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Cofactor Activity of Protein Components of Human Very Low Density Lipoproteins in the Hydrolysis of Triglycerides by Lipoprotein Lipase from Different Sources†

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ABSTRACT: The protein component of very low density lipoproteins of human plasma with carboxyl-terminal glutamic acid was a potent activator of the hydrolysis of triglycerides in a lecithin-stabilized emulsion by highly purified lipoprotein lipase from human and rat post-heparin plasma and cows' milk, and by crude preparations of the enzyme from cows' milk and rat adipose tissue. The protein components with carboxyl-terminal serine and alanine also had slight but detectable activity with enzyme preparations from all sources, except that purified from milk. At high concentrations these two proteins inhibited enzyme activity. Heparin stimulated the activity of both impure and purified preparations of the lipase from cows' milk in the absence of cofactor protein and increased the sensitivity of the enzyme to stimulation by the protein component with carboxyl-terminal glutamic acid. These effects were not observed with purified lipase from rat post-heparin plasma. At high concentrations, the protein components with carboxyl-terminal serine and alanine inhibited the stimulatory activity of the component with carboxyl-terminal glutamic acid. Heparin abolished this effect in the case of the impure lipase from cows' milk, but not with the purified lipases from cows' milk and rat post-heparin plasma. Of three subfractions of the component with carboxyl-terminal alanine, one caused greater stimulation of lipoprotein lipase than the others. This difference could not be attributed to contamination of the former with the component with carboxyl-terminal glutamic acid.

Recent studies have shown that certain polypeptides common to triglyceride-rich lipoproteins (chylomicrons and VLDL)[1] and HDL of human plasma promote the hydrolysis of emulsified triglycerides by impure preparations of lipoprotein lipase in cows' milk protein and extracts of rat adipose tissue (Havel *et al.*, 1970; LaRosa *et al.*, 1970). One polypeptide, R-Glu, was particularly active in this process; a second polypeptide, R-Ala, also had some activity, but it varied among preparations. With enzyme in cows' milk, large amounts of R-Ala were inhibitory and, in addition, they inhibited activation by R-Glu (Havel *et al.*, 1970). Subsequently, evidence was presented to suggest that activity with the milk enzyme of preparations of R-Ala purified by ion-exchange chromatography results from contamination with R-Glu (Brown and Baginsky, 1972). In addition, purified preparations of lipase from post-heparin plasma of dog, rat and man were reported to be activated chiefly by a third polypeptide, R-Ser, and to a lesser extent by R-Glu, while R-Ala was inactive (Ganesan *et al.*, 1971).

We now report a comparative study of the effect of these three polypeptides upon the hydrolysis of triglycerides emulsified with lecithin by purified lipoprotein lipase from human and rat post-heparin plasma, the enzyme from rat adipose tissue and by both the crude and purified enzyme from cows' milk.

Experimental Procedure

Preparation of Lipoprotein Lipase. Lipoprotein lipase was purified from the post-heparin plasma of male human donors or male Sprague–Dawley rats by published techniques (Fielding, 1969, 1970; Nillson-Ehle *et al.*, 1971) to provide an electrophoretically homogeneous product that was free of detectable amounts of lipoprotein polypeptides (Fielding, 1969;

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‡ An Established Investigator of the American Heart Association.
§ Postdoctoral trainee of the U. S. Public Health Service (HE 5251).

[1] Abbreviations used are: VLDL, very low density lipoproteins; HDL, high density lipoprotein; R-Glu, R-Ala, R-Ser, apolipoprotein species with carboxyl-terminal glutamic acid, alanine, and serine, respectively; R_1-Ala, R_2-Ala, R_3-Ala, species of R-Ala with zero, one, and two residues of sialic acid, respectively.

Nillson-Ehle *et al.*, 1971). Lipoprotein lipase in cows' milk protein was lyophilized (Bier and Havel, 1970) and used directly ("crude milk lipase") or purified from fresh skimmed milk (Egelrud and Olivecrona, 1972) with additional purification by gel chromatography on Sephadex G-100 in 1.5 M NaCl–0.005 M sodium Veronal at pH 7.4. Both milk preparations were stored at −20°. Rat epididymal adipose tissue was extracted in acetone (Korn, 1955) and lipoprotein lipase in the soluble product of the partially delipidated tissue was dissolved in 0.05 M ammonia buffer (pH 8.3) before use. Crude and purified milk lipase preparations were also dissolved in the same ammonia buffer. Purified enzymes from human and rat post-heparin plasma, stabilized with 0.5 mM potassium linolenate (Fielding, 1968) in ammonia buffer, were used on the day of preparation.

