Subcellular Distribution of and Epinephrine-induced Changes in Hormone-sensitive Lipase, Phosphorylase, and Phosphorylase Kinase in Rat Adipocytes*

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SUMMARY

A systematic study was carried out of the subcellular distribution of hormone-sensitive lipase, glycogen phosphorylase, and phosphorylase kinase in rat adipocytes and the changes in these enzymes when the cells were incubated with epinephrine. Under the conditions of homogenization used, approximately one-third of the total hormone-sensitive lipase activity was associated with the fat cake floated to the top of a homogenate by low speed centrifugation, although as discussed in detail, this value remains uncertain in view of technical problems faced in assaying lipase tightly bound to fat. Subfractionation of the fat-free homogenate showed that over 80% of the hormone-sensitive lipase was recovered in the soluble fraction and the specific activity of that fraction was considerably higher than it was in particulate fractions. The plasma membrane fraction contained less than 2% and the microsomal fraction less than 3% of the activity in the fat-free homogenate. When adipocytes were lysed, over 80% of the total lipase activity was recovered in the supernatant fraction and less than 20% in the fat cell "ghosts." The distribution of lipase activity among subcellular fractions of the fat-free homogenate was not altered by previous treatment of the adipocytes with epinephrine. Lipase activity in the soluble fraction from control cells was activated by treatment with cyclic adenosine 3',5'-monophosphate-dependent protein kinase and MgATP. When the cells had been previously treated with epinephrine, the percentage of activation by protein kinase was reduced in this and in all fractions. Phosphorylase and phosphorylase kinase activity were found predominantly in the soluble fraction; small amounts (21 and 14%, respectively) were associated with the fat cake assayed after resuspension in Triton X-100. The distribution of these enzymes in cells previously treated with epinephrine was not significantly different from that in control cells. Total phosphorylase activity (assayed in the presence of 5'-AMP) was significantly increased in homogenates prepared from cells previously treated with epinephrine as was the ratio of phosphorylase activities measured in the absence and in the presence of 5'-AMP. On the other hand, there was no change in total phosphorylase kinase activity after epinephrine treatment and no change in the ratio of activities measured at low pH values (6.2 or 6.8) and high pH value (8.2). Further characterization of the phosphorylase and phosphorylase kinase systems in adipose tissue is needed to assess the regulatory role of phosphorylase kinase in this tissue.

Adipose tissue contains at least two different systems that are rapidly activated by epinephrine, one leading to free fatty acid mobilization (1, 2) and one to glycogenolysis (3-6). Both systems are stimulated not only by catecholamines but also by ACTH, glucagon, and many other so-called lipolytic hormones. The significance of the parallel activation of lipolysis and glycogenolysis is not clear nor is it certain that they are functionally or obligatorily linked. It is now well established that activation of hormone-sensitive lipase is effected via adenylyl cyclase and cyclic AMP-dependent protein kinase (7-9). The mechanism of phosphorylase activation in adipose tissue has not been studied in detail but it seems likely that, as in other tissues, this is also mediated by cyclic AMP (6). Further characterization of these two hormonally regulated enzyme systems is essential as a basis for exploring the interrelationships between them and the possibility of differential regulation occurring after the receptor-adenyl cyclase level.

No systematic studies on the subcellular distribution of hormone-sensitive lipase have been reported and the limited data available are conflicting (10-13). In part this may reflect the presence in adipose tissue of several lipases and esterases and the uncertainty regarding criteria for specific assay of hormone-sensitive lipase. Crude extracts of adipose tissue contain, in addition to hormone-sensitive lipase: lipoprotein lipase (14); a high level of activity against monoglycerides and diglycerides (6, 11); and activity against short chain glyceryl esters (esterase) (15, 16). The properties of these enzyme activities suggest that they are at least in part referable to

