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Isolation of Two Distinct Activator Proteins For Lipoprotein Lipase from Ovine Plasma

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Abstract

Two distinct activator proteins for lipoprotein lipase (LPL) have been isolated in approximately equal amounts from ovine plasma. These low molecular weight proteins were readily separated from each other on the basis of size and charge. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis indicated proteins of M_r about 8000 and 5000, with pI in urea-containing gels of about 5·1 and 4·8 respectively. Each of the ovine activator proteins was as effective as human apolipoprotein C-II (apo C-II) in activating LPL, with 1 μg/ml giving near to maximum activation, and in lowering the apparent K_m of LPL for triolein substrate. As the ratio of activator to triolein increased from 0·16 to 5·2 (μg/mg) the apparent K_m fell from about 0·5 to 0·18 mM. Whereas human apo C-II and the two ovine activators were equally effective in stimulating the hydrolysis of triolein, differences were observed between the human and ovine activators when *p*-nitrophenylbutyrate was used as substrate. Unlike human apo C-II, which produced significant inhibition of *p*-nitrophenylbutyrate hydrolysis, the ovine activators were without effect. This suggests that the interaction between the ovine activators and LPL is different from that of human apo C-II.

Introduction

Lipoprotein lipase (EC 3.1.1.34; triacylglyceroprotein acylhydrolase; LPL), bound to the lumenal surfaces of endothelial cells lining the blood capillaries of extrahepatic tissues, determines the rate of hydrolysis of triacylglycerols (TAG) in the plasma. Because of its location and tissue variation in activity, LPL acts as a regulatory enzyme for the uptake of lipid into peripheral tissues. One of the proteins associated with the natural substrates [very low density lipoprotein (VLDL) and chylomicrons] is required for maximal rate of hydrolysis. This protein isolated from human plasma has been designated apolipoprotein C-II (apo C-II) (Havel *et al.* 1970; La Rosa *et al.* 1970).

In our studies on the deposition of fat in ruminant animals of economic importance, we have investigated the regulatory role of LPL in ovine adipose tissue and its activation by component(s) of ovine serum. Recently we have shown that not only do ovine adipose tissues contain very significant levels of LPL activity but that ovine serum is a particularly good source of activator(s) at or below physiological substrate concentrations (Tume *et al.* 1983; Tume and Thornton 1985). Human apo C-II has been widely studied and well characterized (see Quinn *et al.* 1982*a*). Other preparations of LPL activators have been made from egg yolk lipoproteins (Bengtsson *et al.* 1977) and from pig plasma (Jackson *et al.* 1977). Several groups of workers have investigated the various effectors of LPL from bovine serum (Lim and Scanu 1976; Clegg 1978) and plasma (Astrup and Bengtsson 1982). Whereas human, rat and porcine plasma contain only one activator of LPL, bovine plasma and egg yolk lipoproteins have been shown to contain at least two activators. To date, no studies have been reported for ovine serum.

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Compared with human serum, bovine and ovine serum contain relatively low concentrations of both TAG (Tume and Thornton 1985) and VLDL (Nelson 1973), and much of the activator protein is associated with high density lipoprotein (HDL) (Lim and Scanu 1976). Whereas considerable ultracentrifugation time is generally required in the initial steps of the standard isolation procedures for LPL activator proteins, a more rapid technique based on the ability of certain apoproteins to bind to synthetic lipid particles (Erkelens *et al.* 1979; Carlson 1980) has been developed by Astrup and Bengtsson (1982).

In these experiments we have made use of the method described by Astrup and Bengtsson (1982) to isolate, purify and partially characterize two components of ovine plasma that exhibit strong activation of LPL.

Materials and Methods

Preparation of Ovine Plasma

Blood was collected from sheep being slaughtered at a local abattoir. Blood from individual sheep was treated with trisodium citrate (5·8 g/l) and then pooled (10 litres) in a large polyethylene container. On returning to the laboratory the blood was chilled and plasma prepared by centrifugation at about 4°C (1500 *g*, 30 min). The plasma was removed and immediately 5,5′-dithio-bis(2-nitrobenzoic acid) (0·6 mg/ml), phenylmethylsulfonyl fluoride (0·35 mg/ml), sodium azide (0·13 mg/ml) and EDTA (0·37 mg/ml) were added at the concentrations indicated.

Preparation of Washed Intralipid

Intralipid, 20% (w/v) (Kabi Vitrum, Stockholm, Sweden), was washed to remove excess phospholipid as described by Astrup and Bengtsson (1982). The final washed fat cake resulting from 1 litre of Intralipid was resuspended in 0·15 M NaCl, 0·1 M Tris-HCl (pH 7·4) to a total volume of 200 ml.

Binding of Plasma Proteins to Washed Intralipid

The method of Astrup and Bengtsson (1982) was used with some modification. Five litres of plasma were warmed to 25°C, and 200 ml of washed Intralipid suspension added, and the mixture stirred gently for 30 min. Following the binding step, all other procedures were done at 0–4°C. The washed Intralipid containing the bound proteins was isolated by centrifugation (28 000 *g*/90 min). The fat cake was carefully recovered, resuspended in 200 ml 0·15 M NaCl, 0·1 M Tris-HCl (pH 7·4), 10% (v/v) sucrose, layered below 4 volumes of 0·15 M NaCl, 0·1 M Tris-HCl (pH 7·4), and centrifuged again as described. The fat cake was washed at least three times or until no pellet was visible.

