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The Heterogeneity of the Lipoprotein Lipase of Rat Epididymal Adipose Tissue

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Lipoprotein lipase is heterogeneous, and it was suggested that the enzyme in adipose tissue is transformed from a species of mol.wt. approx. 120000 to forms of much higher molecular weight as it is secreted from the fat-cell. This paper demonstrates that the forms of higher molecular weight are probably artifacts. Enzyme preparations were characterized by gel filtration, by density-gradient centrifugation and by affinity chromatography. The results indicate that the enzyme forms of mol.wt. greater than 120000 result from an association of the enzyme with particulate material. It is therefore necessary to reconsider schemes that have recently been proposed for the synthesis and export of lipoprotein lipase.

The hydrolysis of the plasma triacylglycerols (triglycerides) throughthe action of lipoprotein lipase (EC 3.1.1.34, clearing-factor lipase) is considered to be a necessary step in the transfer of their constituent fatty acids into the extrahepatic tissues. The enzyme is believed to act at the luminal surfaces of the capillary endothelial cells, and changes in its activity in particular tissues have been correlated in several instances with alterations in the uptake of triacylglycerol fatty acids by those tissues (Robinson, 1970).

The processes by which changes in lipoprotein lipase activity are brought about are under investigation in this and other laboratories, and evidence has been presented for the involvement of different molecular species of the enzyme. Thus gel filtration of enzyme preparations extracted from defatted rat adipose tissue generally leads to the resolution of three zones of enzyme activity (Garfinkel & Schotz, 1972; Schotz & Garfinkel, 1972; Davies *et al.*, 1974). One of these, which has been designated form a, represents material of very high molecular weight (over 10^6) that is eluted at the void volume of Sepharose 6B, whereas a second, designated form b, is eluted as a peak with an apparent mol.wt. of about 120000. Enzyme activity also reaches a plateau between the two distinct peaks of activity. Form b is believed to be synthesized in the fat-cell and to be the precursor of the higher-molecular-weight forms, which are considered to be transported from the fat-cell to the endothelial cell surface (Robinson *et al.*, 1975; Garfinkel *et al.*, 1976; Nilsson-Ehle *et al.*, 1976).

To study the transformation of form b into form a more readily we have attempted to resolve the various species of lipoprotein lipase by means that were quicker and more sparing of material than gel filtration. However, the results obtained in the course of this work have led us to question the existence of form a as a distinct molecular species. In this paper we report these findings and discuss their implications for the hypothesis that form b is a precursor of the functional enzyme.

Experimental

Materials

Sepharose 6BCL and CNBr-activated Sepharose 4B were obtained from Pharmacia, Uppsala, Sweden. Ultragel AcA22 was obtained from LKB Instruments, South Croydon, Surrey, U.K. Calf intestinal alkaline phosphatase, ovalbumin and chymotrypsinogen were obtained from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Other chemicals were as specified by Davies *et al.* (1974).

Animals

Specific pathogen-free male Wistar-strain rats (A. Tuck and Son, Rayleigh, Essex, U.K.) were used throughout. They were maintained on Oxoid pasteurized diet 41B (Herbert Styles, Bewdley, Worcs., U.K.) and weighed 180–200g in the fed state. They were killed, in either the fed state or after starvation for 24h, by stunning, followed by neck fracture, between 08:30 and 09:30h.

Acetone/ether-dried preparations of adipose tissue

These were made by techniques similar to those previously described (Salaman & Robinson, 1966). Epididymal fat-pads were removed into water at 0°C, rinsed and then blended in water at 0°C (5ml/g wet wt. of tissue). The homogenate was poured into acetone at −18°C (75ml/g wet wt. of tissue) and the

precipitate was collected on filter paper on a Buchner funnel. It was washed with acetone at 20°C (50ml/g wet wt.), then with the same volume of diethyl ether, and finally the preparation was dried under vacuum for 20min. It was stored desiccated at −18°C and used within 48h. The number of fat-pads taken varied between 18 and 52 in different experiments.

