Studies of Triglyceride Biosynthesis in Homogenates of Adipose Tissue

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The mechanism of triglyceride synthesis has been studied in homogenates of chicken liver (1-4) and rat liver (3, 4) and several of the reactions have been investigated individually in some detail. Thus, far, however, no studies on the mechanism of triglyceride synthesis in adipose tissue have been reported. In other experiments we have found that epinephrine, adrenocorticotropic hormone and glucagon appear to inhibit the esterification of C14-palmitate when present during incubation of the epididymal fat pad in vitro (5). Such an action could explain in part the lipid-mobilizing effects of these hormones. Knowledge of the pathways of triglyceride biosynthesis may then be a prerequisite to understanding the mechanism of metabolic and hormonal control of lipid deposition and mobilization. In addition, by studying triglyceride synthesis in a homogenate system in which the sizes of the pools of intermediates are more amenable to measurement and control than they are in the intact tissue, it may be possible to investigate the extent to which the triglycerides in this tissue participate in a true dynamic equilibrium, a question concerning which there is disagreement (6).

Some of the conditions required for the synthesis of triglycerides from free fatty acids and α-glycerophosphate in cell-free homogenates of the epididymal fat pad of the rat are reported below. The pathway suggested by these studies is similar to that proposed by other workers for triglyceride synthesis in the liver (1-4). Experiments in which changes in the size of the free fatty acid pool were determined simultaneously with measurements of the rate of C14-palmitate incorporation demonstrate net synthesis and provide evidence of a true dynamic state.

Experimental Procedure

Male Sprague-Dawley rats weighing 150 to 200 g were decapitated. The epididymal fat pads were rapidly removed and homogenized in 0.15 M potassium chloride at room temperature. Unless otherwise indicated 4 ml of potassium chloride were used for each 1 g of adipose tissue. In some experiments the homogenate was used without further treatment (whole homogenate). The absence of intact cells in one such preparation was established by electron microscopy.1 In other studies the homogenate prepared as above was centrifuged for 10 minutes at approximately 500 x g in a refrigerated centrifuge. This treatment caused, most of the fat to float to the top of the tube and to congeal. Nuclei and cell debris were sedimented to the bottom of the tube. An 18-gauge needle was introduced through the upper layer of congealed fat and the middle layer of slightly turbid solution was aspirated. This fraction is designated defatted homogenate.

Homogenates were supplemented as shown in Table I and incubated at 37° either with 1-C14-palmitate, 1-C14-α-GP,2 or P32-α-GP. Preliminary experiments were carried out in an oxygen atmosphere, later studies in air. Esterification in the system described here proceeds equally well under oxygen, air, or nitrogen. At the end of the incubation the contents of each flask were rinsed into a separatory funnel with three 10-ml portions of extraction mixture (isopropanol:isoctane:1 N sulfuric acid (40:10:1, volume per volume)) (7). The funnel was shaken vigorously and allowed to stand for at least one hour. Isoctane, 18 ml, and H2O, 12 ml, were then added resulting in the separation of two phases. After removal of the aqueous layer the remaining isooctane was washed with 15 ml of alkaline ethanol to remove FFA according to the method of Borgstrom (8). The isooctane phase was washed again with alkaline ethanol and assayed for radioactivity. This fraction, referred to as the neutral lipid fraction, contains essentially all of the triglycerides, diglycerides, cholesterol, and cholesterol esters but, because of their polarity, only a fraction of the monoglycerides and phospholipids. Phosphatidic acid, the only radioactive phospholipid found in any significant amounts in these studies, is completely (>95%) removed from isooctane by the alkaline-ethanol wash. The first alkaline ethanol extract was acidified and the FFA were reextracted from it into isooctane. One aliquot of this was titrated (7) and a second aliquot assayed for radioactivity.