Isolation of Delipidated Proteins

The washed fat cake was transferred to a stainless steel beaker and mixed with 400 ml chloroform until a smooth paste was obtained. A clear solution was formed by adding 800 ml of chloroform–methanol (1 : 1, v/v). However, on standing for 2 h at 0°C a flocculant precipitate formed. This was collected by filtration on a Whatman No. 1 paper, washed with 150 ml chloroform–methanol (2 : 1, v/v), then 100 ml diethyl ether, dried, and stored at −20°C in a desiccator. Usually ≃200 mg dried powder (apoproteins) was obtained per litre of plasma.

Gel Filtration of Apoproteins

Apoproteins (≃400 mg protein) were dissolved in 5 ml of 4 M guanidine-HCl, 0·2 M Tris-HCl, (pH 8·2), 0·1% (w/v) sodium azide, 5 mM dithiothreitol, and applied to a Sephadex G-200 Superfine (Pharmacia) column (2·5 by 100 cm) previously equilibrated in the same buffer. The apoproteins were eluted from the column using the same solvent, and fractions were assayed for activator, pooled, dialysed into 0·01 M Tris-HCl (pH 7·2) and lyophilysed.

Ion-exchange Chromatography of Apoproteins

Peak 3 from the Sephadex G-200 column contained the C group of apoproteins as evidenced by SDS–polyacrylamide gel electrophoresis and high LPL activator activity. These C apoproteins from several Sephadex G-200 runs were further separated on a DEAE-Sephacel (Pharmacia) column (1·6 by 40 cm) using a linear gradient of 0·01–0·2 M Tris-HCl (pH 8·2) in 6 M urea. As soon as practicable, column fractions were tested for LPL activation, as given below, and appropriate fractions pooled and dialysed into 0·01 M Tris-HCl (pH 7·4), so as to minimize carbamylation.

Isolation of Human Apo C-II

Human apo C-II was isolated from recently frozen plasma obtained from a local blood bank. The method used was essentially as described for ovine plasma.

Preparation of Ovine Milk LPL

Lipoprotein lipase was purified from freshly collected ovine milk by affinity chromatography on Sepharose 4B-heparin (Bengtsson and Olivecrona 1977). The eluted enzyme preparation was kept at −20°C in 0·01 M phosphate buffer (pH 7·4), 1·5 M NaCl and 30% (v/v) glycerol at a protein concentration of 0·04 mg/ml. Such preparations were generally diluted 10-fold in 10 mM NH_4OH-NH_4Cl buffer (pH 8·1) just prior to commencement of assays.

Assay of LPL Activity

The determination of LPL activity was carried out essentially as described by Nilsson-Ehle and Schotz (1976). The reaction mixture (0·2 ml) which contained final concentrations of 67 mM Tris-HCl (pH 8·1), 1·5% (w/v) bovine serum albumin, 5·65 mM [^{3}H]triolein (or concentrations indicated) and 8·5% (v/v) serum or dilute column fractions, was pre-incubated for 15 min at 25°C prior to addition of 0·1 ml of dilute LPL preparation. Incubations were done at 37°C for 30–60 min, during which time no more than 8% of the triolein was hydrolysed. All assays were performed in duplicate and tubes containing 10 mM NH_4OH-NH_4Cl (pH 8·1) instead of the enzyme preparation served as controls. Tubes without serum or activator fractions were always included. The [^{3}H]oleic acid released during the reaction was isolated by solvent partition (Belfrage and Vaughan 1969) as modified by Nilsson-Ehle and Schotz (1976). The radioactivity in the upper phase was determined by liquid scintillation counting, using PCS as scintillant.

In some experiments a water-soluble *p*-nitrophenyl ester was used as a substrate for LPL catalysis, using essentially the method described by Quinn *et al.* (1982*b*). The reaction (total volume of 1·0 ml) was performed in a glass cuvette at 25°C, and consisted of 0·1 M sodium phosphate buffer (pH 7·2), 0·5 mM *p*-nitrophenylbutyrate, 0·1 M NaCl, 5 μg heparin and 8 μg ovine milk LPL. Other additions were made as indicated in the text. The hydrolysis of the ester was followed by measuring the absorbance change at 400 nm in a Hewlett-Packard model 8450A UV/VIS spectrophotometer. The rate of the reaction was essentially constant for at least 15 min.

Other Procedures

Urea (Analar grade) was obtained from BDH. Aqueous solutions of 8 M urea were freshly prepared and treated with a mixed-bed resin [Bio-Rad AG501-X8(D), 20–50 mesh] before use. Where necessary, dialysis was performed using Spectra/Por 3 tubing with a molecular weight cut-off of 3500, otherwise Visking tubing was used.

Polyacrylamide gel electrophoresis was usually performed in sodium dodecyl sulfate (SDS) according to Laemmli (1970), but using a linear gradient of 10–22% (w/v) acrylamide. Where indicated, the apoproteins were also separated on alkaline polyacrylamide gels containing urea (Kane 1973). SDS and urea gels were stained at 25°C for 30 min in 0·25% (w/v) Coomassie (PAGE blue 83, BDH Chemicals Ltd, England) in 10% (v/v) acetic acid, 50% (v/v) methanol. Isoelectric focusing was performed in 7·5% (w/v) acrylamide and 6 M urea as described by Pagnan *et al.* (1977) and Mills *et al.* (1984). Ampholines (pH 4·0–6·5) were obtained from LKB, Uppsala, Sweden. Before staining, gels were fixed overnight in 10% (w/v) trichloroacetic acid, 5% (w/v) sulfosalicylic acid. Gels were stained at 60°C for 1 h in 0·1% (w/v) Coomassie in 10% (v/v) trichloroacetic acid.

