Studies on the hormone-sensitive lipase of adipose tissue

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ABSTRACT   Sucrose gradient centrifugation has been used to examine the triglyceride lipases present in extracts of rat epididymal adipose tissue. The aqueous infranatant recovered between the pellet and fat cake of tissue homogenates which had been centrifuged at 40,000 g was shown to contain two types of triglyceride lipase activity. One of these appears in the 15s region and has been identified as the active form of the "hormone-sensitive lipase" believed to be responsible for initiating the hydrolysis of tissue triglyceride stores in response to lipolytic stimuli. The activity of this enzyme was selectively increased in extracts prepared from tissue exposed to epinephrine and decreased in extracts of insulin-treated tissue. The increased lipolytic activity of extracts of tissue from fasted or fasted–refed rats was also found largely in this region. When the tissue was incubated with orthophosphate-^{32}P, radioactivity was incorporated into a protein migrating at 15s.

A second peak of triglyceride lipase activity appeared in the 6s region coincident with the location of the monoglyceride and diglyceride lipase activities. The amount of 6s triglyceride lipase activity did not correlate with changes in the lipolytic activity of the tissue from which the extracts were prepared, and its physiological function remains to be elucidated. The lipoprotein lipase and the short-chain triglyceride lipase ("tributyrinase") each moved more slowly in the gradient than the 6s triglyceride lipase. Both the 6s and 15s enzymes were shown to be present in washed adipocytes isolated from the tissue by collagenase digestion.

SUPPLEMENTARY KEY WORDS   epinephrine · lipolysis · insulin · fasting · adenosine 3′,5′-monophosphate · lipoprotein lipase · free fatty acids

IN RECENT YEARS it has become increasingly apparent that the lipolytic process in adipose tissue plays a key role in the energy metabolism of the whole animal. By regulating the extent of lipolysis in adipose tissue, a variety of hormones exert important influences on the supply of FFA available as fuel for muscle and other tissues of the body. Despite the great interest which has centered on the hormonal regulation of lipolysis, very little is known about the enzymes responsible for these hydrolytic reactions. The enzyme responsible for initiating the hydrolysis of triglycerides stored in adipose tissue in response to lipolytic stimuli has been called the "hormone-sensitive lipase" (1, 2) and evidence suggesting that cyclic AMP may play a role in its regulation has been presented (1, 3–6). Attempts to isolate and purify this enzyme have been hampered by its instability in crude extracts and the presence in these extracts of other enzymes able to hydrolyze glycerides. Thus, although several workers have reported partial purifications of lipases from adipose tissue (7–14), none of the purified enzymes proved to be sensitive to hormonal influences or to be affected by cyclic AMP.

Subsequent to the completion of the work described here, Corbin, et al. (15) and Huttunen, Steinberg, and Mayer (16) reported that the lipase activity in extracts of rat adipose tissue can be enhanced up to twofold by the addition of ATP, magnesium ions, cyclic AMP, and a protein kinase prepared from rabbit skeletal muscle. Huttunen, Aquino, and Steinberg (17) succeeded in purifying a triglyceride lipase 100-fold from rat epididymal adipose tissue extracts and found that the purified

enzyme remained sensitive to activation by the "protein kinase plus cofactors" system.

We have approached this problem not by attempting to purify one of the triglyceride lipases present in crude tissue extracts, but by studying the properties of the lipases present in such extracts. Our goal has been to identify which of the several lipases present is the "hormone-sensitive" species and to obtain some information about its properties which could serve as identification criteria in its subsequent purification. We present here the results of studies done largely by the technique of sucrose gradient centrifugation in which the "hormone-sensitive" enzyme has been partially separated from seven other types of lipolytic activity present in crude tissue extracts.

EXPERIMENTAL PROCEDURES AND MATERIALS

Tissue Source and Handling

Charles River CD rats were obtained from the Charles River Breeding Laboratories, Inc., Wilmington, Mass., and were maintained on Purina Laboratory Chow ad lib. for 1–2 wk prior to their use. Rats in the weight range 150–250 g were selected for most experiments. In experiments in which tissue from fasted–refed animals was used, the rats were fasted for 3 days and then given free access for 2 days to the high-carbohydrate, fat-free diet of Wooley and Sebrell (18), obtained in pelleted form from General Biochemicals, Chagrin Falls, Ohio.

After decapitation of the rats, the epididymal adipose tissue was removed and placed into incubation flasks as described earlier (19). The incubation medium was the Krebs-Ringer phosphate medium of Umbreit, Burris, and Stauffer (20), containing half the recommended calcium, with air as the gas phase. Conditions were selected so that there would be 250–500 mg of tissue segments per ml of medium. For those experiments in which an homogenate was used, the tissue, either freshly excised or following an incubation period, was ground in an all-glass Ten Broeck homogenizer, using approximately 1 ml of ice-cold water per g of tissue. The homogenate was centrifuged for 15–30 min at 40,000 g at 4°C. The semisolid fat cake floating on the surface was lifted off and the aqueous infranatant lying beneath it was recovered for use.