Preparation of Lipoprotein Polypeptides. Very low density lipoprotein obtained by ultracentrifugal flotation (Havel *et al.*, 1955) from the serum of human donors with primary endogenous hyperlipemia was partially delipidated in diethyl ether (Shore and Shore, 1969). The sample was then concentrated by ultrafiltration and by solid dialysis against Dextran-20 (Pharmacia Fine Chemicals, Piscataway, N. J.). Immediately prior to gel filtration, it was dialyzed for 2 hr against 7 M urea in 0.05 M Tris–HCl (pH 8.0). The lower molecular weight components were separated from the β proteins of higher molecular weight on a column of Sephadex G-150, 1.9 × 60 cm at 6–8° in the same buffer using freshly prepared Ultra Pure urea (Schwarz BioResearch, Orangeburg, N. J.). The lower molecular weight fraction was dialyzed for 2 hr and concentrated by ultrafiltration and then dialyzed further against cold, degassed water. The polypeptides in this fraction were then separated by chromatography on DEAE-cellulose in 8 M urea as described previously (Shore and Shore, 1969). Amino acid analysis was used to assess purity and protein concentration of preparations taken for assay in the lipase system. Polyacrylamide gel electrophoresis at pH 8.6 in 10% gels containing 8 M urea was also carried out for characterization of the protein preparations. The polypeptide samples corresponded, within limits of the precision of the amino acid analyzer (1–3%), to results obtained previously (Shore and Shore, 1972).

Assay of Lipoprotein Lipase. To facilitate comparison, assays of each preparation were carried out with single lots of the following reagents: human serum albumin (Cutter Laboratories, Oakland, Calif.), Intralipid, 10% triglyceride–lecithin emulsion (Vitrum AB, Stockholm, Sweden), and sodium heparin (100 IU/mg, Nutritional Biochemicals Corp., Cleveland, Ohio).

For assay of enzyme activity, a buffer–substrate mixture was prepared containing, per 100 ml: 50 ml of modified Krebs–Henseleit buffer (0.141 M NaCl–0.0057 M KCl–0.0030 M $CaCl_2$–0.0014 M $MgSO_4$–0.0014 M KH_2PO_4), 5 ml of Tris-HCl (1.0 M, pH 8.5), 5.3 g of albumin, and 2.4 ml of Intralipid. For each assay, 0.5 ml of water containing the requisite amount of polypeptide to be tested was added to 3.0 ml of buffer–substrate mixture and incubated for 30 min at 37° in a Dubnoff shaker. Ammonia buffer (0.5 ml) containing approximately 5 units of lipoprotein lipase was added (one unit produces 1 μmol of fatty acid/hr when incubated with saturating amounts of emulsified triglyceride under optimal conditions with human serum to supply protein cofactor). A single sample of 0.5 ml was extracted immediately and duplicate samples were extracted at 15, 30, and 60 min in 5 ml of Dole's mixture (Dole, 1956). After separation of the phases, free fatty acids were measured by single-phase titration (Salaman and Robinson, 1961; Kelley, 1965). Enzyme activity was calculated in units (μmol of fatty acid released per hr). In a few cases where the enzyme was fully activated, the rate fell during the second half-hour, so that activity was evaluated from the rate during the first 30 min. Under the conditions described, hydrolase activity against Intralipid–triglycerides in the absence of added cofactor protein, with different preparations of the enzymes, was 5–25% of maximal activity obtained in the presence of serum. Basal activity with other preparations of crude (Brown and Baginsky, 1972) and purified (Egelrud and Olivecrona, 1972) milk lipase is generally less than 15% of maximal stimulated activity, 5–10% with lipase from rat adipose tissue (LaRosa *et al.*, 1970), and 3–5% with purified rat and human post-heparin plasma enzyme (Fielding, 1969, 1970). To take into account minor changes in the activity of enzyme preparations from day to day, values were related to those obtained with the addition of 0.125 ml of serum or 5 μg of R-Glu per ml of medium.