Extraction of lipoprotein lipase

The acetone/ether-dried preparations were homogenized at 25mg/ml at 0°C in aq. 50mM-NH_3 adjusted to pH8.1 with HCl. The homogenate was centrifuged for 30min at 2°C and 26000*g* in the 8×50ml rotor of the MSE High-Speed 18 centrifuge. The supernatant, which contained 44±13% (mean±S.D; $n=20$) of the activity of the homogenate, was used immediately.

Assay of lipoprotein lipase

Lipoprotein lipase activity was determined by measuring the release of fatty acids from an activated triacylglycerol substrate at 30°C. The procedure was that described by Riley & Robinson (1974), but with the assay volume decreased to 2.4ml. Heparin was not present in the assay. One unit of lipoprotein lipase activity is defined as the amount of enzyme releasing 1 μmol of fatty acid/h in this assay.

Gel-filtration chromatography

This was carried out at 4°C on columns (54cm×2.2cm) of Ultragel AcA22 or of Sepharose 6BCL that had been equilibrated with aq. 50mM-NH_3 buffer, pH8.1, in 10% (v/v) glycerol. After the extract of the acetone/ether-dried preparations had been made 10% (v/v) in glycerol, samples (5–15ml) were applied to the columns, which were then eluted with aq. 50mM-NH_3 buffer, pH8.1, in 10% (v/v) glycerol at a rate of 15ml/h. The recovery of enzyme activity from the column was 70–100% of that applied. The proportions of lipoprotein lipase species in the column profiles were quantified by weighing the area of paper under each peak in the profile; the form a and form b peaks were assumed to be symmetrical.

Density-gradient centrifugation

This was carried out at 2°C in the SW 40 rotor of a Beckman L2-65B centrifuge. For separations designed to display both form a and form b enzyme species, extracts of the acetone/ether-dried preparations were centrifuged for 1.5h at 202000*g* on 11ml linear 5–20% (w/v) sucrose gradients underlaid with a 2ml cushion of 2.4M-sucrose. In the absence of the sucrose cushion, form a prepared by column chromatography was pelleted and lost most of its activity; in its presence the activity was recovered quantitatively. All of the sucrose solutions were in aq. 50mM-NH_3 buffer, pH8.1. The integrated field–time needed to clear particles with a particular sedimentation coefficient was estimated by the procedure described in the Beckman centrifuge manual.

Estimations of the molecular weight of the enzyme were made with 13ml linear 5–20% (w/v) sucrose gradients, which were centrifuged for 15–26h at 202000*g* (Martin & Ames, 1961). The marker proteins used were calf intestinal alkaline phosphatase (mol.wt.140000), bovine serum albumin (mol.wt. 68000), ovalbumin (mol.wt.45000) and chymotrypsinogen (mol.wt.25700). The molecular weights of the marker proteins are taken from Darnall & Klotz (1975) and from Weber *et al.* (1972).

Affinity chromatography

Affinity chromatography was carried out on columns (2.6cm×0.7cm) of heparin–Sepharose 4B (Iverius, 1971) that had been equilibrated with aq. 50mM-NH_3 buffer, pH8.1, in 10% (v/v) glycerol. After the enzyme preparations had been applied to the column, it was washed with 10vol. of the same buffer and then eluted successively with 10 column vol. of the buffer containing 0.5M-NaCl and with 10 column vol. of the buffer containing 1.0M-NaCl.

Statistical treatment of results

The data on the proportions of the enzyme species resolved by gel filtration were analysed by non-parametric statistical methods. The significance of the differences between the results obtained with adipose tissue from fed and from starved animals was estimated by using the Kolmogorov–Smirnov two-sample test (Campbell, 1974). The correlation of the proportions of the species present with the tissue enzyme activity was made by Kendall's method of rank correlation (Campbell, 1974).