Sucrose Gradient Centrifugation

The aqueous infranatant was layered onto preformed linear 5 to 20% sucrose gradients of 5- or 30-ml volume. The 30-ml gradients were used only when it was desired to assay each fraction for more than one enzyme, as in the experiment shown in Fig. 2. The centrifugal force

and time were adjusted so that their product was essentially constant, as described in the legend to each figure. Fractions were collected by puncturing the tube bottom and displacing the contents with light mineral oil. It has been shown (21) that the rate of migration in the 5-ml gradient is nearly proportional to the molecular weight of the component. Since hemoglobin was always present in the samples, the position of the lipases could be determined relative to it. Statistical analysis of 13 experiments in which hemoglobin migrated to the same fraction showed that the standard deviation in the position of the 6s lipase was 0.2 fraction. Because of the variation from one preparation to another in absolute levels of lipase activity, we have chosen to display representative experiments rather than attempting to show some form of averaged result. The total number of experiments of each type performed is indicated in the legends.

Triglyceride Lipase Assays (Triolein as Substrate)

Lipolytic activity against long-chain triglycerides was measured by the use of glycerol trioleate-1-^{14}C (Amersham/Searle Corp., Arlington Heights, Ill. or Tracerlab, Waltham, Mass.) or glycerol trioleate-9,10-^{3}H (Amersham/Searle). Carrier triolein was obtained from Mann Research Laboratories, New York. Traces of FFA were removed from both the radioactive and carrier triolein by passage of heptane solutions of each through short columns of Bio-Rad AG1-X8. The substrate emulsion was prepared by mixing solutions of radioactive and carrier triolein in ethyl ether in a heavy-walled tube. The final specific activity was 20–30 μCi/g. The ether was removed under an air stream and sufficient 0.06 M sodium phosphate buffer, pH 7.4, containing bovine serum albumin (83 mg/ml) was added to form a triolein suspension of 30–100 mg/ml. The albumin used was the Fraction V, fatty-acid-poor form distributed by Miles Laboratories, Inc., Kankakee, Ill. The suspension was sonicated using an MSE ultrasonic disintegrator, model 60, operated at 1.6–1.7 ma for 30–120 sec, depending on sample size. The resulting emulsion was generally used immediately, but it could be stored several days before use with only occasional small losses of activity ($< 5\%$).

The sample to be assayed was mixed with one-half its volume of the triolein emulsion and incubated 30 min at 37°C with shaking. Then 2.5 ml of Dole's extractant (22) was added, followed by 2 ml of heptane and 1 ml of water. After vigorous mixing and phase separation, the upper phase was transferred to a scintillation vial. FFA in this sample was separated from unhydrolyzed triolein by a modification of the procedure described by Kelley (23). A quantity of Bio-Rad RG-1-X8 resin, 20–50 mesh, was prepared by consecutive

washes with 5% NaOH, water (to neutrality), iso-propanol, and heptane. Approximately 200 mg of the resin was added to the vial containing the upper phase and the contents were shaken at room temperature for 15 min. The heptane containing the triolein was then removed by aspiration and the resin was washed twice with 5 ml of heptane. Finally, 0.1 ml of NCS (Amersham/Searle) and 10 ml of toluene counting fluid containing 4 g/liter of Omnifluor (New England Nuclear Corp., Boston, Mass.) were added. After standing for several hours at room temperature, the radioactivity was determined using a Picker Nuclear Ansitron II liquid scintillation counter.

This assay proved to be linear with respect to the amount of sample added up to the equivalent of 300 mg of tissue per assay. The reaction was linear with time through 30 min. The radioactive assay gave results equivalent to those obtained with the titration assay of Dole (22) when the assays were directly compared.

Other Lipase Assays

The titration assay was used to measure the tributyrinase and the mono- and diglyceride lipases. Thymol blue was used as indicator while tetrabutylammonium hydroxide served as base (24). The substrate employed in the tributyrinase assay consisted of 100 mg of tributyrin per ml (Sigma Chemical Co., St. Louis, Mo.) in 0.012 M sodium phosphate, pH 7.4, containing 50 mg of albumin per ml. The mixture was emulsified using the Mulsi-Churn apparatus (Mulsijet, Inc., Elmhurst, Ill.). For the mono- and diglyceride lipase assays 120–160 mg of mono- or diolein per ml (K & K Laboratories, Inc., Plainview, N.Y.) was substituted for the tributyrin. Incubation conditions were otherwise the same as for the triolein lipase assays.

Lipoprotein lipase was measured with radioactive triolein as described above except that the substrate preparation was modified to fit the specifications outlined by Robinson (25) and by Korn (26). The substrate consisted of 2–4 mg of triolein per ml (0.3 μCi/ml) suspended in 0.07 M ammonium chloride buffer, pH 8.5, containing 20 mg of albumin per ml and 0.02 M CaCl₂. This mixture was emulsified by sonication as described above and then incubated 30 min at 37°C with an equal volume of fresh rat serum just prior to use. The activity measured with this assay was completely blocked by 3.5 mg of protamine sulfate per ml (27). This agent had no effect on the triglyceride lipase assay.

Chemicals

Orthophosphate-³²P was obtained from New England Nuclear Corp. Beef insulin was the gift of Dr. Otto K. Behrens, Eli Lilly & Co., Indianapolis, Ind., and was used as before (19). Epinephrine bitartrate and sodium deoxycholate were purchased from Sigma Chemical Co. Glycerol kinase, pyruvate kinase, and lactic dehydrogenase were products of Boehringer-Mannheim Corp., New York, and the glucostat reagents were obtained from Worthington Biochemical Corp., Freehold, N.J.