Results

The human VLDL protein R-Glu was by far the most potent activator of hydrolysis of triglycerides by preparations of purified lipoprotein lipase from rat and human post-heparin plasma and cows' milk as well as by the unpurified enzyme of cows' milk and rat adipose tissue (Figure 1). Stimulation was detectable with R-Glu in concentrations as low as 0.01–0.02 μg/ml of incubation medium and it was maximal in the range 1–5 μg of R-Glu/ml (triglyceride: protein = 360–1800:1, w/w). The VLDL proteins R_2-Ala and R_3-Ala also significantly activated the lipases at concentrations as low as 0.1 μg/ml. The VLDL protein R-Ser stimulated lipolysis similarly to R-Ala with all lipases except the purified enzyme from cows' milk, which had a substantially higher basal activity than the other preparations. However, the stimulation by R-Ser and R-Ala was very much less than that produced by R-Glu and it never exceeded 40% of maximal R-Glu stimulated activity. Lipase activity tended to be lower at the highest levels of polypeptide tested in the assay system (20–25 μg/ml or protein: triglyceride = 1:72–90) than at lower levels. This was the case for all the polypeptides tested, and at high levels of R-Ala and R-Ser lipolysis was often less than with no added protein.

Heparin (1 μg/ml) increased hydrolysis of the emulsified triglyceride by both the purified and crude milk preparations in the absence of added VLDL proteins (Figure 2). Heparin increased this basal lipolysis as much as tenfold with some preparations of the crude milk lipase. With the purified milk enzyme, basal lipolysis was shown to be linear with time both in the absence and presence of heparin, so that heparin appeared to activate the enzyme rather than to stabilize it. When heparin was present, the increment in activity produced by R-Glu with the milk lipases was the same as that observed in the absence of heparin, so that maximal lipolysis equalled the sum of that produced separately by heparin and R-Glu. Heparin also increased the sensitivity of both purified and crude milk enzyme preparations to activation by R-Glu. As shown in Figure 2, smaller amounts of R-Glu were required to achieve half-maximal stimulation of lipolysis when heparin was present. Heparin also substantially potentiated the stimulation of lipolysis by R_2-Ala and R_3-Ala but not by R-Ser in the crude milk lipase system (Figure 1). Heparin did not affect the basal- or protein- (R-Glu, R-Ala, or R-Ser) stimulated lipolytic activity of the enzyme purified from rat plasma.

In the crude milk system, preparations of R_1-Ala invariably gave greater stimulation of lipolysis than R_2-Ala and R_3-Ala