In order to permit quantitative estimation of incorporation into phospholipids, in some studies the incubation mixture was extracted with chloroform: methanol, 2:1, 25 ml for each 1-ml sample. The chloroform phase was then passed over a silicic acid column according to the method of Borgstrom (9). The glycerides and free fatty acids were eluted with chloroform; the phospholipid fraction was eluted with methanol. The chloroform eluate was taken to dryness and redissolved in isooctane. The isooctane solution was then washed with alkaline ethanol to remove FFA and an aliquot of the washed isooctane, containing the neutral lipids, was counted. One aliquot of the methanol eluate containing the phospholipids was counted and the remainder was concentrated and chromatographed on silicic acid-impregnated paper by the method of Marinetti and Stotz (10).

1 We acknowledge with thanks the assistance of Dr. Sanford L. Palay in these studies.

2 The abbreviations used are: FFA, free fatty acids; α-GP, α-glycerophosphate.
Incorporation of l-C14-palmitate into Complete system minus orGP...

- Complete system minus Mg++. 35 57
- Complete system minus buffer. 0
- Complete system minus cysteine 51
- Complete system minus NaF. 32 50
- Complete system minus buffer. 0
- Complete system with Tris buffer in place of
  phosphate. 58 93
- Complete system minus creatine phosphate. 58
- Complete system minus ATP plus 2 pmol of ADP and 7.5 pmol of creatine phosphate...
- None. 0
- Buffer only. 2
- Complete system, held at 100° for 5 min... 0

* Each flask contained 2 ml of a 1:5 homogenate of epididymal fat pad in 0.15 M KCl (representing about 400 mg wet weight of adipose tissue) with 10 pmol of aGP, tracer amount (less than 0.01 pmol) 1-C14-palmitate, potassium salt, and the following co-factors: 2 pmol of ATP; 3 pmol of MgCl; 0.1 pmol of CoA; 25 pmol of cysteine; 125 pmol of NaF; 125 pmol of potassium phosphate buffer, pH 7.0; water to make a final volume of 3 ml.

Results

Conditions for Incorporation into Neutral Lipid—Homogenates of adipose tissue incubated with l-C14-palmitate (with or without aGP) but without addition of any cofactors incorporated no radioactivity into neutral lipids beyond the small number of counts found in zero time controls. Boiled homogenates with aGP and all cofactors added likewise did not incorporate palmitate. The supplements that yielded optimal incorporation are seen in Table I. ATP and CoA appeared to be absolute requirements. Synthesis was decreased but not abolished by the omission of cysteine or fluoride. The improved incorporation obtained with addition of creatine phosphate to an ATP-containing system and the ability of creatine phosphate plus ADP to replace completely ATP, indicated the presence of an active transphosphorylase system. The phosphate buffer generally used could be replaced by Tris buffer without significant decrease in activity of the system.

Omission of aGP led to a marked reduction in incorporation but there was always some incorporation without it. In four experiments using whole homogenate, incorporation in the absence of aGP varied from 17 to 36% of that with aGP added. In four experiments with the defatted homogenate these values ranged from 8 to 22%. Dialysis of defatted homogenate was carried out against 0.15 M KCl containing 0.125 M phosphate buffer, pH 7.4, for 8 hours at 4° in an attempt to magnify the effect of omitting aGP by removing endogenous aGP as well as its potential precursors. This dialysis had little effect. It was later shown, however, that only one-third of 1-C14-aGP added to a homogenate could be removed by dialysis under these conditions. The low level of incorporation seen without added aGP could be due to the presence of endogenous aGP in the fat pad. Margolis, using an enzymatic assay, found very low levels of aGP in adipose tissue homogenates, less than 0.2 pmol per gm of tissue (wet weight). It is of interest that the dialysis procedure only reduced the incorporation observed with the full system by about 25%, indicating that no essential factors other than those added as in Table I were removed by dialysis under these conditions.

Incorporation as a function of the concentration of added ATP is shown in Fig. 1 and as a function of the concentration of added aGP in Fig. 2.

