The mechanism of heparin stimulation of rat adipocyte lipoprotein lipase

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ABSTRACT Free fat cells and stromal–vascular cells were prepared from rat adipose tissue by incubation with collagenase. NH_4OH–NH_4Cl extracts of acetone–ether powders prepared from fat cells contained lipoprotein lipase activity but extracts of stromal–vascular cells did not. Intact fat cells released lipoprotein lipase activity into incubation medium, but intact stromal–vascular cells did not. The lipoprotein lipase activity of the medium was increased when fat cells were incubated with heparin, and this was accompanied by a corresponding decrease in the activity of subsequently prepared fat cell extracts. Heparin did not release lipoprotein lipase activity from stromal–vascular cells.

The lipoprotein lipase activity of NH_4OH–NH_4Cl extracts of fat cell acetone powders is increased by the presence of heparin during the assay. This increase is not due to preservation of enzyme activity, but to increased binding of lipoprotein lipase to chylomicrons. Protamine sulfate and sodium chloride have little effect on the binding of lipoprotein lipase to chylomicrons, but they inhibit enzyme activity after binding to substrate has occurred. These inhibitors do, however, inhibit the stimulatory effect of heparin on enzyme–substrate binding.

SUPPLEMENTARY KEY WORDS enzyme–substrate binding · chylomicron–lipoprotein lipase binding · protamine sulfate · sodium chloride

LIPOPROTEIN LIPASE, a hydrolytic enzyme present in adipose and other tissues, is thought to regulate the uptake of chylomicron and lipoprotein triglyceride by these tissues (1–5). Although it is known that lipoprotein lipase is activated by exogenous heparin in vitro (6) and that this activation is almost specific for heparin [it requires a polysaccharide containing *N*-sulfatyl groups (7)], the mechanism and physiological significance of this effect of heparin are not clear. There is indirect evidence that lipoprotein lipase extracted from acetone powders of chicken adipose tissue consists of a protein complexed with endogenous heparin or a similar mucopolysaccharide necessary for enzyme activity (8), and it has been postulated that the heparin-like moiety serves to bind enzyme protein to substrate lipoprotein (9). If so, it is reasonable to expect that activation of lipoprotein lipase by exogenous heparin is due to an effect on enzyme–substrate binding. An alternative possibility is that heparin may act to stabilize the extracted enzyme, an action observed with post-heparin plasma lipoprotein lipase (10).

Exogenous heparin is known also to release lipoprotein lipase from tissue sites in vivo (11) and in vitro (1) but this effect is not specific for heparin, being duplicated in vitro by sodium polymetaphosphate (12), and in vivo by a wide variety of organic and inorganic polyanions [see a recent review (7)]. It has been postulated that these compounds interfere with electrostatic binding of an anionic enzyme to cationic binding sites in tissue (12), but the nature of these sites is unknown and their location in tissue uncertain.

It has been suggested that lipoprotein lipase is associated with the vascular endothelium (13), but more recent evidence has shown that adipose tissue lipoprotein lipase is found in the adipocytes and not in the stromal–vascular components (14, 15). However, conflicting data have also been published (16). In addition, Pokrajac, Lossow, and Chaikoff have shown (15) that fat cells release lipoprotein lipase into incubation medium and that the lipoprotein lipase activity of the medium is increased when fat cells are incubated with heparin. It is not clear from these authors' data whether this effect was due to increased release of lipoprotein lipase or to stabilization or stimulation of released enzyme activity.

Abbreviations: FFA, free fatty acids; KRB, Krebs–Ringer bicarbonate.

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In this investigation, we have studied the cellular origin of lipoprotein lipase in adipose tissue, the effect of heparin on release of the enzyme from fat cells, and the effects of heparin on enzyme stability and enzyme–substrate binding. In addition, we have studied the mechanism by which protamine sulfate and sodium chloride inhibit lipoprotein lipase activity.

MATERIALS AND METHODS

Male Wistar rats weighing 160–200 g, fed ad libitum with Purina Chow, were anesthetized by intraperitoneal injection of sodium pentobarbital, and the lumbar and epididymal adipose tissues were excised. Free fat cells were prepared by incubation of the adipose tissue with bacterial collagenase (Worthington Biochemical Corp., Freehold, N. J.) according to the method of Rodbell (17); glucose, 2 mg/ml, and crystalline zinc insulin (Connaught Medical Research Laboratories, Toronto, Canada), 10 mU/ml, were present during the incubation. The fat cell suspension was strained through a fine-mesh nylon stocking, which separated small tissue fragments and some of the stromal elements. Fat cells and stromal cells were washed three times with 5 volumes of Krebs–Ringer bicarbonate buffer (KRB) containing half the recommended concentration of calcium (18), together with glucose (2 mg/ml) and insulin (10 mU/ml), and then were either suspended in KRB buffer with 5% bovine serum albumin (Brickman and Co., Montreal, Canada) for further incubation, or extracted immediately with acetone and ether. KRB buffer containing 5% bovine serum albumin was dialyzed for 24 hr against KRB buffer, then filtered through a 0.45 μm membrane filter prior to use.