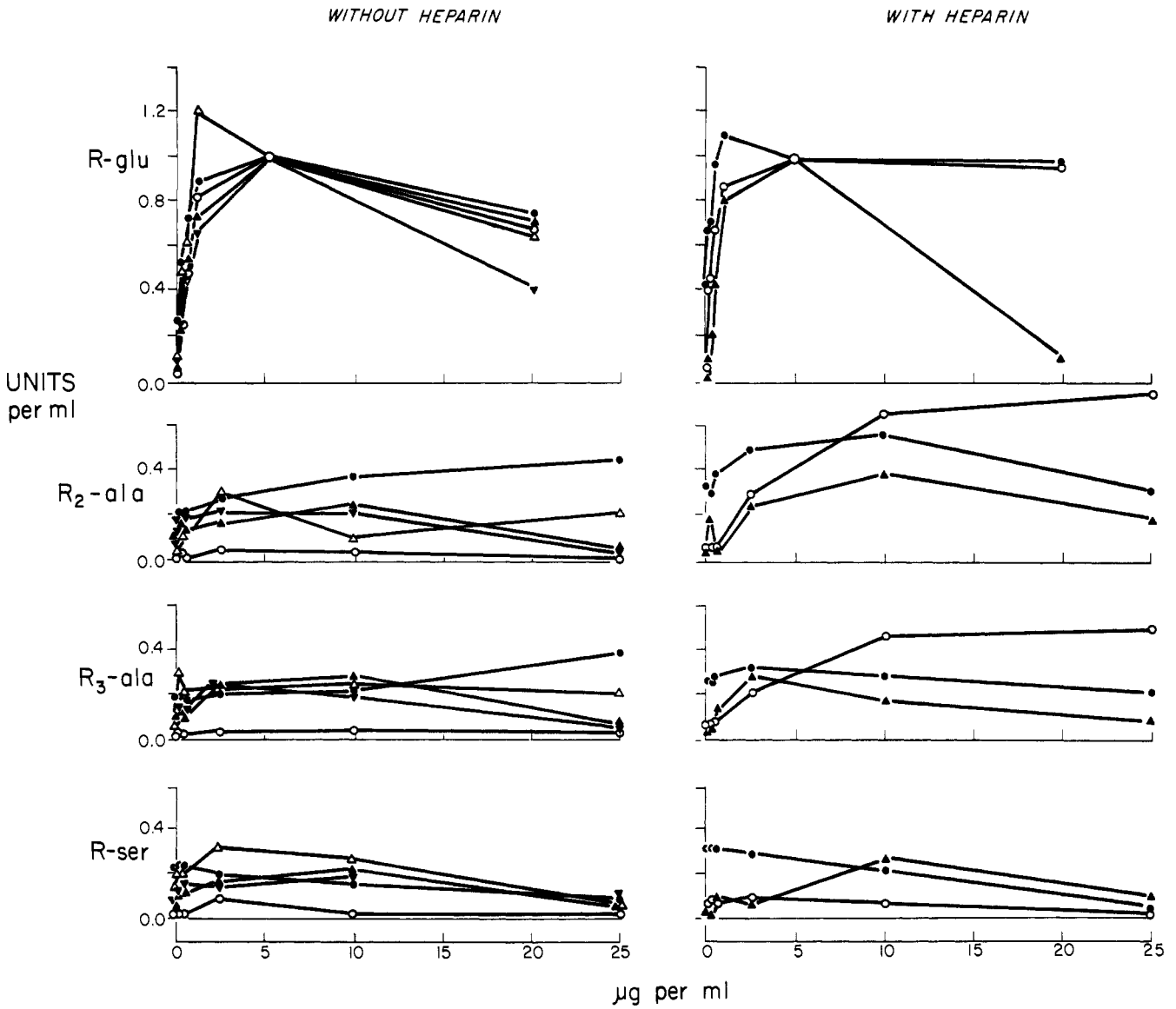

FIGURE 1: The effect of four polypeptide components of human VLDL upon the lipolytic activity of five different preparations of lipoprotein lipase: (○) crude cows' milk lipase; (●) purified cows' milk lipase; (△) purified lipase from human post-heparin plasma; (▲) purified enzyme from rat post-heparin plasma; (▼) lipase from rat adipose tissue. To facilitate comparison all results have been normalized to that observed with R-Glu at 5 μg/ml of medium which has been assigned a value of 1.0. In the studies shown on the right side of the figure, heparin was present at a concentration of 1 μg/ml of incubation medium.

(Figure 3). The elution pattern of R_2-Ala as it emerges from columns of DEAE-cellulose sometimes suggests the presence of a component with a slightly lower elution volume. In two such samples, the early portion of the R_2-Ala peak was collected separately. This fraction had more activity than the main part of the R_2-Ala peak but less than that of R_1-Ala.

The cofactor activity of the R-Ala's was potentiated by heparin with three preparations of crude milk enzyme and with two preparations of the R-Ala subfractions. Higher concentrations of R-Ala subfractions and R-Ser invariably inhibited enzyme activity. Furthermore, these polypeptides generally inhibited activation of the enzyme by R-Glu. This phenomenon was studied further with the purified enzymes from cows' milk and rat post-heparin plasma (Figure 4). In the presence of sufficient R-Glu to produce approximately half-maximal activation (0.5 μg/ml of medium), R_2-Ala and R-Ser progressively inhibited enzyme activity in concentrations exceeding 10 μg/ml except for R-Ser with enzyme from rat plasma. In the presence of heparin, inhibition was similar for both purified enzymes, but no inhibition was observed with crude milk enzyme. This effect of heparin was also observed with other unpurified preparations of the milk lipase. The extent of inhibition in the absence of heparin varied with concentration of R-Glu. Thus, with a constant ratio of R_2-Ala to R-Glu of 5:1, inhibition was slight until the concentration of R-Glu exceeded 2 μg/ml (Figure 3). At higher levels, the extent of inhibition increased to the extent that activity fell below the basal level. Inhibition by large amounts of R-Ala could be overcome by increasing the concentration of R-Glu (Figure 5).

Discussion

These studies indicate that hydrolysis of triglycerides by lipoprotein lipase is strongly activated by the human VLDL protein R-Glu irrespective of the enzyme source—rat or

