Triglyceride, Diglyceride, Monoglyceride, and Cholesterol Ester Hydrolases in Chicken Adipose Tissue Activated by Adenosine 3':5'-Monophosphate-dependent Protein Kinase

CHROMATOGRAPHIC RESOLUTION AND IMMUNOCHEMICAL DIFFERENTIATION FROM LIPOPROTEIN LIPASE*

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Hormone-sensitive lipase and cholesterol ester hydrolase of chicken adipose tissue were markedly activated by adenosine 3':5'-monophosphate (cAMP)-dependent protein kinase (on the average, 235 to 275%; occasionally as much as 1000%). Diglyceride and monoglyceride hydrolases were also activated, but to a lesser extent (60 to 87%). The activation of all four hydrolases was inhibited by protein kinase inhibitor and reversed by the addition of exogenous protein kinase. Following activation by cAMP-dependent protein kinase, all four hydrolases were deactivated in a Mg2+-dependent reaction and then reactivated to or near initial levels on incubation with cAMP and Mg2+-ATP. The reversible deactivation is assumed to reflect activity of one or more protein phosphatases.

The maximum activation obtainable for the four hydrolases decreased when the tissue had been previously exposed to glucagon, indicating that the glucagon-induced activation was probably similar to or identical with the activation demonstrated in cell-free preparations. The pH optima for the four hydrolase activities were similar (7.13 to 7.38). Although the absolute activities and relative degrees of kinase activation differed according to the particular emulsified substrates used, the results do not rule out the possibility that all four hydrolase activities are referable to a single hormone-sensitive hydrolase.

Hormone-sensitive acyl hydrolases were separated from lipoprotein lipase by heparin-Sepharose affinity chromatography. Lipoprotein lipase was active against triolein, diolein, and monoolein, but not cholesterol oleate. Incubation of lipoprotein lipase with exogenous protein kinase, cAMP, and Mg2+-ATP had no effect on any of the three hydrolase activities. Lipoprotein lipase was further purified to homogeneity and used to prepare antiserum in rabbits. The immunoglobulin G fraction from these antisera completely inhibited lipoprotein lipase eluted from heparin-Sepharose columns. However, the hormone-sensitive hydrolase activities (not retained on heparin-Sepharose affinity chromatography) were not inhibited by anti-lipoprotein lipase immunoglobulin G, and anti-lipoprotein lipase immunoglobulin G did not affect the activation process in crude fractions. Thus, hormone-sensitive lipase and lipoprotein lipase, functionally distinct enzymes, have been physically resolved and immunochemically distinguished. Apparently lipoprotein lipase activity is not regulated, at least directly, by cAMP-dependent protein kinase.

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* The abbreviations used are: cAMP, adenosine 3':5'-monophos-
hormone-sensitive lipase was activated 50 to 70% (8). This and other differences in properties raised the question of whether the monoglyceride and diglyceride hydrolases might be distinct from hormone-sensitive lipase, but the evidence did not permit one to rule out the possibility that a single enzyme catalyzes all three glyceride hydrolase activities.

Recently, Pittman and co-workers (6) reported the presence in rat adipose tissue of high levels of a cholesterol ester hydrolase activity that shared many of the properties of hormone-sensitive lipase, including a comparable degree of activation by cAMP-dependent protein kinase. The ratio of hormone-sensitive lipase to cholesterol ester hydrolase activity changed very little during purification and it was suggested that both activities might reside in a single complex or in a single enzyme protein.

Kho and Steinberg have previously shown that hormone-sensitive lipase from chicken adipose tissue is strikingly activated by cAMP-dependent protein kinase—as much as 3- to 8-fold (9). Thus, it seemed attractive to re-examine the relationships among the several acyl hydrolases using this tissue. Furthermore, Huang and Vagelos (10, 11) have succeeded in purifying lipoprotein lipase from chicken adipose tissue to homogeneity and have prepared antibody against it. This provided an opportunity to examine more definitively the possible relationships between lipoprotein lipase and hormone-sensitive lipase of chicken adipose tissue, particularly with respect to their postulated reciprocal regulation by cAMP (10-12) and the possibility that the two enzymes might merely reflect different functional states of a single enzyme protein.

Preliminary reports of some of the present results have been published (13, 14).

