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INTRACELLULAR LOCALIZATION OF LIPOPROTEIN LIPASE ACTIVITY IN RAT HEART [1]

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ABSTRACT

The intracellular localization of a lipoprotein lipase (LPL) activity in rat heart was shown. A crude separation showed the maximal total activity in the nuclear fraction and the maximal specific activity in the microsomal fraction. Each subcellular fraction was purified further and LPL activity remained only demonstrable in the microsomal fraction. A further fractionation of the microsomal fraction was attempted, obtaining the membranous fraction of the sarcoplasmic reticulum in a pure form. This was shown to be the site of the intracellular localization of LPL activity.

RESUMEN

Se demuestra la localización intracelular de la lipoproteína lipasa (LPL) en el corazón de la rata. Una primera separación, mostró la mayor actividad total en la fracción nuclear y la máxima actividad específica en la fracción microsómica. En experimentos posteriores, cada una de las fracciones subcelulares fueron purificadas, y la actividad de la LPL se demostró solamente en la fracción microsómica. Se efectuó un fraccionamiento más fino de la fracción microsómica, obteniéndose las fracciones membranosas del retículo sarcoplásmico en forma pura, que demostró ser el sitio intracelular de actividad de la LPL.

INTRODUCTION

The enzyme lipoprotein lipase (LPL) or clearing-factor lipase which hydrolysis triglycerides of lipoproteins to free faty acids (FFA) and glycerol, is usually defined by the following criteria: it is stimulated by heparin, it is inhibited both by protamine and by NaCl (Korn, 1957).

It has been shown to occur, in heart and lung tissues (Anfinsen *et al.*, 1952), in the endothelium of certain vessels (Robinson, 1963), in adipose tissue (Hol-

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lenberg, 1959; Cherkes and Gordon, 1959; Persson and Hood, 1970) and in the liver (Mayes and Felts, 1968; Naito and Felts, 1970; Felts and Berry, 1970), where it seems to be present in an inactive state.

It is likely that the enzyme is released by heparin to the circulation in man and other mammals (Whayne and Felts, 1970) from different sources, mainly the adipose tissue (Korn, 1955; Korn and Quigley, 1957). However, the behaviour of the enzyme regarding its availability to be released from the tissues by heparin is not the same in all of them. Brady and Higgins (1967), reported the failure to obtain any LPL activity from rat heart when fresh tissue was incubated with heparin. Robinson and Jennings (1965) found a greater total LPL activity in a heart acetone powder than that released from the intact organ on perfusion with heparin. This evidence seems to indicate that in the heart LPL is either in a site not accesible to the heparin or else it is bound in a stronger form than in other tissues.

There have been some attempts to find the intracellular localization of LPL activity in rat heart (Alousi and Mallov, 1964; Gartner and Vahouny, 1966). However, none of these authors have considered, in their cellular fractionations, that the heart has been taken as a example of a methodological problem in the preparations of cellular subfractions (Siekevitz, 1962). On the other hand, different substrates, usually activated fat emulsions from various sources have been used by the different workers, making more difficult the evaluation of these studies.

Calva *et al.* (1962) have shown that LPL from a rat-heart acetone powder extract transforms *in vitro* the human serum Sf 20-400 lipoprotein group into the Sf 0-20 group, according to the ultracentrifugation analysis of the incubation mixture. They have also pointed out that the enzymatic determination is more specific than the colorimetric method for the estimation of the glycerol released by the LPL.

These observations have made highly desirable to study in more detail the intracellular localization of LPL in the heart, using a more specific assay system both from the point of view of the substrate and that of the determination of the reaction product.

METHODS

Male albino rats of the Wistar strain and fed *ad libitum* with regular laboratory chow, weighing from 250 to 300 g were used throughout the experiments.

Preparation of the tissue fractions

The preliminar separation of the various subcellular fractions by differential centrifugation was made according to Cleland and Slater (1953).

The purified preparation of mitochondria was obtained as described by Calva *et al.* (1965). Heart nuclei were prepared following a procedure similar to that deviced by Moulé (1968) for liver nuclei. However, when 2.2 M sucrose solution containing 0.01 M Mg^{++} was used, the preparation was greatly contaminated by mitochondria. It became necessary to raise the sucrose concentration up to 2.6 M (+0.01 mM Mg^{++}) in order to obtain an uncontaminated nuclei preparation.

Two microsomal fractions were prepared with slight modifications, as described for liver (Delhumeau *et al.*, 1965). Fourteen rats were stunned, decapitated and allowed to bleed. Hearts

were quickly removed, rinsed in ice cold 0.25 M sucrose, freed from large vessels and rinsed again until the washing fluid appeared colorless. The excess of moisture was removed with filter paper and the hearts were cut down with scissors in small fragments. All the operations were performed at O°-4°C. The tissue (around 10 g) was weighed and divided into 2 g portions to be homogenized separately in a Potter-Elvehjem homogenized provided with a teflon loose pestle.

