# Reversible protein kinase activation of hormone-sensitive lipase from chicken adipose tissue

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**Abstract** Hormone-sensitive lipase partially purified from adipose tissue of laying hens was markedly activated by cyclic AMP-dependent protein kinase. Activation was approximately 4-fold (ranging up to as great as 10-fold) compared with the much lower degree of activation obtained with analogous preparations from rat and human adipose tissues (59 and 86%, respectively). The partially purified preparations contained adequate endogenous protein kinase activity to effect complete activation with addition of cyclic AMP, ATP, and $Mg^{2+}$. Activation was blocked by protein kinase inhibitor (from rabbit skeletal muscle) but could be restored fully by addition of excess exogenous protein kinase (from bovine skeletal muscle). The fully activated lipase was slowly deactivated by dialysis at 4°C and then rapidly and almost fully reactivated by addition of cyclic AMP and ATP–$Mg^{2+}$. Reactivation was blocked by protein kinase inhibitor. This deactivation–reactivation cycle was rapid at 23°C with dialysis against charcoal and could be demonstrated repeatedly using a single preparation. The reversible deactivation of protein kinase-activated enzyme is presumed to reflect the action of a lipase phosphatase. Lipase prepared from tissue previously exposed to glucagon yielded a much smaller degree of activation than lipase prepared from tissue not exposed to the lipolytic hormone, indicating that the physiological hormone-induced activation is probably similar to or identical with the protein kinase activation demonstrated in the cell-free preparations. Under the conditions of assay used, the partially purified lipase fraction contained diglyceride, monoglyceride, and lipoprotein lipase activities. However, treatment with cyclic AMP-dependent protein kinase had virtually no effect on these lipase activities.

**Supplementary key words** lipoprotein lipase · diglyceride lipase · monoglyceride lipase · glucagon · protein kinase inhibitor · cyclic AMP · lipase phosphatase

The role of cyclic AMP and cyclic AMP-dependent protein kinase in the activation of hormone-sensitive lipase has been established for rat and human adipose tissue (1–10). In both cases, however, the degree of activation observed has been relatively small, from 50 to 100%, compared with the much larger percentage changes in rate of glycerol release that can be induced by hormones, especially in the case of isolated adipocytes from rat epididymal fat pads. We have now found that hormone-sensitive lipase prepared from adipose tissue of laying hens can be activated fourfold and more by cyclic AMP-dependent protein kinase. Lower glyceridase activities are not affected.

A second unresolved problem concerned the reversibility of hormone-sensitive lipase activation. Studies of intact adipose tissue and isolated adipocytes exposed to lipolytic hormones have shown that the rate of lipolysis rapidly returns to basal values when the hormone is removed or its action arrested by addition of the appropriate inhibitor (3, 11, 12). In vivo, again, the lipolytic effects of epinephrine are quite transient (13, 14), but a second sharp rise can be elicited by readministration of the hormone. Clearly, there is a mechanism for rapidly and reversibly deactivating the hormone-stimulated lipase, but attempts to demonstrate this in cell-free systems have been unsuccessful. Tsai, Fales, and Vaughan (15) have described an interesting ascorbic acid-dependent inactivation of hormone-sensitive lipase, but this system causes *irreversible* inactivation; that is, subsequent treatment with cyclic AMP-dependent protein kinase is without effect. We now report the presence in chicken adipose tissue homogenates of a system that rapidly deactivates hormone-sensitive lipase previously activated by protein kinase; the deactivated enzyme can be almost fully reactivated by repeating the incubation with cyclic AMP, ATP–$Mg^{2+}$, and protein kinase.

## MATERIALS AND METHODS

Laying hens (white leghorn) were killed by decapitation, and adipose tissue was dissected from the abdominal

Abbreviations: FFA, free fatty acid(s); cyclic AMP, adenosine 3′,5′-monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid. The nomenclature of the apolipoprotein employed here is based on its COOH-terminal amino acid. In the terminology suggested by Alaupovic et al. (38), apoLp-Glu is defined as apoC-II.

region and around the gizzard. Fresh specimens were also obtained from a local slaughterhouse. The tissues were minced and homogenized for 60 sec at 10–15°C in a Waring Blendor with 2 vol of a buffer containing 0.25 M sucrose, 1 mM EDTA, and 10 mM Tris–HCl, pH 7.4. The homogenate was centrifuged at low speed (1000–5000 *g*) and the bulk of the floating fat cake was removed. The infranatant fluid fraction was filtered through glass wool and centrifuged at 40,000 *g* for 30 min. Any residual floating fat was removed by suction, and the infranatant fluid layer was again filtered through glass wool. Approximately 80% of the lipase activity of the 1000–5000 *g* supernatant fraction was recovered in this fraction. Further centrifugation at 100,000 *g* for 60 min did not reduce lipase activity significantly, indicating that it is a soluble enzyme, like the lipase from rat adipose tissue (16). The 40,000 *g* supernatant fraction (designated $S_{40}$ fraction) was carefully adjusted to pH 5.2 with 0.1 M acetic acid in an ice bath with constant stirring. After 5–10 min, the precipitate formed was collected by centrifugation at 1000 *g* for 10 min. The precipitate was dissolved in homogenizing buffer to one-thirtieth of the original volume of the $S_{40}$ fraction. The pH of the resuspended precipitate was immediately adjusted to 7.0 with 0.2 M Tris–HCl, pH 8.0. This fraction (designated 5.2 p fraction) was used in all the experiments to be described. The recoveries of $S_{40}$ lipase activity in the 5.2 p fraction were close to 100% with three- to fivefold purification. This fraction could be stored at −80°C for as long as 1 month with only about 20% loss in lipase activity. However, as detailed under Results, the degree to which the lipase could be activated by cyclic AMP-dependent protein kinase varied from preparation to preparation. Adipose tissue stored either in the refrigerator for a few days or in the freezer for a few weeks often retained its lipase activity, but its activation by cyclic AMP and ATP–$Mg^{2+}$ was greatly reduced. The reasons for this lability with regard to the activation obtainable are not clear.