**EXPERIMENTAL PROCEDURE**

**Materials**—cAMP-dependent protein kinase was prepared from fresh bovine skeletal muscle through the DEAE-cellulose chromatography column step according to the procedure of Gillman (15). Protein kinase inhibitor was purified from frozen rabbit skeletal muscle through the DEAE-cellulose chromatography step by the method of Walsh et al. (16). Heparin-Sepharose affinity gel was prepared by the method of Iverius (17). Labeled triolein, diolene, and monolein containing [1-14C]oleic acid distributed randomly among the acylated positions, and cholesterol [1-3H]oleate were purchased from Dhsom Products Ltd., North Hollywood, Calif. They were >99% pure as checked by thin layer chromatography. Nonradioactive triolein, diolene, monolein, cholesterol oleate, enolides, and cofactors were purchased from Sigma Chemical Co. A stable emulsion containing a mixture of [14C]oleic acid-labeled triolein (Amersham/Searle) and Intralipid (1%) was specially prepared by Vittum, Sweden (a gift of Drs. Jonas Boberg and W. Virgil Brown). Crude heparin was obtained from Wilson Laboratories; Sepharose 4B from Pharmacia Fine Chemicals; 2% agarose from Bio-Rad Laboratories; adenosine 5'-O-(3-thiotriphosphosphate) from Boehringer-Mannheim Corp.

**Preparation of pH 5.2 Precipitate from Chicken Adipose Tissue**—The procedure was described previously (9) with minor modifications. Laying hens (White Leghorn) were decapitated and adipose tissue was dissected from the abdominal region and from around the gizzard and crop. The fresh tissues were minced and homogenized for 30 s at 10-15" in a Waring Blender with 2 volumes of a buffer solution containing 0.25 M sucrose, 1 mM EDTA, and 10 mM Tris, pH 7.4. The homogenate was centrifuged at 5,000 x g for 5 min at 4°C to remove the bulk of the fat cake and the infranatant fluid was filtered through glass wool and centrifuged at 100,000 x g. Floating fat was sucked off and the fluid was again filtered through glass wool. This was designated the "S100 fraction." The recovery of hormone-sensitive lipase was 80% to 90% from the 5,000 x g infranatant fluid. The S100 fraction was concentrated (30-fold) by precipitation at pH 7.4. The precipitate was resuspended in a small volume of 0.25 M sucrose, micro-mixed, and allowed to homogenize, homogenized briefly and adjusted to pH 7.4 with Tris. This fraction, designated "S2 P," could be stored at -80°C for as long as 1 month without losing hormone-sensitive triglyceride hydrolase activity or response to activation by cAMP-dependent protein kinase. The recoveries of triglyceride, diglyceride, monoglyceride, and cholesterol ester hydrolase activities in the 5.2 P fraction prepared from the S100 fraction were 80%, 97%, 97%, and 74%, respectively.

**Protein Kinase Activating System**—Unless otherwise stated, activation was carried out for 5 min at 30°C. The activation mixture (final volume 0.5 ml) consisted of 0.05 M Tris, pH 7.4; 0.01 mM cAMP; and 5.2 P fraction diluted 1:5 or 1:10 with 1 mM EDTA/10 mM Tris, pH 7.4 (protein content 50 to 100 μg). Exogenous protein kinase was not ordinarily added because there was sufficient endogenous protein kinase to effect maximal activation in 2 to 5 min. In control tubes both ATP and cAMP were omitted.

**Reversible Deactivation System**—The activated enzyme preparation was immediately passed through a Sephadex G-25 column (coarse, diameter 100 x 300 mm) to remove ATP, cAMP, and Mg2+. Aliquots of 0.1 ml were promptly removed and assayed for fully activated hydrolase activities. At the same time, Mg2+ was added to the activated enzyme preparation to a final concentration of 5 mM and incubation was carried out at 30°C. Aliquots of 0.1 ml were removed at time intervals to follow the rate of deactivation. Reactivation of the deactivated enzymes was effected at various time intervals by transferring 0.1 ml to a tube containing 0.5 mM ATP and 0.01 mM cAMP (final concentrations), and incubating for 5 min at 30°C. The reactions were stopped by adding 0.1 ml of 5 N NaOH (final pH 11 to 11.5) (6, 18). The mixture was vortexed vigorously for at least 15 s until a homogeneous suspension of white gel was formed and then centrifuged at 10,000 x g for 10 min with same temperature. [14C]Oleic acid was recovered in the upper aqueous phase. For radioassay, aliquots of the upper phase (1.8 ml) were transferred to vials containing 10 ml of scintillation fluid containing Triton X-100, one-third by volume.

**Lipoprotein Lipase Assay**—Ethinolic substrate mixtures were prepared as described above. Enzyme (0.1 ml) was added to 0.7 ml of substrate solution containing 25 μg/ml of bovine serum albumin, 0.1 M NaCl, 5 mM CaCl2, 25 μl of chicken serum, 1% ethanol, and 50 μM Tris buffer at pH 8.2, and assayed for 30 min at 30°C, during which time the reaction was linear. In some incubations chicken serum was omitted to assess the degree of stimulation by serum.