RESULTS

Characterization of Adipose Tissue Triglyceride Lipase Activity by Sucrose Gradient Centrifugation

Preliminary studies revealed that 75–80% of the triolein-hydrolyzing activity measured at pH 7.4 in water homogenates of adipose tissue could be recovered from the aqueous infranatant appearing between the pellet and the floating fat cake after centrifugation of the homogenate at 40,000 g. Our attention was therefore focused on the lipases present in such infranatants. In order to determine the number and relative sizes of these lipases the infranatant was analyzed by the technique of sucrose gradient centrifugation.

An example of the pattern of lipolytic activity obtained in this way is displayed in Fig. 1 for tissue from both normally fed and fasting animals. Four areas of triglyceride lipase activity may be distinguished in the profiles obtained in the absence of deoxycholate. A major peak of activity is always seen sedimenting just ahead of the endogenous hemoglobin which serves as an internal marker. In Fig. 1 this activity is centered in fraction 13. It will be referred to as the 6s triglyceride lipase. A more rapidly sedimenting activity is also consistently found, which in Fig. 1 is centered in fractions 7–8. The magnitude of this peak is highly variable from experiment to experiment as may be seen from the two examples in Fig. 1. It will be referred to as the 15s lipase. By comparing its position on the gradients with that of the hemoglobin and assuming similar densities, the molecular weight of the 15s lipase may be estimated to be near 500,000 (21). A similar calculation for the 6s enzyme yields a value of 135,000.

Additional lipolytic activity is found associated with the thin fatty layer which floats on top of the gradient. This is presumed to represent lipase which is bound to tissue lipids; it is highly variable in amount, in part because of the difficulty in recovering the lipid material quantitatively. Finally, the lipolytic activity found in the bottom four fractions of the gradient is thought to be enzyme associated with membrane fragments. Evidence to support this view is also shown in Fig. 1. Exposing the 40,000 g infranatant to 0.1% deoxycholate prior to application to the gradient increased its total lipase activity about 10%. As shown in the figure, however, this treatment removed virtually all of the lipase activity from the bottom fractions of the gradients, pre-
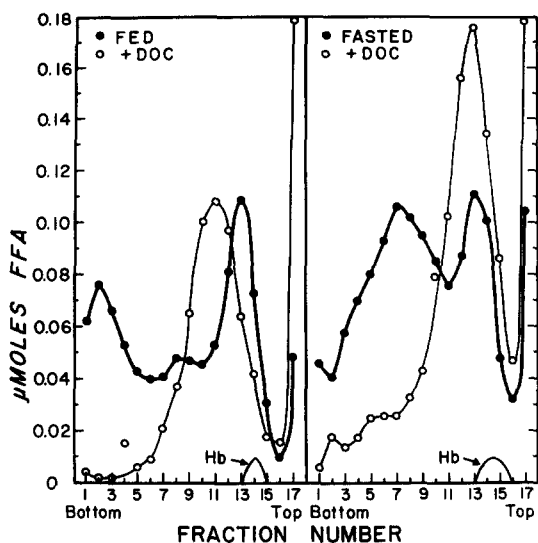
FIG. 1. The distribution of triglyceride lipase activity in extracts of tissue from fed and fasted rats in sucrose gradients, and the effects of deoxycholate. A pool of 2.9 g of tissue, freshly excised from five normally fed rats, was homogenized in 3.0 ml of water. Half of the infranatant obtained after centrifugation for 30 min at 40,000 g at 4°C was made 0.1% in deoxycholate and stored for 30 min on ice. The other half was also stored on ice. The 0.6-ml portions of each half were layered onto 5-ml linear 5–20% sucrose gradients and centrifuged at 4°C for 7.3 hr at 38,000 rpm using a Spinco SW-39 rotor. 0.3-ml fractions were collected and assayed for triglyceride lipase activity as described in the text. The results are expressed as μmoles of FFA produced per total gradient fraction in 30 min. *Hb* designates the position of endogenous hemoglobin. The gradients on the right half of the figure were prepared in an identical manner except that 2.0 g of tissue from four rats fasted for 4 days was employed and the homogenate was prepared in 2.5 ml of water. The results are representative of four such experiments carried out using tissue from fed rats and three experiments using tissue from fasted rats.

sumably by extracting the lipase free of the membranes. Other studies have revealed that considerable lipid material is found in the fractions near the bottom of the gradient, further indicating that membranous material is present.

The action of deoxycholate on the lipases in the infranatant obtained from tissue from fasted rats is not identical to its effect on extracts of normal tissue. From Fig. 1 it can be seen that in the presence of deoxycholate nearly all of the lipase activity in the infranatant from fasted rats is recovered in a single peak coincident with the 6s lipase of the untreated extract. In the normal tissue extract, however, exposure to deoxycholate gives rise to a new peak of activity intermediate between the 6s and 15s regions. No explanation for this difference in behavior between the lipases of tissue from fed and fasted rats has yet been discovered, although it has been a consistent finding.