Extraction of Lipoprotein Lipase

Fat cells were chilled for 5 min in an ice-water bath, then homogenized in 10 volumes of ice-cold acetone by vigorous agitation with a glass stirring rod. The homogenate was filtered through Whatman No. 40 filter paper on a Büchner funnel with gentle suction; the residue on the filter paper was washed rapidly with 50 volumes of acetone at 4°C, 50 volumes of acetone at 25°C, and 50 volumes of diethyl ether at 25°C. The resulting powders were not allowed to dry by suction, but were transferred while still moist with ether to a desiccator and dried in vacuo at 4°C for 1 hr. When stromal cells were assayed, they were homogenized at 4°C in a Potter–Elvehjem homogenizing tube with a Teflon pestle, then treated in the same manner as the fat cells.

After drying, the filter paper and powders were extracted for 1 hr at 4°C with 0.025 M NH_4OH–NH_4Cl buffer at pH 8.2. The filter paper was removed and the suspension centrifuged at 4°C for 10 min at 1200 *g*. The clear supernatant solution was used for lipoprotein lipase assay while the precipitate was recombined with the filter paper for extraction of DNA. Approximately 250 mg of fat cells, from which approximately 1 mg of acetone–ether powder was obtained, were used in the preparation of 1 ml of the NH_4OH–NH_4Cl extract. Preliminary experiments using serial extractions of 1 hr with NH_4OH–NH_4Cl at 4°C showed that 90% of the total soluble enzyme of the acetone–ether powders was extracted in the first hour. No DNA was extracted by the 0.025 M NH_4OH–NH_4Cl buffer.

In some experiments the clear supernatant solution was lyophilized and stored at −20°C. 40% of the enzyme activity was lost during lyophilization but the resulting powders showed no further loss of activity over months of storage.

Extraction of DNA

Tissue residues and filter paper were incubated in 6.0 ml of 0.5 M KOH for 14 hr at 37°C. The filter paper was removed and washed twice with 0.5 M KOH and the washings were combined with the KOH digest. After adjustment of the volume of the digest to 10 ml, DNA was precipitated by the addition of 0.86 ml of 70% perchloric acid. The precipitate was washed twice with 0.5 M perchloric acid at 4°C, then incubated in 0.5 M perchloric acid at 80°C for two 20-min periods. An aliquot of the hot perchloric acid extract was used for DNA determination by a modification of the Dische reaction (19), with sperm DNA (Nutritional Biochemicals Corporation, Cleveland, Ohio) as standard.

Preparation of Substrate

Lymph was collected from the thoracic ducts of mongrel dogs fed safflower oil, defibrinated with a glass stirring rod, and filtered through sterile surgical gauze. After dilution with an equal volume of ice-cold sterile 0.154 M NaCl the lymph was centrifuged for 30 min at 18,000 *g* in the 870 rotor of an International model B20 centrifuge at 4°C, and the butter-like upper layer of chylomicrons was collected. The chylomicrons were washed once by suspension in ice-cold sterile 0.154 M NaCl, separated by centrifugation as above, and stored at 4°C in stoppered glass tubes at an approximate concentration of 500 mg of triglyceride/ml. Chylomicron suspensions so stored were stable and suitable as substrate for lipoprotein lipase for approximately 4 wk without the addition of antibiotics. Chylomicrons prepared in this manner did not require activation by the addition of serum. Chylomicron triglyceride was determined by the method of Van Handel and Zilversmit (20).

Lipoprotein Lipase Assay

This was performed by a modification of the method of Robinson (21). 1 ml of lipoprotein lipase solution was added to 2.0 ml of assay medium prepared to give final concentrations of bovine serum albumin [defatted by the method of Chen (22)], 60 mg/ml; Tris–HCl, 154 mM; chylomicron triglyceride 2.95 mg/ml; and NaCl, 7 mM. The pH of the assay mixture was 8.2. The mixture was shaken at 80 cycles/min at 37°C in a Dubnoff metabolic incubator. Lipoprotein lipase activity was determined by the increase in free fatty acid (FFA) content of 1.0 ml aliquots taken at the beginning and end of 20 min incubation. Control assays, in which 1 ml of NH_4OH–NH_4Cl buffer at pH 8.2 replaced the enzyme solution, were included in each experiment. FFA were extracted and titrated by the method of Dole and Meinertz (23), using the extraction mixture containing 0.04 N H_2SO_4. Lipoprotein lipase activity was expressed as μmoles of FFA produced/hr per 100 μg of DNA.

Binding of Lipoprotein Lipase to Chylomicrons

This was studied by a modification of the method described by Anfinson and Quigley (24). 1.5 ml of an NH_4OH–NH_4Cl extract of acetone–ether powders from fat cells was mixed for 5 min at 4°C with 3.0 ml of assay medium prepared to contain final concentrations of defatted bovine serum albumin, 60 mg/ml; Tris–HCl, 154 mM; chylomicron triglyceride, 2.95 mg/ml; and NaCl, 7 mM at pH 8.2. The chylomicrons were floated by centrifugation at 33,000 *g* for 20 min at 4°C in the SW 39 rotor of a Beckman model L centrifuge, and separated from the infranatant solution first with a spatula, then with a needle and syringe. These chylomicrons were suspended in 4.5 ml of medium prepared to give final concentrations of defatted bovine serum albumin, 60 mg/ml; Tris–HCl, 154 mM; and NaCl, 7 mM at pH 8.2. Fresh chylomicrons were added to the original infranatant fraction to give a final concentration of 2.95 mg of triglyceride/ml. The lipoprotein lipase activity associated with the separated chylomicron and infranatant fractions was assayed as described above.

