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Effect of Nutrition on Subcellular Localization of Rat Fat-Cell Lipoprotein Lipase

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This study supports the possibility for multiple subcellular forms of lipoprotein lipase. 1. The total activity of lipoprotein lipase per g of intact epididymal adipose tissue from fed rats is much higher than that from starved rats. 2. The isolated fat-cells of fed and of starved rats have lipoprotein lipase of almost the same activity per g of fat-pads. The isolated fat-cells of starved rats have a much higher proportion of total activity per g of the intact tissue than do those of fed rats. 3. Under the conditions of homogenization used, only a small proportion of the total activity per g of intact tissue from fed rats was associated with the fat layer which floated to the top of the homogenate during low-speed centrifugation. The different proportions of the specific enzyme activity found in each subcellular fraction are described. 4. Lipoprotein lipase from plasma membranes and microsomal fractions from starved and fed rats was purified by affinity chromatography. 5. The total activity of microsomal lipoprotein lipase per g of intact adipose tissue is enhanced by a normal diet. 6. In intact epididymal adipose tissue from fed rats, the activity per g of tissue of lipoprotein lipase of plasma membranes is much higher than that in the same fraction from starved rats. By contrast, the activities per g of tissue in plasma membranes obtained from starved or from fed rats by collagenase treatment were similar.

In adipose tissue, the main function of lipoprotein lipase (EC 3.1.1.3) is to hydrolyse plasma triacylglycerols and thereby facilitate the uptake of their constituent fatty acids by the tissue. Previous studies (Garfinkel & Schotz, 1972; Davies *et al.*, 1974; Etienne *et al.*, 1976) have shown that acetone-dried powders of purified lipoprotein lipase obtained from adipose tissue of starved rats showed two peaks of activity after gel filtration or heparin–Sepharose chromatography. On feeding the starved rats with fresh cream or glucose, the specific activity of the first peak obtained by heparin–Sepharose chromatography was clearly increased, but the second one remained virtually unchanged.

In agreement with the above authors and with Nilsson-Ehle *et al.* (1976), we have taken into consideration the possibility that one peak is due to an extracellular form of the enzyme, and that the other peak is due to an intracellular form. To investigate this hypothesis we have undertaken subcellular fractionation, localization and characterization of the two enzyme forms.

We have also studied the influence of nutrition on enzyme activity in plasma membranes and in microsomal fractions. Isolated fat-cells were obtained after collagenase treatment (Rodbell, 1964). However, Pokrajac *et al.* (1967) and Cunningham & Robinson (1969) found that collagenase can, under appropriate conditions, inactivate lipoprotein lipase, especially the extracellular form of the enzyme (Nilsson-Ehle *et al.*, 1976). It therefore seemed worth while to investigate the subcellular localization of lipoprotein lipase activity in the whole intact adipose tissue.

Materials and Methods

Male Wistar rats (about 2 months old, and weighing 200–250 g) were either fed *ad libitum* (A.04 pasteurized breeding normal diet; Usine Alimentation Rationnelle, Villemoisson, France) or starved for 15 h. They were decapitated and the epididymal adipose tissue was rapidly excised.

Subcellular fractionation from whole adipose tissue

The epididymal adipose tissue was minced with scissors, strained through gauze and cooled to 4°C. Homogenization was performed with a Potter–Elvehjem homogenizer (Teflon pestle, ten up-and-down strokes, clearance 0.015–0.022 mm, 1800 rev./min) in 3 vol. of 0.25 M-sucrose containing 10 mM-Tris adjusted to pH 7.4 with HCl, and 1 mM-EDTA (disodium salt) at 4°C. The homogenate was diluted with a further 2 vol. of this homogenizing medium and

was centrifuged at 1300*g* ($r_{av.}$ 7cm) for 20min at 4°C (JA 20 rotor, Beckman J.21).