Results

Gel-filtration chromatography of extracts of defatted adipose tissue

In over 30 experiments the enzymic activity of extracts of acetone/ether-dried preparations of rat adipose tissue was resolved by chromatography on Ultragel AcA22 or Sephadex 6BCL into two peaks separated by a plateau (Fig. 1). In accordance with the nomenclature used in earlier papers we have designated the peaks form a and form b (see the introduction section). We propose to refer to the plateau of enzymic activity as form p.

We did 16 experiments using columns of Ultragel AcA22 with preparations from animals in the fed state and 17 with preparations from starved animals. The mean (±S.D.) total lipoprotein lipase activity of homogenates of the acetone/ether-dried preparations from fed rats was 49±15 ($n=16$) units/fat-pad, and

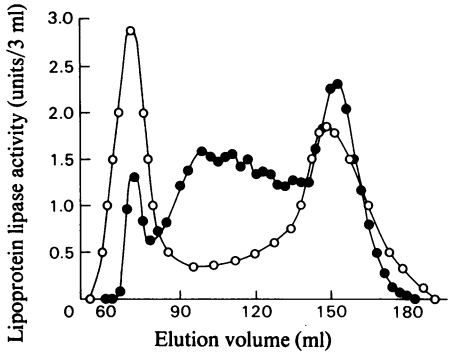

Fig. 1. *Elution profiles of lipoprotein lipase activity*
Extracts of acetone/ether-dried preparations of epididymal fat-pads from fed rats were analysed on Ultragel AcA22 as described in the Experimental section. The results of two separate experiments are shown; in both cases the first peak is at the void volume. Recovery of the applied enzyme activity was 74% (profile a, ○) and 98% (profile b, ●).

that of the homogenates from starved rats was 8 ± 3 ($n = 12$) units/fat-pad.

The proportion of the enzyme activity found as each of the species form a, form p and form b was very variable (Fig. 2), and the data do not obviously fit any of the common distributions. However, non-parametric methods of statistical analysis show that the proportions of each of the enzyme species in the preparations from fed animals are significantly different ($P<0.02$) from each of those found in preparations from starved animals. In particular, the proportions of form a and of form p are greater, and that of form b is smaller in the preparations from fed rats than in those from starved animals. This agrees with previous findings (Schotz & Garfinkel, 1972; Davies *et al.*, 1974; Garfinkel *et al.*, 1976). Further analysis of the data shows that, for the enzyme preparation from starved rats, the proportions of the different enzyme species are related to the total lipoprotein lipase activity of the tissue. For example, as Fig. 3(*a*) shows, there is a significant ($P<0.001$) inverse linear relation between the total activity and the form b activity. For the enzyme preparation from fed rats, however, no such relation exists (Fig. 3*b*).

Density-gradient centrifugation of lipoprotein lipase

Density-gradient centrifugation carried out directly with extracts of acetone/ether-dried preparations of adipose tissue from fed and starved rats revealed no enzyme with a high sedimentation coefficient corresponding to that expected for the form a and form p species. For example, the extract

