Effect of hormones on lipolysis and esterification of free fatty acids during incubation of adipose tissue in vitro*

MARTHA VAUGHAN and DANIEL STEINBERG

Laboratory of Metabolism, National Heart Institute, National Institutes of Health, Bethesda 14, Maryland

[Manuscript received October 10, 1962; accepted January 7, 1963.]

SUMMARY

Using a new approach based on simultaneous measurements of net changes in glycerol and free fatty acids, the rates of lipolysis and of fatty acid esterification were estimated in rat epididymal fat pads under various conditions in vitro. Glycerol release, taken as a measure of the rate of lipolysis, was stimulated by epinephrine, norepinephrine, glucagon, adrenocorticotropic hormone (ACTH), thyroid-stimulating hormone (TSH), and growth hormone (GH). When lipolysis was stimulated by epinephrine, glucagon, ACTH, or TSH, the calculated rate of esterification was also increased, although to a lesser extent, of course. GH, which produced a relatively small increase in the rate of lipolysis, produced no detectable increase in rate of esterification. The stimulatory effect of epinephrine, ACTH, and GH on lipolysis was inhibited by NaF but there was no detectable inhibition of the basal glycerol release from tissues incubated in the absence of hormones. Data are presented suggesting that small amounts of epinephrine (0.3 µg), ACTH (0.12 U), or glucagon (15 µg) are largely inactivated during 30 min of incubation with a fat pad under the conditions employed. The effects of GH and TSH, on the other hand, persisted without diminution for at least 60 min, consonant with the view that the effects of GH on lipolysis in adipose tissue are due to TSH contained in it rather than to ACTH.

It has been shown that when release of free fatty acids (FFA)1 from adipose tissue in vitro is stimulated by epinephrine, ACTH, or glucagon, release of glycerol is also augmented (1-4), suggesting that the rate of lipolysis is increased under these conditions. Increased lipase activity in tissues previously treated with epinephrine or with ACTH has been reported (5-7). The effects of these hormones on fatty acid esterification (glyceride synthesis) are less clear. Leboeuf et al. (2) postulated that the rate of fatty acid esterification is increased when FFA release is stimulated by epinephrine. Their suggestion was based on the finding that, in the presence of epinephrine (or other lipolytic hormone), incorporation of C14 from glucose into glyceride-glycerol was markedly increased. Similar observations have also been reported by other workers (3, 8). The validity of conclusions drawn about triglyceride synthesis from such studies remains uncertain, however, since the specific activity of the immediate precursor of glyceride-glycerol (i.e., α-glycerophosphate) was not known. Ball and Jungas (9) have suggested that the stimulation of oxygen consumption caused by epinephrine (or by other lipolytic hormone) results from increased utilization of ATP as esterification is accelerated, but no direct studies of changes in rates of esterification were reported. Several laboratories, including our own, have made the observation that the incorporation of C14-labeled palmitate into adipose tissue glycerides is markedly decreased by epinephrine, ACTH, or glucagon, and have suggested that triglyceride synthesis is inhibited by these hormones (10-12). Dole (13), on the basis of studies of palmitate-1-C14 incorporation, concluded that epinephrine did not alter the rate of fatty acid esterification. As we have pointed out elsewhere (14, 15), different fractions of adipose
Epiinephrine, ACTH, TSH, and glucagon. If it is assumed that glycerol is employed and the assumptions underlying these experiments have been discussed in detail in an earlier paper (15-17), perhaps due to the absence of glycogen in some circumstances, contain FFA of widely different specific radioactivity. It is not at all clear which, if any, of these is involved in the incorporation of medium fatty acids into tissue triglycerides. Consequently, it may not be valid to equate changes in the rate of incorporation of labeled FFA with changes in the true rate of triglyceride synthesis in adipose tissue. Indeed, the present results show that the rate of FFA esterification is increased in tissues incubated with several of the "lipolytic" hormones.

Glycerol is utilized to only a small extent by adipose tissue (15-17), perhaps due to the absence of glycerokinase (18, 19). If it is assumed that glycerol is formed only from lipolysis of triglycerides and that the only quantitatively important pathways of FFA formation and removal are lipolysis and esterification, respectively, then, by determining net changes in glycerol release and in FFA over a period of incubation, it should be possible to calculate the average rates of lipolysis and of esterification during this time. The methods employed and the assumptions underlying these experiments have been discussed in detail in an earlier paper from this laboratory (20). We have concluded from the studies reported below that the rates of both lipolysis and fatty acid esterification are increased by epinephrine, ACTH, TSH, and glucagon.