**Analytical Methods**—Reactions were stopped by addition of 3 ml of a fatty acid extraction mixture (chloroform/methanol/benzene, 2:2:4/1 containing 0.5 μl of unlabeled oleic acid as carrier, followed by addition of 0.1 ml of 1 N NaOH (final pH 11 to 11.5) (6, 18). The mixture was vortexed vigorously for at least 15 s until a homogeneous suspension of white gel was formed and then centrifuged at 1000 x g for 10 min with same temperature. [14C]Oleic acid was recovered in the upper aqueous phase. For radioassay, aliquots of the upper phase (1.8 ml) were transferred to vials containing 10 ml of scintillation fluid containing Triton X-100, one-third by volume.
Glycerol was determined enzymatically by a modification of the method of Wieland (19, 20). Protein was determined by a modification (21) of the method of Lowry et al. (22).

Preparation of IgG Antibody against Lipoprotein Lipase—Lipo-
protein lipase was purified to homogeneity from butanol powder ex-
tracts of chicken adipose tissue, using DEAE-cellulose chromatog-
raphy, heparin-Sepharose affinity chromatography, Sephadex G-200
chromatography, and a final concentration step on a heparin-Sepha-
rose column. The final preparation showed a single band in sodium
dodecyl sulfate-polyacrylamide gel electrophoresis (M, = 61,000).

Purified enzyme in Freund's adjuvant was used to prepare antiserum
in rabbits from which the IgG fraction was prepared by ammonium
sulfate precipitation and DEAE-Sephadex G-20 chromatography.
IgG was also prepared from the serum of control animals.

RESULTS

Hydrolase Activation in 5.2 P Fraction—All four hydrolase
activities in the 5.2 P fraction were activated on incubation
with ATP, Mg++, and cAMP, although to different degrees
(Table I). The mean activation of triglyceride hydrolase and
cholesterol ester hydrolase in a series of different preparation
was 3 to 4 fold; the activation of the lower glyceride hydrolases
was only 1/10 to 1/8 as great but reproducible and highly sig-
ificant. The pattern was consistent in different preparations,
i.e. the activation of triglyceride and cholesterol, ester hydrol-
ases was always of comparable magnitude and the activation
of the lower glyceride hydrolases was always considerably less.

Activation absolutely required the presence of all three cofactors (cAMP, ATP, and Mg++) omission of any one of the
two abolished activation completely. Activation of none of the
hydrolases was enhanced by addition of exogenous protein
kinase, indicating the presence of sufficient endogenous pro-
tein kinase in the 5.2 P fraction to effect optimal activation.
Addition of protein kinase inhibitor sharply reduced or abol-
ished activation, as shown in Fig. 1, and the inhibition by
protein kinase inhibitor was overcome by addition of exogenous protein kinase.

Among several other cyclic nucleotides tested (cIMP, cGMP,
cAMP, and cUMP) only cIMP could effectively replace cAMP.
The requirement for ATP was also rather specific. GTP (0.5
mM) produced a slight stimulation (90% or less for triglyceride
hydrolase) and no stimulation was observed with ITP. Full
activation was also obtained using a sulfur analog of ATP—
adenosine 5'-O-(3-thiotriphosphate)—but activation was
significantly slower (Fig. 2). Gratecos and Fischer (23) have
reported that phosphorylase activated in the presence of this
ATP analog is relatively resistant to the action of phosphoryl-
ase phosphatase. The apparent greater stability of triglyceride
hydrolase activated in the presence of this analog, as shown in

<table>
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<tr>
<th>Substrate</th>
<th>Hydrolase activity before cAMP-dependent activation</th>
<th>Percentage activation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol free fatty acids/mg/hr</td>
<td></td>
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<tr>
<td>Triglyceride</td>
<td>318 ± 58*</td>
<td>274 ± 29</td>
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<tr>
<td>Cholesterol oleate</td>
<td>78 ± 5</td>
<td>95% ± 10</td>
</tr>
<tr>
<td>Diolein</td>
<td>2535 ± 365</td>
<td>87 ± 13</td>
</tr>
<tr>
<td>Monoglyceride</td>
<td>1482 ± 180</td>
<td>60 ± 6</td>
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</table>

*The values represent mean ± S.E. for 10 separate preparations of
5.2 P fraction. Averages of duplicate determinations were used.
Duplicate determinations agreed to within 10%.

Addition of protein kinase inhibitor sharply reduced or abol-
ished activation, as shown in Fig. 1, and the inhibition by
protein kinase inhibitor was overcome by addition of exogenous protein

FIG. 1. Effect of protein kinase inhibitor on the activation of
diglyceride (DG) (panel A), monoglyceride (MG) (panel B),
and cholesterol ester (CE) (panel C) hydrolases. Aliquots of the
5.2 P fraction were incubated with 5 mM Mg++, 5 mM Mg++,
0.5 mM ATP, and 0.01 mM cAMP for 5 min at 30 °C. Diglyceride
and monoglyceride hydrolases were assayed for 15 min at 30 °C
as described under “Experimental Procedures.” Cholesterol ester
hydrolase was assayed for 30 min at 30 °C. Solid symbols indicate
activation of hydrolases without protein kinase inhibitor (PKI) and without exogenous protein
kinase added. Open symbols indicate activation of hydrolases with
increasing concentrations of purified exogenous protein kinase (0 to 37
µg) added in the presence of a constant amount of protein kinase
inhibitor (11 µg).