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1 The abbreviation used is: cyclic AMP, cyclic adenosine 3',5'-monophosphate.
different enzyme proteins (17, 18) but none has been prepared in pure form. Only recently has hormone-sensitive lipase been partially purified and characterized (19). Thus it is difficult to assess the significance of some of the earlier results. Rizaq (10) studied hormone-sensitive lipase activity in a particulate fraction sedimented from a 10,000 × g supernatant fraction of fat pad homogenates by centrifugation at 105,000 × g for 12 hours. No data were presented on the occurrence or properties of hormone-sensitive lipase in other subcellular fractions. Vaughn et al. (11) found the enzyme to be largely associated with the fat layer after homogenization in 0.15 M KCl. Huttunen et al. (12) showed that the fraction of hormone-sensitive lipase associated with the fat layer was variable, being a function of ionic composition of the homogenizing medium and of temperature at which the homogenate was prepared. When binding to the fat layer was minimized, most of the enzyme was found in the 100,000 × g supernatant fraction prepared from the layer beneath the fat cake. Most recently, Crum and Calvert (13) have suggested that the enzyme is associated predominantly with the plasma membrane. In the present study we have utilized assay conditions shown to be optimal for the assay of partially purified hormone-sensitive lipase (20) and in addition have demonstrated the responsiveness of the enzyme in the various subcellular fractions studied to activation by cyclic AMP-dependent protein kinase.

Activation of phosphorylase has been shown in hormone-stimulated fat pads (3, 21) and adipocytes (22); the phosphorylase activity was measured in the supernatant fraction obtained by low speed centrifugation of homogenates. No attempt has been made to study the subcellular distribution of phosphorylase or its possible association with glycogen particles, such as has been described by Meyer et al. in muscle homogenates (23). Stull and Mayer (24) have recently reported that in skeletal muscle after either electrical stimulation or isoproterenol administration, there can be a dissociation between the conversion of phosphorylase b to phosphorylase a on the one hand and the activation of phosphorylase b kinase on the other. The intimate nature of the phosphorylase activation system in adipose tissue has not been previously studied.

In the present paper we report a systematic study of the subcellular distribution of hormone-sensitive lipase, phosphorylase kinase, and phosphoprotein. Subcellular fractions of rat adipocytes were prepared by methods that have been carefully characterized on morphological and biochemical grounds by Jarett et al. (25-28). In addition, we have studied the response of these several enzymes to epinephrine treatment of adipocytes.

METHODS AND MATERIALS

Cellular Preparations—Epididymal fat pads were excised from fed Sprague-Dawley rats and adipocytes were prepared according to Rodbell (29). The washed cells were resuspended (about 2 mg of cell protein per ml) in Krebs-Ringer bicarbonate buffer (pH 7.4) containing 3% bovine serum albumin (Cohn fraction V) and glucose (2 mg per ml). The suspended cells were equilibrated with 95% O₂-5% CO₂ and incubated for 15 min at 37° prior to the start of each experiment. Aliquots of the fat cell suspension were then resuspended into siliconized vials with and without epinephrine (10 μM) and incubated for exactly 5 min. At the beginning and end of the incubation period an aliquot from each cell suspension was pipetted into trichloroacetic acid (final concentration 5%) for subsequent determination of glycerol and cyclic AMP concentrations. The rest of the cell suspension was transferred to a siliconized tube and centrifuged at 400 × g for 30 s at room temperature. The infranatant solution was aspirated and the cells were washed with 5 to 6 volumes of Medium I (10 mM Tris, pH 7.4, 1.0 mM EDTA; and 0.25 mM sucrose). The cell suspension was resuspended and the packed cells were resuspended in 3 to 4 volumes of the same medium for cell fractionation by a slight modification of the method of McKeel and Jarett (26). The cell suspension, which contained approximately 2 mg of cell protein per ml, was homogenized as originally described and the homogenate centrifuged at 16,000 × g for 15 min at 4° to yield a pellet (P₁), an infranatant fluid (S₁), and a floating fat cake layer which was discarded. S₁ was further centrifuged at 100,000 × g for 70 min to yield the microsomal supernatant fraction while the pellet was gently rinsed, resuspended in Medium I and recentrifuged at 100,000 × g for 70 min to yield the microsomal pellet.