The lipase may also be extracted from the membrane fragments by shaking the infranatant vigorously with an equal volume of isooctane. The infranatant retains

90–100% of its total lipase activity after exposure to this solvent, but exposing the infranatant to a variety of other solvents, including xylene, ethyl ether, acetone, *n*-butanol, and chloroform, caused major losses of total lipase activity. From the effects of deoxycholate and isooctane we conclude that the association of the lipase with membrane fragments is not essential for enzyme activity.

These experiments defined two distinct triolein-hydrolyzing activities present in adipose tissue extracts, the 6s and 15s lipases. To examine the possibility that one or both of these activities was in fact associated with other, previously described, glyceride hydrolases of adipose tissue, the distribution in sucrose gradients of lipoprotein lipase (27), tributyrinase (14), and monoglyceride lipase (11) was examined. Activity against a diglyceride substrate was also tested. The results are summarized in Fig. 2. From the left panel it can be seen that the lipoprotein lipase, although also active against long-chain triglycerides, sediments primarily in a single peak coincident with the hemoglobin and is thus clearly distinct from either the 6s or 15s triglyceride lipases. This location for the lipoprotein lipase peak indicates that the adipose tissue enzyme has a molecular weight similar to that of the postheparin plasma lipoprotein lipase, which was estimated by Fielding (28) to be
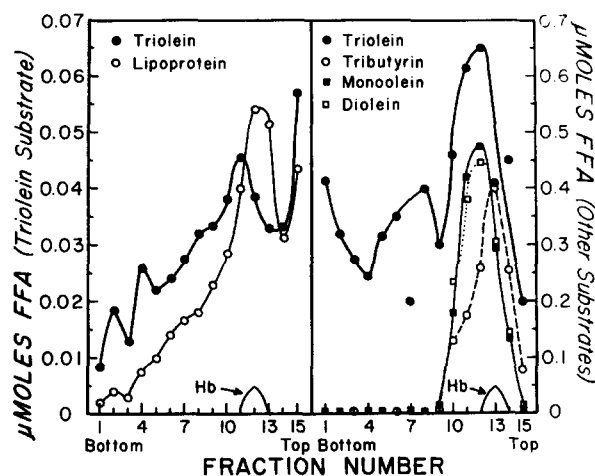

FIG. 2. Relationship of triglyceride lipase activity to other lipases in sucrose gradients. Data in the left panel were obtained using a pool of 5.9 g of tissue removed from six fed rats and homogenized in 6 ml of water. After centrifugation, 1.7 ml of the 40,000 g infranatant was applied to a 30-ml linear 5–20% sucrose gradient. Centrifugation was for 42 hr at 20,000 rpm in a Spinco SW-25 rotor. Fractions were assayed for triglyceride and lipoprotein lipase as described in the text, and the results are expressed as μmoles of FFA per total fraction in 30 min (left ordinate). This experiment was carried out only once. Data in the right panel were obtained from 4.3 g of tissue collected from four fed rats and homogenized in 3.5 ml of water. 2 ml of the 40,000 g infranatant was layered on a 30-ml sucrose gradient and centrifuged for 40 hr at 23,000 rpm. Lipase assays were as described in the text, and the results are given by the right ordinate except for the triglyceride lipase. The results are representative of four such experiments.

72,000. The tributyrinase, or short-chain triglyceride hydrolase, has very little activity toward long-chain glycerides (29) and, as shown in the right panel, is also seen to be smaller than the 6s lipase. However, the mono- and diolein lipolytic activities sediment identically with the 6s enzyme. Although partially purified preparations of the monoglyceride lipase have been reported to exhibit little activity against triglycerides (2, 11, 30), the exact relationship between the 6s lipase and the monoglyceride lipase remains to be clarified. No discrete lipase specific for diglycerides has yet been described for adipose tissue. The diglyceride- and monoglyceride-hydrolyzing activities in the 6s region probably make major contributions to the total fatty acid formation observed with triolein as substrate. Hence, the amount of 6s triglyceride lipase relative to the 15s activity may be exaggerated in the gradient profiles by as much as a factor of three.

Because of the diversity of cells in adipose tissue, and because of the apparent location of most of the lipoprotein lipase at a site in the tissue other than in the fat cells themselves (31), it was of interest to determine whether both the 6s and 15s triglyceride lipases were present in the fat cells. Free fat cells were prepared according to the method of Rodbell (32). The cells were homogenized, and the 40,000 g infranatant fraction of the homogenate was subjected to sucrose gradient centrifugation. The gradient pattern so obtained is compared in Fig. 3 with that obtained concurrently with an infranatant prepared from intact tissue. Both the 6s and 15s lipases as well as some membrane-bound activity are seen to be present in the fat cells themselves. The difference in total lipase activity between the fat cells and the intact tissue results from the loss of fat cells due both to incomplete digestion of the original tissue with collagenase and to loss during the several washes involved.