FIG. 2. Time course for activation of triglyceride (TG) hydrolase.
Aliquots of the 5.2 P fraction (1.08 mg/ml) were incubated at 30 °C
with 5 mM Mg++, 5 mM Mg++, 0.5 mM ATP, and 0.01 mM cAMP
(●), or with 5 mM Mg++, 0.5 mM adenosine 5'-O-(3-thiotriphosphate),
and 0.01 mM cAMP (●). At time intervals indicated, aliquots of 0.1 ml
from each of these incubation mixtures were removed and immediately
added to tubes containing 0.7 ml of [14C]triglyceride
for triglyceride
hydrolase assay.

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Fig. 2, may be on a similar basis. However, the possibility that the analog is itself more resistant to ATPase should be mentioned as a possible second contributing factor. Similar results (i.e., lower rate of activation and greater stability of the activated form) were obtained in studies of diglyceride, monoglyceride, and cholesterol ester hydrolase activation using adenosine 5′-O-(3-thiotriphosphate).

**Kinetic Studies**—Although the lipid emulsions used in this and other studies of lipolytic enzymes yield reproducible results within a given series of assays, it is recognized that preparations made on different occasions are not equivalent, and that kinetic data are difficult to interpret. The availability of a highly stable, finely divided triglyceride emulsion (Intralipid containing added [3H]triolein as described under "Experimental Procedures") allowed us to obtain consistent substrate concentration-activity data. Lineweaver-Burk plots are shown in Fig. 3. The nonactivated enzyme showed an apparent $K_m$ of 0.42 mM and a $V_{max}$ of approximately 125 μmol of free fatty acids/mg/hour. After activation, the $V_{max}$ remained unchanged but the apparent $K_m$ was reduced to 0.1 mM. Attempts to similarly examine the kinetics for cholesterol ester hydrolase and for lower glyceride hydrolases were unsatisfactory in the absence of an analogous small particle, stable emulsion. However, it was shown that the magnitude of the difference between nonactivated and activated enzyme preparations was greater when assayed at lower concentrations of diolein emulsions prepared using ethanol (83% at 0.05 mM versus 28% at 1.0 mM). Thus, a relatively low substrate concentration (0.125 mM) was used routinely in the present studies. The use of higher substrate concentrations in previous studies may well have masked the relatively smaller activation of lower glyceridase activity (9).

**Effect of Hormone Treatment on Activation of Triglyceride, Diglyceride, Monoglyceride, and Cholesterol Ester Hydrolases**—Avian adipose tissues are relatively insensitive to catecholamines, but highly sensitive to glucagon (24, 25). Minces of chicken adipose tissue (about 5 g) were incubated in 10 ml of Krebs-Ringer bicarbonate buffer in the absence or presence of 1 PM glucagon. The incubation was carried out under 95% O2/5% CO2 for 10 min at 37°. At the end of the incubation period, the tissue was homogenized and centrifuged at 40,000 × g for 30 min. After removal of floating fat, the infranatant fluid fraction ($S_{100}$) was assayed for basal hydrolase activities and for cAMP-ATP activation. As shown in Table II, the absolute hydrolase activity in fractions prepared from tissues previously incubated with glucagon was significantly higher and the percentage of increase during cAMP-dependent activation was lower, indicating hormone-stimulated conversion to the activated form during incubation. Similar results have been noted previously in studies of hormone-stimulated rat adipose tissue, but the absolute activity in the supernatant fraction from hormone-treated tissues was often not any greater than that in the fraction from control tissues, possibly attributable to differential losses of activity during preparation of fractions for assay (26).

**pH Activity Profiles of Triglyceride, Diglyceride, Monoglyceride, and Cholesterol Ester Hydrolases**—The activity of all four hydrolases in the 5.2 P fraction was determined as a function of pH, both before and after cAMP-ATP activation (Fig. 4). For ease of comparison, activity of each hydrolase was normalized and expressed relative to that of the activated form at its optimal pH. The pH profiles of the four hydrolases were strikingly similar, with optima in a narrow range from 7.13 to 7.38. It appeared that the optimum for triglyceride hydrolase activity, both the nonactivated and activated forms, and that for the activated form of cholesterol ester hydrolase were slightly higher (7.38 versus 7.13 for the others), but whether this apparent difference is significant or not is uncertain. The data in Fig. 4 show that the greater activation obtained for triglyceride and cholesterol ester hydrolases (panel A) compared to that obtained for diglyceride and monoglyceride hydrolases (panel B) is observed at all pH values.