As shown in Table II, glycerol added in amount equimolar with aGP did not replace it. Attempts to demonstrate glycerophosphokinase activity directly were unsuccessful.4 Monoolein, added to the homogenate in 0.1 ml of ethanol, not only failed to increase incorporation above the level observed in the absence of aGP but actually decreased incorporation probably due to the

4 A. Karmen, unpublished experiments. We acknowledge with thanks the generous assistance of Dr. Arthur Karmen in the paper chromatographic separations of glycerides described here.
It should be noted here that albumin added to unfortified homogenates also markedly suppresses the release of FFA as shown below (Table VI).

**Time Course of 1-C^{14} Palmitate Incorporation**—Incorporation into neutral lipid as a function of time by a whole homogenate, supplemented as described in Table I and incubated at 37\(^\circ\), is shown in Fig. 3. Incorporation remained roughly linear for only 10 minutes, and was essentially completed by 40 minutes. When, at 40 minutes, second additions of 1-C\(^{14}\)-palmitate, 10 \(\mu\)moles of \(\alpha\)-GP, and all of the cofactors used in the complete system were made, esterification was resumed.

Data shown in Table IV are similar, except that in this experiment incorporation during the first 40 minutes was determined in Flask 1, whereas incorporation during the second 40 minutes was determined in Flask 2, to which \(\alpha\)-GP and cofactors were added for each 40-minute period but 1-C\(^{14}\)-palmitate was added only for the second period. Data from Flasks 3 to 7 in this experiment show that none of the combinations of \(\alpha\)-GP and/or one or two cofactors which were tried were as effective as the total mixture (Flask 2) in supporting the incorporation of 1-C\(^{14}\)-palmitate when added with it for the second 40 minutes.

As shown in the last line, when buffer alone was present during the first 40 minutes of incubation (cofactors plus \(\alpha\)-GP and 1-C\(^{14}\)-palmitate added only at the beginning of the second 40 minutes) there was very little C\(^{14}\) incorporated. Incubation under these conditions, as discussed more fully below, leads to a marked in-

| Table II |
|-----------------|-----------------|-----------------|
| **Ineffectiveness of glycerol as precursor for lipid synthesis** |
| Conditions | Time | C\(^{14}\) in neutral lipid |
| Whole homogenate | | |
| Complete system* | 20 | 100 |
| Minus \(\alpha\)-GP | 20 | 40 |
| Minus \(\alpha\)-GP + 10 \(\mu\)moles of glycerol | 20 | 48 |
| Defatted homogenate | | |
| Complete system* | 30 | 100 |
| Minus \(\alpha\)-GP | 30 | 9 |
| Minus \(\alpha\)-GP + 10 \(\mu\)moles of glycerol | 30 | 8 |
| Minus \(\alpha\)-GP + 10 \(\mu\)moles of monoolein | 30 | 2 |

* As in Table I.

**Table III**

| Table III |
|-----------------|-----------------|-----------------|
| **Inhibitory effect of bovine serum albumin on 1-C\(^{14}\)-palmitate incorporation** |
| Inoculation mixture as described for whole homogenate in Table I. |
| Incubation time, 20 minutes. |
| Serum albumin concentration | Total radioactivity in neutral lipids |
| | c.p.m. |
| Experiment A | | |
| 0 % | 16,000 |
| 1 | 2,600 |
| 2 | 1,300 |
| | |
| Experiment B | | |
| 0 3% | 10,200 |
| 3 | 1,600 |
| 3* | 830 |

* Albumin used in this experiment had been treated by the method of Goodman (15) to remove fatty acids.

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P. Goldman, unpublished experiments.
FIG. 3. Time course of incorporation of 1-Cl*-palmitate into neutral lipid. Whole homogenate incubated with cofactors as described in Table I. All supplements added again after 40 minutes in the same amount as at zero time (O—O). Cofactors added at zero time only, 40- to 60-minute points (A—A).