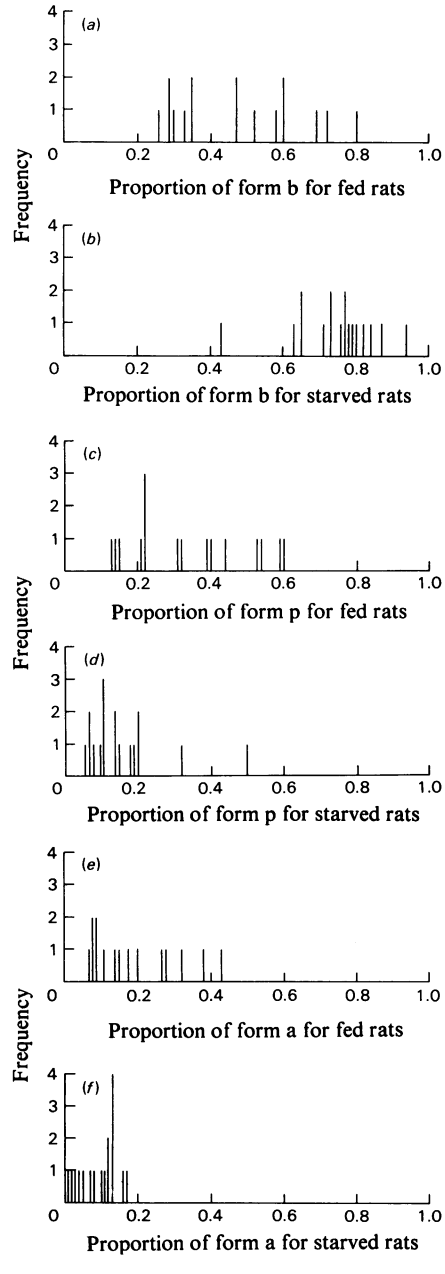

Fig. 2. *Frequency diagrams of the proportion of lipoprotein lipase found as form a, p or b for fed and starved rats*
Extracts of acetone/ether-dried preparations of epididymal fat-pads were prepared from fed and starved animals and fractionated on Ultragel AcA22 as described in the Experimental section. (*a*) Fed, form b; (*b*) starved, form b; (*c*) fed, form p; (*d*) starved, form p; (*e*) fed, form a; (*f*) starved, form a.

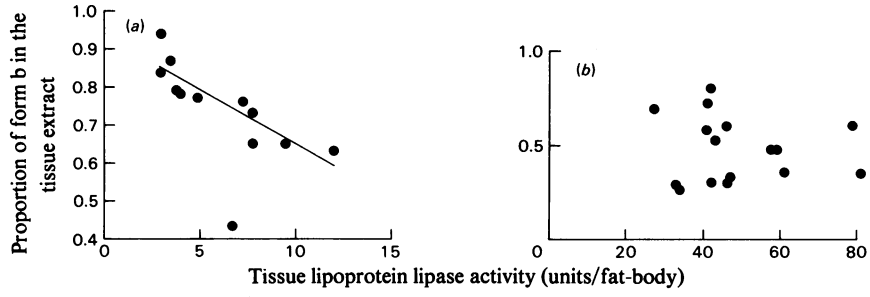

Fig. 3. *Relationship between total lipoprotein lipase activity in extracts of acetone/ether-dried preparations of epididymal fat-pads and the proportion of form b in the extract*

The proportion of form b was determined by chromatography on Ultragel AcA22 as described in the Experimental section. (*a*) Data from starved animals. The correlation coefficient (r), the number of animals (n) and the equation for the regression line are: $r = -0.89$, $n = 11$, $P < 0.001$, $y = 0.94 - 0.0287x$. If the outlier is included $r = -0.66$, $n = 12$, $P < 0.05$. (*b*) Data from fed animals. There is no significant correlation of the proportion of form b with tissue activity.

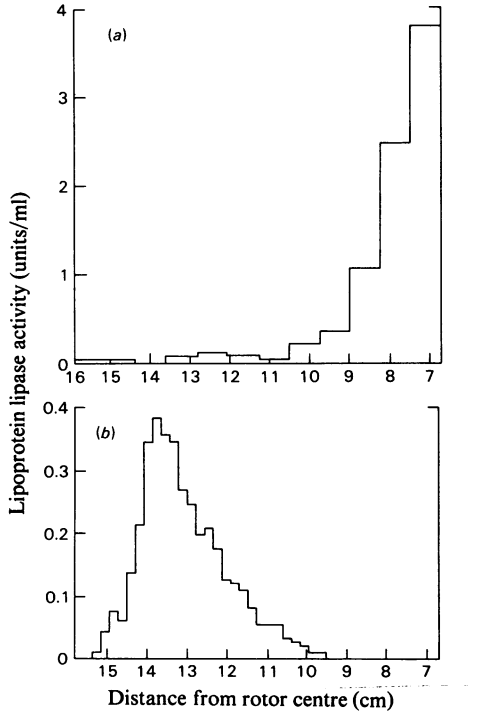

Fig. 4. *Density-gradient centrifugation of an extract of an acetone/ether-dried preparation of epididymal fat-pads from fed rats*

