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Effect of Heparin on the Kinetics of Guinea Pig and Human Postheparin Lipase Activity (37858)

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(Introduced by R. H. Bradford)
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Lipoprotein lipase appears to be a key enzyme in mammalian lipid metabolism. It hydrolyzes the triglycerides contained in very-low-density lipoproteins (VLDL) and chylomicrons. Artificial triglyceride emulsions are hydrolyzed by postheparin lipase, but the reaction depends upon triglyceride substrate activation by specific lipoproteins, i.e., high-density lipoproteins (HDL) (1) and VLDL (2, 3). Evidence has been presented that specific HDL or VLDL peptides of relatively low molecular weights may activate the triglyceride substrate (4–6). The exact mechanism of activation remains unclear. Probably these peptides are an essential component of the protein matrix coating the VLDL and chylomicron surfaces, and they attach similarly to artificial triglyceride emulsions to make them susceptible to hydrolysis by postheparin lipase. Possibly, they cause enzyme–substrate attachment, as suggested by the hyperbolic increase of lipolysis when assay system triglyceride is held constant while HDL is increased (3).

Previous work from this laboratory has shown that the Michaelis-Menten kinetics of triglyceride hydrolysis by postheparin lipase can be modified by heparin *in vitro* (3) [heparin also acts as an essential lipoprotein lipase cofactor (7)]. Rat serum or rat HDL added in increasing amounts to the assay system for postheparin lipase from guinea pigs seems to increase the effective triglyceride substrate concentration (3). In the absence of heparin, an increase in the HDL concentration accelerates the reaction rate, which approaches a limiting velocity (V_{max}) and produces a hyperbolic curve conforming to Michaelis-Menten kinetics. In the presence of heparin, increasing the concentration of HDL produces an S-shaped curve and increases the V_{max}, suggesting that heparin may function as a specific ligand which acts as an allosteric modifier of postheparin lipase.

The experiments described in this report were designed to explore further these triglyceride substrate activation kinetics and to investigate their presence in man.

Experimental Methods. Guinea pig postheparin lipase. Male guinea pigs of the English variety, weighing 600–800 g, were fed a standard guinea pig laboratory diet until 16 hr before the experiment began, during which interval food was withheld. First, heparin from a single lot (Pularin, Evans Medical Ltd., Liverpool, England) was injected into the inferior vena cava of the animal (20 units/kg). Then, blood (15–20 ml) was collected from the abdominal aorta over the next 2–3 min. Pooled blood samples from 10 guinea pigs were allowed to clot at room temperature, and the serum was separated by centrifugation at 1000*g* for 10 min. Aliquots of each serum pool were placed in several test tubes before freezing so that individual aliquots could be used in a single experiment with no refreezing required. Previous work has shown that lipase activity in postheparin serum remains stable for several weeks (Whayne, T. F., Jr., unpublished data). Control samples of guinea pig serum were

also obtained by the procedure described above, except that heparin was not administered.

Human postheparin lipase. Blood was drawn from human subjects 10 min after the intravenous injection of heparin (10 units/kg). The blood was allowed to clot on ice, after which the serum was separated by centrifugation at 100*g* for 10 min. The serum was then ultracentrifuged at 40,000 rpm (1.51×10^8 g-min) for 24 hr using a 40.3 rotor (Beckman Instruments, Inc., Palo Alto, CA) in a Model L2-65B ultracentrifuge. The upper layer (i.e., the VLDL fraction) was then removed with the aid of a tube slicer. The infranatant fraction was mixed thoroughly, reloaded in the ultracentrifuge, and centrifuged 6 hr at 40,000 rpm (3.78×10^7 g-min). The heaviest material was then removed by cutting off the bottom 13 mm of each tube. Lipoprotein electrophoresis of the final top fraction, used as the human postheparin lipase source, was performed on agarose gel in barbital buffer, pH 8.6. No VLDL was present, and HDL contamination was slight. This procedure was utilized to remove as much as possible of the lipoprotein activators (VLDL and HDL) with minimum loss of lipase. Marked enzyme activity was found in the final top fraction. Plain human serum was also obtained by the above technique, but without heparin injection.