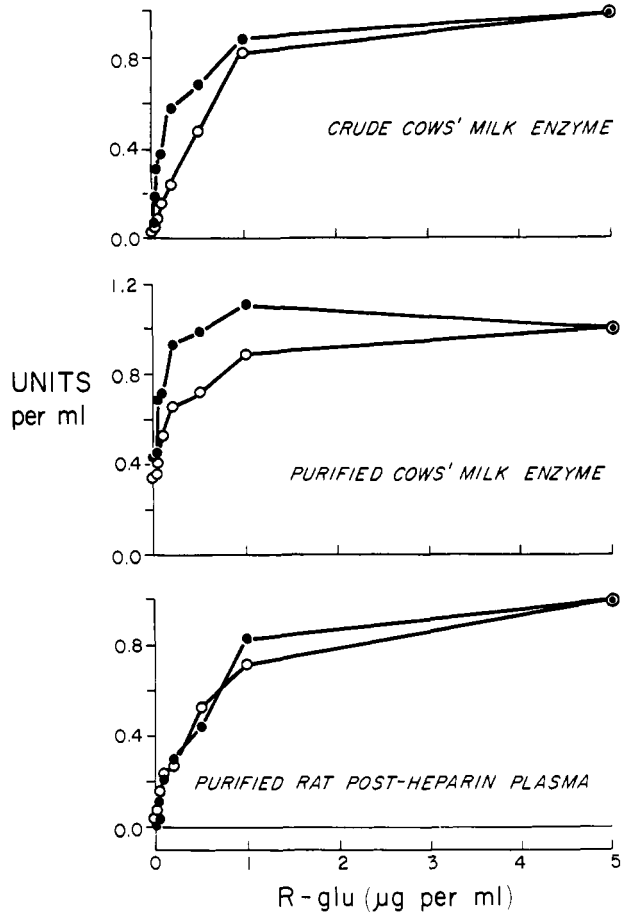

FIGURE 2: The effect of heparin upon R-Glu-stimulated activity of lipoprotein lipase from three sources. In the experiments indicated by the filled circles, heparin was present at a concentration of 1 μg/ml of incubation medium. Values are calculated as in Figure 1.

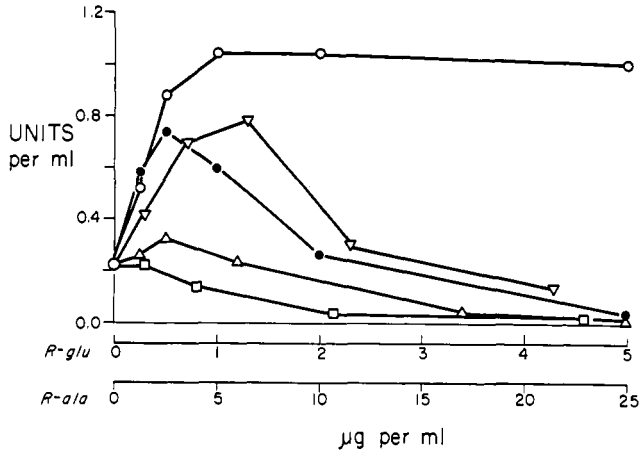

FIGURE 3: The effect of R-Glu and three different subfractions of R-Ala isolated from VLDL of a single subject on the activity of lipoprotein lipase in the protein of cows' milk: (○) R-Glu; (▽) R_1-Ala; (△) R_2-Ala; (□) R_3-Ala; (●) 5:1 mixture of R_2-Ala and R-Glu.

human post-heparin plasma, rat adipose tissue, or cows' milk. Stimulation of lipolysis by the VLDL proteins R-Ser and the R-Ala's was manifestly less in every case, contrary to the report of Ganesan *et al.* (1971) that R-Ser is the major activator for purified post-heparin plasma lipase. Post-heparin plasma contains triglyceride hydrolases other than lipoprotein lipase (Hamilton, 1964; LaRosa *et al.*, 1972) and recent studies indicate that significant amounts of a hepatic lipase that differs from lipoprotein lipase in sensitivity to NaCl, protamine, and diethyl *p*-nitrophenyl phosphate (Fielding, 1972) may be present. Lipase activity with the properties of this hepatic enzyme cannot be detected in the purified plasma lipoprotein lipases used in the present experiments. The hepatic enzyme would not be expected to participate in the initial extrahepatic hydrolysis that accounts for removal of 80–90% of chylomicron triglycerides from the blood (Bergman *et al.*, 1971).

Although R-Glu was by far the most potent of the three polypeptides tested as activator of lipoprotein lipase in this study, both R-Ser and/or the major subspecies of R-Ala had detectable activity with the enzyme from all four sources. It is noteworthy, however, that this activation did not generally exceed the maximal activity observed in the absence of protein cofactor (Figure 1). These consistently low activities may represent a generalized surfactant effect and their functional significance is obscure. By contrast, the cofactor effect of R-Glu provided a rate of lipolysis similar to that seen when Intralipid is activated with whole plasma or when intact native chylomicrons[2] or very low density lipoproteins (Fielding, 1972) are used as substrate for purified lipoprotein lipase.