Reagents

Anhydrous emulsions of [^{3}H]triolein with glycerol and phosphatidylcholine were prepared as described by Nilsson-Ehle and Schotz (1976), and stored at 4°C until used. Tri-[9,10-^{3}H]oleoylglycerol was purchased from Amersham Australia Pty Ltd. Unlabelled triolein was purchased from Calbiochem-Behring Corp., California. Bovine serum albumin (Fraction V, Sigma Chemical Co., St Louis, Missouri) was tested for lipase activity before use. Only those preparations essentially free of lipase activity were used. Heparin (158 U.S.P. J-A units/mg), phenylmethylsulfonyl fluoride, 5,5′-dithio-bis(2-nitrobenzoic acid), and *p*-nitrophenylbutyrate were also purchased from Sigma Chemical Co., St Louis, Missouri. Electrophoresis calibration kits for molecular weight determinations of polypeptides were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. PCS scintillant was obtained from Amersham Corp., Arlington Heights, Illinois.

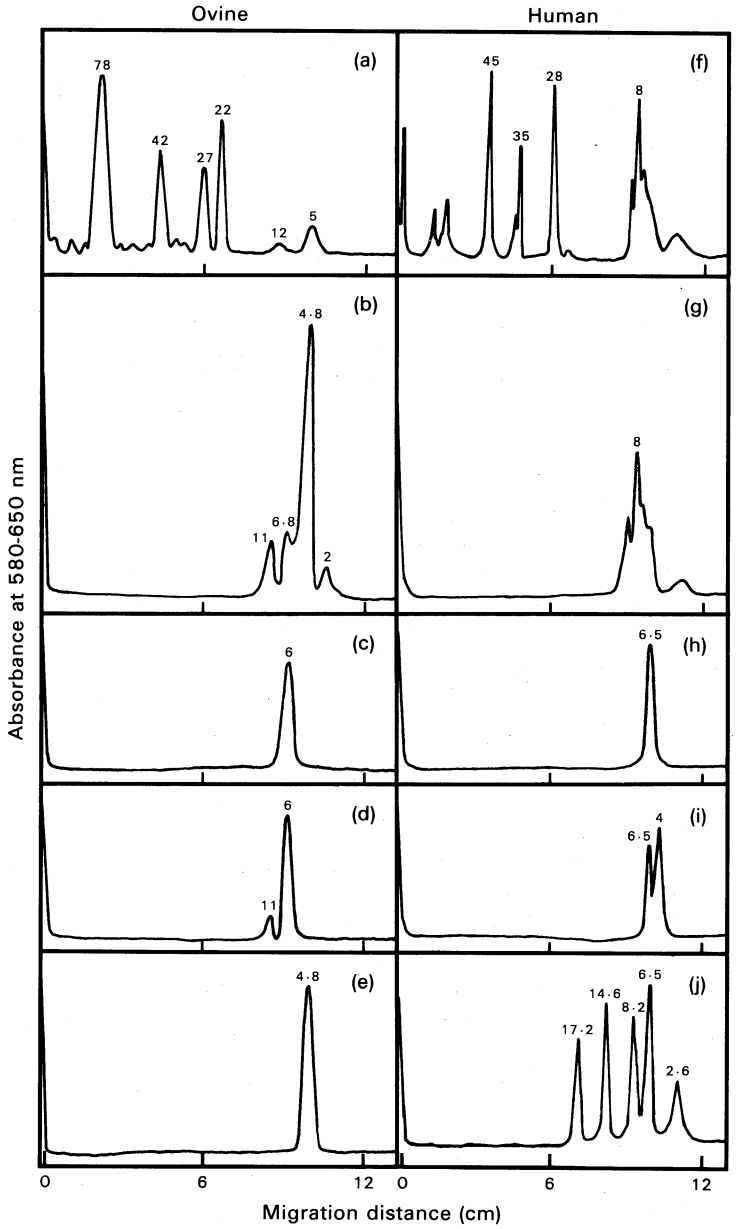

Fig. 1. SDS polyacrylamide gradient gel electrophoresis of proteins bound to washed Intralipid from ovine (*a*)-(*e*) and human (*f*)-(*i*) plasma. Total protein bound from ovine plasma (*a*) and human plasma (*f*); C group of apoproteins (peak 3) from Sephadex G-200 [(*b*) and (*g*)]; ovine OV_4 (*c*); ovine OV_5 (*d*); ovine OV_6 (*e*); human HU_2 (*h*) and human HU_3 (*i*); low molecular weight standards (*j*). The calculated molecular weight ($\times 10^{-3}$) of the individual proteins are indicated on the major peaks.