Cyclic AMP-dependent protein kinase was prepared from fresh bovine skeletal muscle through the DEAE-cellulose chromatography column step according to the procedure of Gilman (17). Protein kinase inhibitor was purified from frozen rabbit skeletal muscle through the DEAE-cellulose chromatography step by the methods of Walsh et al. (18). Apoprotein with COOH-terminal glutamic acid (apoLp-Glu) was purified from very low density lipoproteins of human plasma (19) and kindly provided by Dr. W. V. Brown of our Division.

Labeled triolein, diolein, and monoolein containing [1-$^{14}$C]oleic acid distributed randomly among the acylated positions were purchased from Dhom Products, Ltd., North Hollywood, Calif. These substrates were >99% pure by thin-layer chromatography. Nonradioactive triolein, diolein, and monoolein were purchased from Sigma Chemical Co., St. Louis, Mo. Details of the preparation of substrates have been described elsewhere (20). Briefly, a mixture of solvent-free labeled and unlabeled triolein was sonicated in 5% gum arabic. Diolein emulsions were prepared in the same way. Because monoolein did not yield a homogeneous suspension with gum arabic, it was prepared by mixing with 10 mM taurodeoxycholate, pH 7.0, using a Vortex mixer.

### Activation of hormone-sensitive lipase

Unless otherwise stated, activation was carried out in a 13 × 100 mm disposable glass test tube for 10 min at 30°C in a final volume of 0.2 ml containing enzyme (0.1–0.25 mg of protein of the 5.2 p fraction), 5 mM $Mg^{2+}$, 0.5 mM ATP, 0.01 mM cyclic AMP, 1 mM theophylline, 1 mM dithiothreitol, 0.5 mM EGTA, and 50 mM Tris–HCl, pH 8.0 (optimum pH for lipase activation). This is hereafter referred to as the complete system. In control tubes, both ATP and cyclic AMP were usually omitted, and this is referred to as the incomplete system. In studies of deactivation, theophylline was omitted.

### Lipase assay

Immediately after 10 min of incubation with complete or incomplete system, 0.6 ml of a substrate mixture containing 1 μmole of triolein emulsion (in 5% gum arabic), 20 mg of bovine serum albumin (Armour fraction V), and 40 μmoles of sodium phosphate buffer, pH 6.8, was added (final pH approx. 7.0) and incubated for 30 min at 30°C. The rate of fatty acid release in all cases remained constant over the assay period and was linearly related to enzyme concentration over the range studied (up to 25 μl of 5.2 p fraction, about 0.25 mg of protein).

The reaction was stopped by adding 3 ml of a fatty acid extraction mixture (chloroform–methyl alcohol–benzene 2:2.4:1) containing 0.3 μmole of nonradioactive oleic acid as carrier, followed by addition of 0.1 ml of 1 N NaOH (final pH 11–11.5). The mixture was shaken vigorously in a Vortex mixer for at least 15 sec and then centrifuged at 2500 rpm at room temperature in an International centrifuge for 10 min. An aliquot of the upper phase (1.8 ml), containing radioactive sodium oleate, was transferred to a scintillation vial, and 10 ml of scintillation fluid (4 g of 2′,5′-diphenyloxazole [PPO] and 0.1 g of 1,4-bis-2[4-methyl-5-phenyloxazolyl]-benzene [dimethyl POPOP] in 1 l of toluene containing Triton X-100, one-third by volume) was added.[1] Radioactivity was determined in a Packard Tri-Carb liquid scintillation counter. Quenching, which was generally constant (approx. 30%), was corrected for. Recovery of added [$^{14}$C]oleic acid was approximately 80%. Reproducibility of duplicate assays was excellent, generally within less than 5%.

[1] Pittman, R. C., and D. Steinberg. Unpublished data.

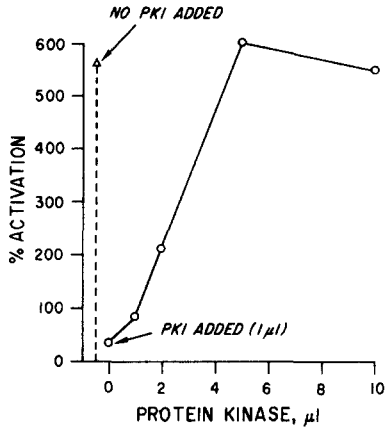


**Fig. 1.** Effect of protein kinase inhibitor on the activation of chicken hormone-sensitive lipase. Lipase in the 5.2 p fraction was incubated with complete or incomplete system (see Materials and Methods) for 10 min at 30°C followed by lipase assay for 30 min at 30°C; △, lipase activation due to endogenous protein kinase with no protein kinase inhibitor (PKI) added; ○, lipase activation in a series of incubations containing a constant amount of PKI with the addition of the indicated amounts of exogenous protein kinase purified from bovine skeletal muscle.