Lipoprotein fractionations. Lipoprotein fractions were prepared as described previously (3). HDL was prepared from rat serum; VLDL was prepared from normolipidemic human serum. One part of 0.25 *M* disodium ethylenediaminetetraacetate (EDTA) and 0.5 *M* phosphate buffer (pH 7.5), was added to 49 parts of serum to give a serum EDTA concentration of 0.005 *M* and a phosphate buffer concentration of 0.01 *M*. To inhibit bacterial growth, polymyxin B sulfate (Aerosporin, Burroughs Wellcome and Co., Research Triangle Park, NC) was added to give a serum concentration of 25 units/ml. Densities were adjusted with a NaCl–KBr solution (8). This solution was altered to contain 0.005 *M* EDTA and 0.01 *M* phosphate buffer, pH 7.5. VLDL was obtained from human serum by flotation in a 40.3 rotor at a solvent density of 1.006 for 18 hr at 40,000 rpm (1.13×10^8 g-min). HDL was obtained from rat serum after removal of VLDL and low-density lipoproteins by flotation at a density of 1.21 for 24 hr at 40,000 rpm (1.51×10^8 g-min). After flotation, the rat HDL or human VLDL was separated from the infranatant solution with a tube slicer (Beckman Instruments, Inc.). The contents of the tube above the blade were removed. The HDL and VLDL fractions were then washed for 24 hr by flotation at densities of 1.21 and 1.006, respectively. The HDL and VLDL were then dialyzed for 24 hr against 0.15 *M* NaCl containing 0.005 *M* EDTA, 0.01 *M* phosphate buffer (pH 7.5), and 25 units/ml of polymyxin B sulfate. This was stored at 4° until use. (Lipolytic activity remains stable in such lipase preparations for several weeks).

Postheparin lipase assay system. Lipase activity in guinea pig or human postheparin serum was assayed in duplicate. The assay system consisted of the following: 0.75 ml of triglyceride substrate (1 part 10% Intralipid, A. B. Vitrum, Stockholm, Sweden, 1 part 0.15 *M* NaCl, and 8 parts 0.154 *M* NaCl); 0.375 ml of 1.35 *M* Tris buffer, pH 8.6; 1.125 ml of a 15% (w/v) solution of bovine albumin (Sigma, St. Louis, MO), pH 8.6; 0.5 ml of 0.025 *M* NH_4OH adjusted to pH 8.6 with HCl; and 0.5 ml of guinea pig or human postheparin serum. In addition, one or more of the following test additives were used to make a volume of 0.45 ml test additives/incubation: 0 to 0.45 ml of 0.15 *M* NaCl, native rat HDL adjusted to a final concentration of 0 to 0.2 mg/ml, human VLDL adjusted to a final concentration of 0 to 0.4 mg/ml, and 0 to 0.05 ml of a heparin solution with a final heparin concentration ranging from 0.4 to 25.6 units/ml. The final calcium concentration in all assay systems was adjusted to 2.5 μmoles/ml. This calcium concentration (which is physiologic) was shown previously to be optimal for the lipolytic reaction of lipoprotein lipase (9, 10). Incuba-