TABLE IV
Effect of preincubation of homogenate on esterification of 1-Cl*-palmitate

Whole homogenate incubated as described in Table I.

<table>
<thead>
<tr>
<th>Flask No.</th>
<th>Additions during first 40-min. incubation</th>
<th>Additions during second 40-min. incubation</th>
<th>Final neutral lipid radioactivity (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Full system with 1-Cl*-palmitate (see Table I)</td>
<td>None, stopped first 40-min. incubation</td>
<td>73,000</td>
</tr>
<tr>
<td>2</td>
<td>Full system without 1-Cl*-palmitate</td>
<td>1-Cl*-palmitate plus all cofactors</td>
<td>73,400</td>
</tr>
<tr>
<td>3</td>
<td>Full system without 1-Cl*-palmitate</td>
<td>1-Cl*-palmitate added</td>
<td>5,500</td>
</tr>
<tr>
<td>4</td>
<td>Full system without 1-Cl*-palmitate</td>
<td>1-Cl*-palmitate plus ATP and Mg++</td>
<td>24,700</td>
</tr>
<tr>
<td>5</td>
<td>Full system without 1-Cl*-palmitate</td>
<td>1-Cl*-palmitate plus CoA</td>
<td>7,950</td>
</tr>
<tr>
<td>6</td>
<td>Full system without 1-Cl*-palmitate</td>
<td>1-Cl*-palmitate plus ATP, Mg++, and αGP</td>
<td>19,000</td>
</tr>
<tr>
<td>7</td>
<td>Full system without 1-Cl*-palmitate</td>
<td>1-Cl*-palmitate plus αGP</td>
<td>5,600</td>
</tr>
<tr>
<td>8</td>
<td>Buffer only</td>
<td>1-Cl*-palmitate plus all cofactors</td>
<td>9,100</td>
</tr>
</tbody>
</table>

increase in FFA concentration and the low palmitate incorporation may be related to this (decreased precursor specific activity and/or enzyme inactivation).

Concentration and Specific Radioactivity of FFA during Incubation—The time course of changes in neutral lipid radioactivity, FFA radioactivity, FFA specific radioactivity, and FFA concentration in a fully supplemented homogenate is shown in Fig. 4. The radioactivity in the FFA decreased rapidly for 10 minutes, then more gradually, being rather close to a mirror image of the neutral lipid radioactivity curve. The net concentration of FFA decreased by about 35% in this experiment. It was shown in parallel experiments that the homogenate system used (without CO₂ present) incorporates virtually no acetate into FFA and that oxidation of 1-Cl*-palmitate is negligible under these conditions. It can be concluded that the disappearance of FFA probably represents net synthesis of lipid.

The specific radioactivity of the FFA fraction fell during the course of the incubation. Since there is apparently no de novo fatty acid synthesis under these conditions, a continuing lipolysis can be inferred. Even during active incorporation, in some experiments accompanied by a net decrease in FFA, in other experiments with little or no net change in FFA concentration, there is a simultaneous breakdown of triglyceride (see also Table IV).

Effects of Added FFA—The effect of adding extra FFA to the homogenate was variable, particularly in experiments with

-Fig. 4. Time course of changes in FFA concentration and specific activity during incorporation of 1-Cl*-palmitate into neutral lipid. Defatted homogenate supplemented as in Table I.

TABLE V
Effect of increasing initial FFA concentration on net changes in FFA and 1-Cl*-palmitate incorporation

Defatted homogenate incubated as described in Table I including creatine phosphate.