### METHODS

Epididymal fat pads were obtained from male Sprague-Dawley rats that had been permitted free access to food until they were decapitated. Incubation of tissues and quantification of FFA and of glycerol in medium and in tissues was carried out as described previously (20). The method of Dole (21) was used for determination of FFA. Glycerol in medium was determined, using Korn's modification (22) of the method of Lambert and Neish (23). This was checked with the previously described enzymatic method in which glycerol is first converted to α-glycerophosphate and the latter assayed using α-glycerophosphate dehydrogenase (20). The enzymatic method was employed for measurement of glycerol in tissues. For quantification of mono- and diglycerides, tissues were homogenized in 3 ml water. A 2-ml sample of homogenate was immediately extracted with 50 ml of a mixture of chloroform-methanol 2:1. The lipid fraction was subjected to chromatography on Unisil. Samples of the glyceride classes obtained from the columns were taken for determination of hydroxamate-forming ester by the method of Snyder and Stephens (24). Epinephrine and norepinephrine solutions were prepared from the respective bitartrates. Concentrations are expressed in terms of the free base. Glucagon (Lot 258-234B-54-2) was supplied by the Eli Lilly Company through the courtesy of Dr. O. K. Behrens. ACTH (Aethar) and bovine serum albumin were purchased from the Armour Pharmaceutical Company. TSH was kindly provided by Dr. Peter Condliffe. The purified bovine growth hormone preparation (NIH-GHO-3) prepared by Dr. A. E. Wilhelmi was a gift of the Endocrinology Study Section, National Institutes of Health.

### RESULTS

Factors Affecting the Rate of Glycerol Release. The effects of several hormones on release of glycerol from adipose tissue are summarized in Table 1. As shown, epinephrine, norepinephrine, ACTH, glucagon, TSH, and GH all stimulated glycerol release at the concentrations used.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Conc.</th>
<th>No. of Pairs</th>
<th>Glycerol Release</th>
<th>Change Due to Hormone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>µmole/g/hr</td>
<td></td>
</tr>
<tr>
<td>Epinephrine, 0.1 µg/ml</td>
<td>12</td>
<td>1.3</td>
<td>+3.0 ± 0.32</td>
<td></td>
</tr>
<tr>
<td>Norepinephrine, 0.1 µg/ml</td>
<td>3</td>
<td>1.4</td>
<td>+3.0 ± 0.51</td>
<td></td>
</tr>
<tr>
<td>ACTH, 0.04 U/ml</td>
<td>15</td>
<td>1.4</td>
<td>+3.6 ± 0.23</td>
<td></td>
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<tr>
<td>Glucagon, 5 µg/ml</td>
<td>3</td>
<td>2.4</td>
<td>+3.2 ± 0.38</td>
<td></td>
</tr>
<tr>
<td>TSH, 10 µg/ml</td>
<td>4</td>
<td>1.3</td>
<td>+2.4 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>TSH, 25 µg/ml</td>
<td>8</td>
<td>1.4</td>
<td>+3.8 ± 0.42</td>
<td></td>
</tr>
<tr>
<td>GH, 200 µg/ml</td>
<td>6</td>
<td>1.1</td>
<td>+1.6 ± 0.13</td>
<td></td>
</tr>
</tbody>
</table>

* Paired tissues incubated for 1 hr in Krebs' bicarbonate medium containing bovine serum albumin, 30 mg/ml, one of each pair with and one without the indicated hormone.
† Mean of differences between paired tissues ± standard error.
Sodium fluoride (2 × 10⁻² M), which had little effect on glycerol release in the absence of added hormone, caused a marked decrease in glycerol release when ACTH or epinephrine was present in the medium (Table 2). At concentrations of sodium fluoride below 10⁻² M, there was little inhibition. The lack of effect of 6 × 10⁻⁴ M fluoride in the presence of epinephrine is shown in Table 2. Glycerol release was also inhibited by 10⁻² M sodium fluoride in the presence of GH (0.1 mg/ml).

Caffeine, 2.5 × 10⁻⁴ M, caused a small but significant stimulation of glycerol release. In the presence of an amount of epinephrine (0.03 μg/ml) that itself had only a slight effect, the effect of caffeine was significantly greater (Table 3).