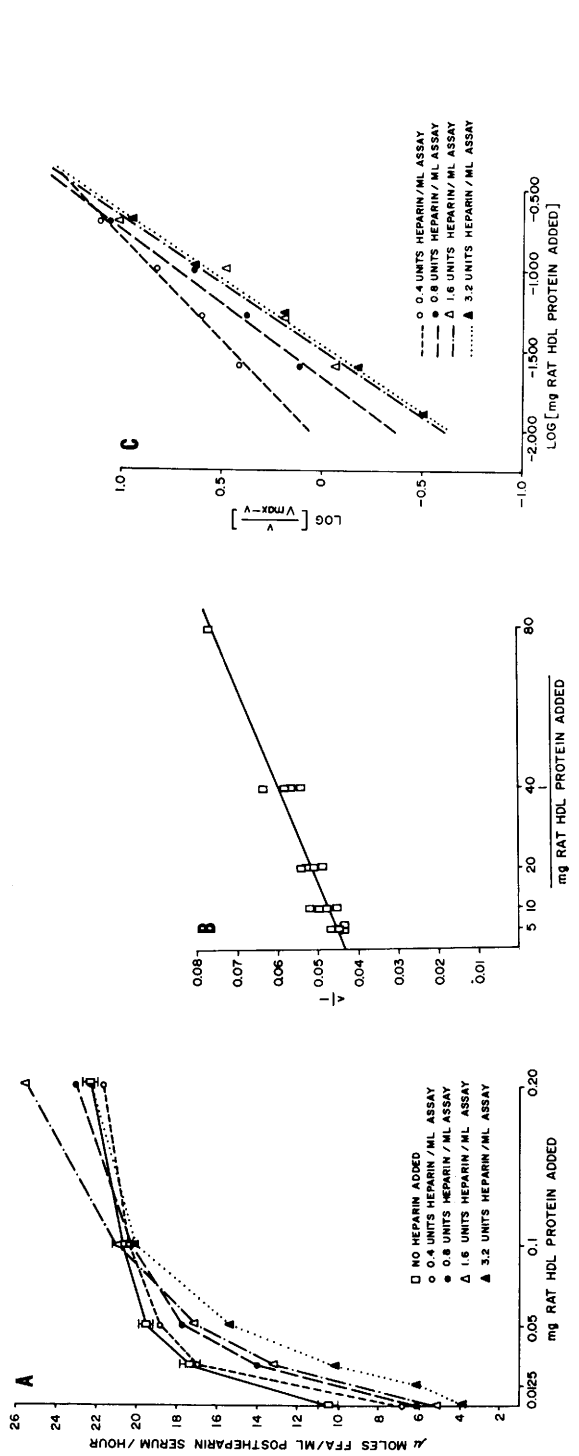

FIG. 1. Effect of rat HDL on postheparin lipase activity of guinea pig postheparin serum with increasing concentrations of *in vitro* heparin. (A) The effective triglyceride substrate saturation curves (obtained by increasing the triglyceride substrate activator, rat HDL) with *in vitro* heparin concentrations of 0 to 3.2 units/ml. Four curves, each with duplicate assays at each point, were obtained with no *in vitro* heparin. However, the curves were plotted using single points plus standard errors. (B) The Lineweaver-Burk plot obtained from the assays with no heparin. (C) The Hill plots for the assays with *in vitro* heparin.

tions were carried out at 37° for 1 hr. Free fatty acids were extracted by the method of Dole (11), as modified by Trout, Estes and Friedberg (12). These fatty acids were then titrated by a modification of the method of Salaman and Robinson (13). One unit of postheparin lipase activity is equivalent to 1.0 μmole of free fatty acid released/ml of postheparin serum/hr. Titrations were usually made on aliquots removed at 0 and 60 min from the assay system. The rate of release of free fatty acids was linear over a 60-min interval, tested with the addition of rat HDL or human VLDL to the guinea pig postheparin lipase assay system and with the addition of human VLDL to the human lipase assay system. Each point was assayed in duplicate with titrations in quadruplicate. For each heparin curve, a curve without was obtained.

Effect of rat HDL on lipase activity of guinea pig postheparin serum with increasing concentrations. Two pools of guinea pig postheparin serum were assayed as described (Figs. 1A and 2A). As the concentration of heparin *in vitro* was raised, the activation curve for postheparin lipase shifted to the right. The effective triglyceride substrate refers to the triglyceride emulsion (Intralipid) present in fixed concentration and activated by increasing amounts, of lipoprotein activator. Lineweaver-Burk plots were made of the data obtained in the absence of heparin (Figs. 1B and 2B). The points produced a linear regression ($P < 0.01$), demonstrating conformity to a Michaelis-Menten hyperbola (14). The points for each curve when heparin was added *in vitro* were plotted separately according to the linear form of the Hill equation (Figs. 1C and 2C) (14). The points for each Hill plot produced a linear regression ($P < 0.01$), indicating straight-line relationships and conformity to cooperative sigmoid kinetics.