<table>
<thead>
<tr>
<th>Flask No.</th>
<th>FFA added (μEq per flask)</th>
<th>Zero time (c.p.m.)</th>
<th>20 min. (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0,4</td>
<td>0,4</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>1,5</td>
<td>0,7</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>97,000</td>
<td>12,000</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>25,000</td>
<td>28,000</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>200</td>
<td>20,400</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>200</td>
<td>12,900</td>
</tr>
</tbody>
</table>

*1.2 μEq of FFA prepared by saponification of epididymal fat pad lipids.
Bovine serum albumin, which inhibits incorporation of palmitate of I-C14-palmitate continues under these conditions, Table I). Additions that when sodium fluoride alone is omitted from the mixture there is a net increase in FFA (although esterification is the usual cofactor mixture. The results of Experiment 2 show in this comparison that the specific radioactivity of the FFA changes rapidly, most rapidly near the beginning of the incubation. Thus, measurements of initial rate are hampered. Preliminary experiments show that after three successive centrifugations with removal of the top fat layer each time, the sensitivity of the system to FFA increases. Addition of a very small amount of the congealed fat layer to the defatted homogenate seems to exert a protective action against the inhibitory action of added FFA. The variability from experiment to experiment may be due to the variable degree to which the tissue fat is removed. The nature of the material "protecting" the homogenate has not been determined.

Production of FFA during incubation of adipose tissue homogenate—Changes in the specific radioactivity of FFA during incubation (Fig. 4, Table V) suggest that fatty acid is formed from triglyceride during incubation of adipose tissue homogenates. In the absence of cofactors, there is no synthesis of glycerides as shown above, and there is a net increase in FFA (Table VI). In Experiments 1 and 2 there was no net change in FFA concentration when the incubation was carried out in the presence of the usual cofactor mixture. The results of Experiment 2 show in addition that when sodium fluoride alone is omitted from the mixture there is a net increase in FFA (although esterification of 1-C14-palmitate continues under these conditions, Table I). Bovine serum albumin, which inhibits incorporation of palmitate

**Table VI**

Production of FFA during incubation of whole adipose tissue homogenate

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Additions</th>
<th>Time (min)</th>
<th>Net change in FFA µEq/flask</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Complete system*</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>20</td>
<td>+1.5</td>
</tr>
<tr>
<td>2</td>
<td>Complete system</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Complete system minus NaF</td>
<td>20</td>
<td>+1.4</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>60</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>Albumin, 30 mg per ml</td>
<td>60</td>
<td>+0.4</td>
</tr>
<tr>
<td></td>
<td>FFA-free albumin, 30 mg</td>
<td>60</td>
<td>+0.3</td>
</tr>
</tbody>
</table>

* Conditions as described in Table I except that no phospho-creatine was included in the complete system in Experiments 1 and 2.

Kornberg and Pricer noted that the synthesis of phosphatidic acid in guinea pig liver homogenates was inhibited by added FFA (16). Because of the dilution of labeled fatty acid, calculations must, of course, take into account the relative precursor specific radioactivities in control and experimental flasks. This difficulty does not exist in experiments in which the incorporation of P32-labeled or C14-labeled αGP was studied and found also to be inhibited by the addition of FFA. Quantitative evaluation of the effect in experiments employing 1-C14-palmitic acid is made difficult by the fact that the specific radioactivity of the FFA changes rapidly, most rapidly near the beginning of the incubation. Thus, measurements of initial rate are hampered. Preliminary experiments show that after three successive centrifugations with removal of the top fat layer each time, the sensitivity of the system to FFA increases. Addition of a very small amount of the congealed fat layer to the defatted homogenate seems to exert a protective action against the inhibitory action of added FFA. The variability from experiment to experiment may be due to the variable degree to which the tissue fat is removed. The nature of the material "protecting" the homogenate has not been determined.