Plasma membranes and a crude microsomal fraction were obtained by a modification of the procedure of MacKeel & Jarett (1970). Most of the fat layer which floated to the top of the homogenate was discarded and the supernatant (S_1) was centrifuged again at 21000*g* ($r_{av.}$ 7cm) for 20min at 4°C to give supernatant S_{21}.

The resulting pellet (P_{21}), which contained mitochondria and plasma membranes, was homogenized in 8ml of homogenizing medium in a Potter–Elvehjem homogenizer at 1200 rev./min (Teflon pestle, three up-and-down strokes, clearance 0.010–0.015mm). The pellet (P_{21}) was then placed on top of a discontinuous density gradient composed of 10ml of 9% (w/v) Ficoll (Sigma Chemical Co., St Louis, MO, U.S.A.), layered over 10ml of 15% (w/v) Ficoll, both in homogenizing medium. After centrifugation at 53000*g* ($r_{av.}$ 9.3cm) for 45min (SW 25.1 rotor), plasma membranes were collected from the top of the gradient, and the mitochondria fraction from the pellet. The intermediate layer was heterogeneous and contained several enzyme activities [5′-nucleotidase activity (EC 3.1.3.5) and a high acid phosphatase activity (EC 3.1.3.2)]. The fractions were diluted with 3vol. of homogenizing medium and centrifuged at 105000*g* ($r_{av.}$ 5.9cm) for 20min at 4°C (40 rotor; Spinco L50).

The crude microsomal pellet was obtained by centrifugation of supernatant S_{21} at 105000*g* ($r_{av.}$ 5.9cm) for 60min at 4°C. The pellet was resuspended in 10ml of homogenizing medium without EDTA and the final centrifugation was at 105000*g* ($r_{av.}$ 5.9cm) for 60min at 4°C. This pellet is referred to as the microsomal fraction.

Isolation of fat-cells

Isolated fat-cells were prepared by crude collagenase (EC 3.4.24.3); (*Clostridium histolyticum*; Worthington Biochemical Corp., Freehold, NJ, U.S.A., type CLS, 157 units/mg) treatment of adipose tissue (Rodbell, 1964).

Preparation of subcellular fractions from isolated fat-cells

The isolated fat-cells were homogenized as described by Wolf *et al.* (1975) by using a Swinny (Millipore Corp., Bedford, MA, U.S.A.) device (Laudat, 1972). Subcellular fractions were prepared as described above for intact adipose tissue.

Acetone/diethyl ether-dried fat-layer preparation

When enzyme activity was to be assayed in the fat-layer, acetone/diethyl ether-dried preparations were made first. The fat-layer, which floated to the top of the homogenate by centrifugation at 1300*g* ($r_{av.}$ 7cm) for 20min at 4°C, was mixed directly into 500ml of acetone at 4°C and filtered on Buchner funnels. The precipitate was washed with 1500ml of acetone and then with 2000ml of diethyl ether at about 20°C. It was dried under a reduced pressure of N_2 and stored at 4°C. Within 12h, the preparation was suspended in 0.05M-NH_4Cl adjusted to pH8.6 with aq. 0.05M-NH_3 with shaking at 4°C. The enzyme was assayed in the supernatant after centrifugation at 3000*g* ($r_{av.}$ 7cm) for 15min at 4°C.

Lipoprotein lipase assay

Lipoprotein lipase activity was assayed with a synthetic glycerol tri[1-^{14}C]oleate substrate emulsified in the presence of Triton X-100 (Greten & Walter, 1973).