(*a*) A sample of the extract (0.4ml) was analysed on a linear sucrose gradient by centrifugation for 1½h at 202000*g* with a cushion of 2.4M-sucrose, as described in the Experimental section. The results are typical of six experiments. (*b*) A sample of the extract (0.3ml) was analysed on a linear sucrose gradient by centrifugation for 26h at 202000*g* as described in the Experimental section. Similar results were obtained in 28 experiments with preparations both from fed and from starved rats.

used to obtain the column profile b shown in Fig. 1 was simultaneously analysed by density-gradient centrifugation, with the result shown in Fig. 4(*a*). More prolonged sedimentation has been carried out on 28 separate extracts prepared from the adipose tissue of both fed and starved rats. In all cases a single peak contained the whole of the applied activity, and, by comparison with the rates of sedimentation of marker proteins, the molecular weight of this species is in the region of 120000 (Fig. 4*b*).

These results suggest that there is only one species of lipoprotein lipase in extracts of acetone/ether-dried preparations and that it is similar in size to form b. The possibility that a high-molecular-weight species of the enzyme is dissociated during centrifugation is excluded by the finding that preparations of form a do not disaggregate under the same conditions. Thus, when extracts of acetone/ether-dried preparations were fractionated on columns of Sepharose 6BCL and the enzyme recovered as form a was analysed by density-gradient centrifugation, in all cases about 75% of the applied form a activity was recovered at the cushion of 2.4M-sucrose (Fig. 5). The sedimentation coefficient of this material must be greater than 240S. The profiles were also unaltered when glycerol, which is used as a routine to stabilize the enzyme during the column fractionations, was used to form the gradients.

Gel-filtration chromatography of defatted adipose-tissue extracts after their high-speed centrifugation

The absence of any enzyme activity corresponding to form a or form p after density-gradient centrifugation of extracts of the acetone/ether-dried preparations could be accounted for if the form a species were formed only on the gel-filtration columns. This might occur either through aggregation of the enzyme or through its association with particulate

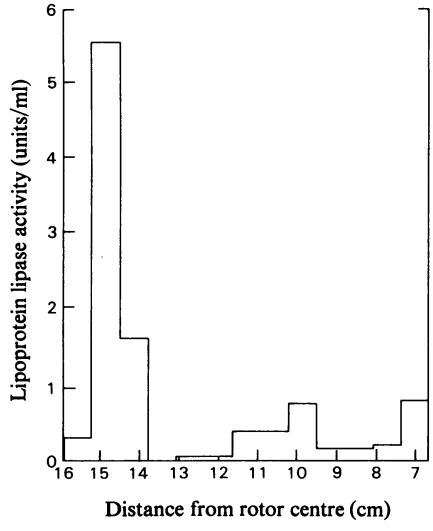

Fig 5. *Density-gradient centrifugation of form a*
After fractionation of an extract of an acetone/ether-dried preparation of epididymal fat-pads from fed rats on a column of Ultragel AcA22, 27ml of the fractions containing form a was concentrated to 2ml by using the Amicon P10 ultrafiltration cell. A sample (0.5ml) was analysed on a sucrose gradient as described in the Experimental section; the recovery of applied enzyme activity was 109%. The results are typical of three experiments.

material present in the extract. To distinguish between these possibilities, extracts were centrifuged for 4h at 110000$g_{av.}$ in the SW39 rotor of a Beckman L2-65B centrifuge at 2°C before column fractionation. These conditions were calculated to precipitate from the extract all material with a sedimentation coefficient greater than 30S.