Production of FFA during incubation of adipose tissue homogenate—Changes in the specific radioactivity of FFA during incubation (Fig. 4, Table V) suggest that fatty acid is formed from triglyceride during incubation of adipose tissue homogenates. In the absence of cofactors, there is no synthesis of glycerides as shown above, and there is a net increase in FFA (Table VI). In Experiments 1 and 2 there was no net change in FFA concentration when the incubation was carried out in the presence of the usual cofactor mixture. The results of Experiment 2 show in addition that when sodium fluoride alone is omitted from the mixture there is a net increase in FFA (although esterification of 1-C14-palmitate continues under these conditions, Table I). Bovine serum albumin, which inhibits incorporation of palmitate

**Figure 5.** Neutral lipid synthesized by whole homogenate chromatographed on silicic acid by a modification of the method of Hirsch and Ahrens (11) using diethyl ether in petroleum ether, 1 to 100%. Plotted are µEq of hydroxamate-forming ester per ml of effluent and c.p.m. per ml of effluent. Break in the curve is due to loss of fractions.

**Table VII**

<table>
<thead>
<tr>
<th>Total radioactivity and specific radioactivities of neutral lipid fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Tube No.</td>
</tr>
<tr>
<td>29</td>
</tr>
<tr>
<td>Cholesterol esters (?)</td>
</tr>
<tr>
<td>111 thru 202</td>
</tr>
<tr>
<td>Triglycerides</td>
</tr>
<tr>
<td>Diglycerides</td>
</tr>
<tr>
<td>Monoglycerides</td>
</tr>
</tbody>
</table>

The results of Experiment 2 show in addition that when sodium fluoride alone is omitted from the mixture there is a net increase in FFA (although esterification of 1-C14-palmitate continues under these conditions, Table I). Bovine serum albumin, which inhibits incorporation of palmitate in the full system (Table III), likewise inhibits release of FFA in the unfortified homogenate, as shown at the bottom of Table VI.

Nature of Lipids Synthesized—The neutral lipids synthesized by whole homogenate supplemented as in Table I were fractionated on a silicic acid column by the method of Hirsch and Ahrens (11), except that a gradient elution from 1 to 3% diethyl ether in petroleum ether was used rather than switching to 4% diethyl ether. This was done to obtain subtraction of groups of triglycerides of different fatty acid composition for other purposes. The elution pattern of radioactivity and hydroxamate-forming esters shown in Fig. 5. Lipid fractions isolated as described in text from whole homogenate incubated under conditions outlined in Table I.
and diglycerides. The identification of the material in these peaks was confirmed, using chromatography on silicic acid-impregnated paper by the method of Karmen. As can be seen in Table VII the total amounts of radioactivity incorporated into triglyceride and diglyceride were similar, 40 and 53% of the total, respectively. The specific radioactivities, however, were widely different, 21.4 and 1570 c.p.m. per μEq hydroxamate-forming ester, respectively. In a second experiment using whole homogenate, the total radioactivities in triglyceride and diglyceride were 3080 and 3230 c.p.m. respectively and the specific radioactivities were 4.65 and 254 c.p.m. per μEq, respectively. The magnitude of the differences in specific radioactivity is undoubtedly exaggerated by the large excess of preformed triglyceride in the tissue. In two experiments with defatted homogenate the radioactivity was equally distributed between triglyceride and diglyceride with a small percentage in monoglyceride.

In Table V the total radioactivities incorporated into the triglyceride and diglyceride with a small percentage in monoglyceride. The RF of this material on silicic acid impregnated paper was compatible with that of monoglyceride. Its specific radioactivities, however, were widely different, 21.4 and 1570 c.p.m. per μEq hydroxamate-forming ester, respectively. In a second experiment using whole homogenate, the total radioactivities in triglyceride and diglyceride were 3080 and 3230 c.p.m. respectively and the specific radioactivities were 4.65 and 254 c.p.m. per μEq, respectively. The magnitude of the differences in specific radioactivity is undoubtedly exaggerated by the large excess of preformed triglyceride in the tissue. In two experiments with defatted homogenate the radioactivity was equally distributed between triglyceride and diglyceride with a small percentage in monoglyceride.