## RESULTS

### Characteristics of the activation system

Hormone-sensitive lipase activity was markedly stimulated by preincubation with cyclic AMP, ATP, and $Mg^{2+}$. The activation absolutely required $Mg^{2+}$, ATP, and cyclic AMP, as shown in **Table 1.** Theophylline was not essential, presumably because the cyclic AMP concentration used here ($1 \times 10^{-5}$ M) was far in excess of the $K_m$. It was included routinely (except in the studies of deactivation) to ensure that cyclic AMP levels were always comparable.

TABLE 1. Cofactor requirements for activation of hormone-sensitive lipase

| Addition | Lipase Activity | Percentage Activation[a] |
|---|---|---|
| | *nmoles FFA/mg protein/hr* | |
| Incomplete system[b] | 508 | |
| Incomplete system + ATP, 0.5 mM | 521 | 3 |
| Incomplete system + cyclic AMP, 0.01 mM | 508 | 0 |
| Incomplete system − theophylline | 508 | 0 |
| Complete system[c] | 1319 | 160 |
| Complete system − theophylline | 1325 | 160 |

[a] Lipase activation is expressed as the percentage increase in lipase activity over that of enzyme incubated with the standard incomplete system.

[b] Incomplete system: 5 mM $Mg^{2+}$, 1 mM theophylline, 1 mM dithiothreitol, 0.5 mM EGTA, 50 mM Tris–HCl, pH 8.0, and enzyme in a total volume of 0.2 ml. This mixture was incubated at 30°C for 10 min, followed by lipase assay for 30 min at 30°C.

[c] Complete system: incomplete system, as above, plus 0.5 mM ATP and 0.01 mM cyclic AMP.

Activation was not enhanced by addition of exogenous protein kinase. However, evidence that endogenous protein kinase was indeed responsible for the activation is presented in **Fig. 1.** Addition of protein kinase inhibitor blocked activation almost completely (35% in the presence compared to 600% in the absence of protein kinase inhibitor). Protein kinase inhibitor itself at the concentration used had no direct effect on lipase activity. The inhibition of activation due to added protein kinase inhibitor was abolished by adding back enough protein kinase to exceed the binding capacity of the protein kinase inhibitor present (Fig. 1).

Activation of lipase was very rapid when high cofactor concentrations were used (5 mM $Mg^{2+}$, 0.5 mM ATP, and 0.01 mM cyclic AMP). Maximum activation was reached in less than 2 min. In order to obtain a measurable time course of activation, the cyclic AMP concentration was lowered to $5 \times 10^{-7}$ M, and aliquots were removed for lipase assay at 15-sec intervals, as shown in **Fig. 2,** *A*.

The dependence of activation on magnesium acetate concentration is shown in Fig. 2*B;* a maximum rate of activation was obtained at about 0.5 mM magnesium acetate. When 5 mM $CaCl_2$ was substituted for 5 mM magnesium acetate, no activation was observed. $Co(NO_3)_2$ and $MnCl_2$ were about one-fifth as effective as magnesium acetate. Moreover, all three divalent cations at 5 mM directly inhibited lipase activity (in the absence of $Mg^{2+}$): $Mn^{2+}$, 64%; $Ca^{2+}$, 63%; and $Co^{2+}$, 90%. Effects of the companion anions have not been systematically studied.

The dependency of the extent of activation on concentrations of ATP and cyclic AMP is shown in Fig. 2*C* and Fig. 2*D*, respectively. The ATP and cyclic AMP concentration curves were hyperbolic, and saturation was reached at about $2 \times 10^{-4}$ M for ATP and at about $1 \times 10^{-6}$ for cyclic AMP. Half-maximal concentrations for ATP and cyclic AMP were approximately $2.5 \times 10^{-5}$ M and $4 \times 10^{-7}$ M, respectively.

In contrast to the enzyme from rat (21) and from man (10), hormone-sensitive lipase from chicken adipose tissue showed a very sharp pH optimum in phosphate buffer (**Fig. 3**). The optimum for both the activated and nonactivated lipases was at 7.0. Tris–HCl was a poor buffer for assay of lipase activity but did not inhibit activity in the presence of 0.05 M phosphate buffer (tested up to 12.5 mM). At pH 8.2, lipase activity was generally 10% or less of the activity at pH 7.0 with phosphate buffer.