Results

Proteins Bound to Intralipid Particles

Ovine LPL activator was rapidly bound to the washed Intralipid particles. More than 90% of the activity present in the original plasma was removed with the washed lipid particles within 30 min and this did not change with longer incubation. Washing the protein-bound Intralipid essentially resulted in no loss of activator. An identical binding procedure was used for the human plasma and, in each case, approximately 200 mg of proteins were bound to the washed Intralipid particles per litre of plasma. SDS-polyacrylamide gel electrophoresis of the delipidated proteins (Figs 1*a*, 1*f*) revealed that at least 7–10 major proteins were bound to the Intralipid particles and, in the case of the ovine preparation

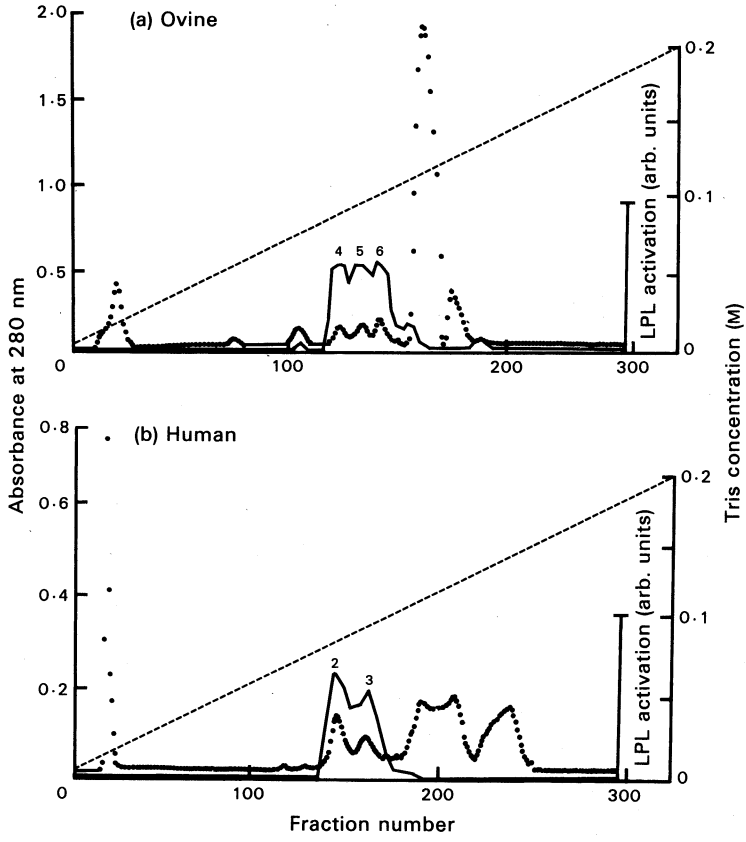

Fig. 2. Separation of the C group of apoproteins on DEAE-Sephacel: (*a*) ovine; (*b*) human. The apoproteins were eluted from the column with a linear gradient of 0·01–0·2 M Tris-HCl, pH 8·2, in 6 M urea. An aliquot of each fifth fraction was assayed for LPL activation (solid line) as described under Methods.

(Fig. 1*a*), relatively small amounts of the C group of apoproteins (M_r *c.* 10 000) were bound compared with those higher molecular weight proteins. An unknown protein of M_r 78 000 was the major component, but significant amounts of M_r 42 000, 27 000 and 22 000 proteins were also present. These latter components were tentatively identified as being ovine equivalents of human apoproteins A-IV, A-1 and D. In contrast to ovine plasma, the C group of apoproteins were by far the major components bound to Intralipid from human plasma, with lesser amounts of M_r 45 000 (apo A-IV), 35 000 (apo E) and 28 000 (apo A-1) (see Fig. 1*f*). Separation of the apoproteins from the higher molecular weight

components was achieved by gel chromatography on Sephadex G200. Usually three major peaks were obtained with each type of preparation, and the C group of apoproteins were shown to be present in the third peak by SDS gradient gel electrophoresis (Figs 1*b*, 1*g*). Analysis of the column fractions confirmed that peak 3 contained the activators of LPL.

The C group of apoproteins (peak 3) from ovine plasma consisted of at least four proteins of M_r 11 000, 6800, 4800 and 2000 with the M_r 4800 component accounting for about 90% of the total. On the other hand, the C group of apoproteins from human plasma were predominantly of higher molecular weight.

DEAE-Sephacel was then used to separate these proteins on a gradient of 0·01–0·2 M Tris-HCl (pH 8·2) in 6 M urea. About eight protein peaks were obtained with the ovine preparation, three of which gave activation of LPL (OV_4, OV_5, OV_6). These three peaks eluted from the column with about 0·1 M Tris-HCl (Fig. 2*a*).

Further analysis of the three active peaks demonstrated that only two activator proteins were present. Ovine fractions OV_4 and OV_6 ran on SDS-polyacrylamide gradient gels with apparent M_r of 6000 and 4800 respectively (Figs 1*c*, 1*e*). Ovine fraction OV_5 contained essentially the protein of OV_4 contaminated with a small amount of M_r 11 000 proteins.

Human C group apoproteins were resolved by DEAE-Sephacel into about six protein peaks, the activator being present in two peaks that eluted with about 0·1 M Tris-HCl (Fig. 2*b*). Fraction HU_2 gave essentially a single band of apparent M_r 6500 (Fig. 1*h*), but fraction HU_3 contained the M_r 6500 activator contaminated with a lower molecular weight component (Fig. 1*i*). These two components were separated by isoelectric focusing, eluted and tested for LPL activation, but only the protein equivalent to HU_2 was active.