As shown in Fig. 5 and Table VII there was a small amount of radioactivity eluted with 1% ethyl ether in petroleum ether, coincident with a very small amount of hydroxamate-positive material. The specific radioactivity of the material was extremely high. Cholesterol esters are normally eluted at this point but no positive identification of this small amount of material was obtained. Another small fraction of the radioactivity was eluted with 100% diethyl ether, which normally elutes monoglycerides. The RF of this material on silicic acid impregnated paper was compatible with that of monoglyceride. Its specific radioactivity was lower than that of the diglycerides and higher than that of the triglycerides.

Synthesis of Phosphatidylcholine.—In order to assess the extent of incorporation into phospholipids a series of experiments was done in which, at the end of the usual incubation, the total lipid was extracted with chloroform-methanol (2:1, volume per volume) and chromatographed by the method of Borgstrom (9). An aliquot of the methanol eluate was counted (phosphatidic acid radioactivity). The material eluted with chloroform was taken to dryness, redissolved in isooctane, and treated with alkaline ethanol to remove FFA. An aliquot of the washed isooctane was counted (neutral lipid radioactivity). In six experiments with whole homogenate the radioactivity present in phospholipid averaged 17% (range, 5 to 22%) of the total in both fractions. When the phospholipid was chromatographed on silicic acid paper (10), most of the radioactivity (75 to 91%) ran with an RF similar to that of reference phosphatidic acid.

The synthesis of phosphatidic acid was also demonstrated in defatted homogenates incubated with P32-labeled αGP, plus cofactors as described above. All of the radioactivity in the phospholipid fraction isolated from such an incubation was recovered in one spot (RF similar to known phosphatidic acid) after chromatography on silicic acid-impregnated paper. When this material was subjected to mild alkaline hydrolysis (17) and then chromatographed (ethyl acetate:acetic acid:water, 3:3:1, volume per volume) (18) the radioactivity was found in one spot with an RF similar to that of αGP. Phosphatidic acids remain at the origin in this system. The positions of the products of hydrolysis of other phospholipids have not been determined. According to known pathways of biosynthesis of phosphatidylcholine, phosphatidyl serine, and phosphatidyl ethanolamine, however, the P32 of αGP32 is lost as inorganic phosphate prior to the addition of the nitrogenous base.

In other studies the incorporation of C14-labeled αGP into phospholipid and into neutral lipid was determined after 3 and 30 minutes of incubation as shown in Table VIII. At 3 minutes phosphatidic acids accounted for most of the lipid synthesized from C14-αGP. At 30 minutes the neutral lipid fraction contained about 70% of the total lipid C14. Similar experiments using 1-C14-palmitate also demonstrated a larger percentage of counts in neutral lipid after 30 minutes than after 3 minutes. Even in the 3-minute incubations, however, there was a preponderance of radioactivity in neutral lipid.

**DISCUSSION**

The requirements of the adipose tissue homogenate for triglyceride synthesis are very similar to the requirements demonstrated by Weiss and Kennedy (1) and by Tietz and Shapiro (3) for triglyceride synthesis in liver homogenates. The data reported above are compatible with triglyceride synthesis by a pathway essentially similar to that proposed by these authors for synthesis in liver:

\[ \text{Phosphatidic acid} + \text{diglyceride} + \text{phosphate} \rightarrow \text{phosphatidylcholine} \]

\[ \text{Glycerol} + \text{Phosphatidic acid} \rightarrow \text{glycerophosphatidic acid} \]

\[ \text{Glycerophosphatidic acid} \rightarrow \text{glycerophosphokinase} \]

\[ \text{Glycerol} + \text{Phosphatidic acid} \rightarrow \text{glycerophosphatidylcholine} \]

Disappearance of fatty acid incorporated into other phospholipids was very small. Because of the large quantity of preformed unlabeled triglyceride present in the homogenate the isolation of diglyceride of specific radioactivity 50 or more times that of the triglycerides

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**Table VIII**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Time (min)</th>
<th>Percentage of glyceride C14 in Neutral lipid</th>
<th>Phospholipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>39</td>
<td>61</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>68</td>
<td>53</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>76</td>
<td>24</td>
</tr>
</tbody>
</table>