### Reversible deactivating system

The very large percentage activation obtainable with the chicken lipase suggested that it might be a good system in which to study the postulated reversible deactivation of

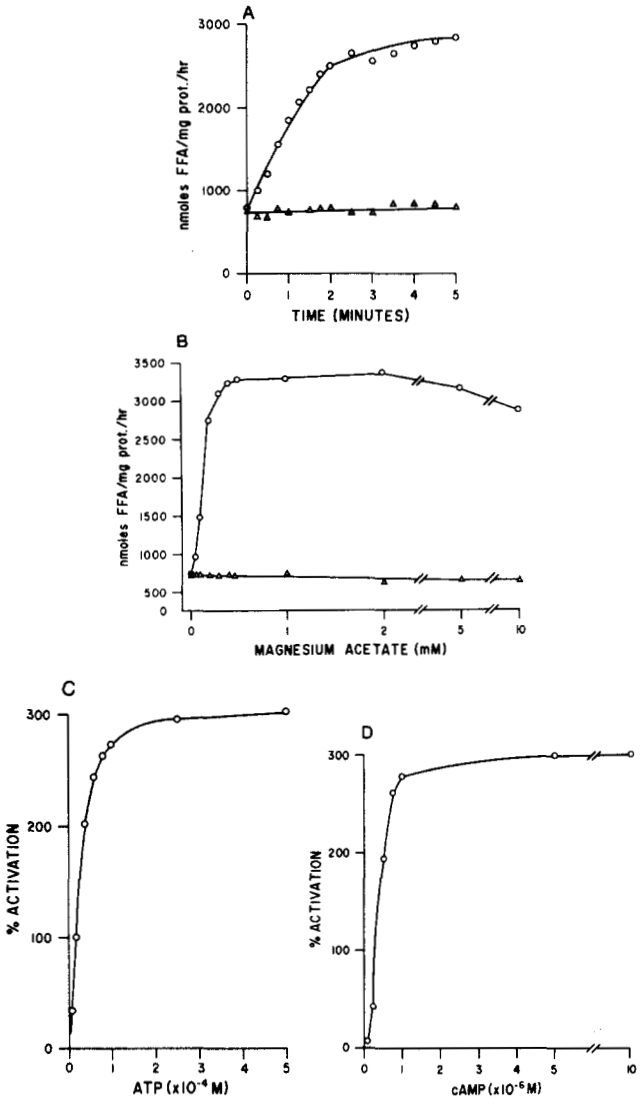


**Fig. 2.** Kinetics of lipase activation. *A*, time course of lipase activation with complete (○) or incomplete (△) system. The cyclic AMP concentration used here was reduced from $1 \times 10^{-5}$ M to $5 \times 10^{-7}$ M. *B*, concentration dependence with regard to magnesium acetate (0.5 mM ATP and 0.01 mM cyclic AMP). *C*, concentration dependence with regard to ATP (5 mM $Mg^{2+}$ and 0.01 mM cyclic AMP). *D*, concentration dependence with regard to cyclic AMP (5 mM $Mg^{2+}$ and 0.5 mM ATP). In the studies summarized in panels *B*, *C*, and *D*, the 5.2 p fraction was incubated with complete or incomplete system for 5 min at 30°C, followed by lipase assay for 30 min at 30°C.

the activated form. Dialysis of the activated lipase for 5 hr at 4°C against a large volume of buffer caused a large decrease in lipase activity, from 2900 down to 700 nmoles of FFA/mg of protein/hr (**Fig. 4**). Upon subsequent incubation with addition of 5 mM $Mg^{2+}$, 0.5 mM ATP, and 0.01 mM cyclic AMP, there was more than a threefold increase in activity, although the level reached was somewhat less than that reached originally (Fig. 4). When either ATP or cyclic AMP was omitted from the reactivating mixture, lipase activities remained the same as in the control tube containing $Mg^{2+}$ alone. The reactivated enzyme was then again subjected to dialysis against fresh buffer for another 5 hr. Again, lipase activity decreased to the basal value, and incubation with cofactors stimulated lipase activity 3.6-fold. This process was repeated. After an additional 5-hr dialysis, the activity prior to reactivation had dropped well below the original basal level and reactivation increased lipase activity by only 84%. While reactivation was evident at all time intervals, the absolute values reached fell progressively with time. This, together with the decrease in basal activity at 15 hr, suggested that there was, in addition to reversible deactivation, some progressive irreversible inactivation or denaturation of the lipase.

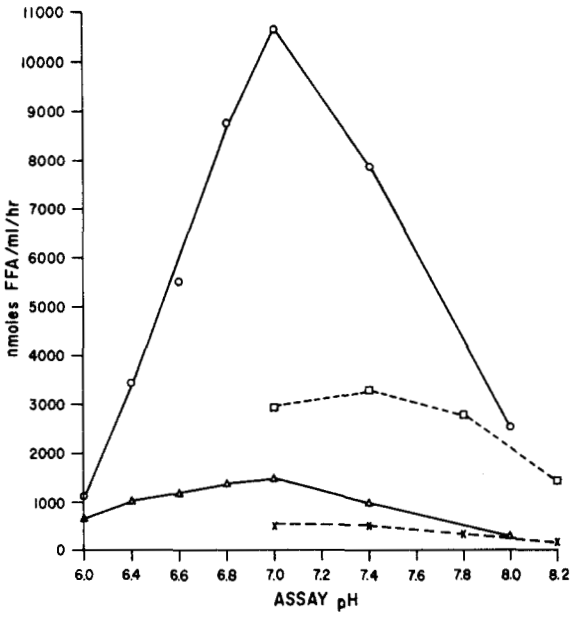


**Fig. 3.** pH–activity curves for the activated and nonactivated forms of chicken hormone-sensitive lipase. Lipase in the 5.2 p fraction was first incubated with complete or incomplete system for 10 min at 30°C, followed by lipase assays for 30 min at 30°C with 40 μmoles of sodium phosphate buffer (activated lipase, ○; nonactivated lipase, △) or with Tris–HCl buffer (activated lipase; □; nonactivated lipase, ×) at the indicated pH values.