Effect of human VLDL on lipase activity of guinea pig postheparin serum with increasing concentrations of heparin. The effect of *in vitro* heparin on the interaction of the postheparin serum lipase of guinea pigs with the triglyceride substrate activated by human VLDL was studied as described above. Another pool of guinea pig postheparin serum was used (Fig. 3A–C) and the curves responded in the same way.

Effect of human VLDL on lipase activity of human postheparin serum with increasing concentrations of heparin. Human postheparin lipase with most of the VLDL and HDL removed by centrifugation was used to study the effect of *in vitro* heparin on the interaction of the human enzyme with the triglyceride substrate activated by human VLDL (Fig. 4A–C). The results resembled those obtained with guinea pig postheparin serum.

Discussion. Whayne and Felts (3) previously showed that when either rat serum or HDL was added to the postheparin lipase assay system of guinea pig postheparin serum in increasing amounts, activity approached a limiting velocity and followed a hyperbolic curve typical of Michaelis–Menten kinetics. This suggested that rising concentrations of either whole serum or HDL increased the effective substrate concentration by converting the triglyceride emulsion to an activated state. With heparin present, both rat HDL and rat serum produced similar S-shaped curves and both raised the maximum rate of hydrolysis (V_{max}). These data were the first to suggest that heparin may function as a specific ligand acting as an allosteric modifier of lipoprotein lipase and altering the kinetics of interaction of lipase with the effective triglyceride substrate. More evidence is needed to prove whether heparin functions as an allosteric modifier of lipoprotein lipase and to show the significance of this metabolic phenomenon in man. Such proof would represent the first proved instance of "fine" metabolic control for a major enzyme of lipid metabolism. Ultimately, pure lipoprotein lipase, pure triglyceride substrate, and pure triglyceride substrate activation factors must be employed. This study has yielded additional data in support of this control function for heparin, and the phenomenon has been demonstrated with human postheparin lipase.