**Reversible Deactivation**—We have previously shown that hormone-sensitive triglyceride hydrolase from chicken adipose tissue can be deactivated in a Mg2+-dependent reaction and that this is reversible on incubation with CAMP and Mg2+-ATP (9, 27). Using similar methods it was shown that all four hydrolases in the 5.2 P fraction show reversible deactivation (Fig. 5). A 5.2 P fraction was activated in the usual way, the

Table II

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Hydrolase activity in homogenate</th>
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<tbody>
<tr>
<td></td>
<td>Before cAMP-dependent activation</td>
</tr>
<tr>
<td></td>
<td>nmol free fatty acids/mg/hr</td>
</tr>
<tr>
<td>Triolein</td>
<td>76</td>
</tr>
<tr>
<td>Cholesterol oleate</td>
<td>11</td>
</tr>
<tr>
<td>Diolein</td>
<td>860</td>
</tr>
<tr>
<td>Monolein</td>
<td>546</td>
</tr>
<tr>
<td></td>
<td>102</td>
</tr>
</tbody>
</table>
preparation was then immediately passed through a Sephadex G-25 column to remove nucleotides and Mg²⁺, and aliquots of 0.1 ml were taken for assay. The activation for the triglyceride, cholesterol ester, diglyceride, and monoglyceride hydrolases initially were 600%, 700%, 90%, and 85%, respectively. The specific activities shown at zero time in Fig. 5 represent the activities in the Sephadex G-25 effluent. Aliquots of 0.1 ml were removed at the time intervals indicated for direct assay. The time course for deactivation was similar for all four hydrolases, although the initial rate appeared to be somewhat greater for deactivation of triglyceride and cholesterol ester hydrolases.

To effect reactivation, aliquots of 0.1 ml were incubated with ATP-cAMP for 5 min at 30°. For all four hydrolases there was a clear cut reactivation, in most cases restoring activity to levels close to those of the original activated preparations.

Separation of Lipoprotein Lipase from Hormone-sensitive Triglyceride Hydrolase—Heparin-Sepharose affinity chromatography has been successfully employed for purification of lipoprotein lipase from a variety of sources (28–30), including chicken adipose tissue (14, 31). As shown in Fig. 6, most of the lipoprotein lipase in the 5.2 P fraction was retained when the column was loaded and eluted with 0.5 M NaCl, emerging only after the eluent was shifted to 1.5 M NaCl. Fractions were always diluted just prior to assay to reduce the NaCl concentration to 0.1 to 0.15 M. Specific enzyme activity was 3180-fold.

![Fig. 4](image-url)  
**Fig. 4.** Effect of pH on hydrolase activities in the 5.2 P fraction with and without prior cAMP-ATP activation. Aliquots of the 5.2 P fraction were incubated for 5 min at 30° in a buffer containing 1 mM EDTA, 10 mM Tris, pH 7.4; and either 5 mM Mg²⁺ alone (open symbols) or 5 mM Mg²⁺, 0.5 mM ATP, and 0.01 mM cAMP (closed symbols). Triglyceride, cholesterol ester, diglyceride, and monoglyceride hydrolase activities were then determined as described under “Experimental Procedures” but at the final pH values indicated. A, Triglyceride (TG) circles) and cholesterol ester (CE) (triangles) hydrolases. B, Diglyceride (DG) (circles) and monoglyceride (MG) (triangles) hydrolases. Activities have been normalized and expressed relative to that of the activated form at its optimal pH (set equal to 100).

![Fig. 5](image-url)  
**Fig. 5.** Deactivation and reactivation of triglyceride (TG), cholesterol ester (CE), diglyceride (DG), and monoglyceride (MG) hydrolases. Samples of the 5.2 P fraction were activated with cAMP-ATP in a standard 5-min incubation as described under “Experimental Procedures.” This fully activated enzyme fraction was then chromatographed on Sephadex G-25 to remove nucleotides and Mg²⁺. Mg²⁺ was then added to the desalted activated enzyme fraction to a final concentration of 5 mM, and the deactivation process was followed during incubation at 30°. At the time intervals indicated, aliquots were removed for assay of hydrolase activities (○). Reactivation was effected at intervals by incubating 0.1 ml of the reaction mixture with 0.5 mM ATP and 0.01 mM cAMP for 5 min at 30° (● — △).