In an attempt to speed up the reversible deactivating process relative to the irreversible process, an aliquot of fully activated lipase was dialyzed at 23°C against a buffer solution containing charcoal to adsorb ATP and cyclic AMP. Aliquots were removed at intervals and assayed after a 10-min incubation with $Mg^{2+}$ alone or with ATP–$Mg^{2+}$ plus cyclic AMP, as shown in **Fig. 5.** The initial activation at zero time yielded a 300% increase in activity. During a 5-min dialysis, this preparation lost activity nearly but not quite to the basal level. At this time there was still significant ATP and cyclic AMP present, as shown indirectly by activation obtainable on addition of either $Mg^{2+}$ and cyclic AMP (+45%) or of ATP–$Mg^{2+}$ alone (+81%) (data not shown). Activation on addition of both ATP–$Mg^{2+}$ and cyclic AMP, however, was 160%

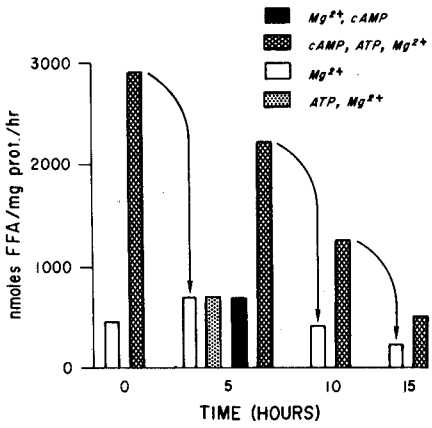


**Fig. 4.** Reversible deactivation of chicken hormone-sensitive lipase. A 1:5 dilution of the 5.2 p fraction (5 ml) was made with a buffer solution containing 1 mM EDTA and 20 mM Tris–HCl, pH 7.4. $Mg^{2+}$ was added to a final concentration of 5 mM. For basal lipase assays, duplicate aliquots of 0.1 ml were removed and incubated for 10 min at 30°C and then assayed for 30 min at 30°C (*open bar*). To the remaining 4.8 ml, ATP and cyclic AMP were added to final concentrations of 0.5 mM and 0.01 mM, respectively, and the mixture was incubated for 10 min at 30°C. Duplicate aliquots of 0.1 ml were removed for lipase assay (*hatched bar*). The large volume of fully activated lipase was immediately transferred to dialysis tubing and dialyzed at 4°C against 1 l of 20 mM Tris–HCl, pH 7.4, containing 2 mM $Mg^{2+}$ and 1 mM EDTA. After 5 hr, aliquots of 0.1 ml were incubated for 10 min at 30°C with 5 mM $Mg^{2+}$ only (*open bar*), with 5 mM $Mg^{2+}$ plus 0.5 mM ATP (*stippled bar*), or with 5 mM $Mg^{2+}$ plus 0.01 mM cyclic AMP (*solid bar*). To 3 ml of the dialyzed enzyme was added 5 mM $Mg^{2+}$, 0.5 mM ATP, and 0.01 mM cyclic AMP. After incubation for 10 min at 30°C, duplicate aliquots of 0.1 ml were removed for lipase assay (*hatched bar*). The lipase reactivated by incubation with all three cofactors was again transferred to dialysis tubing, and the entire process was repeated after an additional 5 hr of dialysis except that the control incubations with ATP–$Mg^{2+}$ and $Mg^{2+}$–cyclic AMP were omitted. The activated lipase was subjected to still another 5-hr dialysis and then reactivated in the presence of all three cofactors. The arrows indicate the degree of deactivation of the activated lipase over each 5-hr dialysis period.