The pellet from the 16,000 × g centrifugation of the crude homogenate (P₁) was resuspended in Medium I and centrifuged at 100,000 × g for 10 min to yield a pellet containing nuclei and large cell debris (P₂). The supernatant fluid from this centrifugation was centrifuged at 17,000 × g for 20 min to yield a pellet (P₃) which was resuspended in 8 ml of Medium I and placed on a discontinuous Ficoll gradient consisting of 10 ml of 15% Ficoll beneath 10 ml of 9% Ficoll, both in 0.25 M sucrose. The gradient was centrifuged in a Beckman SW 25.1 rotor at 24,000 rpm for 45 min. The plasma membranes banded at the sample-9% Ficoll interface and the mitochondria formed a pellet at the bottom of the tube. All particulate fractions were resuspended in 1 to 2 ml of Medium I. Protein content of the various subcellular fractions was determined by the method of Lowry et al. (30).

Preparation of Fat Cell “Ghosts” — Fat cell ghosts were prepared as described by Birnbaumer et al. (31) except that the lysing medium contained only 10 mM Tris, pH 7.4, and 1 mM EDTA. This preparation was carried out at 0°. Five milliliters of the lysing medium was added to approximately 1.7 × 10¹⁰ fat cells in a plastic tube. The tube was inverted 20 times, 3 s per cycle. The lysate was collected by floating the released fat by centrifugation at 200 × g for 1 min and aspirating the turbid infranatant material. To the remaining fat plus intact fat cells, 5 ml of the fresh lysing medium was added and the procedure was repeated three additional times. The lysate was pooled and centrifuged at 900 × g for 15 min to yield a pellet (fat cell ghosts) and the supernatant fluid. The fat cell ghosts were then homogenized in 2 ml of lysing medium prior to enzyme assays.

Assay Procedures—Hormone-sensitive lipase was assayed by the [¹⁴C]glycogen method of Huttunen and Steinberg (20), the free fatty acid being isolated by the method of Kelley (32). When hormone-sensitive lipase was activated with skeletal muscle protein kinase prior to assay, the activation system was as previously described (20), except that an ATP-regenerating system was added. Oneweih milliliter of the activation mixture was added to 0.1 ml of the sample. The final mixture during activation contained: 10 mM Tris, pH 7.4; 1.0 mM dithiothreitol; 0.5 mM EGTA; 0.25 mg of bovine serum albumin per ml; 2.5
RESULTS

Distribution of Triglyceride Lipase Activity—An initial series of experiments was performed to determine the fraction of triglyceride lipase activity associated with the bulk of storage fat floating to the top of a homogenate of isolated adipocytes. The homogenate was centrifuged at room temperature for 1½ min at 400 × g. The infranatant fraction was aspirated and the floated fat cake was rehomogenized in a volume of Medium I equal to that of the infranatant fraction. The data in Table I illustrate that about 35% of the homogenate activity was determined by the [14C]triolein assay associated with the fat cake. An aliquot of the fat cake resuspended in Medium I was re-centrifuged and the activity in the infranatant solution was assayed. Only about 4% of the original fat cake activity was found in this extract, indicating that the lipase activity associated with the fat cake was tightly bound. In a later section this fat bound enzyme is discussed in more detail.

The distribution of triglyceride lipase activity among the various subcellular fractions after removal of the fat cake is shown in Table II. By far the largest fraction of triglyceride lipase activity (more than 80%) was recovered in the S1 fraction and most of this was in the microsomal supernatant fraction. Only minor amounts of activity were recovered in the various particulate fractions. The activity associated with the microsomes was not reduced when this fraction was resuspended in a large volume of Medium I and recentrifuged at 160,000 × g, suggesting that it was not simply adsorbed or trapped.