![Fig. 6](image-url)  
**Fig. 6.** Chromatography of the 5.2 P fraction on a heparin-Sepharose affinity column. NaCl was added to 6 ml of the 5.2 P fraction to yield a final NaCl concentration of 0.5 M. The sample was then centrifuged at 10,000 x g for 15 min at 4°. The fat cake and sediment were discarded and the fluid layer was loaded on a heparin-Sepharose affinity column (1.5 x 40 cm) previously equilibrated with a buffer solution containing 0.5 M NaCl, 20% glycerol, and 10 mM Tris, pH 7.4. The flow rate was 35 ml/hour and fractions of 1.4 ml were collected. Triglyceride (TG) hydrolyase activity (0.1-ml aliquots) was assayed in each tube as shown (●). Tubes were pooled as indicated into Fractions I, II, and III. At 190 ml effluent volume, the NaCl concentration of the eluent was increased to 1.5 M to elute the lipoprotein lipase (LPL). The latter was assayed at pH 8.2 with addition of serum as described under “Experimental Procedures” (○) and the values are indicated by the ordinate scale on the right.
increased over that in the original 5.2 P fraction. The pooled fractions of lipoprotein lipase were immediately frozen and stored at -80°C for later immunochemical studies. This lipoprotein lipase fraction was also active against diolein and monoglyceride but addition of serum did not enhance lower glyceride hydrolase activities to the same extent. Thus, diglyceride hydrolase activity was increased 2.2-fold, monoglyceride hydrolase activity 5.5-fold, but triglyceride hydrolase activity over 50-fold. There was no detectable cholesterol ester hydrolase activity. Incubation of enzyme from this fraction with cAMP, Mg2+-ATP, and exogenous protein kinase under standard activation conditions had no effect on triglyceride, diglyceride, or monoglyceride hydrolase activity assayed in the presence of serum at pH 8.2 (Table III).

The triglyceride hydrolase activity not bound by the affinity column was eluted partly in the void volume peak but a large fraction was somewhat retarded and appeared as a broad retained peak (Fig. 6). The tail of this peak overlapped the tubes containing pigment, probably hemoglobin, from the 5.2 P fraction. The void volume peak was designated Fraction I; the second peak was divided into an early fraction (Fraction II), which was clear and colorless, and a late fraction (Fraction III), which was clear but pink. Fractions I, II, and III were dialyzed for 14 hours against two changes of a buffer solution containing 20% glycerol, 1 mM EDTA, and 10 mM Tris, pH 7.4.

Fractions I, II, and III were assayed for lipoprotein lipase activity at pH 8.2 with and without serum. There was no serum effect, indicating little or no contamination with lipoprotein lipase. All four of the hydrolases were present in each of these fractions and all were activated by cAMP-ATP. The degree of activation of triglyceride, cholesterol ester, and diglyceride hydrolases in the lipid-rich void fraction (Fraction I) was greater than that in the original 5.2 P fractions; activation in the triglyceride hydrolase in this fraction was somewhat retarded and appeared as a broad peak. Fractions I and II, which had been stored in 20% glycerol, were dialyzed for 2 hours to eliminate potential interference of glycerol with the antibody reaction. As shown in Table IV, the antibody had no significant inhibitory effect on the triglyceride hydrolase in these fractions. This was true whether or not the preparation had been previously activated, i.e. neither the nonactivated nor the activated hormone-sensitive triglyceride hydrolase was inhibited by the antibody against lipoprotein lipase. Nor did the antibody have any effect on the other three hydrolase activities in Fractions I, II, or III (data not shown).

Effects of the antibody on triglyceride and cholesterol ester hydrolase activities in a 5.2 P fraction were also examined. Total triglyceride hydrolase activity was inhibited 36% by the 20 min incubation with antibody; cholesterol ester hydrolase was unaffected. The results suggest that a little over one-third of the triglyceride hydrolase activity in this fraction assayed under the conditions optimal for hormone-sensitive triglyceride hydrolase (pH 7.0, no serum added) is attributable to lipoprotein lipase. There was no antibody inhibition of cholesterol ester hydrolase activity, consonant with the observation that it appears that even the retarded fractions still contained more protein kinase to allow full activation using the standard 5 min activation procedure. Rates of activation were not determined. In each column fraction, as in the 5.2 P fraction, triglyceride and cholesterol ester hydrolase activities were enhanced to a greater extent than diglyceride and monoglyceride hydrolase activities.

Immunoechemical Studies—Larger quantities of highly purified lipoprotein lipase were prepared from butanol powder of chicken adipose tissue and used to produce antibody in rabbits, as described under “Experimental Procedures.” The potency of this antibody in inhibiting the triglyceride hydrolase activity of lipoprotein lipase (purified by heparin-Sepharose chromatography) is shown in Fig. 7. Using a 20 min exposure to antibody at 30°C prior to assay, 50% inhibition was obtained with antibody of about 5 μg/IgG (25 μg/ml of assay mixture) and virtually complete inhibition of lipoprotein lipase activity was obtained with 40 μg.