In their description of allosteric transi-

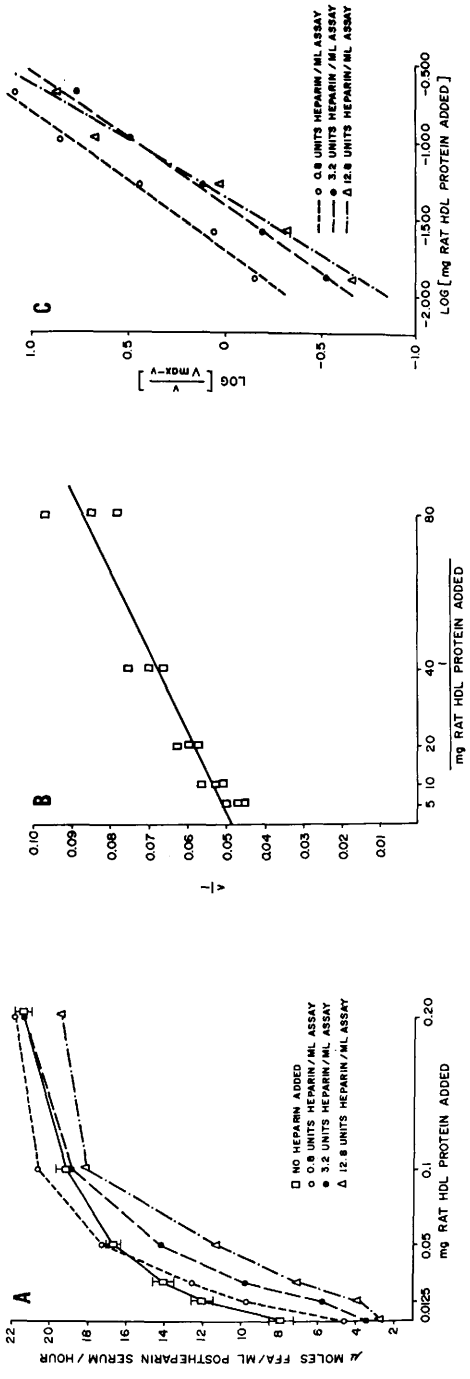

FIG. 2. Effect of rat HDL on postheparin lipase activity of guinea pig postheparin serum with increasing concentrations of *in vitro* heparin. (A) The effective saturation curves for triglyceride substrate (obtained by increasing the triglyceride substrate activator, rat HDL) with *in vitro* heparin concentrations of 0 to 12.8 units/ml. Three curves, each with duplicate assays at each point, were obtained with no *in vitro* heparin. However, the curves were plotted using single points plus standard errors. (B) The Lineweaver-Burk plot obtained from the assays with no heparin. (C) The Hill plots for the assays with *in vitro* heparin.

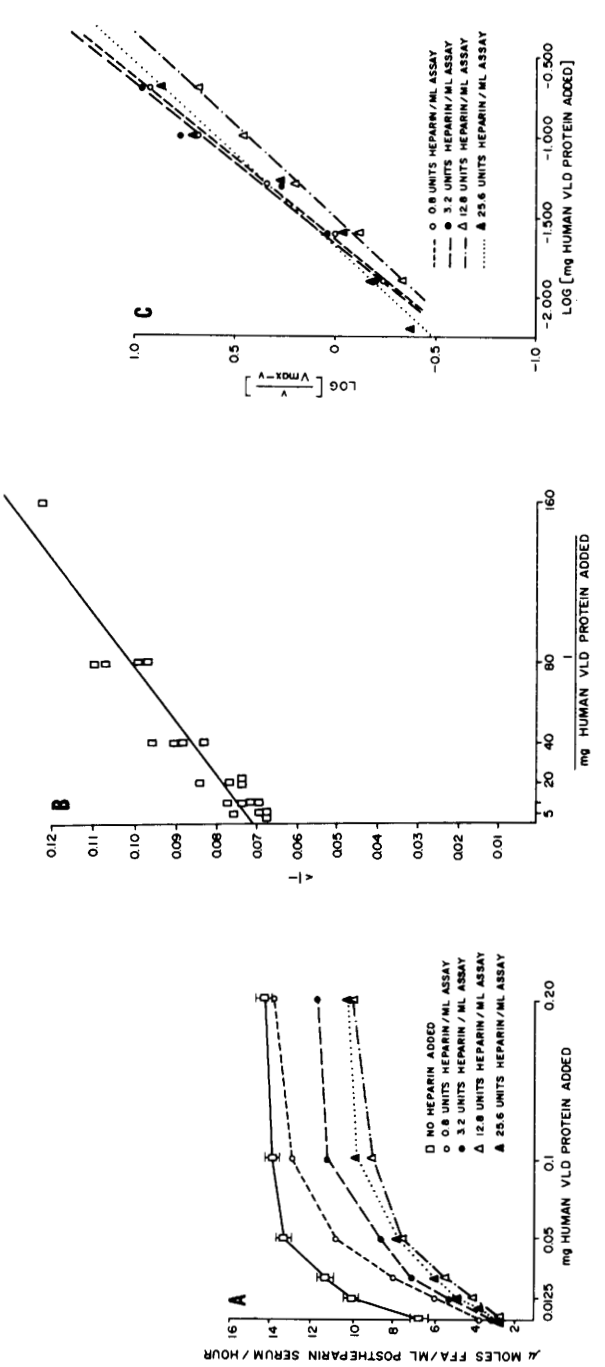

FIG. 3. Effect of human VLDL on postheparin lipase activity of guinea pig postheparin serum with increasing concentrations of *in vitro* heparin. (A) The effective triglyceride substrate saturation curves (obtained by increasing the triglyceride substrate activator, human VLDL) with *in vitro* heparin concentrations of 0 to 25.6 units/ml. Four curves, each with duplicate assays at each point were obtained with no *in vitro* heparin. However, the curves were plotted using single points plus standard errors. (B) The Lineweaver-Burk plots obtained from the assays with no heparin. (C) The Hill plots for the assays with *in vitro* heparin.

