipolytic action. Our data indicated that glyceride synthesis is a highly saturable process and that NE does not directly alter kinetics of the pathway. The specific findings are discussed in detail below.

### Fatty acid mixing across the cell membrane

Uptake of [$^{3}$H]oleate indicated that exchange between extracellular and intracellular FA was very rapid, owing to the low concentrations of medium ubFA. Previous reports have suggested compartmentation of cell FA (7, 8, 25). Our data on kinetics of FA mixing documented this directly. We estimated that, in basal cells, about 2% of cell FA mixed with exogenous FA and was accessible to FA-esterifying enzymes. This was derived from three lines of evidence. First, accumulation of labeled free FA in the cell ceased within 0.5 min despite the fact that initial uptake (nmol/min) was about one-fifth of cell FA. Second, exogenous free FA in the cell at steady state (Fig. 1, middle panel) was very low compared to measured cell FA and was only diluted 2-fold by endogenous FA (as indicated by the [$^{3}$H]oleate/[$^{14}$C]glucose incorporation ratios). Third, synthesis of DG and PL at 60 min, calculated from specific activity of cell FA ([$^{3}$H]FA / total cell FA), greatly exceeded their chemically measured amounts.

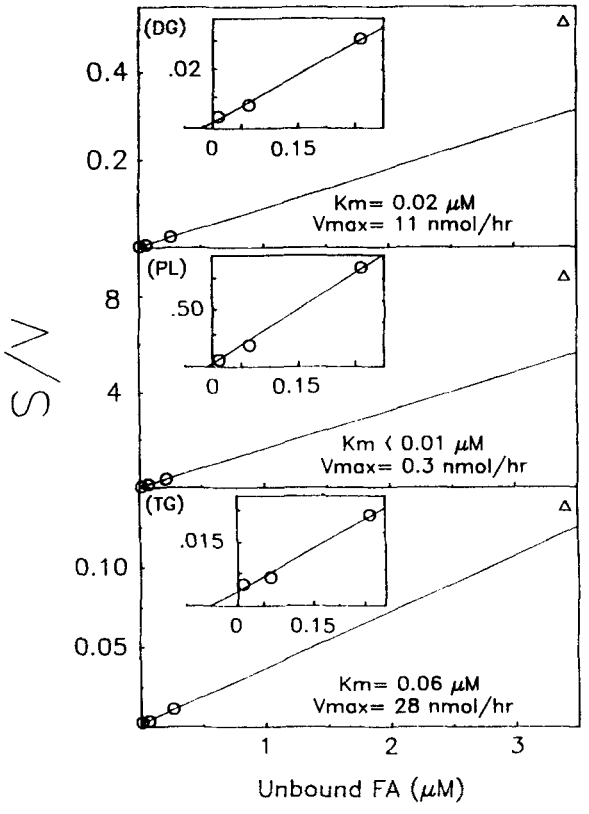


**Fig. 6.** Hanes plot of [$^{14}$C]glucose incorporation into DG, PL, and TG. Data from Fig. 5 were analyzed as S/v versus S plots, where v = nmol incorporated by $10^5$ cells at 60 min and S = medium unbound FA; Basal, ○; NE-treated, △.

In NE-treated cells, intracellular [$^3$H]FA increased to about 5 nmol per $10^5$ cells. The [$^3$H]/[$^{14}$C]incorporation ratios indicated an equal rise in the amount of diluting endogenous FA. This meant that metabolically inactive cell FA (about 9 nmol per $10^5$ cells) remained identical in basal and NE-treated cells. Thus, FA derived from lipolysis constituted the fraction of cell FA that exchanged with and diluted exogenous FA on its way to esterification.

### High-turnover pools of DG and PL

The heterogeneity of cell glycerides has been previously noted (7–9). It was demonstrated in our studies by the two phases (fast and slow) for $^3$H and $^{14}$C incorporations into DG and PL (Fig. 2). The similarity in incorporation patterns for [$^3$H]oleate and [$^{14}$C]glucose indicated that the rapid and slow turnover phases involved complete hydrolysis–resynthesis and that recycling by substitution of one FA moiety did not contribute significantly to the rates. The linear phase of incorporation from 5- to 60 min indicated that the major fraction of these lipids turned over slowly. Estimates of the size of the high-turnover DG and PL pools from the fast incorporation phases of Fig. 2 are consistent with this last interpretation. For example, in basal cells, the rapid-turnover DG pool amounted to 0.6 nmol per $10^5$ cells (about 0.4% of cell DG) and was expanded by NE to about 1.5 nmol (0.8% of cell DG). Similarly the rapid-turnover PL pool (0.06 nmol per $10^5$ cells) constituted only 0.6% of cell PL and was expanded by NE to 0.25 nmol (2.8% of cell PL). The changes in PL turnover would reflect the effects of NE to accelerate breakdown of membrane PL (26). However, a significant part of the NE effect on high-turnover PL appeared to involve recycling rather than hydrolysis–resynthesis. This was deduced from the observation that the effect of NE to enhance the early phase of [$^3$H]oleate incorporation into PL (Fig. 2) was not matched by a similar enhancement of [$^{14}$C]glucose incorporation (Fig. 3).