Incorporation of 1-C14-αGP into neutral lipid and into phospholipid

Defatted homogenate incubated with supplements as described in Table I except for omission of 1-C14-palmitic acid and the addition of 0.32 μmole of 1-C14-L-αGP.
does not permit of any firm conclusions regarding the pathway of triglyceride synthesis. The difference found does rule out
the possibility that the diglycerides isolated arise primarily from
breakdown of the triglycerides examined and is compatible with
Reaction 4 which has been demonstrated directly in other
studies. Results of experiments such as those summarized in
Table VIII showing what appears to be a progressive transfer of
incorporated radioactivity from phospholipid to neutral lipid
with increasing time of incubation again are compatible with
this series of reactions. In addition, phosphatidic acid phospho-
phatase activity (Reaction 3) has been demonstrated directly in
homogenates of the epididymal fat pad.

Incubation of homogenates without addition of cofactors and
substrates is accompanied by a significant increase in FFA and
of glycerol, by definition evidence of lipase activity. Recent
studies in other laboratories indicate there may be several
lipases in adipose tissue. Korn has demonstrated that this tissue
is rich in lipoprotein lipase (19), an enzyme that under certain
conditions catalyzes transesterification (20). Thus, it might be
anticipated that some incorporation would occur independent of
a supply of energy donors, particularly in the whole homogenate
which contains so much triglyceride. The apparent absence of
any such exchange incorporation was unexpected. Either the
lipases active in these homogenates do not catalyze transesterifi-
cation or the conditions employed were not suitable.

On the other hand, when fully fortified homogenates are in-
cubated there is rapid incorporation of C14-palmitic acid into
glycerides and frequently a net decrease in FFA. The fact that
the specific radioactivity of the FFA drops considerably during
incubation of fortified homogenates indicates that glyceride
breakdown continues simultaneously with synthesis, i.e. that
there is active turnover. If such a dynamic state exists also in
the whole adipose tissue, as is indicated by a number of isotopic
studies in intact animals, an increase in the rate of release of FFA
could be effected either through an acceleration of the rate of
breakdown of triglycerides or by inhibition of the rate of esterifi-
cation:

\[
\text{FFA} \xrightarrow{k_1} \text{triglycerides} \xrightarrow{k_2}
\]

If there were an increase in \( k_1 \) with no change (or a lesser change)
in \( k_2 \), the net effect would be to decrease the level of FFA at the
expense of the triglyceride pool. The well documented inhibitory
effect of glucose on fatty acid release, for example, may be
due to acceleration of triglyceride synthesis as has been suggested
by other workers (21, 22). Since adipose tissue does not utilize
free glycerol to any significant extent, the maintenance of a steady
state in the presence of continuing lipolysis requires a constant
supply of substrate from which ATP can be generated and from
which energy for the esterification process can be derived.

Conversely, the stimulation of fatty acid release demonstrated
for several hormones could be brought about in part through
inhibition of triglyceride synthesis. Preliminary results from
this laboratory have been reported showing that epinephrine,
glucagon and adrenocorticotropic hormone do in fact inhibit the
incorporation of C14-palmitic acid into triglycerides of intact
adipose tissue (5).

SUMMARY

1. Requirements for the synthesis of neutral lipids by cell free
homogenates of rat epididymal adipose tissue are described.

2. All of the observations in the complete system and in studies
of single reactions are compatible with the pathway of glyceride
synthesis proposed earlier by other workers for the system from
liver, involving the formation of phosphatidic acid from fatty
acyl coenzyme A derivatives and α-glycerophosphate followed by
conversion of this to diglyceride, which is then esterified to form
diglyceride.

3. In homogenates of adipose tissue lipolysis goes on simul-
taneously with esterification. This occurs even when there is a
net decrease in free fatty acids during incubation.

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Studies of Triglyceride Biosynthesis in Homogenates of Adipose Tissue
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