Binding of Heparin-^{35}S to Chylomicrons

1.5 ml of NH_4OH–NH_4Cl extracts of acetone powders of fat cells or stromal cells was mixed for 5 min at 4°C with 3.0 ml of assay medium prepared to contain final concentrations of defatted bovine serum albumin 60 mg/ml; Tris–HCl, 154 mM; chylomicrons, 2.95 mg of triglyceride/ml; NaCl, 7 mM; and heparin-^{35}S, 5 μg/ml (specific activity approximately 0.7 mc/g, Calbiochem, Los Angeles, Calif.). The chylomicrons were separated from the infranatant fluid after centrifugation and resuspended in 4.5 ml of medium prepared to give final concentrations of defatted bovine serum albumin, 60 mg/ml; Tris–HCl, 154 mM; and NaCl, 7 mM at pH 8.2. The chylomicrons were then washed twice by centrifugation, separation, and resuspension in the above medium, and finally suspended in 1.0 ml of the above medium. 1 mg of nonradioactive heparin (British Drug Houses, Toronto, Canada) was then added as carrier to each chylomicron suspension. 10 ml of acetone at 4°C was added to each suspension, and the mixtures were centrifuged at 4°C for 10 min at 1200 *g*. The resulting precipitates were washed twice with acetone at 4°C, dried overnight in vacuo, and dissolved in 1.0 ml of 1 M KOH. 0.5 ml of the KOH solution was taken for counting in a Nuclear-Chicago model 720 scintillation counter using a dioxane scintillator (25). The efficiency was 70%. Heparin-^{35}S standards were counted directly, and also carried through the acetone precipitation procedure. No radioactivity was present in the acetone washes and recovery of radioactivity was 94–98%. Protein was determined on the enzyme extracts by the method of Lowry, Rosebrough, Farr, and Randall (26).

RESULTS

Kinetic Studies with Lipoprotein Lipase Extracted from Free Fat Cells

The initial studies, performed with NH_4OH–NH_4Cl extracts of acetone–ether powders from fat cells, were designed to establish the kinetics of hydrolysis of dog chylomicron triglyceride by lipoprotein lipase, and to assess the effects of heparin (5 μg/ml) on the reaction. As indicated in Fig. 1, production of fatty acids was linear for 40 min whether or not heparin was present, although

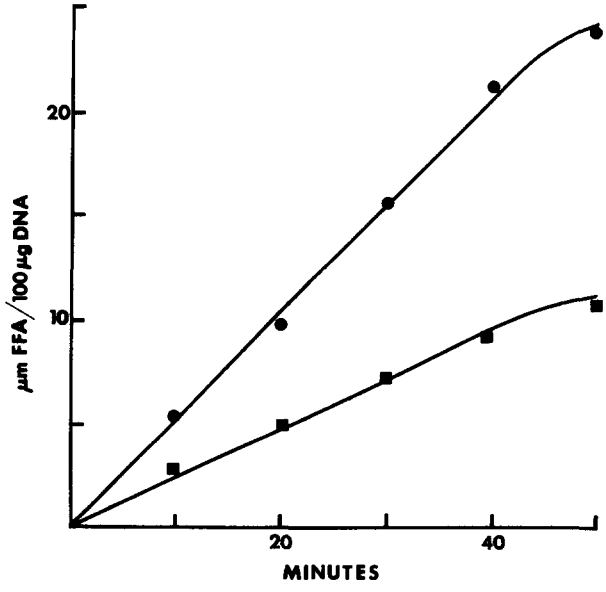

FIG. 1. Time course of hydrolysis of chylomicron triglyceride by lipoprotein lipase extracted from fat cells. ■, no heparin; ●, heparin, 5 μg/ml.

the rate of the reaction was increased by heparin. As shown in Fig. 2, the reaction rate was proportional to enzyme concentration and was increased in the presence of heparin. Augmentation of enzyme activity by heparin was consistently observed, although the increment varied from 20 to 110% with different batches of enzyme. The concentrations of heparin that were maximally effective were from 1 to 10 μg/ml, although an effect could be detected with as little as 0.05 μg/ml.

Cellular Location and Release of Lipoprotein Lipase

Table 1 compares the lipoprotein lipase activity of extracts of acetone–ether powders of fat cells and stromal cells prepared from the same adipose tissue pool. Two representative experiments are shown. The fat cell extracts were 15–50 times more active than the stromal cell extracts, which contained barely detectable amounts of lipoprotein lipase. The fat cell extracts were consistently stimulated by the presence of heparin during the assay, whereas the stromal cell extracts were not. The presence of heparin (15 μg/ml) during the extraction of stromal cell or fat cell acetone powders did not increase the amount of lipoprotein lipase in solution. In other experiments stromal cell extracts were combined with the fat cell extracts to determine whether the low lipase activity of the stroma was due to the presence of a known inhibitor, such as residual collagenase (15), or chymotrypsin derived from mast cells (27). However, it was found that extracts of stromal cells had no effect on the lipoprotein lipase activity of fat cell extracts.