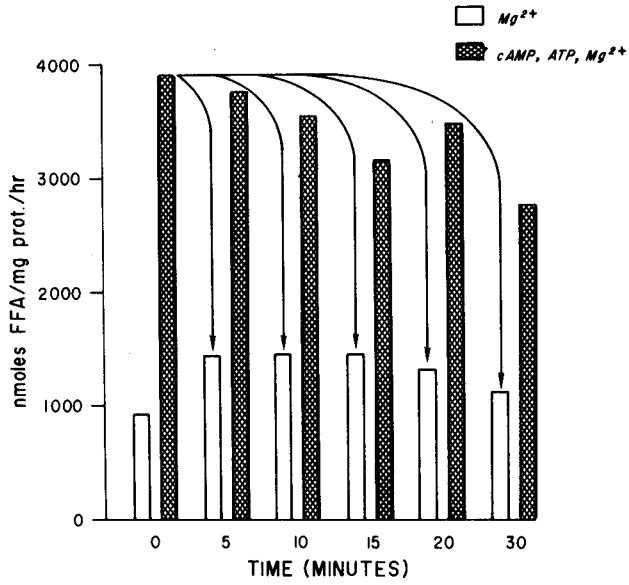


**Fig. 5.** Rapid deactivation of chicken hormone-sensitive lipase. 7.5 ml of a 1:5 dilution of the 5.2 p fraction as described in Fig. 4 was incubated with 5 mM $Mg^{2+}$, 0.5 mM ATP, and 0.01 mM cyclic AMP for 10 min at 30°C. Another aliquot of the diluted enzyme was treated in the same manner with addition of $Mg^{2+}$ only. The lipase activities after this initial incubation are indicated as zero-time values. The fully activated lipase was then immediately transferred to dialysis tubing open at the top and immersed in 500 ml of 2 mM $Mg^{2+}$, 1 mM EDTA, and 20 mM Tris–HCl, pH 7.4, containing 1 g of charcoal and incubated at 23°C. The charcoal was kept in suspension using a magnetic stirrer. At intervals over a 30-min period, duplicate aliquots of 0.1 ml were removed and incubated with 5 mM $Mg^{2+}$ alone (*open bars*) or with 5 mM $Mg^{2+}$, 0.5 mM ATP, and 0.01 mM cyclic AMP (*hatched bars*) for 10 min at 30°C, followed by lipase assays.

(Fig. 5). By 30 min, the removal of ATP and cyclic AMP was evidently complete because at this time no reactivation was obtained unless all three essential cofactors were added back.[2] Addition of protein kinase inhibitor prevented reactivation as effectively as it blocked the initial activation process (see above). Fully activated enzyme was freed of $Mg^{2+}$, ATP, and cyclic AMP by gel filtration (Sephadex G-25). Deactivation in this preparation was markedly stimulated on addition of 5 mM $Mg^{2+}$ (data not shown).

### Effect of prior hormone treatment

Chicken adipose tissue minces were incubated for 30 min in medium with or without $1 \times 10^{-6}$ M glucagon, and the $S_{40}$ fractions were then prepared and studied for activation. As shown in **Table 2,** the percentage activation of lipase obtained was significantly reduced in the $S_{40}$ fractions prepared from tissue previously treated with hormone. This decrease in percentage activation presumably indicates that a large fraction of the nonactivated lipase had been converted to its activated form under the action of glucagon and by a mechanism identical with or closely related to that effected by the protein kinase system (9, 10).

### Effects on the hydrolysis of diolein and monoolein

In the case of rat (22) and human (10) adipose tissue, the rate-limiting step in the release of FFA under almost all circumstances is the hydrolysis of the first ester bond of the triglyceride molecule. As shown by the data in **Table 3,** the same appears to be true in chicken adipose tissue. Diglyceride and monoglyceride lipase activities were about ten times greater than that of triglyceride lipase, but there was little or no activation of these lower glyceridases by the protein kinase system under conditions that tripled triglyceridase activity.

### Effects on lipoprotein lipase

Previous studies on human and rat adipose tissue fractions (10) have shown no changes in lipoprotein lipase ac-

[2] Subsequent studies show that deactivation proceeds, although more slowly, without dialysis, indicating degradation of ATP and cyclic AMP in the system used here.

TABLE 2. Decrease in the activation of hormone-sensitive lipase prepared from tissue previously treated with glucagon

| Expt. No. | Glucagon Treatment | Percentage Activation |
|---|---|---|
| 1 | − | 179 |
| | + | 67 |
| 2 | − | 331 |
| | + | 126 |
| 3 | − | 108 |
| | + | 66 |

Pieces of minced adipose tissue (5–10 mm) were incubated with and without $1 \times 10^{-6}$ M glucagon in Krebs-Ringer bicarbonate, pH 7.4, with 3% bovine serum albumin for 30 min at 37°C under 95% $O_2$:5% $Co_2$. The tissues were immediately homogenized as described under Materials and Methods, and 0.2 ml of the $S_{40}$ fraction was incubated with complete or incomplete system, followed by lipase assay for 30 min at 30°C.

tivities due to cyclic AMP-dependent protein kinase. However, it was necessary to examine that possibility in the context of the present work.

The conditions used for assay of hormone-sensitive lipase, pH 7.0 in 0.05 M phosphate buffer without addition of serum activator, should sharply reduce any contribution of lipoprotein lipase. As shown in Fig. 3, activity at pH 8.2 (the reported optimum for lipoprotein lipase) in the absence of serum activator was only about 10% of that at pH 7.0. However, when serum was added and the assay carried out at pH 8.2, there was a marked increase in lipase activity. As shown in **Table 4,** this activity had the properties expected of lipoprotein lipase, being inhibited 98% by 1 M NaCl and 78% by protamine (23). In contrast, the activity assayed at pH 7.0 in the absence of serum activator was, if anything, enhanced by 1 M NaCl and not affected by protamine. EDTA also affected oppositely the activities measured under the two sets of conditions.

To test whether kinase activation alters lipoprotein lipase activity, preparations previously incubated with either the incomplete or the complete system were assayed with and without addition of serum activator. As shown in **Table 5,** total activity even at pH 7.0 was increased by addition of either serum or apoLp-Glu. However, the increment in activity due to addition of serum was almost exactly the same whether kinase activation (incubation with complete system) had taken place or not. This implies that kinase activation did not affect lipoprotein lipase activity. The *absolute* increment in apparent hormone-sensitive lipase activity brought about by incubation with the complete system was the same whether assayed with or without serum activator. The percentage activation, of course, decreased. In other words, the results indicate that the hormone-sensitive lipase in the preparation was kinase-activated while the lipoprotein lipase activity remained unchanged; the latter activity, in the presence of

TABLE 3. Comparative activation of tri-, di-, and monoglyceride lipase activities

| Substrate | Lipase Activity after Incubation with: Incomplete System | Lipase Activity after Incubation with: Complete System | Percentage Activation |
|---|---|---|---|
| | *nmoles FFA/mg protein/hr* | | |
| Triolein, 1 mM | 440 | 1375 | 213 |
| Diolein, 1 mM | 5668 | 6563 | 16 |
| Monoolein, 1 mM | 4325 | 4535 | 5 |

serum activator, behaved in a sense as a "blank" or background of lipase activity unaffected by protein kinase.