The IgG antibody was then used in maximally inhibitory amounts (40 μg per assay) to test for inhibition of acyl hydrolases in the early column fractions. Fractions I and II, which had been stored in 20% glycerol, were dialyzed for 2 hours to eliminate potential interference of glycerol with the antibody reaction. As shown in Table IV, the antibody had no significant inhibitory effect on the triglyceride hydrolase in these fractions. This was true whether or not the preparation had been previously activated, i.e. neither the nonactivated nor the activated hormone-sensitive triglyceride hydrolase was inhibited by the antibody against lipoprotein lipase. Nor did the antibody have any effect on the other three hydrolase activities in Fractions I, II, or III (data not shown).

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Effects of IgG antibody against lipoprotein lipase on triglyceride hydrolase activity in two fractions not retained by a heparin-Sepharose affinity column

Aliquots of 0.2 ml from Fractions I and II (Fig. 6) were incubated with or without cofactors (cAMP and Mg²⁺-ATP) at 30°. After 5 min, IgG antibody against lipoprotein lipase or control IgG from normal serum were added and incubation was continued for 15 min at 30°, followed by triglyceride hydrolase assay. Overnight incubation with IgG did not cause any further inhibition, nor did centrifugation following incubation with IgG.

<table>
<thead>
<tr>
<th>Heparin-Sepharose fraction</th>
<th>IgG added</th>
<th>Triglyceride hydrolase activity (nmol free fatty acid/mg/hr)</th>
<th>Percentage activation (%)</th>
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<tr>
<td>I</td>
<td>Control</td>
<td>74</td>
<td>290</td>
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<td>IgG (40 µg)</td>
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</tr>
<tr>
<td></td>
<td>IgG (40 µg)</td>
<td>70</td>
<td>347</td>
</tr>
<tr>
<td></td>
<td>Anti-lipoprotein lipase</td>
<td>65</td>
<td>434</td>
</tr>
</tbody>
</table>

Discussion

The present results demonstrate for the first time the activation of lower glyceride hydrolases in cell-free preparations of adipose tissue. Like the activation of hormone-sensitive triglyceride lipase, the activation was totally dependent on addition of cAMP, but there was sufficient endogenous protein kinase in the fractions used to support activation. The dependency on protein kinase was established by adding protein kinase inhibitor and showing that activation then occurred only with addition of exogenous protein kinase, an approach used in similar fashion by Corbin et al. (32) in studies of crude fractions of rat adipose tissue. Fully activated diglyceride and monoglyceride hydrolases were deactivated in Mg²⁺-dependent reactions, as previously demonstrated for the hormone-sensitive triglyceride hydrolase of chicken adipose tissue (9). Furthermore, prior treatment of the intact adipose tissue with glucagon resulted in cell-free preparations that then yielded a lower degree of activation, implying that the hormone-stimulated activation in the intact cell occurs by the same mechanism demonstrated in the cell-free preparations. Thus, in all major respects the activation of diglyceride and monoglyceride hydrolases paralleled that of triglyceride hydrolase, with the important exception that the degree of activation was considerably less. This is undoubtedly one reason that activation of lower glyceride hydrolases has been overlooked previously (8, 9). In the present studies, using a very finely divided, phospholipid-stabilized triglyceride emulsion (Intralipid), we have shown that activation decreases the $K_v$ but does not affect the $V_{max}$ for hormone-sensitive triglyceride hydrolase. In previous studies we used high, saturating concentrations of triolein emulsions stabilized with gum arabic and observed increases in the apparent $V_{max}$. The reasons for this different behavior of the activated enzyme with respect to these different substrate preparations are not clear. The much greater surface area offered by the Intralipid preparation must be relevant. In any case, it may be important to be aware of the phenomenon when studying the activation of lipolytic enzymes. In the present studies we reduced the concentration of lower glycerides used (from 1 mM to 0.125 mM) and enhanced the apparent degree of protein kinase-dependent activation.

If in rat adipose tissue the relative activation of diglyceride and monoglyceride hydrolases is, as in the chicken adipose tissue, much less than that for triglyceride hydrolase, it would be difficult to demonstrate. In rat adipose tissue, activation of the triglyceride hydrolase only enhances activity by about 50%, and thus the activation of the lower glyceride hydrolases might be 10 to 15% by analogy with the present results in chicken adipose tissue. In fact, the data of Heller and Steinberg (8) show that there was activation of this degree in some experiments, but because of the variance it was not considered significant.