When intact fat cells and stromal cells were incubated separately in KRB–albumin buffer, with and without heparin, lipolytic activity was consistently present in the media that had contained fat cells, and the presence of heparin during the period of fat cell incubation increased this activity (Table 2). Little lipolytic activity was present in the media that had contained stromal cells, whether or not heparin was present during the incubation.

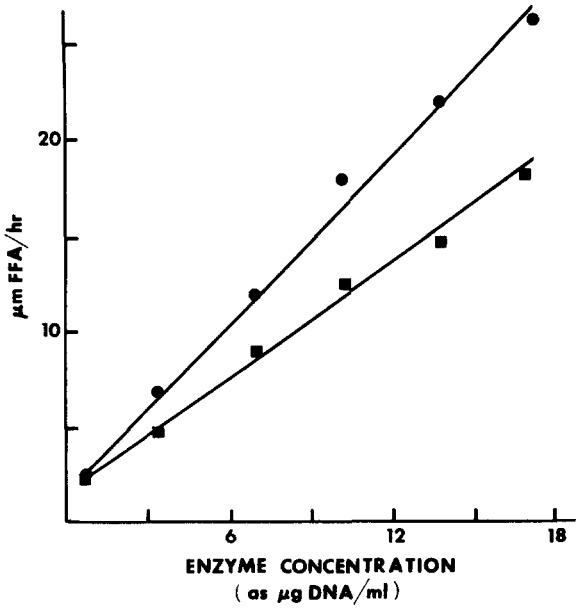

FIG. 2. Effect of lipoprotein lipase concentration on the rate of hydrolysis of chylomicron triglyceride. Lipoprotein lipase extracted from fat cells was diluted with 0.025 M NH_4OH–NH_4Cl buffer at pH 8.2, and assayed by measurement of FFA released from chylomicrons. Enzyme concentration is expressed as equivalent amounts of fat cell DNA (μg/ml). ■, no heparin; ●, heparin, 5 μg/ml.

These results suggest that heparin enhances the release of lipase from fat cells in a fashion analogous to its known effect on intact adipose tissue (1). However, proof of this supposition requires that the increase in enzyme activity in the medium be associated with a reduction of the enzyme activity remaining in the cells. This was studied by determining the activity of acetone–ether powders of fat cells that had been incubated in buffer, with and without heparin. Preliminary studies demonstrated one complicating feature; brief exposure of fat cells to heparin resulted in activation of the enzyme in the extracts of subsequently prepared acetone–ether dried powders, possibly because a small amount of

TABLE 1 LIPOPROTEIN LIPASE ACTIVITY OF NH_4OH–NH_4Cl EXTRACTS OF ACETONE–ETHER POWDERS FROM FAT CELLS AND STROMAL CELLS

Expt.	Assay	Lipoprotein Lipase Activity			
		Stromal Cells		Fat Cells	
		μmoles FFA/hr per total extract	*μmoles FFA/hr per 100 μg DNA*	*μmoles FFA/hr per total extract*	*μmoles FFA/hr per 100 μg DNA*
1	Control	3.7	1.9	46.5	45.1
	Heparin*	2.2	1.1	67.6	65.1
2	Control	1.4	0.5	63.6	69.5
	Heparin*	4.5	1.6	110.0	120.0

Lipoprotein lipase activity was assessed by the release of FFA from chylomicrons. Each experiment was done with the pooled lumbar and epididymal adipose tissues of four rats.
* 5 μg/ml of assay medium.

TABLE 2 RELEASE OF LIPOPROTEIN LIPASE FROM INCUBATED FAT CELLS AND STROMAL CELLS

	Lipoprotein Lipase Activity of Medium	
	Stromal Cells	Fat Cells
	μmoles FFA/hr per 100 μg DNA	
Incubation without heparin	0, 0.10	19.3, 19.7
Incubation with heparin	0, 0.05	25.3, 25.7

Intact fat cells and stromal cells were incubated for 10 min at 37°C in KRB–albumin buffer with glucose, 2 mg/ml; insulin, 10 mU/ml; and when added, heparin, 20 μg/ml. After removal of the cells, 1.0 ml of incubation medium was assayed for lipoprotein lipase activity. Heparin, 6.7 μg/ml, was added to all assays except those in which the incubation medium already contained heparin. Values are given for two experiments.

heparin was carried through into the extract. To obviate this difficulty, we added heparin in optimal concentration to all assay systems. The results of these studies are shown in Table 3. Incubation of fat cells with heparin increased the medium lipoprotein lipase activity and caused a corresponding reduction in the activity of fat cell acetone–ether powders. Total activity (medium plus cells) of the system in which intact cells had been exposed to heparin did not differ significantly from that of the system in which heparin had been omitted from the medium containing the fat cells.

These results demonstrate that heparin increased the release of lipoprotein lipase from fat cells, but they differ from the results of another recent study in which no reduction of the residual fat cell activity was observed (15). However, in the latter investigation no allowance was made for the activating effect of heparin in the medium on the residual fat cell enzyme.