Three such experiments, each with preparations from the adipose tissue of both fed and starved rats, were carried out and in every case over 97% of the enzyme in the supernatant was found by gel filtration to be present as form b. The recovery of enzyme activity in the supernatant solution was between 64 and 67%. This is entirely accounted for by the initial uniform distribution of the enzyme in the centrifuge tube, which results in the pelleting of some of the enzyme. Thus there was a similar recovery of enzyme activity when the supernatant solution was centrifuged a second time. It appears therefore that both the form a and the form p species result from an irreversible association during the column chromatography of the enzyme with particulate material present in the extracts of acetone/ether-dried preparations.

Affinity chromatography of form a and form b species

Heparin–Sepharose affinity chromatography has been used in the purification of lipoprotein lipase from a number of sources (Olivecrona *et al.*, 1971; Greten & Walter, 1973; Bensadoun *et al.*, 1974; Iverius & Ostlund-Lindquist, 1976). In the present work it has been used to study the binding of the form a and form b fractions eluted from the gel-filtration columns. In nine experiments the proportion of form b bound was 0.97±0.07 (mean±S.D.), whereas in four experiments the corresponding proportion of form a bound was 0.22±0.09.

All of the bound form a was eluted by buffers containing 0.5M-NaCl. The form b, on the other hand, was eluted in two stages, part by buffers containing 0.5M-NaCl and the rest by buffers containing 1.0M-NaCl. A similar pattern of elution of lipoprotein lipase from heparin–Sepharose columns has been observed with the enzyme from heart muscle (Twu *et al.*, 1975).

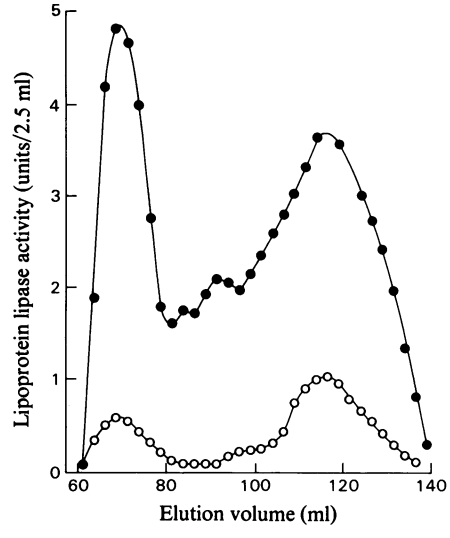

Fig. 6. *Effect of previous affinity chromatography on the distribution of lipoprotein lipase on gel-filtration columns*
An extract of an acetone/ether-dried preparation of epididymal fat-pads from fed rats was made as described in the Experimental section. A portion of the extract was fractionated directly on a Sepharose 6BCL gel-filtration column. A second portion was loaded on to a heparin–Sepharose 4B affinity column and the eluate from this was then fractionated on the Sepharose 6BCL column. Samples (2.5ml) of the eluates from the gel-filtration column were assayed for lipoprotein lipase activity. Form a accounts for 32% of profile a (●) and for 23% of profile b (○). The conditions of the column fractionations are described in the Experimental section. The results are typical of eight experiments.

The low efficiency of form a binding may simply be due to the large size of the particles with which the enzyme is associated, since these will be excluded from the gel and hence from access to most of the heparin ligands. It suggests, however, that, if the form a species is already present in the extracts of the acetone/ether-dried preparations, the proportion of the enzyme activity bound by the affinity columns should be low in such extracts when they are prepared from the adipose tissue of fed rats, where the proportion of form a is relatively high. Moreover, the fraction not bound should be enriched in form a. In fact, in eight experiments with extracts prepared from the epididymal fat-pads of fed rats the percentage of the enzyme activity that was bound to the affinity column was $85\pm8\%$ (mean$\pm$S.D.). Gel-filtration chromatography of the enzyme activity that was not bound demonstrated that the proportion of form a was either unchanged or less than in the original extract (Fig. 6). These results therefore support the view that form a is formed during gel filtration and is not present as such in the extracts of the acetone/ether-dried preparation.