In light of the above data, triglyceride lipase activity in subsequent studies was measured only in selected fractions. Table III summarizes the data on distribution of activity found among the various subcellular fractions in a series of experiments. Over 60% of the total activity in control cells was recovered in the P1 and S1 fractions as measured by the [14C]triolein assay. This is consistent with the data in Table I indicating that the fat cake contained about 35% of the lipase activity of the homogenate. The plasma membranes accounted for only about 1% of the homogenate activity and the microsomes about 3%. The microsomal supernatant fraction ac-

### Table I

| Subcellular fraction     | Total activity | Percentage of total homogenate activity |
|-------------------------|---------------|----------------------------------------|
| Total homogenate        | 9887          | 100                                    |
| Infranatant fraction     | 5590          | 56                                     |
| Fat cake                | 3461          | 35                                     |
| Fat cake extract        | 376           | 4                                      |

*Total activity in 7.5 ml of homogenate prepared from 2.6 ml of packed cells in Medium I (see "Methods and Materials").
Activation of Triglyceride Lipase by Cyclic AMP-dependent Protein Kinase—In preliminary experiments, activation by protein kinase was examined with the system described by Huttunen et al. (20). This yielded consistent activation of the enzyme in the microsomal supernatant fraction but little or no activation in other fractions. Previous studies by Jarett and McKeel (25) showed that the crude homogenate and the various particulate fractions contained an active Mg++-ATPase but that the microsomal supernatant fraction did not, suggesting that ATP might be rate-limiting in the case of the particulate fractions. When an ATP-regenerating system was added to the system previously used, a consistent and rapid activation was observed in all the fractions, reaching a maximum by 8 min. All subsequent assays included the ATP-regenerating system.

The triglyceride lipase in control fat cell homogenates was activated 56% by cyclic AMP-dependent protein kinase (Table III). The enzyme associated with the S1 fraction, the microsomal fraction and the microsomal supernatant fraction was activated to about the same extent while the enzyme in the P1 and plasma membrane fractions was only activated about 50%.

The degree of activation of lipase in the total homogenate, the S1 fraction and the microsomal supernatant fraction was of the same magnitude as that found by Huttunen et al. (20) with purified hormone-sensitive lipase. This would indicate that the triglyceride lipase activity measured in the present experiments represents primarily hormone-sensitive triglyceride lipase activity with very little contribution from or interference by lipoprotein lipase or other lipases.

As shown in Table III and discussed below, fractions prepared from cells previously treated with epinephrine were activated to a much lesser extent by protein kinase than were corresponding fractions from control cells.

**Table II**

| Subcellular fraction | Total activity | Specific activity |
|----------------------|---------------|------------------|
|                      | mg FFA/hr/mg protein | mg FFA/hr/mg protein |
| P1                   | 2.160         | 75               |
| P2                   | 174           | 36               |
| P3                   | 928           | 38               |
| S1                   | 426           | 82               |
| Mitochondria         | 110           | 12               |
| Microsomal supernatant | 17,880      | 318              |
| Microsomes           | 9,940         | 294              |
|                      | 1,676         | 235              |

* Total activity in 25 ml of fat-free infranatant fluid from homogenate prepared from 8 ml of packed cells in 35 ml of Medium 1.

**Table III**

| Subcellular fraction | Percentage of total homogenate activity | Percentage of total homogenate activity | Percentage of total homogenate activity | Percentage of total homogenate activity |
|----------------------|----------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|
|                      | Control cells                          | Epinephrine-treated cells              | Control cells                          | Epinephrine-treated cells              |

| Subcellular fraction | Percentage of total homogenate activity | Percentage of total homogenate activity | Percentage of total homogenate activity | Percentage of total homogenate activity |
|----------------------|----------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|
|                      | Control cells                          | Epinephrine-treated cells              | Control cells                          | Epinephrine-treated cells              |

| Subcellular fraction | Percentage of total homogenate activity | Percentage of total homogenate activity | Percentage of total homogenate activity | Percentage of total homogenate activity |
|----------------------|----------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|
|                      | Control cells                          | Epinephrine-treated cells              | Control cells                          | Epinephrine-treated cells              |

* Significantly different from control at p < 0.01.
The ghosts and 900 × g infranatant fraction from 10 ml of cell suspension containing 1.7 × 10⁶ cells were prepared by a modification of the method of Birnbaumer et al. (19) (see "Methods and Materials") and lipase activity was measured with [14C]triolein. The amount of radioactivity associated with P₁, plasma membrane, or microsomal fractions from epinephrine treatment of the adipocytes prior to homogenization and experiments [14C]triolein was added in an equal amount and released as oleic acid was determined and converted to (Table III). However, recovery of activity in the S₁ fraction values shown in Column 7 would have been expected (Value A). Had the added triolein all been derived from the original 1.7 × 10⁶ cells. The fat cake after the final wash was discarded.