Effect of Heparin on Lipoprotein Lipase Activity

In the following experiments the effect of heparin on the activity of extracted lipoprotein lipase was investigated. Initially the possibility was explored that heparin enhanced the activity of lipoprotein lipase by reducing the rate of decay of enzyme activity. Extracts of fat cell acetone–ether powders were incubated for various periods at 37°C without substrate, and in the presence and absence of heparin; substrate was then added and enzyme activity was assayed in the presence of optimal concentrations of heparin. As indicated in Fig. 3 *a*, no effect of heparin on the decay of lipoprotein lipase activity was observed.

Further proof that the effect of heparin on enhancement of lipoprotein lipase activity was due to activation of enzyme rather than to an effect on enzyme stability was obtained by an experiment in which a fat cell extract was added to the usual assay medium, and the mixture then divided into three portions, each of which

TABLE 3 RELEASE OF LIPOPROTEIN LIPASE FROM INCUBATED FAT CELLS

Incubation	Lipoprotein Lipase Activity		
	Medium	Fat Cells	Total
	μmoles FFA/hr per 100 μg DNA		
Control	20.1 ± 1.5	101.9 ± 3.0	122.0 ± 3.0
With heparin	25.0 ± 1.8*	92.6 ± 2.3*	117.3 ± 3.6†

Fat cells were incubated for 10 min at 37°C in KRB–albumin buffer containing glucose, 2 mg/ml; insulin, 10 mU/ml; and when added, heparin, 20 μg/ml. After separation of the cells, 1 ml of incubation medium was assayed for lipoprotein lipase activity. Acetone–ether powders of the cells were prepared, extracted, and assayed as described in the text. Heparin, 6.7 μg/ml, was added to all assays except those in which the incubation medium already contained heparin. Results are the mean ± SEM of 12 experiments.

* P ("t" test) for difference from control, <0.05.

† NS.

was incubated at 37°C with shaking. In one portion, fatty acid production over the first 10-min of incubation was determined. To one of the other two portions heparin was added at 10 min and in both these portions fatty acid production from 10 to 20 min was measured. The data are shown in Figure 3 *b*. In the absence of heparin, lipolysis was as rapid over the second 10-min incubation period as over the first, and the addition of

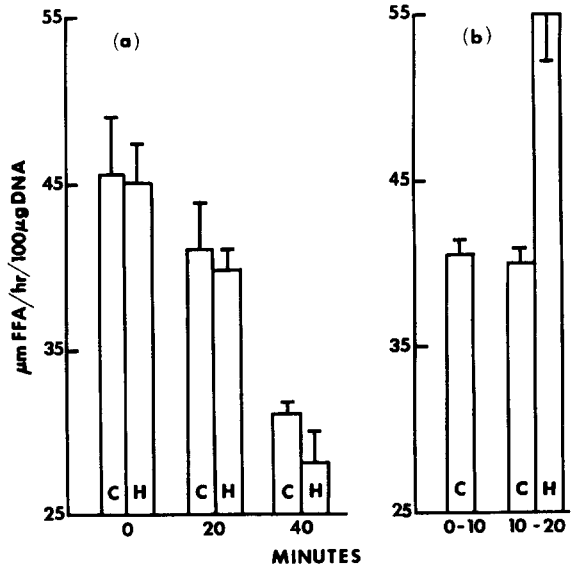

FIG. 3. (*a*) Effect of heparin on the decay of lipoprotein lipase activity. NH_4OH–NH_4Cl extracts of fat cell acetone–ether powders were incubated at 37°C in the presence and absence of heparin, 15 μg/ml, and then assayed for lipoprotein lipase activity at the times shown. All assays contained heparin, 5 μg/ml. *C*, control; *H*, incubation with heparin. The means of two experiments with the range are shown. (*b*) Stimulation of lipoprotein lipase activity by heparin. Extracts of fat cell acetone–ether powders were mixed with assay medium, divided into three portions, and incubated at 37°C. FFA production was measured from 0 to 10 min in one flask and from 10 to 20 min in the other two. Heparin, 5 μg/ml was added to one of the latter flasks at 10 min. *C*, control; *H*, heparin added at 10 min. The means of two experiments with the range are shown.

heparin at 10 min increased the activity considerably above that seen during the initial 10 min interval. These results would only have been obtained had heparin activated the enzyme and are incompatible with an effect of heparin on enzyme stability.

Effect of Heparin on Binding of Lipoprotein Lipase to Chylomicrons

To determine whether heparin stimulated lipoprotein lipase activity by influencing the binding of the enzyme to its substrate, fat cell extracts were briefly mixed with chylomicrons in the presence and absence of heparin; the chylomicrons were then separated by centrifugation and the lipolytic activities associated with the separated infranatant and chylomicron fractions were assayed. All the assay systems contained optimal concentrations of heparin. These data are shown in Table 4. Although the presence of heparin during the mixing period had no effect on the total lipoprotein lipase activity (infranatant plus chylomicron-bound), it markedly influenced the distribution of activity between the two fractions by increasing the proportion associated with the chylomicrons.