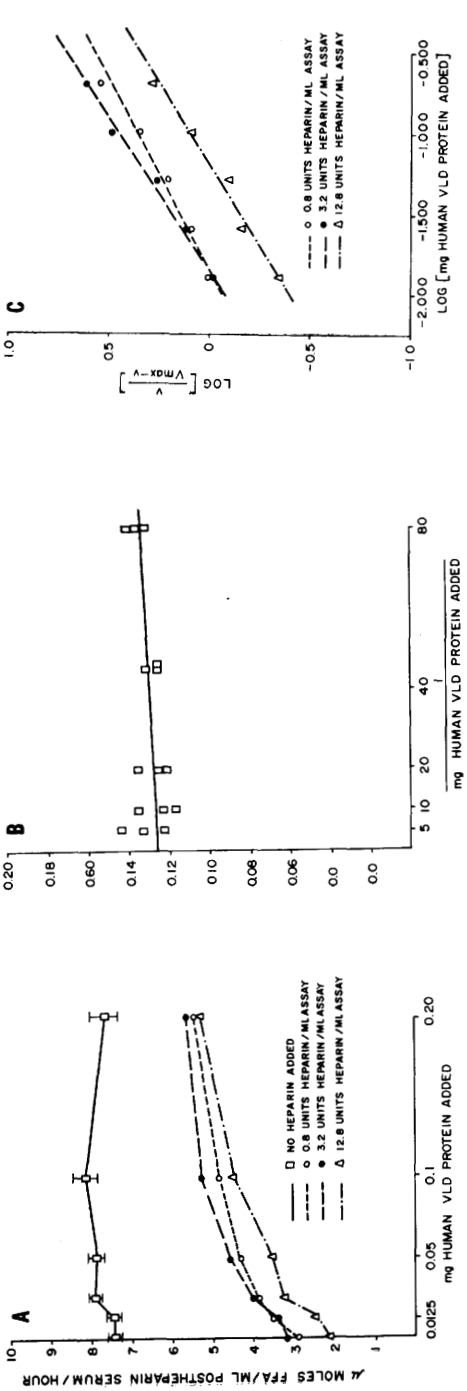

FIG. 4. Effect of human VLDL on postheparin lipase activity of human postheparin serum with increasing concentrations of *in vitro* heparin. (A) The effective triglyceride substrate saturation curves (obtained by increasing the triglyceride substrate activator, human VLDL) with *in vitro* heparin concentrations of 0 to 12.8 units/ml. Three curves, each with duplicate assays at each point, were plotted from single points plus standard errors. (B) The Lineweaver-Burk plot obtained from the assays with no heparin. (C) The Hill plots for the assays with *in vitro* heparin.

tions, Monod, Wyman and Changeux (15) describe a symmetry model for enzyme subunits and state that the cooperative effect of the allosteric ligand (heparin in this study on postheparin lipase) is more marked when the allosteric constant L is large. The value of this constant is 1.0 for a Michaelis-Menten hyperbola. As the value of this constant is increased by the ligand, the hyperbolic velocity–substrate concentration curve is altered to a sigmoid relationship and shifted to the right. As the concentration of the allosteric ligand is increased, the velocity–substrate saturation curve (or what may be called the substrate saturation curve) shifts progressively to the right. In such a case, the allosteric ligand is functioning as a heterotropic inhibitor to alter and increase the cooperative homotropic interactions of the substrate with its enzyme. According to Koshland (16), the symmetry model predicts Michaelis-Menten kinetics when all subunits are already in the active state. On the other hand, the sequential model of Koshland assumes that such kinetics will be observed when no changes in the subunit interactions are brought about by the binding ligand. If changes result, a range of conditions exist, from the simplest sequential model (with distortion of the neighboring subunits along with the subunit bearing the ligand) to a final model in which the distortion of one subunit is so tightly coupled to the remaining subunits that all change together. Which model is correct does not matter; the kinetic observations are identical. The substrate saturation curve shifts to the right as homotropic enzyme–substrate interactions are altered by the inhibitory ligand (heparin).