Glycerol trioleate containing [1-^{14}C]oleate in all three positions was purchased from Commissariat à l'Energie Atomique, Gif-sur-Yvette, France (CMM 274). The assay system contained the following components in a final volume of 1ml: 200μmol of Tris adjusted to pH8.6 with HCl; 1.8nmol of glycerol tri[1-^{14}C]oleate (2.2×10^5 d.p.m.); 750nmol of non-radioactive glycerol trioleate (Sigma Chemical Co.); 5mg of bovine serum albumin (fatty acid-poor; bovine albumin powder, fraction V Armour; Serlabo, Paris, France); 15μl of aq. 1% (v/v) Triton X-100; 100nmol of $CaCl_2$, 100μmol of NaCl; 50μl of normal human serum and various concentrations of enzyme solution. Incubations were for 30min at 37°C in a shaking water bath. Total lipids were extracted by the method of Dole (1956). The radioactive non-esterified fatty acids were isolated by a modification of the method of Kelley (1968). A 3ml portion of the upper phase of each sample was transferred to a low-potassium glass counting vial containing 0.50g of an anion-exchange resin (AG1 X8; 20–50 mesh, OH^- form, Bio-Rad Laboratories, Richmond, CA, U.S.A.). The resin was washed twice with 5ml of n-heptane. A portion (1ml) of a mixture containing 28g of KOH, 250ml of Triton X-100 and 250ml of methanol was added to the resin and incubated for 30min at 70°C. The radioactivity of the fatty acids was determined in an Intertechnique SL_{31} liquid-scintillation counter in 10ml of toluene containing 5.0g of 2.5-diphenyloxazole/litre and 0.3g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre. The counting efficiency was about 90%. The results were corrected for quenching by the channels-ratio method. Enzyme activities are expressed as μmol of non-esterified fatty acids liberated/h per ml of enzyme solution. The reaction rate decreased if more than 12% of the substrate was hydrolysed. All results were therefore obtained from assays maintained within these limits. Lipoprotein lipase was characterized by its sensitivity to serum activator and its inhibition by 1M-NaCl.

Analytical methods

5'-Nucleotidase activity (EC 3.1.3.5) (plasma-membrane marker) was measured as described by Dixon & Purdom (1954). NADH–cytochrome *c* reductase activity (EC 1.6.2.4) (microsomal marker) was measured by the method of Mackler (1967) in the presence of rotenone (30μg). These measurements were made by using an Acta III spectrophotometer (Beckman Instruments, Gagny, France), which allows graphic monitoring of the reaction as a function of time.

Proteins were determined by the standard method of Lowry *et al.* (1951) or, for lower protein concentrations, by the method of Schaffner & Weissmann (1973).

Purification of lipoprotein lipase from microsomal fractions and plasma membranes

The solubilization of the enzymes from the microsomal fraction and plasma membranes by shaking for 12h in 0.05M-NH_4Cl/aq. 0.05M-NH_3 buffer, pH8.6, increased the specific activity of the enzyme with a yield of 80±8% (mean±S.D. for six independent experiments). The microsomal fraction and the plasma membranes were centrifuged at 105000*g* ($r_{av.}$ 5.9cm) for 1h at 4°C.

The supernatants were immediately applied to a column (7cm×1cm) of Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) covalently linked with heparin (Wilson laboratories, Chicago, IL, U.S.A.) prepared as described by Iverius (1971). The column was equilibrated with 0.5M-NaCl containing 5mM-sodium barbital, pH8.6, and 20% (v/v) glycerol at 4°C. Elution was carried out with a discontinuous NaCl gradient of increasing molarity (30ml of 0.5M; 50ml of 0.7M; 70ml of 1.16M) in 5mM-sodium barbital buffer, pH8.6, containing 20% (v/v) glycerol. Fractions (5ml) were collected and assayed for lipoprotein lipase activity.

Results

Lipoprotein lipase activities in intact adipose tissue and isolated adipocytes

The enzyme activity per g of fat-pads in homogenates of intact tissue from starved rats was about 42% of that measured in fed rats (see Table 1). In starved rats, the isolated adipocytes contain 80±10% (mean±S.E.M. for four experiments) of the total enzyme activity in the intact tissue. In fed rats, only 40±5% (mean±S.E.M.) of the total enzyme activity is recovered in the isolated adipocytes. Moreover, the isolated fat-cells of fed and of starved rats had lipoprotein lipase of almost the same total activity per g of tissue. These results confirm those of Cunningham & Robinson (1969) and Nilsson-Ehle *et al.* (1976), and may indicate a change in subcellular enzyme distribution in starved or fed rats.