Further experiments were performed to determine whether heparin would activate the enzyme once the enzyme had been bound to chylomicrons. Enzyme and chylomicrons were mixed in the absence of heparin, and the chylomicron and infranatant fractions subsequently obtained were assayed with and without heparin. No activation of chylomicron-bound enzyme occurred, although the infranatant fraction was stimulated by heparin (Table 5).

Although these results suggest that heparin increases the binding of lipoprotein lipase to chylomicrons, similar results could have been obtained if the fat cell extracts had contained two forms of the lipase, one not requiring heparin for binding to chylomicrons and another binding to chylomicrons only in the presence of heparin. This possibility was next explored. Enzyme that had been bound to chylomicrons in the absence of heparin was precipitated with acetone and ether by the same method used to prepare acetone–ether powders of fat cells. The reextracted enzyme was then tested for affinity for chylomicrons in the presence and absence of heparin. Heparin increased the proportion of the total enzyme activity bound to the chylomicrons from 58 to 93%, and while it failed to stimulate the activity of enzyme bound to chylomicrons in the absence of heparin, it significantly enhanced the activity of the infranatant fraction. Thus heparin affected the reextracted enzyme in the same fashion as it did the enzyme in the original fat cell extract. These results are compatible with the presence of a single lipase in the original enzyme preparation.

TABLE 4 EFFECT OF HEPARIN ON THE BINDING OF LIPOPROTEIN LIPASE TO CHYLOMICRONS

	Total Lipoprotein Lipase Activity	Total Activity Bound to Chylomicrons
	μmoles FFA/hr per 100 μg DNA	%
Control	75.7	59.5 ± 3.7
Heparin	75.1	81.0 ± 3.4*

NH_4OH–NH_4Cl extracts of fat cell acetone–ether powders were mixed for 5 min at 4°C with assay medium containing chylomicrons, in the presence and absence of heparin, 5 μg/ml. The chylomicrons were separated by centrifugation and the lipoprotein lipase activities bound to chylomicrons and remaining unbound in the infranatant were assayed separately. All assays were done in the presence of heparin, 5 μg/ml. The results are the means ± SEM of five experiments. "Total Activity" is the sum of chylomicron bound and infranatant lipoprotein lipase activity.

* *P* ("*t*" test) for difference between means, <0.01.

TABLE 5 EFFECT OF HEPARIN ON CHYLOMICRON-BOUND AND INFRANATANT LIPOPROTEIN LIPASE ACTIVITY

Fraction	Stimulation by Heparin
	%
Chylomicron-bound	4.3 ± 2.9
Infranatant solution	24.6 ± 4.1

NH_4OH–NH_4Cl extracts of fat cell acetone–ether powders were mixed for 5 min at 4°C with assay medium containing chylomicrons. The chylomicrons were collected by centrifugation, then the chylomicron-bound and infranatant lipoprotein lipase activities were assayed separately. Heparin, 5 μg/ml, was added to one of each pair of assays. The results are the means ± SEM of three experiments.

Binding of Heparin-^{35}S to Chylomicrons

In studying the mechanism by which heparin enhanced the binding of lipoprotein lipase to chylomicrons, attempts were made to determine whether heparin formed part of the enzyme–chylomicron complex, and if so, whether the first step in this process entailed the binding of heparin to enzyme or to chylomicrons. In these experiments NH_4OH–NH_4Cl extracts of fat cell acetone powders were mixed with the usual assay medium, to which heparin-^{35}S was added. The chylomicrons were then separated from the mixture and the radioactivity associated with this fraction was determined. In the control experiments, 0.025 M NH_4OH–NH_4Cl replaced the cell extracts. The results are shown in Table 6. In the control experiments a small amount of heparin was recovered in the chylomicron fraction, but when active extracts of fat cell acetone powders were used, there was a marked increase in the heparin content of this fraction. This effect, however, was not specific for active enzyme preparations, as extracts of stromal cells which were de-

TABLE 6 BINDING OF HEPARIN-^{35}S TO CHYLOMICRONS

Extract	Lipoprotein Lipase Activity	Heparin-^{35}S Bound
	μmoles FFA/hr per 100 μg DNA	*μg*
None	0	0.12
Fat cell	70.6	1.03
Fat cell (Heated)	2.8	2.15
Stromal cell	1.4	1.01

NH_4OH–NH_4Cl extracts of acetone powders of fat cells and stromal cells were mixed with assay medium containing chylomicrons and heparin-^{35}S, 5 μg/ml. The chylomicrons were separated by centrifugation and washed, and the bound heparin-^{35}S was determined. In some cases fat cell extracts were heated to 70°C for 5 min to inactivate lipoprotein lipase prior to mixing with assay medium. All extracts contained 0.93 ± 0.13 mg of protein per ml. The results are the means of two experiments.

void of lipolytic activity produced similar effects, while the greatest increments in heparin binding were observed with fat cell extracts in which lipoprotein lipase had been inactivated by heating. Hence, binding of heparin to chylomicrons was increased in the presence of tissue extracts, but was not dependent on the presence of active lipase in these preparations.

Inhibition of Lipoprotein Lipase Activity by Protamine Sulfate and Sodium Chloride

Although sodium chloride and protamine sulfate are well recognized inhibitors of adipose tissue lipoprotein lipase, the mechanism of their action is not known. This subject was explored in the following experiments.