Calculation of the Rate of Esterification of FFA. In earlier studies (10), one fat pad from each rat was used to determine the amount of FFA and glycerol in the tissue at zero time. The other fat pad was incubated 1 hr and the tissue and medium were analyzed for both components at that time. Thus, net changes in FFA and glycerol during the incubation period were directly determined. In order to assay the effects of hormones on rates of lipolysis and of esterification, both fat pads from each rat were incubated, one with and one without the hormone. For the purpose of calculating these rates, it was assumed that the tissue contained 1.0 μmole glycerol/g and 1.0 μEq FFA/g at zero time. These values are within the range usually observed in tissues from rats of the size and nutritional state that were used in the pertinent experiments.³ Table 4 contains data from a single pair of fat pads and demonstrates the method of calculation. It is important to note that the zero time value assumed equally affects the rates calculated for control and for hormone-treated tissues. Thus, it does not affect the difference between them. As shown in the third and fourth lines of Table 4, if zero time values of 2.0 μmoles/g instead of 1.0 μmoles/g are assumed for glycerol and for FFA, this alters the calculated esterification rates but does not influence the magnitude of the hormone effect on the calculated rate of esterification.

Although the lower glycerides constitute only a very small percentage of the total lipids of adipose tissue, it was necessary to consider the possibility that some of the glycerol released might derive from monoglycerides. As long as the fraction of glycerol derived from lower glycerides remains the same in control and hormone-treated tissues, the hormone effects

³ For example, during the course of the experiments reported here, fat pads analyzed immediately after removal from a group of 6 normal rats (not fasted) contained 0.7 ± 0.20 μmoles glycerol/g tissue and 1.1 ± 0.17 μEq FFA/g tissue.
TABLE 4. Sample Calculations to Demonstrate That Differences in Calculated Rates Between Paired Tissues Are Independent of the Concentrations of Glycerol and FFA Assumed for the Tissue at Zero Time

<table>
<thead>
<tr>
<th>ACTH, 0.4 U/ml</th>
<th>Glycerol (µmole/g tissue)</th>
<th>Rate of Lipolysis† (µEq FFA/g/hr)</th>
<th>FFA (µEq/g tissue)</th>
<th>Rate of Esterification (µEq FFA/g/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min*</td>
<td>60 min</td>
<td>Net</td>
<td>0 min*</td>
</tr>
<tr>
<td>+</td>
<td>1.0</td>
<td>0.7</td>
<td>1.3</td>
<td>3.9</td>
</tr>
<tr>
<td>0</td>
<td>2.0</td>
<td>0.3</td>
<td>0.9</td>
<td>3.7</td>
</tr>
<tr>
<td>+</td>
<td>2.0</td>
<td>5.0</td>
<td>15.0</td>
<td>3.7</td>
</tr>
</tbody>
</table>

* Assumed zero time values.
† It has been assumed that 3 moles of FFA were formed with each mole of glycerol produced. See text for details.

in the hormone-treated than in the control tissue. Available information suggests that no significant fraction of the glycerol released into the medium is derived from α-glycerophosphate. It has been reported (3) that the glycerol released from tissues incubated with C14 glucose and epinephrine contained essentially no radioactivity, good evidence that it did not come from α-glycerophosphate.

Effects of Hormones. Rates of lipolysis and of esterification in the experiments of Table 5 and of Fig. 1 were calculated on the basis of the assumptions discussed above. The calculated rates in the control tissues are of the same order as those observed in earlier studies in which the zero time values were not assumed but were determined. Further, in control tissues, when calculated with these assumptions, the rate of esterification was roughly equal to the rate of lipolysis. This correlation, shown graphically in Fig. 1, was also observed in the earlier experiments. In the tissues treated with epinephrine, ACTH, TSH, or GH, there was only a rough correlation between the rates of lipolysis and esterification. The effects of the hormones on the rates of these processes calculated from the differences between paired control and hormone-treated tissues are summarized in Table 5. The rate of esterification was significantly increased by epinephrine, ACTH, and TSH, but not, of course, to the same extent as was the rate of lipolysis. Growth hormone (200 µg/ml) had a smaller effect on lipolysis than did the other hormones and had no effect on esterification. The effects of glucagon (5 µg/ml) were compared directly with those of ACTH in paired tissues in order to make it easier to demonstrate any quantitative differences in the effects of the hormones on the two processes. The data are not included here since no such differences were observed.