Identification of the 15s Lipase as the Physiologically Regulated Depot Fat Lipase

Experiments were next carried out to determine the effects of hormones and of dietary manipulations on the 6s and 15s triglyceride lipases. Early reports (1, 33, 34) established that homogenates prepared from adipose tissue previously incubated with epinephrine contained enhanced lipase activity. The effect of exposing the tissue to epinephrine prior to homogenization on the pattern of lipase activity in sucrose gradients was therefore examined. An exposure time of 10 min was chosen in order to allow ample time for full expression of the epinephrine effect (2) and yet not allow sufficient time for excessive accumulation of FFA in the tissue. The result of such an experiment is shown in Fig. 4. The tissue incubated as a control yielded an infranatant displaying the usual 6s (fraction 13) and 15s (fraction 10) activities along with
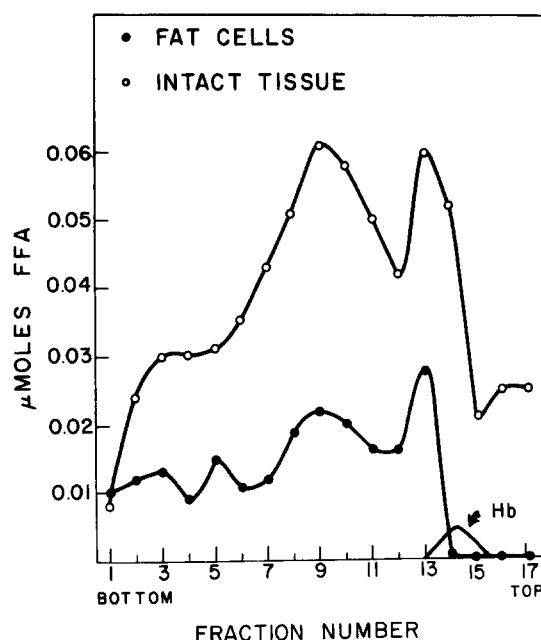

FIG. 3. Distribution of triglyceride lipase of fat cells in a sucrose gradient. Free fat cells were prepared from about 1.5 g of tissue removed from two fed rats. The washed cells were suspended in 1.3 ml of Krebs-Ringer phosphate medium containing 1% albumin and incubated at 37°C for 20 min in a plastic vial. The vial contents were then homogenized and centrifuged at 40,000 g for 15 min. About 1.2 g of intact tissue was incubated simultaneously in the same medium, then homogenized in 1 ml of this medium and centrifuged as above. Samples of 0.5 ml of each infranatant were applied to 5-ml sucrose gradients and centrifuged at 26,000 rpm for 16.5 hr. Results are expressed as in Fig. 1.

more rapidly sedimenting activity presumably associated with membrane fragments. The homogenate prepared from tissue exposed to epinephrine for 10 min contained more lipase activity in every fraction recovered from the gradient, but the increase was especially pronounced in the 15s region. This can be most clearly seen from the difference curve also plotted in the figure. Apparently it is the 15s lipase whose activity is selectively increased in response to this lipolytic hormone, with only very little change in the activity of the 6s species.

Fasting for periods in excess of 24 hr leads to increased rates of lipolysis in adipose tissue (35, 36). This increase may also be demonstrated in homogenates prepared from tissue of fasting animals (1). Data suggesting that the 15s lipase may also be responsible for the increased lipolysis during fasting has already been shown in Fig. 1. The relative amount of the 15s lipase compared with the 6s lipase was much greater in the extract prepared from tissue of fasted animals. To explore this relationship further, rats were fasted for 2, 3, or 4 days and 40,000 g infranatants prepared from each were subjected to gradient centrifugation. The results summarized in Fig. 5 demonstrate that the activity of the 15s enzyme relative to the 6s enzyme increases with increasing length
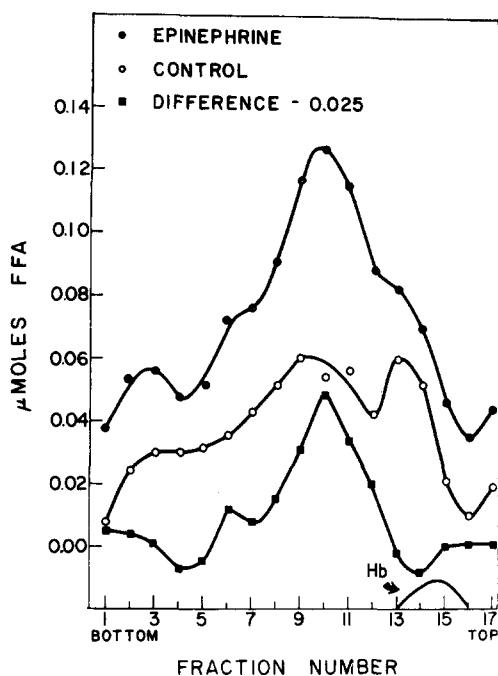
FIG. 4. Alteration in triglyceride lipases caused by exposure of the tissue to epinephrine. Approximately 1.4 g of tissue removed from a group of four fed rats was incubated in each of two flasks containing 2.7 ml of Krebs-Ringer phosphate medium. After 60 min of incubation at 37°C, 0.3 ml of additional medium or of an epinephrine solution was added to give a final epinephrine concentration of 60 μM. 10 min later the tissues were removed to ice-cold 0.15 M NaCl, blotted, and homogenized in 1.3 ml of water. Portions of 0.5 ml of the 40,000 g infranatants prepared from each homogenate were applied to 5-ml sucrose gradients and centrifuged for 7.5 hr at 37,000 rpm. 11 such experiments were carried out.
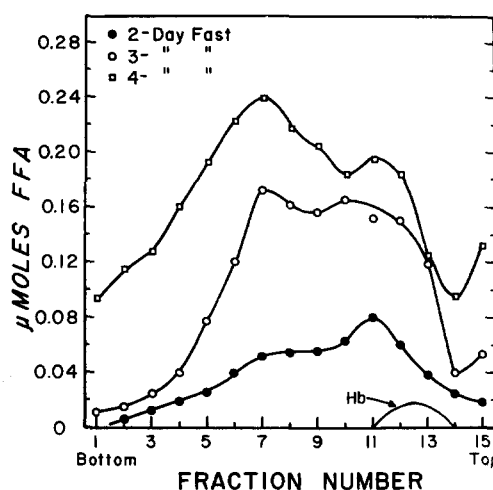