Ordinarily, hormonal stimulation of lipolysis in rat adipose tissue is not accompanied by accumulation of lower glycerides (33). However, Scow et al. (34, 35) have shown that there is an accumulation of diglyceride in rat parametrial fat pads perfused with ACTH and Wästern (36) has reported an increase in lower glyceride content of rabbit adipose tissue after administration of epinephrine in vivo. Shafrir (37) has noted increases in monoglyceride hydrolase activity of a microsomal fraction prepared from rat adipose tissue first incubated to reduce basal lipolysis and then exposed to epinephrine. Taken together these data suggest that diglyceride and monoglyceride hydrolase activities are enhanced along with triglyceride hydrolase activity, but to a lesser extent. If, as seems quite possible from the present studies and previous studies (34-37), the diglyceride and monoglyceride hydrolase activities actually reside in the same hormonally-regulated lipase, regulation of free fatty acid mobilization is still to be understood in terms of the conversion of the nonactivated form to the activated form of this relatively nonspecific acyl hydrolase. However, there may be situations in which the further breakdown of the lower glycerides is effected by separate enzymes, and it would be important to know whether these are under hormonal regulation.

The tendency for lipoprotein lipase and hormone-sensitive lipase to vary in a reciprocal fashion and, in particular, the inverse changes in these enzyme activities in response to lipolytic hormones, $N^\omega$,-dibutyryl adenosine 3':5'-monophosphate cAMP, and inhibitors of phosphodiesterase, have led to the speculation that lipoprotein lipase might also be under control by cAMP (10-12). The hypothesis is attractive in that it would be analogous to the "push-pull" control of phosphorylase and glycogen synthase. We have previously reported negative results with regard to modification of lipoprotein lipase by cAMP-dependent kinase (9, 30) but those
studies were carried out with mixtures of lipoprotein lipase and hormone-sensitive lipase. In the present studies these two activities were completely resolved and the highly purified lipoprotein lipase was shown to be unaffected (neither activated nor deactivated) by incubation with exogenous protein kinase under conditions that markedly increased the activity of hormone-sensitive lipase.

The triglyceride hydrolase activity of the purified lipoprotein lipase was completely inhibited by IgG antibody isolated from rabbits immunized with homogenous lipoprotein lipase. Titration with antibody showed that the diglyceride and monoglyceride activities of the lipoprotein lipase were inhibited in a completely parallel fashion, indicating that the lower glyceride hydrolase activities reside in the same enzyme protein. On the other hand, the partially purified hormone-sensitive lipase fractions (not retained by the heparin-Sepharose column) were not inhibited at all even by high concentrations of the IgG antibody. Furthermore, the presence of the specific IgG during activation did not inhibit that process. These results make it most unlikely that lipoprotein lipase is directly regulated by cAMP-dependent protein kinase; they do not rule out the possibility that cAMP is involved, directly or indirectly, in regulation of lipoprotein lipase in some other fashion. For example, several investigators have suggested that lipoprotein lipase levels are regulated by tissue levels of free fatty acids and that this could explain the reciprocal relationships observed between levels of lipoprotein lipase and hormone-sensitive lipase (39, 40).

Cholesterol ester hydrolase activity has previously been demonstrated in adipose tissue from man (5) and rat (6). The enzyme from rat adipose tissue was activated to about the same degree as the triglyceride hydrolase of that tissue, i.e. about 50% (6). The enzyme from chicken adipose tissue in the present studies was again activated to a degree comparable to that for the triglyceride hydrolase, i.e. 200 to 300%. These two activities did not separate during the limited purification carried out here; both showed a similar pattern of reversible deactivation; they had the same pH profile; and the responses to prior treatment of the tissue with glucagon were similar. The absolute activity of triglyceride hydrolase was considerably greater than that of cholesterol ester hydrolase when expressed in terms of total free fatty acids released per unit of time (Table I). However, since the splitting of the first ester bond in triolein is probably rate-limiting, and the rate of further hydrolysis of diolein and monolein is so much greater, the total nanomoles of free fatty acids released by triglyceride hydrolase should probably be divided by 3 to allow a more meaningful comparison with the activity of cholesterol ester hydrolase. Moreover, the impossibility of providing these insoluble substrates in truly equivalent concentrations makes it difficult to draw conclusions from the apparent differences in absolute activity, no matter how they are expressed. From the data available it seems that the two hydrolase activities may reside in a single enzyme protein, but attempts to resolve the activities physically should be continued. The physiologic role of cholesterol ester hydrolase in adipose tissue remains obscure. The concentration of cholesterol in adipose tissue is relatively low, but because of the mass of the adipose tissue organ the total amount of cholesterol stored in depot fat is very large (41). Only a small fraction of the adipose tissue cholesterol is present in ester form (41, 42) but it may be essential to hydrolyze that cholesterol ester during fatty acid mobilization to prevent the relative build-up of the ester. Alternatively, it has been speculated by Kovanen et al. (42) that cholesterol ester might in some way protect triglyceride from the action of lipase. In that case, the cholesterol ester hydrolase activity might be essential in exposing the triglyceride in fat droplets, and thus have a cooperative affect in optimizing lipolysis.

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