The differences in cofactor specificity between the enzymes purified from post-heparin plasma in the present study and those reported by Ganesan *et al.* (1971) may be due to the purification of different enzymes. The method of purification used by Ganesan *et al.* leads us to suspect that the enzyme they prepared may not be lipoprotein lipase as ordinarily defined (Robinson, 1970). Their method for purification of lipase from post-heparin plasma (Ganesan *et al.*, 1971) was described as a modification of that of Fielding (1969), but it differs from it in the use of a mixture of ethanol and diethyl ether to extract lipid and deoxycholate from the partially purified enzyme. This solvent mixture almost completely inactivates the enzyme prepared by the original method. In some instances, triglyceride–gum arabic (which is a poor substrate for purified lipoprotein lipase (Fielding, 1972)) was substituted for triglyceride–lecithin and the final step of purification involved electrophoresis on polyacrylamide gel, sometimes in the presence of 8 M urea. Electrophoresis under these conditions completely inactivates plasma lipoprotein lipase as defined in this study. Unfortunately, the results of Ganesan *et al.* do not include data on the percentage recovery of lipase from plasma by this modified method. The lipoprotein lipase prepared by the original method, used in the present research, contained no lipoprotein polypeptides detectable by electrophoresis and had no greater basal lipase activity than the enzymes from milk or adipose tissue. The nature of the substrate used in assaying triglyceride hydrolase activity by Ganesan *et al.* (triglyceride–gum arabic) could also influence the interaction of the enzyme with the complex of substrate and protein cofactor. Intralipid appears to be an appropriate substitute for the natural substrate in *in vitro* studies of lipoprotein lipase. Like chylomicrons, it is stabilized principally by a monolayer of lecithin and readily takes up low molecular weight polypeptides from plasma or HDL (Havel *et al.*, 1973) and the kinetics of elimination of its constituent triglycerides from the blood are similar (Carlson and Hallberg, 1963).

The greater activity of the minor component, R_1-Ala, compared to that obtained with the two R-Ala components, at

[2] C. J. Fielding, in preparation.