Heparin *in vitro* has effects on postheparin lipase consistent with these models of the conformational aspects of enzyme regulation. In each experimental condition studied, a higher concentration of heparin shifted the effective triglyceride substrate saturation curve to the right. Whayne and Felts (3) described a possible allosteric effect of heparin on guinea pig postheparin lipase. This enzyme source was used because of the marked susceptibility of its artificial triglyceride substrate to activation by rat HDL. Further evidence of a possible allosteric effect of heparin on lipoprotein lipase has been obtained in the same system in this study by the increasing cooperativity demonstrated as *in vitro* heparin is increased. Next, the kinetics were demonstrated using the same enzyme system but a different triglyceride substrate activator, human VLDL. Finally it has been possible to show the same alteration in the allosteric constant by increasing concentrations of heparin using human postheparin lipase, with human VLDL as triglyceride substrate activator. Therefore, similar metabolic control of postheparin lipase is present in man. This means that a physiologic body compound, heparin, may be able to control triglyceride hydrolysis by lipoprotein lipase so that, over a range sensitive to regulatory control, the rate of hydrolysis of the substrate is finely regulated by the concentration of the substrate.

In the assay system, the triglyceride concentration is maintained at a constant level. The HDL or VLDL activator is varied and changes the effective triglyceride substrate concentration. The observation must be made that with no added activator, postheparin lipase activity is present. The explanation for this is undoubtedly that some triglyceride substrate activator is present in the case of both guinea pig and human postheparin serum (see *Experimental Methods*). However, although enzyme activity is not zero with no added HDL or VLDL, this seems not to alter the basic kinetic observations. Eidels and Preiss (17) have reported on the regulation of citrate synthase in *Rhodopseudomonas capsulata.* In showing the effect of AMP concentration on the activity of citrate synthase in the presence of varying levels of DPNH, they discovered a sigmoidal shift of enzyme activity to the right as enzyme activity was increased by AMP. All of the sigmoid curves conformed to linear Hill plots, although enzyme activity was not near zero with no added AMP, showing an allosteric regulatory effect by DPNH on the AMP deinhibition of the enzyme.

In conclusion, the data reported in this study further suggest lipoprotein lipase as a regulatory enzyme and show the kinetics are obtainable with the human enzyme. Such regulatory control may be important to the function of this key enzyme of lipoprotein metabolism and to a possible relationship to atherosclerosis. This appears especially significant because metabolism of VLDL by lipoprotein lipase is the major source of low-density lipoproteins. Lipoprotein lipase is a major constituent lipolytic enzyme of postheparin lipase. The next obvious requirement is application of a purified liproprotein lipase preparation, resembling those already described (18, 19) to lipase kinetic studies along with purified apoprotein lipase substrate activators (4–6).

Summary. Lipoprotein lipase plays a major role in mammalian lipid metabolism. It is the key enzyme for hydrolysis of triglycerides contained in very-low-density lipoproteins (VLDL) and chylomicrons. Triglyceride hydrolysis by this enzyme requires that peptides be present on the chylomicron and VLDL surfaces. When artificial triglyceride emulsions are used in a postheparin lipase system, triglyceride hydrolysis depends upon emulsion activation by these same peptides. When triglyceride concentration is held constant in the assay system, an increase in high density lipoproteins or VLDL produces a Michaelis-Menten hyperbolic increase in lipase activity. Previous work has suggested that heparin added to the assay system modifies the kinetics of interaction of postheparin lipase with its triglyceride substrate. The result is a sigmoid substrate saturation curve, with heparin apparently acting as an allosteric modifier of the lipase. This study supports the role of heparin as an allosteric modifier of postheparin lipase. The postheparin lipase substrate saturation curves shift to the right as heparin concentration increases in the assay system. Moreover, the results indicate an allosteric effect of heparin on human postheparin lipase. Because of the importance of triglyceride hydrolysis in lipoprotein metabolism, abnormalities in its regulatory control could be important in the relationship of lipoproteins to atherosclerosis.

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