When assays were carried out at pH 8.2 and in the presence of serum (conditions optimal for lipoprotein lipase), the percentage increment due to the incubation with the complete activation system was 11% in one case and 4% in the other (Table 5, experiments 4 and 5). The absolute increments appear to be larger than in the absence of serum, but these represent small differences between large numbers and are not considered significant in view of the limited precision of the assay. Thus, it appears that the activation of hormone-sensitive lipase, assayed under conditions optimal for that enzyme, can be readily measured with little or no interference from lipoprotein lipase and that the lipoprotein lipase present is not influenced by the protein kinase activation system.

## DISCUSSION

The present results demonstrate for the first time in a cell-free preparation a reversible activation–deactivation cycle for hormone-sensitive lipase. The activation is ATP-

TABLE 4. Effects of NaCl, protamine, and EDTA on lipoprotein lipase and hormone-sensitive lipase activities

| | Relative Activity[a]: Lipoprotein Lipase[b] | Relative Activity[a]: Hormone-sensitive Lipase |
|---|---|---|
| None | 100 | 100 |
| NaCl, 1 M | 2 | 185 |
| Protamine, 125 μg/ml | 22 | 101 |
| EDTA, 0.14 M | 56 | 144 |

[a] Activities of lipoprotein lipase and hormone-sensitive lipase assayed without additions were arbitrarily set at 100 (absolute activities of lipoprotein lipase and hormone-sensitive lipase were 1750 and 188 nmoles of FFA/mg of protein/hr, respectively).

[b] Lipoprotein lipase was assayed with substrate mixture containing 40 μmoles of Tris–HCl buffer, pH 8.2, 20 mg of bovine serum albumin, 1 μmole of triolein emulsion in 5% gum arabic, 50 μl of chicken serum, and 0.1 ml of enzyme solution in a total volume of 0.8 ml with NaCl at a final concentration of 0.11 M. The substrate mixture was preincubated at 30°C for 30 min, and lipase activity was assayed over 30 min at 30°C. Hormone-sensitive lipase (not previously activated) was assayed at pH 7.0 without serum added, as described under Materials and Methods.

TABLE 5. Effects of the protein kinase activation system and of lipoprotein lipase activators on hormone-sensitive lipase and lipoprotein lipase activities in chicken adipose tissue extracts.

| Expt. No. | pH of Assay | Addition | Incomplete System | Complete System | Absolute Increment in Activity Due to Protein Kinase | Percentage Activation Due to Protein Kinase |
|---|---|---|---|---|---|---|
| | | | *nmoles/ml/hr* | | | |
| 1 | 7.0 | None | 538 | 1,319 | 781 | 145 |
| | 7.0 | ApoLp-Glu, 25 μg | 2,112 | 2,927 | 815 | 38 |
| | | Increment due to apoLp-Glu | 1,574 | 1,608 | | |
| 2 | 7.0 | None | 317 | 1,279 | 962 | 303 |
| | 7.0 | Chicken serum, 25 μl | 521 | 1,458 | 937 | 180 |
| | | Increment due to serum | 204 | 179 | | |
| 3 | 7.0 | None | 3,797 | 9,632 | 5,835 | 154 |
| | 7.0 | Chicken serum, 25 μl | 16,077 | 21,875 | 5,798 | 36 |
| | | Increment due to serum | 12,280 | 12,243 | | |
| 4 | 8.2 | None | 1,292 | 1,971 | 679 | 52 |
| | 8.2 | Chicken serum, 25 μl | 25,114 | 27,858 | 2,744 | 11 |
| 5 | 8.2 | None | 425 | 1,140 | 715 | 168 |
| | 8.2 | Chicken serum, 50 μl | 27,125 | 28,303 | 1,178 | 4 |

Assays at pH 7.0 were done in phosphate buffer, those at pH 8.2 in Tris buffer.

dependent and catalyzed by cyclic AMP-dependent protein kinase, as in the case of the rat adipose tissue enzyme (5, 6). In the latter case, the protein kinase activation was shown to be accompanied by transfer of the terminal phosphate of ATP to the partially purified enzyme (7), and it was postulated that a lipase phosphatase would effect the deactivation. The $Mg^{2+}$-dependent, reversible deactivation of chicken hormone-sensitive lipase described above most probably reflects the activity of such a lipase phosphatase. However, definitive proof of this will require further purification of the chicken enzyme so that phosphorylation–dephosphorylation can be correlated with changes in activity.