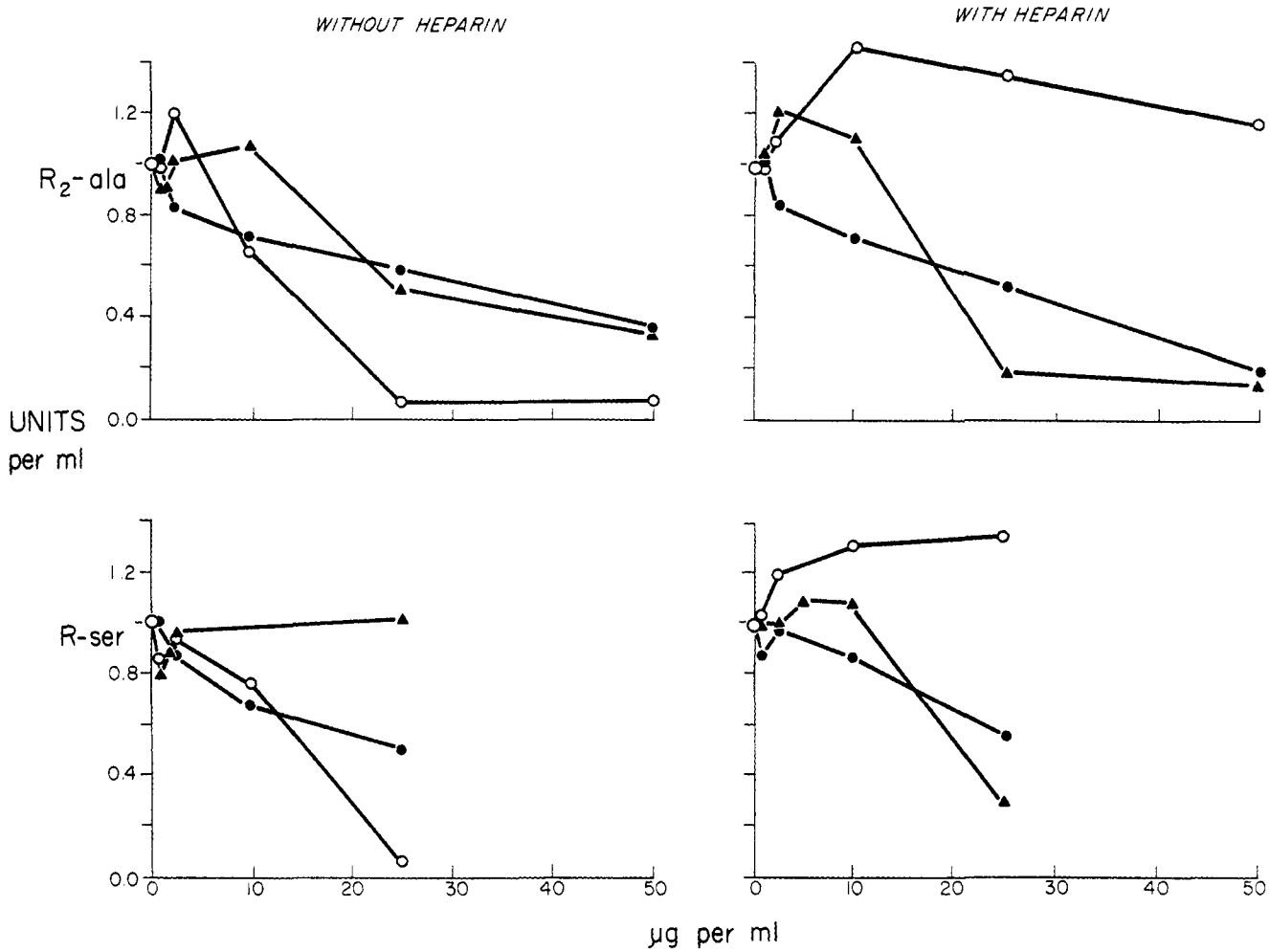

FIGURE 4: Effect of R_2-Ala and R-Ser upon R-Glu-stimulated activity of three preparations of lipoprotein lipase. In all cases, R-Glu was present at a concentration of 0.5 μg/ml of incubation medium and, in the studies shown on the right side of the figure, heparin was present at a concentration of 1 μg/ml: (○) crude cows' milk lipase; (●) purified cows' milk lipase; (▲) purified lipase from rat post-heparin plasma. The activity observed with R-Glu alone has been assigned a value of 1.0.

first sight seems to be consistent with the suggestion that the cofactor property of this fraction results from contamination with R-Glu. This possibility is supported by the presence of a variable amount of isoleucine in R_1-Ala since R-Glu contains 12 mol of isoleucine/10,000 g while it is absent from R_2-Ala and R_3-Ala. However, comparison of critical amino acid ratios, such as tyrosine:phenylalanine, of various preparations of R_1-Ala with those of R_2-Ala or R_3-Ala and R-Glu indicates that contamination with R-Glu cannot account for the variable amount of isoleucine in R_1-Ala. For the preparation of R_1-Ala used in the experiment shown in Figure 3, the observed ratios of these amino acids indicate that a mixture of R-Glu with material with the composition of R_2-Ala or R_3-Ala could not exceed 3%. Since the activity of this preparation of R_1-Ala at low concentrations was only slightly less than that of a 1:5 mixture of R-Glu and R_2-Ala, the cofactor property of R_1-Ala evidently cannot be explained solely by contamination with R-Glu. The chemical basis of this anomalous behavior of R_1-Ala cannot yet be assessed. It could be related to lack of sialic acid, which is present in R_2-Ala and R_3-Ala (Brown *et al.*, 1970), or it could reflect microheterogeneity of amino acid composition. However, the variable content of isoleucine in R_1-Ala and the presence of material with substantial cofactor activity that emerges from DEAE-cellulose columns just before the main peak of R_2-Ala in some preparations of apo-VLDL suggest that an as yet uncharacterized polypeptide, distinct from R-Glu species, usually present in very small amounts, may account for the apparent cofactor activity of R_1-Ala. Given the small amount of protein recovered in R_1-Ala, such activity can account for only a very small fraction of that contributed by R-Glu in native VLDL.