Time Course of Hormone Effects. The rates of lipolysis and esterification as calculated represent the mean of the rates for the entire period of incubation. These rates in tissues incubated under control conditions often are not constant for an hour and the rates in the hormone-treated tissues were not expected to be. In Table 6 are summarized data from three pairs of tissues that were incubated in medium containing ACTH, 0.04 U/ml. One of each pair was removed after 20 min, the other after 40 min, and medium and tissue
were analyzed for FFA and glycerol. Comparison of changes in glycerol with those of FFA in the second period shows that esterification had essentially stopped by 20 min. In order to determine whether these findings were due to inactivation of ACTH, the rates during consecutive 30-min periods were determined in two groups of tissues, to one of which ACTH (0.04 U/ml) was added a second time after 30 min. As seen in Fig. 2, when ACTH was added a second time at 30 min, the rate of lipolysis was approximately equal in the two periods; in the tissues to which there was no second addition of ACTH, there was no lipolysis during the second 30 min. In both groups of tissues, the rate of esterification was essentially zero during the second 30 min. The findings with epinephrine (0.1 μg/ml added once or twice) were qualitatively similar except that, when epinephrine was added twice, the rate of esterification was maintained during the second 30 min along with the rate of lipolysis.

The effect of glucagon (5 μg/ml added once at zero time) was also significantly less in the second 30 min than it was in the first. In three pairs of tissues, lipolysis in the second period was 6.4 ± 1.4 and esterification was 2.8 ± 1.4 μEq FFA/g tissue, in contrast to calculated rates of 15.1 ± 2.5 and 6.3 ± 1.4 μEq FFA/g, respectively, during the first period (calculated with the assumption that the tissue at zero time contained 1.0 μmole/g glycerol and 1.0 μEq/g FFA). In contrast, the effects of GH or of TSH (which were very different in magnitude with the particular concentrations of each employed) did not tend to decline in the second 30 min of incubation. In Fig. 3, data from three experiments with each hormone are presented individually since there was some variability in the time course. It is apparent that the time course of the effects of these hormones on lipolysis is different from that of ACTH, epinephrine, or glucagon.

**TABLE 6. TIME COURSE OF EFFECT OF ACTH, 0.04 U/ml**

<table>
<thead>
<tr>
<th>Expt No.</th>
<th>Net Change in Glycerol (μmole/g tissue)</th>
<th>Net Change in FFA (μEq/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0–20 min</td>
<td>20–40 min</td>
</tr>
<tr>
<td>1</td>
<td>+2.4</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>+1.8</td>
<td>+0.8</td>
</tr>
<tr>
<td>3</td>
<td>+1.5</td>
<td>+0.5</td>
</tr>
</tbody>
</table>

* Pairs of tissues incubated in Krebs' bicarbonate medium containing bovine serum albumin, 30 mg/ml, and ACTH, 0.04 U/ml, one of each pair incubated for 20 min and the other for 40 min before analysis of tissue and medium for FFA and glycerol.
† Calculated assuming that at zero time tissue glycerol content = 1.0 μmole/g and FFA content = 1.0 μEq/g.
‡ Calculated from data for tissue incubated 40 min and paired contralateral tissue incubated for 20 min.

**DISCUSSION**

The present studies confirm previous reports (1-4) that epinephrine, ACTH, and glucagon stimulate the rate of glycerol production in adipose tissue. A similar
effect of TSH and of a purified growth hormone preparation has also been demonstrated. If it is accepted that the rate of glycerol production faithfully reflects the rate of triglyceride breakdown in adipose tissue, the effects of these several hormones can properly be designated “lipolytic.”

Attempts to assess the rate of fatty acid esterification as affected by the hormones that stimulate triglyceride breakdown have led to conflicting results as pointed out in the Introduction (10–13). The studies reported above employed a “balance method” based on the measurement of simultaneous net production of glycerol and FFA during incubation (20). The results show that when lipolysis was stimulated by epinephrine, ACTH, glucagon, or TSH, the rate of fatty acid esterification was also increased. Glyceride breakdown was, of course, increased to a greater extent than was glyceride resynthesis, leading to the observed net release of free fatty acid. The overall effect of the hormones, then, is to increase the turnover of adipose tissue glycerides while effecting an increased rate of mobilization of free fatty acids. Most probably the primary effect of the hormones is on the rate of lipolysis, the increased rate of triglyceride synthesis being secondary to this or related to other metabolic effects of the hormones on adipose tissue. Our findings support the conclusion of Leboeuf et al. (2) that esterification is accelerated by epinephrine and other lipolytic hormones. This conclusion is contrary to that drawn from our earlier work (10) and from some other studies measuring the rates of incorporation of labeled fatty acid from the medium (11–13). Elsewhere, we have presented the reasons for doubting the validity of measurements of incorporation of labeled fatty acids as a measure of triglyceride synthesis in adipose tissue (14, 15). It is now felt that the decreased incorporation observed under the influence of hormones is artifactual, presumably secondary to dilution of the entering labeled fatty acids by unlabeled material at some point prior to incorporation into the depot triglycerides.