Enzyme activity in the fat-layer

After homogenization, most of the lipoprotein lipase activity in fed rats was not discarded with the fat-layer extract which contained only 3±0.6% (mean±S.D. for three independent experiments) of the total lipoprotein lipase activity of the intact tissue. Thus during homogenization the enzyme does not bind to cellular triacylglycerols.

Lipoprotein lipase in subcellular fractions

The total activity of lipoprotein lipase per g of fat-pads in homogenates of intact tissue from fed rats is almost 2.5-fold higher than that from starved rats. However, in both cases the microsomal fraction contains an almost equal amount (10%) of the total enzyme activity. On the other hand, the total enzyme activity in the microsomal fraction from fed rats is about 2.5-fold higher compared with that from starved rats (Table 1).

The total lipoprotein lipase activity per g of fat-pads in plasma membranes from intact tissue was about 2% of the total homogenate enzyme activity in starved rats, and about 5% in fed rats. Moreover, the lipoprotein lipase activity in the plasma membranes was about 5-fold higher in fed rats than in starved rats. On the other hand, after collagenase treatment, plasma membranes obtained from fed or starved rats have similar enzyme activities (Table 1). It is noteworthy that the values of these latter plasma-membrane activities are in the same range as those found in intact adipose tissue from starved rats.

Lipoprotein lipase from microsomal fractions had the highest specific activity (Table 2). The total enzyme activity could be inhibited by 1M-NaCl. The percentage inhibition was higher for microsomal fractions (90±10%) than for plasma membranes (50±10%). This is similar to results obtained with isolated fat-cells (Wolf *et al.*, 1975). Incubation without serum gave lipoprotein lipase with a similar low specific activity (15±5%) for both subfractions (all values are means±S.D. for three separate experiments).

The cytosol of adipose tissue contains, in addition to lipoprotein lipase, a hormone-sensitive lipase with a high specific activity (Huttunen *et al.*, 1970; Khoo *et al.*, 1972). Moreover, Wolf *et al.* (1975) have shown that lipase activity titrated in cytosol is hardly inhibited by 1M-NaCl and neither is it sensitive to serum. These results suggest that the lipase with a high specific activity found in the cytosol is not only due to lipoprotein lipase.

As indicated in Table 2, extraction with 50mM-NH_4Cl/aq. 50mM-NH_3 buffer, pH8.6, increased the

Table 1. *Lipoprotein lipase activity in subcellular fractions from intact adipose tissue*

Fractionation from intact tissue was performed as described in the Materials and Methods section. Plasma membranes from isolated fat-cells were prepared by incubating epididymal fat-pads in the medium used for the isolation of fat-cells from intact tissue. Incubation was performed in silicone-treated flasks at 37°C for 20min under a vigorous mechanical agitation (5mg of collagenase per g of tissue was added in 3ml of incubation medium). The flasks were then centrifuged for 1min at 1000*g* ($r_{av.}$ 7cm) and the infranatant was removed. The layer of adipocytes floating at the top of the centrifuge tube was collected and washed twice with 10ml of the sucrose/Tris/HCl/EDTA medium, pH 7.4. Homogenization was performed by using a Swinny device. Plasma membranes were prepared as described for intact adipose tissue (see the text). All values have been normalized to correspond to 1 g of intact tissue and are given as means ± S.E.M. for the numbers of experiments in parentheses. Probability of significance of difference between mean for starved rats and mean for fed rats was computed by Student's *t* test, and *P* values are from Fischer's tables. Abbreviation: N.S., not significant, $P > 0.1$.

	Lipoprotein lipase activity (μmol of non-esterified fatty acids/h per g of tissue)		
	Starved rats	Fed rats	*P*
Homogenate	4.40 ±0.60 (4)	10.50 ±0.8 (3)	<0.02
Cytosol	2.20 ±0.30 (4)	7.50 ±0.6 (3)	<0.02
Plasma membranes	0.095±0.03 (4)	0.54 ±0.10 (3)	<0.05
Microsomal fraction	0.430±0.10 (4)	1.10 ±0.20 (3)	<0.1
Plasma membranes of isolated fat-cells	0.100±0.10 (3)	0.110±0.10 (3)	N.S.