Studies in vivo and in vitro in many species show that the lipolytic response to hormones is short-lived (3, 12, 13). Using isolated rat adipocytes and assessing the fraction of hormone-sensitive lipase in the nonactivated form by measuring the percentage activation obtained in partially purified preparations, it was found that the epinephrine-activated enzyme can revert (under the influence of insulin) to the nonactivated form with 5 min.[3] Thus, it is clear that the conversion of activated lipase to nonactivated lipase can be very rapid. The rates of deactivation demonstrated here in cell-free fractions are compatible with the postulate that the reaction observed accounts for the rapid reversion of lipolysis to basal values in hormone-treated cells.

The marked activation obtainable with the chicken enzyme, up to 4- and 10-fold over basal, considerably facilitated the study of the deactivation process. Hormone-sensitive lipase from turkey and pigeon adipose tissue was also found to be highly activated by cyclic AMP-dependent protein kinase.[3] Attempts to study reversible deactivation in rat adipose tissue extracts have been severely hampered by the limited activation observed. Also, rat adipose tissue contains an ATP–$Mg^{2+}$ and ascorbic acid-dependent *irreversible* inactivating system that may complicate studies in crude fractions (15, 24). We have not observed such ATP-dependent inactivation in the chicken adipose tissue fractions studied. In light of the present findings, the question of reversible deactivation in rat tissue can be reexamined, perhaps utilizing fractions of chicken adipose tissue enriched in lipase phosphatase.

Several explanations for the enhanced activation obtainable with avian adipose tissue enzyme come to mind. First, the difference in the specific activities of the activated and nonactivated forms of lipase may be much greater than it is for the rat enzyme. Were this the case, we might expect a greater percentage increment in lipolysis on hormonal stimulation of the intact tissue. The available data on this subject are inconclusive. Avian adipose tissue shows a pattern of hormonal responsiveness quite different from that of mammals, being relatively insensitive to catecholamines but highly sensitive to glucagon (25–29). Parenthetically, it should be noted that insulin has little or no antilipolytic effect and under some conditions actually enhances lipolysis (25, 26, 30–32), as does insulin at high concentrations in rat adipose tissue (33, 34). The magnitude of the response to glucagon stimulation varies with the age of the bird (35) and varies from species to species

[3] Khoo, J. C., and D. Steinberg. Unpublished observations.

(25). Isolated fat cells from mature cockerels showed basal glycerol release rates of about 0.05 μmole/hr/mg of DNA, and this was increased threefold by saturating levels of glucagon (27). In contrast, the basal glycerol release rate in fat cells from immature birds (28–56 days old) was lower and the magnitude of the glucagon effect much greater, 30- to 150-fold (26). Results were the same in tissue from males and females. In these same studies the rate of release of glycerol from intact pieces of adipose tissue was increased only 4- to 30-fold. While these hormonal effects may indeed be somewhat greater than those seen in rat adipose tissue, the data are too incomplete to be helpful at this stage.

Second, the marked protein kinase activation of the chicken lipase relative to the limited activation of the rat lipase could be due to the occurrence, during preparation of subcellular fractions, of either factitious activation of the rat enzyme or deactivation of the chicken enzyme. This latter possibility is certainly supported by the very rapid deactivation that can occur in chicken enzyme fractions under appropriate conditions (Fig. 5). Factitious activation during homogenization and fractionation of the rat enzyme, though not ruled out, has yet to be demonstrated explicitly.

A third possibility is that the observed difference in the activities of the two forms of hormone-sensitive lipase depends on the presence of some yet unidentified effector(s) playing a role analogous to that of glucose-6-phosphate in the glycogen synthase system (36) or of 5′-AMP in the phosphorylase system (37). If such an effector were present at very different concentrations in the rat and chicken cell fractions studied, it might account for the apparent difference between the two systems. An inhibitor could equally well account for the difference. Ruling against these possibilities is the fact that the percentage activation obtained with the rat enzyme was more or less the same using either the crude 100,000 *g* supernatant fraction or the enzyme purified 100-fold from it (21).

Finally, the larger effect obtained may relate in some way to the high levels of sex hormones in laying hens. We studied 40,000 *g* supernatant fractions from five cockerels, which have much less available adipose tissue than laying hens, and found that the mean percentage activation of the lipase was only 69%.

Except for the greater magnitude of the effect, the properties of the activation system for hormone-sensitive lipase in chicken adipose tissue are like those in rat (7) and human (10) tissues. The absolute requirement for ATP–$Mg^{2+}$ and cyclic AMP, the total inhibition by protein kinase inhibitor from skeletal muscle, and the absence of concurrent activation of monoglyceride and diglyceride lipase activities in the preparations speak for a specific protein kinase-catalyzed activation, presumably linked to enzyme phosphorylation as discussed above. Thus the "cascade" triggering hormone-induced lipolysis involves the following steps: (*1*) hormone–receptor interaction; (*2*) increased adenylate cyclase activity; (*3*) increased cyclic AMP levels; (*4*) increased levels of active protein kinase (catalytic unit); (*5*) increase in the fraction of activated hormone-sensitive lipase; (*6*) increased rate of hydrolysis of the first ester bond in triglycerides; (*7*) increased rate of FFA release from triglycerides and lower glycerides, the latter not being under direct hormonal regulation. This system, now demonstrated in three very different animal species, is probably the common basic system operative in higher animals. Whether the lipase phosphatase activity inferred to be responsible for lipase deactivation is itself metabolically regulated remains to be determined.

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