FIG. 5. Effect of fasting on the distribution of triglyceride lipase in a sucrose gradient. Tissue freshly excised from two rats fasted for 2 days (0.92 g), 3 days (0.49 g), or 4 days (0.80 g) was homogenized in 1 ml of water. The homogenates were centrifuged for 30 min at 40,000 g at 4°C, and 0.5-ml samples of each of the resulting infranatants were applied to 5-ml sucrose gradients. After centrifugation for 16.5 hr at 28,000 rpm, fractions were collected and assayed for triglyceride lipase as described in the text. Values for the 3- and 4-day fasted animals have been adjusted to represent the same amount of tissue as used with the 2-day fasted rats. Hb designates the distribution of endogenous hemoglobin. 12 such experiments were performed.

of the fasting period. Thus the data suggest that the 15s enzyme also plays a major role in the enhanced lipolysis in tissue from fasted animals. The significance of the absolute rise in 6s activity with fasting shown in Fig. 5 is not clear. Since different rats were necessarily employed to obtain each of the gradient profiles shown in the figure, reliable quantitative comparisons between profiles cannot be made.

Adipose tissue removed from rats which have been fasted for 3 days and then refed for 2 days on a high-carbohydrate diet has an even higher rate of basal lipolysis than tissue from fasted rats, and this rapid lipolysis is very sensitive to the antilipolytic action of insulin (37, 38). Infranatants were therefore prepared from tissue of refed rats after it had been incubated in vitro with or without insulin. Since the onset of action of insulin appears to be slower than that of epinephrine (39), we chose to allow a full hour of exposure to insulin prior to homogenization. The gradient patterns obtained are shown in Fig. 6. For tissue from refed rats incubated without insulin the 15s lipase (fraction 8) is the dominant activity. Only relatively small amounts of the 6s enzyme

(fraction 13) are present. In the particular example shown here, there is also a peak of membrane-bound activity in fraction 5. This membrane-associated activity does not sediment to the same location from experiment to experiment and often is nearly absent. The profile obtained from the tissue of refed rats exposed to insulin shows a striking decrease in the 15s enzyme relative to the 6s enzyme. The membrane-associated activity, however, was not altered by the insulin treatment. These features of the insulin treatment are seen most clearly from the difference curve shown at the base of the figure. Insulin had a similar effect on tissue obtained from normally fed rats, though the decrease in the 15s region was less pronounced in this case.

Orthophosphate-^{32}P Incorporation Studies

The above results suggested that it is the 15s enzyme whose activity is subject to physiological regulation. If this conclusion is correct, and if the lipase activity of adipose tissue is regulated in a manner analogous to that of muscle or liver glycogen phosphorylase, it might be anticipated that the 15s lipase would be subject to a phosphorylat`on–dephosphorylation sequence associated with its physiological regulation. Strong evidence to support this hypothesis has appeared since the completion of this work (15–17). Experiments were therefore performed to test whether ^{32}P-labeled inorganic phosphate might be incorporated into a protein migrating at the
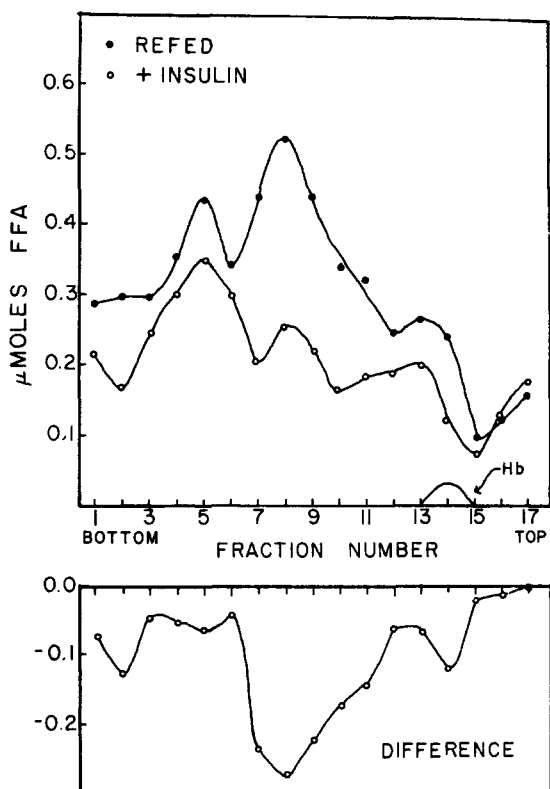
FIG. 6. Effect of insulin on the distribution of triglyceride lipase activity of extracts of tissue from fasted–refed rats in a sucrose gradient. Two portions of tissue, each about 0.9 g, were obtained from three rats which had been fasted and refed. The tissues were incubated for 1 hr in 3 ml of Krebs-Ringer phosphate medium with or without 2 mU of insulin/ml. Following homogenization in 1 ml of water and centrifugation at 40,000 g, 0.5-ml samples of the infranatants were layered onto 5-ml sucrose gradients and centrifuged for 16.5 hr at 25,000 rpm. Fractions were collected and assayed for triglyceride lipase as described in the text. The difference curve represents the results of subtracting the control values from the values of the insulin-treated tissue. The experiment was performed three times with similar results.