Table 2. *Comparison of different subfractions separated from whole adipose tissue of starved rats*

Fractions S_1 and S_{21} and the extraction of the lipoprotein lipase from the microsomal fractions and from the plasma membranes are defined in the Materials and Methods section. Values are means ± S.E.M. for four separate experiments.

	Specific activity		
Fraction	5′-Nucleotidase (nmol of P_i/min per mg of protein)	NADH–cytochrome *c* reductase (nmol of cytochrome *c* reduced/min per mg of protein)	Lipoprotein lipase (μmol of fatty acids liberated/h per mg of protein)
S_1	40±10	80±20	1.40±0.3
S_{21}	35±10	110±20	1.50±0.3
Cytosol	15± 5	20± 5	1.25±0.3
Mitochondria	30±10	50±10	0.30
Plasma membranes	250±50	55±10	2.20±0.4
Extracted from plasma membranes	110±20	0	3.50±0.4
Microsomal fraction	80±20	470±40	2.20±0.4
Extracted from microsomal fractions	40±10	10± 5	4.60±0.4

specific activity of lipoprotein lipase by more specifically solubilizing the enzyme, in particular the microsomal enzyme.

Lipoprotein lipase purification in subcellular fractions

Heparin–Sepharose chromatography was used to isolate lipoprotein lipase from the soluble extract. Proteins without affinity for heparin were eluted first with 0.5M-NaCl and then with 0.7M-NaCl buffer. On elution with 1.16M-NaCl buffer, microsomal and plasma-membrane fractions isolated from intact adipose tissue always showed two peaks of lipoprotein lipase activity.

The first peak of activity eluted from the microsomal extract of starved rats contained four times more lipoprotein lipase activity than did the second one (Fig. 1). The first peak of activity from the microsomal extract of fed rats contained 10 times more enzyme activity than the second. The first fraction showed a 70±10% inhibition by 1M-NaCl buffer,