Molecular Weights of Activator Proteins

Ovine plasma thus contains two activator proteins, one of similar apparent molecular weight to the activator from human plasma and the other somewhat smaller. The molecular weight of human apo C-II determined from the amino acid sequence is 8916 (Hospattankar *et al.* 1984), but on the SDS gradient gel system used here, it ran corresponding to a molecular weight of 6500 according to the reference standards used (cyanogen bromide fragments of sperm whale myoglobin). Because of this discrepency in molecular weight, the mobility of individual activator proteins relative to the reference peptide mixture was investigated using homogeneous gels (Laemmli 1970) over a range of acrylamide concentrations 10, 15, 20 and 25% (w/v) (data not given). At an acrylamide concentration of 10% (w/v), OV_4 and HU_2 had apparent M_r of 8300 and 8600, but at the higher concentrations ($\simeq$20%) of acrylamide which we used in the gradient gels, the apparent M_r fell to about 6400 and 7000 respectively. The apparent M_r of OV_4 was always less than HU_2 and that of the second activator of ovine plasma (OV_6) also decreased with increasing acrylamide concentration, from 5200 to 4800.

Fig. 3 shows the various activator proteins from ovine and human plasma when separated using a variety of techniques. Gradient gel electrophoresis (Fig. 3*a*) in the presence of SDS clearly demonstrates the difference in molecular weight between the two ovine activators OV_4 and OV_6. However, with this system, it was not possible to separate mixed samples of ovine OV_4 with the human activator HU_2 although the band was somewhat broadened. When run individually, the apparent molecular weight of OV_4 was always less than the human activator. Using alkaline-urea gels (Fig. 3*b*) (Kane 1973) the ovine activator OV_4 was well separated from the human activator and the lower molecular weight ovine activator OV_6 migrated between the two. The greatest resolution of the various activators was obtained by iso-electric focusing in urea-containing polyacrylamide gels (pH 4–6·5) (Fig. 3*c*). Ovine activators OV_4 and OV_6 and human activator had pI of 5·1, 4·8 and 4·5 respectively. The absolute values varied somewhat between successive runs but the relative mobilities of the proteins remained constant.

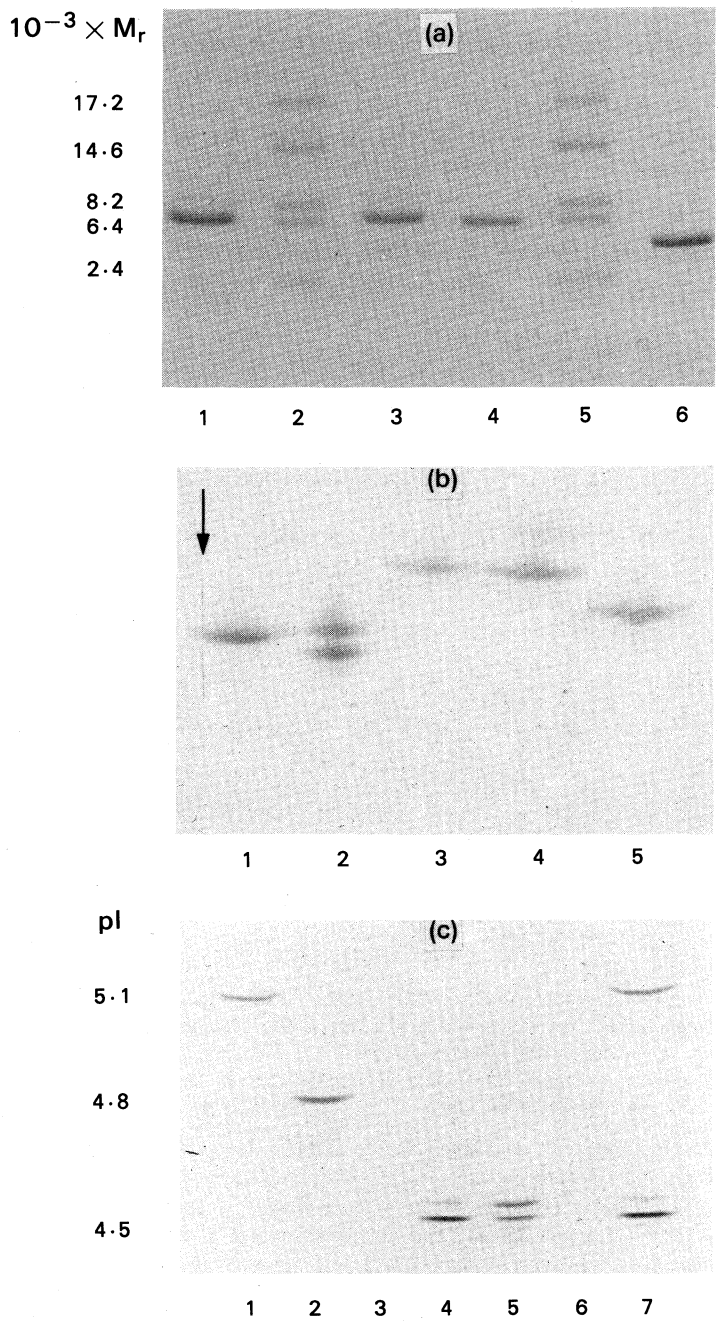

Fig. 3. Electrophoretic separation of ovine and human activator proteins. (*a*) SDS-polyacrylamide gradient gel electrophoresis. Lane 1, ovine OV_4 + human HU_2; 2, low molecular weight standards; 3, human HU_2; 4, ovine OV_4; 5, low molecular weight standards; 6, ovine OV_6. (*b*) Alkaline-urea polyacrylamide gel electrophoresis. Lane 1, human HU_2; 2, human HU_3; 3, ovine OV_4; 4, ovine OV_5; 5, ovine OV_6. Arrow indicates direction of migration. (*c*) Isoelectric focusing on polyacrylamide gels. Lane 1, ovine OV_4; 2, ovine OV_6; 3, blank; 4, human HU_2; 5, human HU_3; 6, blank; 7, ovine OV_4 + human HU_2.