**TABLE IV**

Distribution and specific activity of triacylglycerol lipase in adipocyte ghosts and 900 × g supernatant fraction

| Subcellular fraction | Total activity | Percentage of total activity | Specific activity | Percentage activation by protein kinase and cyclic AMP |
|----------------------|----------------|-----------------------------|------------------|------------------------------------------------------|
|                      | mg FFA/hr      | % mg FFA/mg protein/hr      |                  | %                                                  |
| 900 × g supernatant  | 3560           | 83                          | 582              | 73                                                  |
| Ghosts               | 740            | 17                          | 494              | 41                                                  |

*Total activities calculated for the entire pellet fraction (ghosts) and the entire 900 × g supernatant fraction were derived from the original 1.7 × 10⁶ cells. The rate of hydrolysis so calculated is shown in Column 6. Had the increment in free fatty acid release due to added triolein all been derived from the added substrate without dilution of specific activity by endogenous substrate the values shown in Column 7 would have been expected (Value A). Had the added triolein mixed completely with the endogenous unlabeled triglyceride the values shown in Column 8 would have been expected (Value B).

**TABLE V**

Hydrolysis of endogenous triacylglycerol substrate and of added triolein by total homogenates

Net free fatty acid (FFA) release was measured directly in the crude total homogenate (including the fat cake) with and without the addition of 0.74 mg per ml of unlabeled triolein. In parallel experiments [14C]triolein was added in an equal amount and the release of [14C]oleic acid was measured. The triglyceride contribution from the homogenate (endogenous substrate) was 5 to 12.5 mg per ml of assay mixture. The amount of radioactivity released as oleic acid was determined and converted to nanomoles per mg based on the specific activity of the [14C]triolein. The rate of hydrolysis so calculated is shown in Column 6. Had the increment in free fatty acid release due to added triolein all been derived from the added substrate without dilution of specific activity by endogenous substrate the values shown in Column 7 would have been expected (Value A). Had the added triolein mixed completely with the endogenous unlabeled triglyceride the values shown in Column 8 would have been expected (Value B).

**TABLE VI**

Specific activity, total activity, and subcellular distribution of phosphorylase and phosphorylase kinase

Phosphorylase was assayed in the presence of 5'-AMP. Phosphorylase kinase was assayed at pH 8.2. Both enzymes were measured without prior dilution. The subcellular fractions are described in Table II and under "Methods and Materials." One unit of phosphorylase forms 1 amole of glucose 1-phosphate from glycogen per min at 30⁰C. One unit of phosphorylase kinase converts 1 unit of muscle phosphorylase b to a in 1 min at 30⁰C.

was significantly and consistently lower in homogenates prepared from epinephrine-treated cells than in homogenates prepared from control cells. Recoveries in the microsomal supernatant fraction prepared from epinephrine-treated cells also tended to be lower but the difference was not statistically significant.

The degree of protein kinase activation of lipase in all fractions prepared from epinephrine-treated cells was significantly less than in corresponding fractions from control cells (Table III). These findings are consistent with earlier results both in crude fractions and with purified hormone-sensitive lipase (9, 20).

**Lipase Activity Associated with Fat Cake—Measurement of lipase activity in the crude, triglyceride-rich homogenate or in the fat cake itself poses special problems. Previous studies show that the rate of release of fatty acids from endogenous substrate in crude homogenates is increased by prior treatment of fat pads with epinephrine (11). In the present studies the rate of release of free fatty acids from endogenous triglycerides was increased 113 ± 12% (n = 9) in homogenates from epinephrine-treated cells as compared with controls. When, however, lipase activity was measured in terms of the rate of release of [14C]oleic acid from added [14C]triolein, the increase due to epinephrine treatment was only 17 ± 5% (n = 15). Evidently the added radioactive substrate and the endogenous substrate are not equivalent.