15s position in the sucrose gradients. Two groups, each about 600 mg, of epididymal fat bodies were incubated in 3 ml of incubation medium (0.05 M Tris–Cl, pH 7.45, 0.11 M NaCl, 0.1 mM phosphate) containing 75 μCi of orthophosphate-^{32}P for 2 hr at 37°C. In one vessel epinephrine and theophylline were added to give concentrations of 5.1 μM and 10 mM, respectively, for the final 10 min of the incubation. The tissues were rinsed in ice-cold 10 mM sodium phosphate, pH 7.4, three times, then homogenized in 1 ml of 0.1 M NaF–10 mM EDTA, pH 7. After centrifugation at 40,000 g for 30 min, 0.5-ml portions of the infranatants were passed through a Bio-Gel P-60 column of total volume 8.8 ml. The column was eluted with the fluoride–EDTA mixture, and material emerging in the void volume, presumably including all the large proteins of the sample, was recovered. 0.6-ml portions from each column eluate were layered onto 5-ml sucrose gradients and centrifuged 14.5 hr at 29,000 rpm
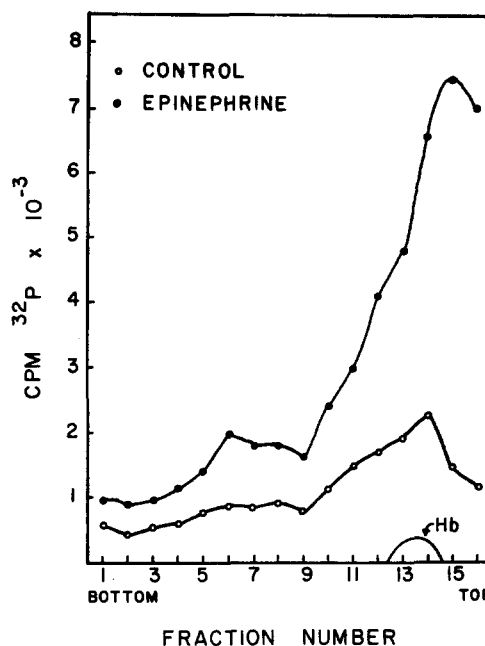

FIG. 7. Distribution of alkali-labile phosphate-^{32}P in sucrose gradients. The preparation of tissue extracts and procedures for centrifugation and ^{32}P analysis are described in the text. The results are representative of three such experiments.

using a Spinco SW-50.1 rotor. 0.3-ml fractions were collected and 0.1-ml portions of each were assayed for ^{32}P. A small peak of radioactivity was revealed in the 15s region of the gradients of both control and epinephrine-treated samples.

On the assumption that the protein-bound ^{32}P of greatest interest would be alkali-labile, another 0.1-ml portion of each fraction was subjected to alkaline hydrolysis according to the method of Crestfield, Smith, and Allen (40). Any traces of inorganic phosphate-^{32}P present in the fractions were first removed by adding 10 μg of carrier phosphate and excess acid ammonium molybdate to each and extracting with isobutanol (41). This extraction procedure was repeated following the alkaline hydrolysis to recover any inorganic phosphate released thereby. The distribution in the sucrose gradient of this alkali-labile phosphate-^{32}P is shown in Fig. 7. A shoulder of radioactivity is clearly visible in the 15s region (fractions 6–7), especially in the sample prepared from tissue exposed to epinephrine. Analysis of gradient fractions from this region by polyacrylamide disc gel electrophoresis followed by radioautography of the gels revealed the presence of two bands containing ^{32}P in a form which was alkali-labile. Whether one or the other of these bands represents the active form of the depot fat lipase cannot be decided until a purified preparation of the enzyme is available. Assays of sucrose gradient fractions for glycogen phosphorylase activity (42) revealed that this enzyme would sediment to fraction 9. In other

experiments it was found that neither epinephrine nor insulin had any effect on the incorporation of orthophosphate-^{32}P into adipose tissue total protein.