Effectiveness of Activators

The relative effectiveness of the ovine and human activators for the stimulation of LPL was determined using a variety of assay conditions. Ovine milk LPL was incubated at 37°C with triolein at a final concentration of 5·6 mM with either no activator added or with increasing amounts of each of the purified activators. Fig. 4*a* shows that between 0·1 and 3·0 μg ml, each activator behaved identically, approaching maximum effect at about 1·0 μg/ml. The concentration of activators giving half-maximum velocity was about 0·2 μg/ml. In the second experiment shown (Fig. 4*b*), the concentration of each activator was constant and equal to the amount of LPL present, but the substrate concentration was varied from 0·16 to 5·95 mM triolein. Each activator was equally effective over the range studied.

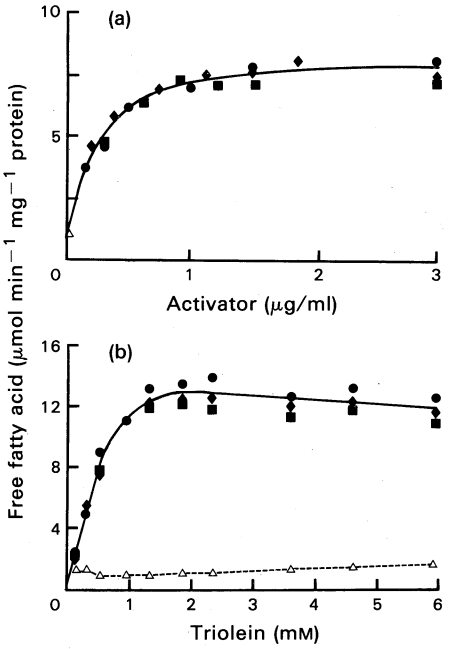

Fig. 4. Effect of activator proteins on LPL activity. LPL was assayed as described under Methods using (*a*) 0·2 μg LPL per 0·2 ml reaction volume (1 μg/ml) with either no activator or activator at the concentrations indicated, or (*b*) 0·25 μg LPL with 0·25 μg of activator with substrate concentrations of 0·16–5·95 mM triolein. ■ Ovine OV_4. ◆ Ovine OV_6. ● Human HU_2. △ No activator added.

In a third series of experiments (Fig. 5), the activation of LPL was measured at various ratios of the different activator proteins (OV_4, OV_6 and HU_2) to triolein (0–5·2 μg activator protein/mg triolein). For each of the activators OV_4, OV_6 and HU_2, a ratio of 1 μg activator/mg triolein gave essentially full activation at all of the substrate concentrations tested. When the data was plotted according to Lineweaver–Burk, a series of straight lines were obtained for each ratio (μg activator/mg triolein) indicating that the hydrolysis of the substrate by LPL obeys Michaelis–Menten kinetics (data not shown). Fig. 5*d* shows the effect of this ratio on the apparent K_m values calculated from these lines. It was not possible to calculate an apparent K_m value for that measured in the absence of activators as LPL activity per milligram of protein was very low (0·18–0·25 μmol fatty acid/min). However, as the amounts of ovine activators were increased from 0·16 to 5·2 μg per mg triolein, the apparent K_m fell from *c.* 0·5 mM to *c.* 0·18 mM. Human activator protein behaved in a similar manner. As the ratio of activators increased there was a small increase in $V_{max.}$ which did not change with ratios above 1·0.

It is evident that the ovine activator OV_4 has a similar molecular weight to the human activator HU_2, and is equally effective in stimulating LPL activity, but differs in its pI on isoelectric gels and its mobility on alkaline-urea gels. Whereas human apo C-II markedly stimulates the hydrolysis of long-chain triacylglycerides by LPL, it is not required, and has been shown to inhibit LPL activity, when the water-soluble substrate *p*-nitrophenylbutyrate is used (Quinn *et al.* 1982*b*). Therefore the effect of the various activator proteins on the hydrolysis of *p*-nitrophenylbutyrate by ovine milk LPL was determined. The rate of the LPL reaction with *p*-nitrophenylbutyrate as substrate was 0·12 ± 0·01 μmol/min per milligram LPL (mean ± s.e.m.; $n = 31$) at 25°C, only about 1% of the rate obtained