To explore this further, studies were done comparing net free fatty acid release in total homogenates with and without the addition of exogenous triolein substrate. As shown in Table V, net release was increased by addition of extra substrate (triolein) both to control homogenates and to homogenates from epinephrine treated cells. This occurred although the increment in substrate concentration (0.74 mg per ml) was ± 0.9 to 32.6 ± 2.1 pmoles per mg protein (n = 14). Epinephrine treatment of the adipocytes prior to homogenization did not cause a change in the percentage of lipase activity associated with P₁, plasma membrane, or microsomal fractions (Table III). However, recovery of activity in the S₁ fraction was significantly and consistently lower in homogenates prepared from epinephrine-treated cells than in homogenates prepared from control cells. Recoveries in the microsomal supernatant fraction prepared from epinephrine-treated cells also tended to be lower but the difference was not statistically significant.

The degree of protein kinase activation of lipase in all fractions prepared from epinephrine-treated cells was significantly less than in corresponding fractions from control cells (Table III). These findings are consistent with earlier results both in crude fractions and with purified hormone-sensitive lipase (9, 20).
small relative to the concentration of triglyceride contributed by the homogenate itself (approximately 12.5 mg per ml). To determine the extent to which the added exogenous substrate equilibrated with endogenous triglycerides, parallel incubations were carried out in which \[^{14}C\]triolein of known specific radioactivity was added to aliquots of whole homogenate at the same net concentration (0.74 mg per ml). The data shown in Table V suggest that the added substrate was hydrolyzed with little or no dilution by the large excess of endogenous substrate. The observed free fatty acid radioactivity released was converted to nanoequivalents assuming no dilution with endogenous unlabeled triglyceride. Those results are shown in Column 6 (observed). If the increment in net release (Column 5) indeed occurred without any mixing, one would have expected the maximum amount of radioactivity to be released in the incubations with \[^{14}C\]triolein; Column 7 shows the expected values expressed in nanoequivalents. At the other extreme, if labeled and unlabeled substrate mixed completely, much less radioactive oleic acid would have been released and the calculated values are shown in Column 8. The results suggest that the degree of mixing was very limited although somewhat variable from experiment to experiment.

**Distribution of Phosphorylase and Phosphorylase Kinase**—As shown in Table VI, the phosphorylase activity of the fat cell homogenate was recovered only in Fraction S1 and in the microsomal supernatant fraction; the particulate fractions (P1 and microsomes) were found to be devoid of activity. However, only two-thirds of the phosphorylase activity in the original homogenate was accounted for. Phosphorylase kinase was also found to be primarily associated with the S1 and microsomal supernatant fractions; 5% or less was recovered in P1 and in the microsomal fraction. Only one-half of the original activity was accounted for. Assay of the fat cake posed difficulties; when it was rehomogenized in 0.5% Triton X-100, 21% of the phosphorylase and 14% of the phosphorylase kinase activity were found in this fraction.

**Effect of Epinephrine on Phosphorylase and Phosphorylase Kinase Activity**—Epinephrine treatment of adipocytes did not alter the distribution of either phosphorylase or phosphorylase kinase among the adipocyte subcellular fractions and only the activity in total fat-free homogenates (low speed centrifugation) was subsequently measured. Total phosphorylase activity (measured in the presence of 5'-AMP) in whole homogenates prepared from epinephrine-treated adipocytes was increased almost 2-fold \((p < 0.01)\) over that from nontreated cells (Table VII). This activation was accompanied by a marked increase in the relative activity assayed in the absence of 5'-AMP \((p < 0.01)\). In contrast, epinephrine treatment of adipocytes resulted in no change in total phosphorylase kinase activity, nor in the ratio of activities assayed at low pH (6.2 or 6.8) and at high pH (8.2) (Table VII).