The action of these inhibitors in the complete assay system is shown in Table 7. With these concentrations, sodium chloride was a more effective inhibitor of the unstimulated enzyme than was protamine, but both agents completely abolished the stimulation of activity produced by heparin. Higher concentrations of protamine caused a greater inhibition of enzyme activity but these concentrations produced visible precipitation in the enzyme preparations.

To determine whether these compounds acted by irreversibly altering chylomicrons, the usual assay medium was incubated for 5 min at 4°C in the presence and absence of sodium chloride (0.5 M) or protamine sulfate (10 μg/ml), with 0.025 M NH_4OH–NH_4Cl replacing the enzyme preparation. The chylomicrons were separated from the assay medium by centrifugation and suspended in fresh albumin–Tris medium, after which fat cell extracts were added. Assays, done in the presence and absence of heparin, demonstrated that the prior exposure of chylomicrons to these inhibitors did not affect the subsequent rate of triglyceride hydrolysis.

In the next experiments the effects of protamine sulfate and sodium chloride on the binding of lipoprotein lipase

TABLE 7 EFFECTS OF PROTAMINE SULFATE AND SODIUM CHLORIDE ON LIPOPROTEIN LIPASE ACTIVITY

Additions to Assay	Lipoprotein Lipase Activity	
	Expt. 1	Expt. 2
	μmoles FFA/hr per 100 μg DNA	
None	40.5	39.0
Protamine	30.2	31.2
NaCl	8.3	8.5
Heparin	68.2	70.7
Heparin + protamine	40.0	43.2
Heparin + NaCl	5.4	4.4

The lipoprotein lipase activity of NH_4OH–NH_4Cl extracts of fat cell acetone powders were assayed in the presence and absence of protamine sulfate, 10 μg/ml, or sodium chloride, 0.5 M. Assays were done with and without heparin, 5 μg/ml.

to chylomicrons were determined. Extracts of fat cell acetone–ether powders were mixed with these inhibitors in the presence and absence of heparin, then the usual assay medium containing chylomicrons was added. The chylomicrons were separated by centrifugation and the lipoprotein lipase activity bound to the chylomicrons and that remaining in the infranatant were assayed separately without further additions of heparin or inhibitors. The results are shown in Table 8. In Experiment 1, when no heparin was present during the binding period, protamine and sodium chloride had little effect on the binding of lipoprotein lipase activity to chylomicrons. Total lipoprotein lipase activity, the sum of chylomicron-bound and infranatant activity, was reduced by sodium chloride because of marked inhibition of the activity of the infranatant fraction, unavoidably assayed in the presence of the inhibitor. There was no apparent effect of residual protamine on the activity of the infranatant fraction, probably because a slight degree of inhibition by protamine, comparable to that observed in the intact system (see Table 7), would not be detected with the small amount of enzyme activity present in the infranatant fraction.

When these experiments were repeated with heparin present during the binding period (Experiments 2 and 3, Table 8), the increase in binding of lipoprotein lipase to chylomicrons caused by heparin was completely inhibited by both protamine and sodium chloride.

In further experiments, shown in Table 9, the inhibitors were added to the assay of chylomicron-bound lipoprotein lipase. There was substantial inhibition of enzyme activity whether or not heparin had been present during the binding period. The degree of inhibition was comparable to that obtained when the inhibitors were added to a reaction mixture in which there had been no prior separation of bound and unbound enzyme.

It is evident from these results that the predominant effects of protamine sulfate and sodium chloride in the absence of heparin occurred after the formation of lipo-

TABLE 8 EFFECTS OF PROTAMINE SULFATE AND SODIUM CHLORIDE ON BINDING OF LIPOPROTEIN LIPASE TO CHYLOMICRONS

Expt. No.	Additions in Binding Period	Lipoprotein Lipase Activity			
		Series 1		Series 2	
		Total	Bound to Chylomicrons	Total	Bound to Chylomicrons
		μmoles FFA/hr per 100 μg DNA			
1	Control	28.2	21.6	30.2	19.3
	Protamine	27.7	20.2	30.8	18.8
	NaCl	19.3	17.7	18.1	16.3
2	Control	31.6	23.0	34.7	25.9
	Heparin	36.8	32.8	38.6	34.8
	Heparin + protamine	32.2	24.4	35.4	28.6
3	Control	28.1	20.3	29.3	20.3
	Heparin	30.8	27.3	32.1	29.2
	Heparin + NaCl	19.2	16.8	17.6	14.1

NH_4OH–NH_4Cl extracts of fat cell acetone powders were mixed with protamine sulfate, sodium chloride, and heparin as indicated. Assay medium containing chylomicrons was added, and the mixture incubated for 5 min at 4°C. The final concentrations of inhibitors were: protamine, 10 μg/ml; sodium chloride, 0.5 M; and heparin, 5 μg/ml. The mixtures were centrifuged, the chylomicrons were separated, and the lipoprotein lipase activity associated with the chylomicrons, and that remaining in the infranatants, were assayed separately. No additions of inhibitors or heparin were made to the assays. "Total activity" is the sum of chylomicron-bound and infranatant lipoprotein lipase activity.