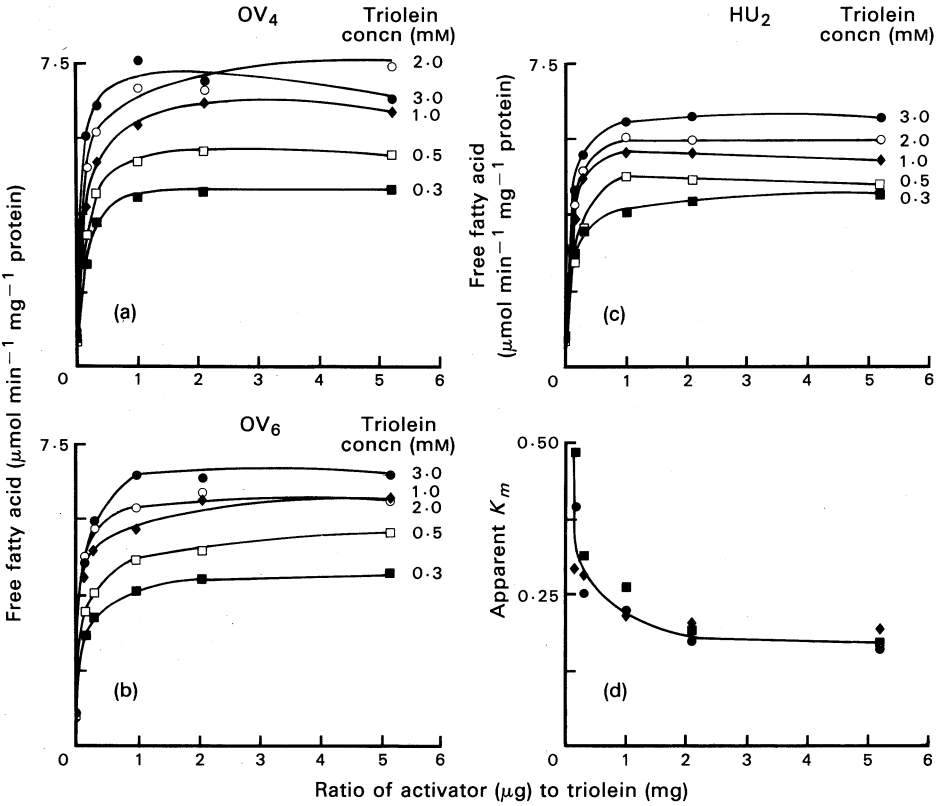

Fig. 5. LPL activity as a function of activator protein and triolein concentrations. LPL was assayed as described under Methods using 0·33 μg LPL per 0·2 ml reaction volume with triolein concentrations of 0·3 mM (■), 0·5 mM (□), 1·0 mM (◆), 2·0 mM (○), 3·0 mM (●) with (*a*) ovine activator OV_4, (*b*) ovine activator OV_6 or (*c*) human activator HU_2. The apparent K_m of LPL for triolein is shown (*d*) for each of the activator proteins OV_4 (■), OV_6 (◆), HU_2 (●) at various ratios of activator to substrate concentrations.

with triolein but comparable to that reported by others (Quinn *et al.* 1982*b*) for the soluble substrate. The effect of the activator proteins on the hydrolysis of *p*-nitrophenylbutyrate expressed as a percentage relative to the reaction rate in their absence is given in the following tabulation (values are expressed as mean ± s.e.m. and the number of assays are indicated in parentheses):

OV_4 (50 μg/ml)	100 ± 1·2 (4)
OV_6 (50 μg/ml)	103 ± 0·5 (3)
HU_2 (30 μg/ml)	67·3 ± 6·1 (4)

Human activator was almost as effective at 10 μg/ml, but similar concentrations of ovine activators were without effect.

Discussion

Human fraction HU_2 shows all the characteristics of apo C-II. It has an apparent molecular weight similar to that reported for C-II. It stimulates LPL hydrolysis of long-chain TAG and causes inhibition of LPL activity when *p*-nitrophenylbutyrate is used as the reaction substrate. It is an important functional property of apo C-II that it can readily transfer between different lipoproteins (Havel *et al.* 1973; Eisenberg 1978). In a similar way, apo C-II present in serum added to *in vitro* assays of LPL readily transfers to the lipid particles of the TAG emulsion (La Rosa *et al.* 1970). This binding of various plasma proteins and apolipoproteins to Intralipid has been studied in some detail (Erkelens *et al.* 1979; Carlson 1980). Astrup and Bengtsson (1982) used a phospholipid-depleted Intralipid preparation as the initial step for the large-scale preparation of activator proteins from bovine plasma. This technique offers many advantages over the other procedures which can only deal with small volumes and involve considerable time in the ultracentrifuge. Furthermore, the present study shows that the technique is particularly well suited to those species (e.g. sheep) that have very low plasma concentrations of VLDL. In such cases the bulk of their readily transferable activators reside in the HDL fraction. In contrast, it has been determined that only 50% of the total apo C-II was present in the VLDL-free serum from normotriglyceridemic humans (Kashyap *et al.* 1977).

Carlson (1980) showed that Intralipid particles bound essentially only apo C-II and apo C-III when incubated with human serum or VLDL-free serum, but in the work reported here with ovine and human plasma, the C group of apoproteins represented only a small amount of the total proteins bound to the washed Intralipid particles (about 9 and 25% respectively). Astrup and Bengtsson (1982) have also shown that many proteins other than the C group of apoproteins were bound from bovine plasma. In other studies we have used both serum and plasma from sheep and humans with various amounts of Intralipid and this has not significantly altered the type and quantity of proteins bound (R. K. Tume *et al.*, unpublished observations). Even though other proteins were bound to the Intralipid particles, gel chromatography effectively separated these proteins from the C group of apoproteins, allowing their complete separation on DEAE-Sephacel.