The balance method used here is based on several premises. The first is that glycerol released by lipolysis is neither re-incorporated into triglycerides nor oxidized, or otherwise metabolized to any important extent. The validity of this assumption is supported by the work of Shapiro et al. (16), Lynn et al. (3), and Cahill et al. (17), as well as by studies of glycerol-C14 utilization done in our laboratory under the same conditions used in the present experiments (15). A second premise is that net breakdown of mono- and diglycerides accounts for very little of the observed net increase in glycerol. Data have been cited above to support this assumption. A third premise is that the amounts of fatty acid degraded are small relative to the amounts released from glycerol and re-esterified during incubation. Results in our laboratory show that 10% or less of added labeled fatty acid is oxidized in the course of a 1-hr incubation with adipose tissue. Rates calculated by this balance method will be in error to the extent that these premises represent approximations. In particular, it must be kept in mind that under unusual metabolic conditions the data supporting the premises may be inapplicable. In the experiments reported, however, the errors involved should be small and certainly the qualitative results should be valid.

In the present studies, a general correlation between the rates of breakdown and resynthesis was demonstrable when data from 60-min incubations were plotted as in Fig. 1. A similar correlation observed in tissues incubated under control conditions has been reported (20). Furthermore, in almost all tissues, changes in the rate of esterification paralleled changes in the rate of lipolysis with time. The failure of esterification to
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keep pace with lipolysis in the presence of the hormones apparently is not due to any absolute lack of substrate or of energy for triglyceride synthesis. The experiments in which a hormone was added a second time after 30 min of incubation, when lipolysis and esterification were at a very low level, indicated that the tissue mechanisms responsible for augmentation of lipase activity and for restoration of esterification were intact at this time. The lag in esterification relative to lipolysis may be due in part to the fact that the large fraction of FFA formed in lipolysis that escapes into the medium is unavailable for rapid re-esterification and/or influencing the rate of this process. Perhaps, also, elevation of the concentration of FFA within the tissue has deleterious effects on the enzymes of esterification or on other processes upon which glyceride synthesis is dependent (9).

Both TSH (25) and growth hormone (26) have been shown to stimulate release of FFA. Hollenberg et al. (6), however, observed no increased lipase activity in fat pads incubated with a growth hormone preparation (20 μg/ml), whereas increased lipase activity was demonstrable in tissues incubated with ACTH under similar conditions. They concluded that the stimulation of release of FFA produced by growth hormone might be mediated in some other fashion. The data reported above indicate that lipolysis of triglycerides in adipose tissue is increased by 200 μg/ml of the growth hormone preparation employed. Hollenberg and co-workers (6) also reported that addition of sodium fluoride to the incubation medium inhibited the effects of ACTH (or of epinephrine) on release of FFA and on tissue lipase activity. Consonant with their findings, we observed that 2 × 10⁻¹ M sodium fluoride, which did not interfere with glycerol release in the absence of hormone, markedly diminished glycerol release in the presence of epinephrine, ACTH, or GH. This suggests that the lipase responsible for triglyceride breakdown in the intact tissue is not appreciably inhibited by fluoride under these conditions but, rather, that fluoride in some way interferes with the production of increased lipase activity by the above hormones.

The mechanism by which caffeine and epinephrine interact in the stimulation of lipase activity is unknown. Caffeine inhibits the enzymatic cleavage of 3'5' AMP (27), the accumulation of which is enhanced by epinephrine in a particulate fraction of adipose tissue cells (28). The relationship of 3'5' AMP to the lipase system, however, remains to be elucidated.

Results of studies with incubation periods of 30 and 60 min indicate that small amounts of epinephrine (0.3 μg), ACTH (0.12 U), or glucagon (15 μg) are largely inactivated during 30 min of incubation with fat pads under the conditions employed. The effects of TSH and of GH, the latter in concentrations having a relatively small effect on lipolysis and no effect on esterification, persisted without significant diminution for 60 min. These findings are compatible with the suggestion of Ball and Jungas (9) that the effects of GH preparations of FFA release are not due to contamination with ACTH, but may be due to contamination with TSH.

REFERENCES