A 10% homogenate was prepared in 0.88 M sucrose in the following way: to each 2 g portion of tissue 7 ml of the sucrose solution were added, and homogenized for a minute by short ups and downs of the pestle (4 per second); then, 11 ml more of the sucrose solution were added and the homogenization continued for 30 seconds (one up and down per second). The homogenate was filtered through a double gaze to remove the course fragments and around 20% of the tissue was lost but the procedure was gentle enough to decrease the breakage of some structures and thus the contamination of the fractions. The homogenates was centrifuged for 20 minutes at 24,000 x g max. (International Centrifuge PR-2). The sediment containing unbroken cells, cell debris, nuclei and mitochondria, was discarded, and the supernatant used to obtain the two microsomal fractions as is shown in Fig. 1.

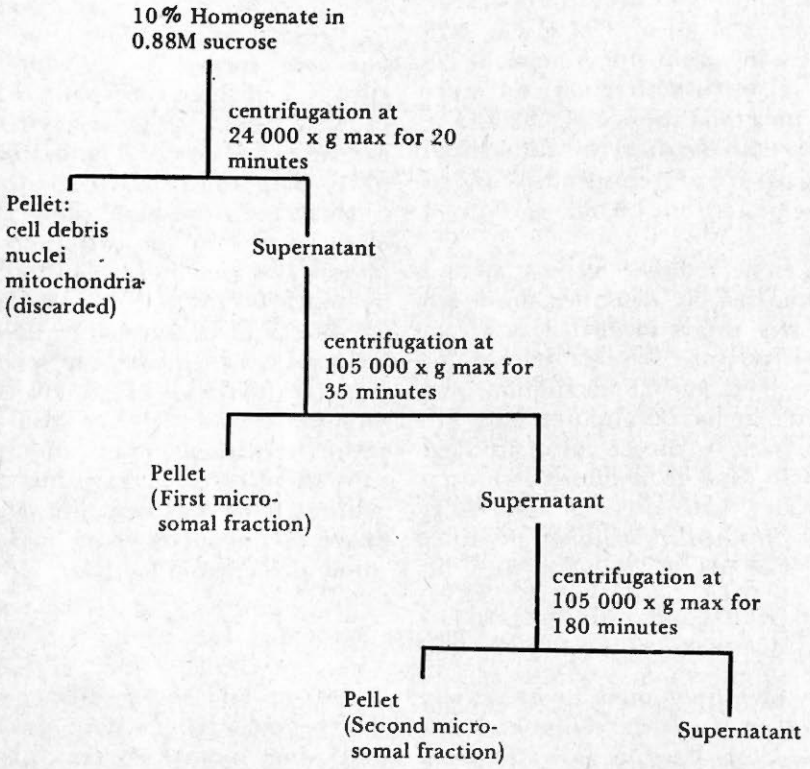

Fig. 1. Preparation of microsomal fractions from rat heart tissue.

The two microsomal fractions were subjected to a further fractionation according to the technique desbribed by Chauveau *et al.* (1962). Each one was resuspended separately in 6 ml of a 1.65 M sucrose solution and centrifuged for 20 hours at 105,000 x g. Three portions were obtained: a surface jelly-like pellicle, a clear supernatant fraction and a pellet. In the first microsomal fraction the yield of the surface layer was higher and the pellicle could be easily detached and removed by means of a fine stainless steel spatula. On the contrary, the pellicle from the second fraction was thinner and stuck to the wall of the tube all way down and even reached the pellet.

Standard incubation conditions

The incubation mixture contained: 1.5 ml of 1 M glycine-Na buffer, pH 9.45, 0.015 ml of heparin (Calbiochem, 1000 u/ml), 0.3 ml of 2 M $CaCl_2$, 0.75 ml of human serum lipoproteins Sf 20-400, 0.6 ml of tissue fraction and water to make up a total volume of 3.0 ml. The tissue fraction obtained by differential centrifugation was resuspended, unless otherwise stated, in 1.0 ml. of 0.25 M NH_4OH.

An acetone powder extract of rat heart, prepared as described by Korn (1955), was always incubated as a control. The mixture was maintained for 1 hour at 37°C, and 0.8 ml aliquots were taken at 0, 30 and 60 minutes. Each aliquot was received in 0.8 ml of ice cold 15% $HClO_4$ and then diluted with 2.4 ml of water, and after 15 minutes at 0°C was filtrated (Whatman #2 filter paper). The pH of 2.4 ml of each filtrate was adjusted to 9.0 with about 85 µl of 10 N KOH and the precipitate was spun down in the cold. A 1.0 ml of the supernatant was taken for the enzymatic determination of glycerol at 25°C in a UNICAM automatic recording spectrophotometer.

Protein determination was made by the Folin-Ciocalteau method. Lipoproteins Sf 20-400 from human serum were isolated according to De Lalla *et al.* (1954).