The present results extend earlier observations of the inhibition of lipoprotein lipase by large amounts of R-Ala (Havel *et al.*, 1970; Brown and Baginsky, 1972) and show that this phenomenon is shared by R-Ser and is common to the purified enzyme from several sources. In addition, both R-Ser and R-Ala inhibited activation of purified enzymes from rat post-heparin plasma and cows' milk. This effect was not simply related to the ratio of R-Glu to R-Ala or R-Ser but rather was also a function of the absolute concentration of inhibitory polypeptide. Furthermore, inhibition of a constant amount of R-Glu by increasing amounts of R-Ser and R_2-Ala was similar, suggesting that the effect, like the activation with these polypeptides at lower concentrations, may be a nonspecific result of adsorption of the polypeptide to the surface of the emulsion particles. In keeping with this interpretation, R-Glu itself partially inhibited enzyme activity at similar concentrations to those proving inhibitory with R-Ala and R-Ser

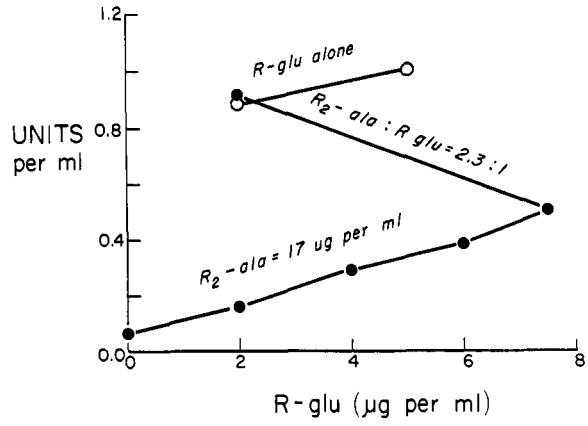

FIGURE 5: Effect of R_2-Ala upon R-Glu-stimulated activity of lipoprotein lipase in the protein of cows' milk. At a concentration of R-Glu in the incubation medium of 2 µg/ ml, 4.6 µg/ml of R_2-Ala had no effect, but 17 µg/ml almost completely inhibited the activity of R-Glu. Inhibition by the higher concentration of R_2-Ala decreased with increasing concentrations of R-Glu. At a constant ratio of R_2-Ala:R-Glu of 2.3:1, inhibition increased with increasing concentration of cofactor protein (see also Figure 3).

(Figure 1). However, unlike Brown and Baginsky (1972), who used triglyceride–gum arabic substrate and different concentrations of polypeptides, we observed that inhibition of crude milk enzyme by R-Ala could be overcome by increasing amounts of R-Glu (Figure 5). The mechanism of the inhibition is uncertain and its biological significance cannot be evaluated from data at hand.

In previous work with the milk enzyme, Iverius *et al.* (1972) found that heparin can stabilize the enzyme under certain conditions and enhance its activity under others. In the present study, the effects of heparin evidently cannot be ascribed to a stabilization of the enzyme, since the fatty acid release observed was linear with time both in the presence and in the absence of heparin. Activation by heparin was a function of the state of purity and source of the enzyme. Heparin increased the basal activity of the unpurified and purified milk enzymes as well as the sensitivity of their response to R-Glu. Heparin did not increase the basal or R-Glu stimulated activity of the enzyme purified from rat post-heparin plasma (Figure 2), although it does increase enzyme activity when whole serum provides the cofactor.[2] Iverius *et al.* (1972) similarly found that with skimmed milk as the enzyme source and human serum as the activator a marked inhibition of the enzyme activity was observed that could be relieved by heparin. The effect of heparin upon the activation of lipolytic activity in cofactor-poor guinea pig post-heparin plasma by rat serum or HDL (Whayne and Felts, 1970) also differs from its effect on the enzyme from milk. Although heparin can affect the activity of lipoprotein lipase under some conditions and it has been suggested to be an allosteric modifier of the enzyme (Whayne and Felts, 1970), this effect may depend upon the presence of a factor other than the enzyme itself and the cofactor protein.[3] Nevertheless, the present results show detailed agreement between the protein cofactor properties of highly purified and crude lipoprotein lipase from various sources in several mammalian species. They therefore suggest that the lipases released from the endothelial site (post-heparin plasma enzyme), derived from tissue parenchymal cells (milk enzyme (Hamosh and Scow, 1971)), and presumably containing both of these elements (whole adipose tissue), although differing in stability (Fielding, 1968–1970; Egelrud and Olivecrona, 1972), have the same active site. Since these properties have been observed with a substrate closely resembling the native lipoprotein substrates of the enzyme, they may assist in the design of further studies of the role of lipoprotein polypeptides in the transport of triglycerides.

[3] Elimination by heparin of the inhibitory effect of R-Ala and R-Ser upon activation of the enzyme in cows' milk protein may be of use in evaluating the activator property of mixed apoprotein in native lipoproteins.

Acknowledgments

We thank Barbro Carlander, Berith Emanuelsson, Åsa Gradin, and Edwin White for expert technical assistance.

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