TABLE 9 EFFECT OF PROTAMINE SULFATE AND SODIUM CHLORIDE ON CHYLOMICRON-BOUND LIPOPROTEIN LIPASE

Additions to Assay	Lipoprotein Lipase Activity			
	Bound Without Heparin		Bound With Heparin	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
	μmoles FFA/hr per 100 μg DNA			
None	19.4	12.8	28.4	27.8
Protamine	15.3	8.5	16.1	21.2
NaCl	3.6	2.4	3.0	3.0

NH_4OH–NH_4Cl extracts of fat cell acetone powders were mixed for 5 min at 4°C with assay medium containing chylomicrons, in the presence and absence of heparin, 5 μg/ml. The chylomicrons were separated after centrifugation, resuspended, and assayed for lipoprotein lipase activity. Protamine sulfate, 10 μg/ml, or sodium chloride, 0.5 M, were added to the assays as indicated.

protein lipase–chylomicron complexes, and that these inhibitors had little effect on the binding of the enzyme to chylomicrons.

DISCUSSION

Cellular Location and Release of Lipoprotein Lipase

The results of this investigation are in agreement with the observations of Rodbell (14) and Pokrajac et al. (15) in establishing that fat cells isolated from adipose tissue contain lipoprotein lipase and that the stromal–vascular components do not. They are at variance with the results of Ho et al. (16), who were unable to demonstrate lipoprotein lipase activity in free fat cells. However, these authors assayed lipoprotein lipase by measuring the increase in free fatty acid content of a mixture of fat cells and substrate. No correction was made for the extensive uptake and esterification of free acids by intact fat cells that undoubtedly occurred, hence fat cell lipoprotein lipase activity was probably significantly underestimated.

The evidence that lipoprotein lipase in adipose tissue is predominantly associated with the fat cells appears to be at variance with electron microscopic and histochemical evidence that suggests that chylomicron triglyceride hydrolysis, the reaction thought to be catalyzed by lipoprotein lipase, occurs at the luminal surface of the capillary endothelium (28, 29). It is important to determine if these discrepant pieces of information can be reconciled for, if they cannot, the entire concept of lipoprotein lipase regulation of triglyceride assimilation by adipose tissues will have to be reassessed. As it has been shown that collagenase inactivates lipoprotein lipase (15), it is possible that enzyme produced in and released from the fat cells, but active at the capillary endothelium or in the extracellular fluid, was totally inhibited during preparation of the free fat cells. It is also possible that the electron microscopic demonstration that chylomicrons are confined within capillaries in adipose tissue does not imply that hydrolysis occurs at this level. Chylomicrons could be dispersed into smaller units as they pass through the endothelial cells, with triglyceride hydrolysis occurring at the fat cell membrane. There is, of course, very adequate evidence that

hydrolysis of lipoprotein triglyceride occurs during assimilation of this substrate by isolated adipose tissue slices (4, 30).

Effect of Heparin, Protamine Sulfate, and Sodium Chloride on Lipoprotein Lipase Activity

The experiments reported here demonstrate that exogenous heparin stimulates the activity of lipoprotein lipase in solution by increasing the binding of enzyme to chylomicrons, and that heparin has no effect on either the stability of the extracted enzyme, or on enzyme activity after the formation of enzyme–chylomicron complexes has occurred. It is not yet clear whether exogenous heparin is included in the enzyme–chylomicron complex, but if, as postulated by Korn and Quigley (9), chicken adipose tissue lipoprotein lipase contains endogenous heparin which is involved in binding of the enzyme to chylomicrons, it is possible that exogenous heparin activates rat adipocyte lipoprotein lipase by forming additional binding sites on the enzyme molecule. Alternatively, heparin may stimulate enzyme activity by allosteric activation of existing binding sites.

In the absence of exogenous heparin, protamine sulfate and sodium chloride had little effect on the binding of lipoprotein lipase activity to chylomicrons, but inhibited enzyme activity after formation of the enzyme–chylomicron complex. It is evident that the effects of these inhibitors on lipoprotein lipase are reversible under the conditions used in these experiments, because exposure of the enzyme to protamine and sodium chloride during the binding period had little effect on the enzyme activity of lipoprotein lipase–chylomicron complexes that were subsequently separated from the inhibitor-containing infranatant. The results do not, however, exclude the possibility that protamine and sodium chloride act reversibly on the chylomicrons.

Further, these observations imply that if protamine and sodium chloride act on the enzyme and not on the substrate, they must act at catalytic rather than at binding sites on the enzyme molecule and the data are not compatible with the thesis that protamine acts on a heparinoid necessary for enzyme–substrate binding (6, 9). If these inhibitors in fact act on a heparin-like moiety of the enzyme, then this component must be involved in enzymatic activity after binding to chylomicrons has occurred. Conversely, if a heparin-like moiety is necessary for binding the enzyme to chylomicrons, these inhibitors do not affect the function of this component. Although protamine sulfate and sodium chloride block the stimulatory effect of exogenous heparin on binding of lipoprotein lipase activity to chylomicrons, this action is not necessarily similar to an effect on an endogenous heparinoid component, as the inhibitors may, as postulated by Korn and Quigley (9), prevent the binding of exogenous heparin to the enzyme molecule.

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