We have demonstrated that ovine plasma contains two distinct activators of LPL having apparent M_r 8300 and 5200 (homogeneous 10% SDS polyacrylamide gels). These low molecular weight proteins were readily separated from each other on the basis of size and charge. Although the presence of isoproteins or molecular variants have been described in several species (Lim and Scanu 1976; Havel *et al.* 1979), only in hens egg yolk lipoprotein has any significant size difference been demonstrated (Bengtsson *et al.* 1977). The activator components from egg yolk were of similar size to those of ovine plasma, but each egg yolk activator component existed in several charge variants. Attempts to purify these components resulted in loss of activity. Furthermore, the component of hen's yolk lipoprotein of M_r 9000 was in about 10-fold excess of M_r 5000 component whereas the ovine activators were present in about equal amounts.

Human plasma apo C-II exists as a single species of molecular weight 8916 (Hospattankar *et al.* 1984) but a minor isoelectric variant form has been shown to exist (Havel *et al.* 1979). Bovine plasma contains two isoproteins of simliar apparent molecular weight 8000–10 000 (Lim and Scanu 1976; Clegg 1978) which can readily be separated by ion-exchange chromatography. Lim and Scanu (1976) and Astrup and Bengtsson (1982) have shown that these bovine activator proteins differ in amino acid composition.

Despite the apparent variability between the activators of different species and between activators within a species, activators show no species specificity when used with a wide variety of lipoprotein lipases (Wallinder *et al.* 1982; Bengtsson and Olivecrona 1977). It is likely therefore that each of these activator proteins has certain regions of specific structural similarities which are highly conserved.

Each of the ovine activators proved to be at least as effective as the human apo C-II in stimulating LPL activity with 1 μg/ml giving near maximal activation. Values reported

for maximum activation by apo C-II vary widely between various groups, but commonly range from 1 μg/ml (Matsuoka *et al.* 1981; Wallinder *et al.* 1982) to more than 40 μg/ml (Kinnunen *et al.* 1977). The relationship between the apparent K_m and the activator protein–triolein ratio was the same for all three activator preparations and was similar to that reported for human LPL with apo C-II (Schrecker and Greten 1979). The two activator proteins obtained from egg yolk lipoproteins by Bengtsson and Olivecrona (1977) gave half-maximal stimulation at concentrations of 1–2 μg/ml. In contrast, much higher final concentrations were used in two studies on bovine activator proteins. Lim and Scanu (1976) used up to 50 μg/ml of each of their activators D1 and D3 and even then obtained respectively only 58 and 18% activation compared with added bovine or human lipoproteins. Clegg (1978) used activator concentrations of 20–25 μg/ml, and did not obtain more than about 63% of the rate measured in the presence of added serum. Although it is not possible to make absolute comparisons of the effectiveness of the various activators due in part to the different conditions used for assay by various workers, it is clear that each of the ovine activators isolated in this study is at least as active as any human preparation so far reported, and much more active than those isolated from bovine plasma.

There are certain experimental conditions in which LPL is fully active even in the absence of apo C-II. Usually the substrates in these cases are either water-soluble esters or short-chain triacylglycerols, but they appear to be hydrolysed by the same site. Rapp and Olivecrona (1978) demonstrated that tributyrin was hydrolysed by LPL at a high rate whether or not apo C-II was present. Activator protein was not required for hydrolysis of *p*-nitrophenyl esters (Egelrud and Olivecrona 1973) and also caused significant inhibition of LPL (Quinn *et al.* 1982*b*). Apo C-II was the only apoprotein tested that caused such inhibition. It has been suggested that apo C-II interacts with LPL in such a way that the enzymes' catalytic turnover rate with a water-soluble substrate is reduced. In the present study we used *p*-nitrophenylbutyrate as substrate in order to compare the ovine activators with human apo C-II. Human apo C-II did cause inhibition of LPL activity but we were unable to observe any change with either of the ovine activators. This suggests that the interaction between each ovine activator and LPL is different from that observed with human apo C-II.

We are interested in the origin, function and structure of these two ovine activators, particularly as they differ so much in molecular size. The smaller activator is likely to contain some 30 amino acid residues fewer than the larger activator (and human apo C-II), yet it still functions as effectively. Studies on synthetic fragments or cyanogen bromide fragments of human apo C-II have indicated that two distinct functional domains exist in the entire molecule; one for binding to lipid (residues 43–51, Morrisett *et al.* 1977; Catapano *et al.* 1979), another for the catalytic activation of LPL (residues 56–78, Kinnunen *et al.* 1977; Catapano *et al.* 1979); both are required for maximal activation. It must be assumed that each of the ovine activators has these two functional regions since they are both as effective as the human apo C-II preparation. The ovine activators therefore offer a unique opportunity to investigate structure–function relationships. The proteins can be relatively easily isolated in pure form and are stable with time of storage.

We are currently determining the sequence of amino acids in each of the ovine activator proteins. Such investigations would determine the similarity of the sequence in the functional regions in the two activators, and establish whether or not the smaller activator has a shorter *N*-terminal sequence prior to the lipid-binding region. Should the sequence be completely different then it might suggest a different site of synthesis of each activator such as liver and intestine.

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