DISCUSSION

The sucrose gradient centrifugation studies described here have provided evidence that the 15s triglyceride lipase is the active form of the hormone-sensitive lipase. The amount of lipolytic activity sedimenting in this region of the gradient correlated well with the extent of lipolysis occurring in the tissue from which the extract was prepared. Also, stimulating lipolysis by exposing the tissue to epinephrine increased specifically the activity of the 15s lipase. The antilipolytic action of insulin was associated with a decrease in the amount of 15s lipase activity. In both the fasted and fasted–refed dietary states, when the tissue lipase activity is high relative to that of the fed animal, the predominant form of lipase activity on the gradient was the 15s enzyme. Note that the molecular weight estimate of 500,000 for this species derived from the sedimentation velocity may be in serious error. If in fact the depot fat lipase contains substantial quantities of lipid material bound to it, as has recently been suggested (12, 13, 17), its density may be reduced sufficiently to slow its rate of migration. For example, if the density of the lipase were 1.085 (17) rather than 1.34 as assumed here, the molecular weight estimate would be increased from 5×10^5 to 6×10^6. This is in reasonable agreement with the estimate of 7.2×10^6 given by Huttunen et al. (17) for the molecular weight of their purified lipase. The presence of lipid in the 15s region of the gradient was experimentally verified and ^{32}P was incorporated into this lipid during a 2-hr exposure to orthophosphate-^{32}P. Variations in the amount of lipid associated with the lipase may account in part for the breadth of the 15s lipase peak observed on the gradients.

The experiments in which crude fractions were exposed to deoxycholate suggested that much of the lipolytic activity of adipose tissue homogenates is associated with membranous material. Other workers have reported that substantial quantities of triglyceride lipase either sedimented at 100,000 g (43, 44) or floated into the fat cake (2, 45). These activities may represent the 15s enzyme associated, respectively, with additional membrane fragments or with more lipid. Quite possibly the amount of such association may depend critically on the exact conditions of homogenization.

Alternatively, in the native state the 6s enzyme may be associated with membranes and in this form might possess quite different properties, perhaps coming under hormonal influence. The only support we have found for this possibility is that the enzyme extracted from the 100,000 g pellet by deoxycholate migrated in the 6s region of the gradient. However, changes in the activity of the 6s triglyceride lipase did not correlate with those of the 15s enzyme and the activity of the 6s enzyme does not appear to be modified by hormones.

Increases in the 15s lipase activity in response to epinephrine were not accompanied by decreases in lipolytic activity in any other region of the gradient. Thus, if epinephrine causes the conversion of a nonactivated form of the 15s depot fat lipase to a more active form, the nonactivated form either must have no measurable activity by our assay or must coincide on the gradient with the position of the active 15s enzyme. Work of Chlouverakis (46) showed that fructose diphosphate stimulated the lipase activity both in intact adipose tissue and in homogenates. It seemed possible that fructose diphosphate might be a cofactor for the nonactivated form of the lipase in the way that AMP serves for phosphorylase b (47) and glucose-6-phosphate for glycogen synthetase D (48). However, we have not been able to obtain consistent effects of fructose diphosphate on the triglyceride lipase activity of either fresh or aged infranatants. Moreover, assaying each of the gradient fractions obtained from tissue from fed rats in the presence of a boiled extract of adipose tissue failed to reveal any new peaks of lipase activity.

Some evidence in support of an activation process for the 15s species involving enzyme phosphorylation was obtained in the orthophosphate-^{32}P incorporation studies. It was shown that incubation of the tissue with phosphate-^{32}P resulted in the incorporation of ^{32}P into a substance migrating at 15s in the sucrose gradient. Since this ^{32}P was alkali-labile, the substance was probably a protein. The magnitude of this incorporation appeared to be increased when the tissue was exposed to epinephrine for 10 min prior to homogenization. We have been unable to modify the activity of the lipases present in 40,000 g infranatants by incubating them with ATP, magnesium, and cyclic AMP as described by Rizack (1) and recently by Tsai, Belfrage, and Vaughan (6), or by the addition of calcium ions or epinephrine. We have observed approximately twofold increases in infranatant triglyceride lipase activity following the addition of ATP, cyclic AMP, magnesium, and a crude protein kinase preparation from rat liver[1] in confirmation of recent reports (15, 16).

The changes which occur in the lipases of the tissue during fasting remain to be clarified. We have documented elsewhere (49) that insulin fails to affect lipolysis in the tissue from fasted rats under conditions in which both the glucose uptake and glycogen phosphorylase are altered by this hormone. The normal response of

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[1] Wise, L. S., and R. L. Jungas. Unpublished experiments.

phosphorylase suggests that tissue levels of cyclic AMP were altered by insulin in the tissue from fasted rats. It therefore appears that the lipolytic system of the tissue from fasted rats becomes less responsive to changes in the cyclic AMP level of the tissue. Epinephrine, too, exerts a much smaller lipolytic effect in such tissue (37). The present data suggest that the same 15s lipase is responsible for lipolysis in the tissue from fasting rats as is active in the presence of epinephrine. These findings have led us to the hypothesis that the lipase activation during fasting results from a gradual decrease in the activity of an inactivating enzyme, perhaps a phosphatase, responsible for restoring the active lipase to a nonactivated form. Consequently, the active form of the enzyme accumulates without the necessity for a stimulus from a lipolytic hormone (50). This might explain why lipolysis in the tissue from fasted animals is not inhibited by anaerobic incubation (37), whereas lipolysis in normal tissue is severely inhibited by these conditions of ATP starvation. It has also been our experience that the lipase activity of infranatants of tissue from fasted rats is more stable to storage at room temperature than is the case with extracts of tissue from fed animals. To date, however, our attempts to obtain quantitative data on the amounts of inactivating enzyme present in extracts of tissue from fed and fasted rats have been unsuccessful.

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