### Saturation kinetics of glyceride synthesis

In basal cells, labeled glucose accurately estimated rates of synthesis for all glycerides. Relating lipid incorporation to medium ubFA concentration indicated that pathways for synthesis of DG, PL, and TG were highly saturable. In contrast, relating label incorporation to total FA in the medium (6, 27) underestimates substrate concentration, since ubFA does not change in proportion with total FA, and could characterize the process as unsaturable (27). The $K_m$ for TG synthesis (about 0.06 μM) was in the physiological range while that for PL (<0.01 μM) appeared lower. This meant that physiological changes in blood FA will modulate synthesis of TG. Synthesis of PL would be largely saturated under physiological conditions and thus independent of blood FA. The high saturability of all pathways for FA esterification indicated that small increases in glyceride hydrolysis can rapidly result in accumulation of free FA in the cell. This would inhibit further lipolysis unless the lipolyzed FA could be released (28). Lipolyzed FA, as previously discussed, exchanges with medium ubFA and its net release will reflect the molar ratio of FA:BSA in the medium. Thus, lowering of medium FA (and of ubFA) should favor both less TG deposition and more TG hydrolysis.

### Regulation of glycerolipid synthesis by NE

Saturation and substrate dependence of glycerolipid synthesis should be considered in assessments of pathway regulation from labeled precursors. In the case of adipocytes, NE and numerous other agents produce changes in FA release and in medium FA:BSA ratio, and hence ubFA. This will influence incorporation rates. As we have shown, the effects of NE on glucose incorporation into DG, PL, or TG depended on the FA:BSA ratio (ubFA) of basal cell incubations, which was quite variable. If the basal FA:BSA ratio (and hence ubFA) was low, esterification pathways were unsaturated and substrate-dependent, so the effect of NE to raise the FA:BSA ratio and ubFA dominated, producing an enhancement of incorporation rates. On the other hand, if the basal FA:BSA ratio were high enough for ubFA to saturate esterification, other

effects of NE predominated. For example, a reduction in incorporation rates was observed in the case of DG and PL at similar saturating concentrations of ubFA. This reflected acceleration by NE of the hydrolysis of labeled DG and PL. Thus, in order to obtain precise estimates of synthesis for DG and PL in NE-treated cells, glucose incorporation rates had to be adjusted for label loss from product breakdown. Incorporation into TG, as a result of the large TG pool, was not affected by label loss from TG hydrolysis and adequately reflected TG synthesis.

### Limitations of the studies

The $K_m$ values we report for synthesis of glycerolipid might be overestimated. There is evidence to suggest that FA in aqueous solution begins to associate below the critical micellar concentration (29, 30). Since the FA monomer is the species that binds albumin, our calculations of unbound FA, which did not correct for FA dimerization, would overestimate the concentration of monomeric ubFA. The occurrence of FA dimerization below the critical micellar concentration is still disputed (31); however, if it is a real phenomenon, levels of ubFA that produce saturation of glycerolipid synthesis would be lower than those we report. This, however, does not affect data interpretation. For example, we have recalculated data of Fig. 6 based on dissociation constants for stearate without and with correction for aqueous association of the FA (constants for oleate being unavailable). When dimerization was taken into consideration the $K_m$s for DG, PL, and TG synthesis were lowered about 6-fold, however the effect of NE treatment was identical for all three lipids.

### Summary

In conclusion, we could find no evidence for acute regulation of FA esterification enzymes by NE. This does not exclude acute effects by other hormones or long-term regulation by NE and other factors. The experiments described in this report provide a framework for estimating direct regulation of glycerolipid synthesis amidst complications introduced by changes in glycerolipid hydrolysis. The methodological approach should be useful for investigating direct regulation of synthesis by other agents (for example insulin, refs. 32–34) or by metabolic or disease states (fasting or diabetes).

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