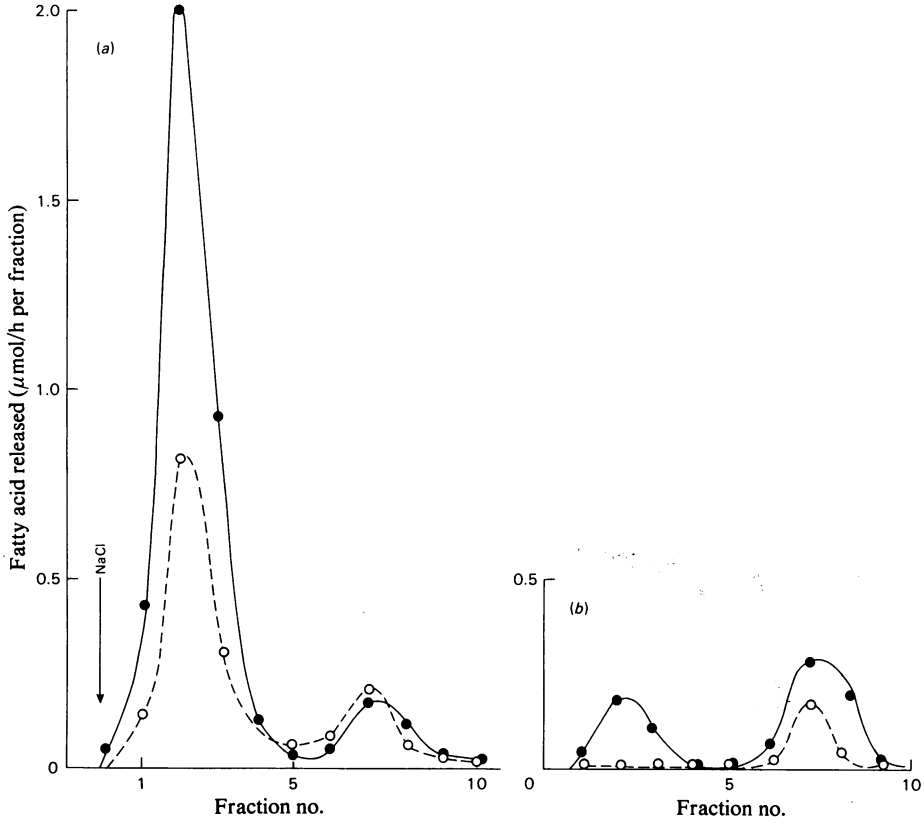

Fig. 1. *Heparin–Sepharose chromatography of microsomal fractions (a) and plasma membranes (b) in starved (○) or in fed (●) rats*

The NH_4Cl/NH_3 extract of each subcellular fraction was applied to a column (7cm × 1cm) of heparin–Sepharose as described in the Materials and Methods section. Fractions (5ml) containing lipoprotein lipase activity were eluted with 1.16M-NaCl buffer. Enzyme activity is expressed as μmol of non-esterified fatty acid/h per fraction. The results are normalized for 10g of adipose tissue.

Table 3. *Purification of lipoprotein lipase from microsomal fractions of adipose tissue from starved rats*

The total NH_3/NH_4Cl extract was applied to heparin–Sepharose (see the Materials and Methods section). The results are given for 122g of adipose tissue (100 starved rats).

	Protein (mg)	Volume (ml)	Total enzymic activity (μmol of non-esterified fatty acids/h)	Specific activity (μmol of non-esterified fatty acids/h per mg of protein)	Purification (fold)	Yield (%)
Homogenate	420	220	600	1.4	—	—
Supernatant S_1	370	220	550	1.5	—	—
Microsomal fraction	22	10	56	2.6	1	100
Sample subjected to heparin–Sepharose chromatography	10.6	10	48	4.5	1.8	86
Tube number 2	0.023	5	10	440	170	18
Peak 1 total	0.086	20	17	200	75	30
Tube number 7	0.008	5	2.5	310	120	4.5
Peak 2 total	0.022	20	5.0	225	100	9

and the second a 40±10% inhibition. When serum was added, lipoprotein lipase activity was increased by 75±10% for the first peak and by 60±10% for the second peak (mean±S.D. for three separate experiments).

For the plasma membranes, the second peak of activity eluted represents the main fraction which possessed lipoprotein lipase activity (Fig. 1). This second peak was slightly increased when animals were fed on a normal diet.

The sequential purification steps of lipoprotein lipase from microsomal fractions of adipose tissue from starved rats are summarized in Table 3.

Discussion

Pokrajac *et al.* (1967) found that collagenase can, under appropriate conditions, inactivate lipoprotein lipase. In fed rats, the total activity of the enzyme per g of fat-pads in the plasma membranes from the intact tissue was high. This appears to be predominantly due to an enzyme activity which decreased when the intact tissue was treated with collagenase. In starved animals, the enzyme activity in the intact adipose tissue is markedly lower and is similar to the activity in the isolated fat-cells. Moreover, the specific activities of $(Mg^{2+}+Na^{+}+K^{+})$-dependent adenosine triphosphatase and of 5′-nucleotidase (plasma-membrane enzyme marker) were also lower in fractions separated from isolated fat-cells as compared with those separated from whole adipose tissue (Giacobino & Chmelar, 1975). This suggests that the enzyme inactivated by collagenase is extracellular either associated with the fat-cells or with the stroma-vascular tissue (Cunningham & Robinson, 1969). Ho *et al.* (1967) reported that the stroma-vascular cells of adipose tissue had a lipase activity with some of the characteristics of lipoprotein lipase. It is likely that this extra-adipocytic contamination may have a particularly high lipoprotein lipase activity in fed rats, and it may be the cause for the high total activity per g of fat-pads observed in plasma membranes from the intact adipose tissue.