**DISCUSSION**

A large fraction of the hormone-sensitive lipase in rat adipose tissue can be recovered in a large, phospholipid-rich "particle" of high molecular weight (approximately \(7 \times 10^6\)) (19). This has suggested the possibility that the enzyme in the intact cell might be associated with a lipid-rich matrix. Electron microscopic evidence for an ordered complex of filaments (44) or a fenestrated envelope of thin cytoplasm (45) surrounding the central fat droplet has been presented, although these structures have never been fully characterized. Localization of hormone-sensitive lipase at the perimeter of the fat droplet would seem to be functionally advantageous. One might postulate that the vigorous homogenization needed to disrupt intact adipose tissue might result in fragmentation of a membrane-like structure present in the intact cell. However, the present studies were carried out with the gentlest available technique for disrupting isolated adipocytes (26) and again the hormone-sensitive lipase was found predominantly in the cytosol, 89% of it if one neglects the enzyme associated with the fat cake. Less than 2% was associated with the plasma membrane and the specific activity in this fraction was rather low. Furthermore, lysis of the cells to prepare ghosts by the method of Birnbaumer et al. (31) released over 80% of the total hormone-sensitive lipase into the supernatant fraction, only 17% being recovered in the ghosts. These results strongly suggest to us that hormone-sensitive lipase is predominantly a cytosolic enzyme.

The nature of the enzyme activity associated with the fat cake is difficult to evaluate. Previous studies have shown that the fraction of enzyme activity bound to the fat cake depends in part on the conditions used for preparing the homogenate (11, 12). In the present studies, using a sucrose-EDTA-Tris medium and homogenizing at room temperature, about one-third of the hormone-sensitive lipase was bound to the fat layer. Washing procedures did not remove the lipase from the fat and attempts to remove lipase from the fat by solvast ex traction procedures have in the past been unsuccessful (46).

All that can be said is that a significant fraction of hormone-sensitive lipase is associated with the fat layer and that the absolute enzyme activity in that fraction is markedly increased.
when the adipocytes have been exposed to epinephrine. This may account for the observed decrease in lipolytic activity in the microsomal supernatant fraction in epinephrine-treated cells. Whether that enzyme is particle-bound and trapped in the fat layer or whether it represents firmly adsorbed "soluble" enzyme cannot be determined.

The results of the studies in which exogenous triolein substrate was added to whole homogenates are compatible with the conclusion that the enzyme tightly bound to endogenous substrate acts on it exclusively (i.e. is already saturated with substrate) and that the exogenous substrate is hydrolyzed by lipase in other fractions of the whole homogenate. At the very least, the data indicate that the added exogenous substrate does not mix uniformly with the endogenous substrate during the assay. The results indicate the potential hazards involved in relying on measurements of the rates of hydrolysis of added radioactive triglyceride to assay lipase activity in mixtures containing significant amounts of endogenous unlabeled triglyceride.

The present results confirm and extend previous studies on cyclic AMP-dependent protein kinase activation of hormone-sensitive lipase (20). Activation was shown with the enzyme in each of the subcellular fractions. When the adipocytes had previously been exposed to epinephrine, the percentage activation effected by protein kinase treatment was sharply reduced, indicating that the activation process in the intact cell is very probably the same as or closely related to that effected by protein kinase.

Both phosphorylase and phosphorylase kinase were found almost exclusively in the soluble fractions. Since, however, as much as 50% of the total activity was lost during the preparation of the subcellular fractions, the presence of these enzymes in other fractions cannot be completely ruled out. Prior treatment of adipocytes with epinephrine gave a clear-cut increase in total phosphorylase activity, as well as an increase in the ratio of activities at low and high pH values change as a result of epinephrine treatment. These results raise the question of whether there are in adipose tissue mechanisms for epinephrine stimulation of phosphorylase that do not depend upon a concurrent transformation of phosphorylase kinase to an activated form. However, neither phosphorylase nor phosphorylase kinase in adipose tissue has been previously purified or characterized. The methods used for assay here have been drawn from previous experience with these enzymes in heart (39) and skeletal muscle (24). Consequently, interpretation of the result must be guarded. The methods for preparing phosphorylase kinase for assay or the conditions necessary to distinguish between activated and nonactivated forms may be quite different in adipose tissue from those in muscle or heart. This question is under further investigation.

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