Discussion

The above results strongly suggest that the high-molecular-weight species of lipoprotein lipase that have been previously postulated as being present in adipose tissue are artifacts and that they originate through the binding of the enzyme to particulate material during gel filtration. A similar situation has been reported for the hexokinase of rat brain (Wilson, 1972). Thus the whole of the activity of rat brain hexokinase is eluted at the void volume of Sepharose G-200 columns, although density-gradient centrifugation of the same preparation indicates a mol.wt. of about 100000.

The view that form a is a distinct species of lipoprotein lipase has been supported hitherto from evidence that the proportion of form a is higher in enzyme preparations derived from fed rats than in those from starved rats. This has been confirmed in the present work (Fig. 2), but we believe that it can be accounted for on the basis of form a being an artifact. Thus, in adipose tissue from starved rats, the proportion of form b is significantly and negatively correlated with the tissue total enzyme activity (Fig. 3*a*). This could signify that the quantity of particulate material that can bind lipoprotein lipase is not limiting in such extracts, so that the proportion of form b decreases as the total enzyme activity increases. On the other hand, for preparations of higher total activity from fed rats, we would suggest that the quantity of particulate material does limit the extent of binding of the larger amounts of enzyme present. The considerable variation in the proportions of form b found when extracts from fed rats are fractionated (Fig. 3*b*) could well be accounted for by differences in the degree of homogenization of the acetone/ether-dried preparations, and hence in the particle content of the extracts. From Fig. 3 it appears that, in general, the limit of the binding capacity occurs somewhere between 20 and 30 units of tissue activity/fat-pad and results in some 50% of form a being converted into a high-molecular-weight form under the conditions of the present study.

If the lipoprotein lipase species form a and form p are indeed artifacts, then schemes for the synthesis and export of the enzyme from the fat-cells that have been presented (Robinson *et al.*, 1975; Nilsson-Ehle *et al.*, 1976) must be reconsidered. At the present time it seems essential to propose only the existence of the enzyme species of mol.wt. about 120000, which is synthesized in the fat-cell and exported as such to the functional site at the capillary endothelial-cell surface, where it may be bound by a heparin-like glycosaminoglycan (Robinson, 1963; Olivecrona *et al.*, 1971). However, the activity of the enzyme is under hormonal control (see Robinson, 1970), and increases in its activity occur through both protein-synthesis-dependent and protein-synthesis-independent mechanisms (Wing & Robinson, 1968; Stewart & Schotz, 1971; Cryer *et al.*, 1973). Moreover, it has been shown that actinomycin D does not prevent the induction of the enzyme under appropriate conditions *in vivo* or *in vitro* (Eagle & Robinson, 1964; Schotz & Garfinkel, 1965; Garfinkel *et al.*, 1967; Wing & Robinson, 1968).

The studies with actinomycin seem to exclude control at the transcriptional level. However, it could clearly be exerted through regulation of the translation of the mRNA for lipoprotein lipase and thereby of the rate of synthesis of the enzyme protein. The enzyme at the endothelial-cell surface is much less stable than that within the fat-cell (Cunningham & Robinson, 1969; Davies & Robinson, 1973); its activity in the tissue at any given time could therefore simply depend on the balance between its synthesis and degradation. On the other hand, the possibility of post-translational modification certainly cannot be excluded. Thus, apart from evidence that hormones may control the proportions of two forms of the enzyme of similar molecular weight in adipose tissue (Davies *et al.*, 1974), the lipoprotein lipases of pig adipose tissue and of cow's milk (Bensadoun *et al.*, 1974; Iverius & Ostlund-Lindquist, 1976) have been shown to be glycoproteins. Activation by a protein-synthesis-independent mechanism could therefore occur through the completion of unglycosylated enzyme protein, as for thyroglobulin (Herscovics, 1970).

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