The solubilized lipoprotein lipase (NH_4Cl extract) was further purified by heparin–Sepharose chromatography. During this purification step, the enzyme could be dissociated into two active fractions. This has already been observed with acetone-dried powders of human or rat adipose tissue (Etienne *et al.*, 1974, 1976). Garfinkel & Schotz (1972, 1973), Davies *et al.* (1974) and Cryer *et al.* (1975) in a related series of studies separated by gel filtration, two molecular species (named a and b) of lipoprotein lipase with different cellular locations. Nilsson-Ehle *et al.* (1976) described two high-molecular-weight forms (a and i) of an enzyme which predominate in extracts of intact rat adipose tissue and correspond to an extracellular lipoprotein lipase. They also described a low-molecular-weight form (b) of this enzyme, which predominates in isolated fat-cells and corresponds to an intracellular form.

The first fraction eluted on affinity chromatography, which is the main component of the microsomal fractions, may be conjectured to be the intracellular form [corresponding to the b fraction of Schotz & Garfinkel (1972)]. The active form of the enzyme requires activation by serum, and is inhibited by 1 M-NaCl.

The main component of plasma membranes eluted in peak 2 may represent the extracellular form of the enzyme [corresponding to the a or i fraction of Nilsson-Ehle *et al.* (1976)]. This fraction was activated by serum, like the main fraction eluted from microsomal fractions, but was only partially inhibited by 1 M-NaCl.

The plasma-membrane fractions prepared by us from crude mitochondrial fractions had a leaflet structure, whereas those prepared by Avruch & Wallach (1971) from crude microsomal fraction formed vesicles (Giacobino & Chmelar, 1975). The 5′-nucleotidase contamination in microsomal fractions could be explained by the fact that part of the isolated plasma membranes forms vesicles and co-sediments with microsomal particles. Re-isolation of these plasma-membrane vesicles by hypo-osmotic shock showed that they were essentially identical with plasma-membrane leaflets. Both contained the same 5′-nucleotidase and lipoprotein lipase (A. Vanhove & C. Wolf, unpublished work). This contamination by plasma membranes may correspond to the second minor peak of material eluted during affinity chromatography of the microsomal fractions.

The characterization of the two subcellular fractions is consistent with experiments which showed that the first fraction (eluted by 1.16 M-NaCl from acetone-dried powders of rat adipose tissue) has the characteristics of lipoprotein lipase (Etienne *et al.*, 1976). The second fraction has these characteristics to a lesser extent. Nielsson-Ehle *et al.* (1976) showed that only the lower-molecular-weight portion of fraction b has the characteristics of the lipoprotein lipase in acetone-dried powder extracts of intact tissue.

Several authors (Cunningham & Robinson, 1969; Garfinkel & Schotz, 1973; Nilsson-Ehle *et al.*, 1976), as well as us, found that lipoprotein lipase activities vary with the nutritional state of the animal. Etienne *et al.* (1976) showed an increase in specific activity in the first fraction eluted (peak 1) from heparin–Sepharose chromatography after dietary intake of either lipids or glucose. By contrast, the specific activity of the second fraction (peak 2) remained virtually unchanged, irrespective of the diet. These results might suggest that the differences in sensitivity of the various fractions to diet may be connected with their subcellular origin. In the present study, the

lipoprotein lipase activity in plasma membranes and in purified microsomal fractions were studied in relation to the nutritional state. Compared with starved rats, the results with fed rats showed an increase in the total activity per g of tissue of lipoprotein lipase in the first peak eluted from microsomal fractions, but only a small difference in the second fraction.

We propose the following hypothesis on the effect of nutrition on the lipoprotein lipase activity of different subcellular fractions. Fed rats have a higher intracellular lipoprotein lipase activity than starved rats. This increase in the microsomal lipoprotein lipase activity may be due to an increase in intracellular synthesis of lipoprotein lipase. A portion of this newly synthesized enzyme may be displaced towards the enzyme's extracellular sites (the adipocyte plasma membranes and the stroma-vascular cell). This may explain the observed increase in plasma-membrane lipase activity.

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