Chemical determinations of the microsomal factions

Each one of the microsomal pellets and the surface jelly-like pellicles were treated by the method described by Schmidt and Thannhauser (1945). In the first step proteins and nucleic acids were precipitated and washed once with 5% ice-cold $HClO_4$. Then, the precipitate was treated twice with ice-cold ethanol and three times with a mixture of ethanol-ether (3:1) at 65°C in order to extract the lipids. The combined extracts were concentrated to dryness at room temperature by blowing a current of nitrogen over the extract and phosphorus was determined in the residue by Macheboeuf and Del Sol method. To the lipid free material 1 M NaOH solution was added and the mixture incubated 18 hours at 37°C. Proteins were solubilized and RNA hydrolyzed. A sample was taken to determine nitrogen with the Nessler reagent after acid digestion. RNA was determined as phosphate in the supernatant by a modification of Mejbaum method.

RESULTS

A very high lipoprotein lipase activity was found in the nuclei, unbroken cells and cell debris fraction separated from rat homogenates according to the method of Cleland and Slater (1953), although the greatest specific activity was detected in the microsomal fraction (Table 1). This result is in general agreement with

those reported by Alousi and Mallov (1964) and by Gartner and Vahouny (1966). No activity was apparent in the final supernatant fraction and practically none in the mitochondrial fraction.

TABLE 1

LIPOPROTEIN LIPASE ACTIVITY DISTRIBUTION IN CRUDE FRACTIONS OF RAT HEART HOMOGENATES *

Subcellular fraction	Total activity nmoles of glycerol/hr/g. of fresh tissue	Specific activity nmoles glycerol/hr/ mg. protein
Total homogenate *	3220	38.1
Nuclei, unbroken cells, cell debris, etc.	2415	32.4
Mitochondria	46.8	3.84
Microsomal fraction	810	131
Final supernatant *	0.0	0.0

* Tested directly. The other fractions were resuspended in 0.025 M NH_4OH to make up a volume of 1.0 ml. as described in the text.

It seemed important to attempt a further purification of each subcellular fraction, in order to define the localization of LPL activity. A nuclear fraction separated from a heart tissue homogenate prepared in 2.6 M sucrose 0.01 mM Mg^{++} solution and appearing uncontaminated in the electron microscope showed no lipoprotein lipase activity, although the yield of this cell fraction was low (Fig. 2).

In the case of mitochondria, they were prepared with practically no contamination by other cellular particles, as shown by the electron microscope (Fig. 3); they had no LPL activity whatsoever. Since maximal specific activity was found in the microsomal fraction (Table 1) a further fractionation was undertaken as described above.

The LPL activity in the first microsomal fraction represent 71.1% of the total microsomal activity and also had the highest specific activity (Table 2). The absolute values of the enzyme activity varied from one experiment to another (high standard deviations). However, the relative distribution (%) of the activity among fractions remained fairly constant.

The first microsomal fraction represents 84.8% of the phospholipid phosphorus (P-PL) contained in both microsomal fractions, and it also shows to be richer in RNA phosphorus (P-RNA) (64.9%), although the relation $\frac{\text{P-RNA}}{\text{N}}$

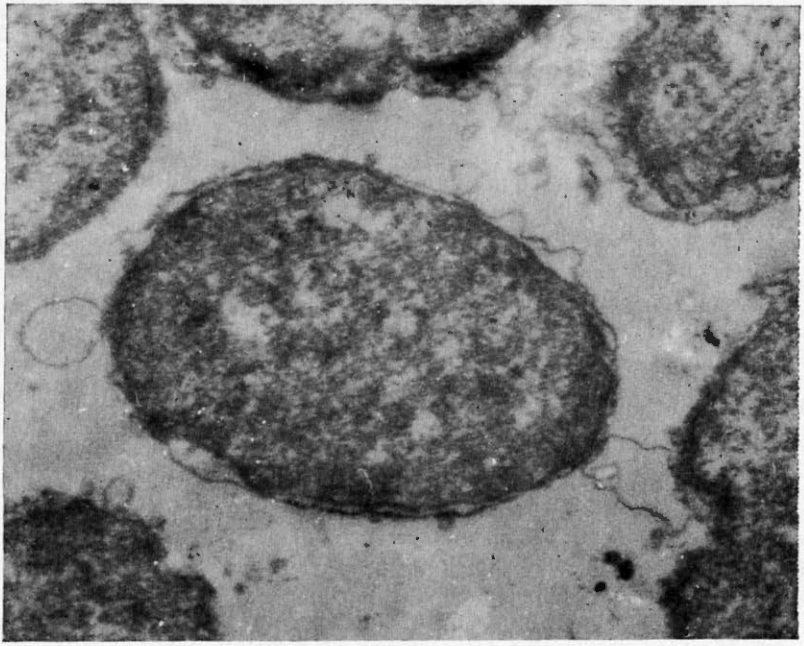

Fig. 2. Purified nuclear fraction (x 25 200).

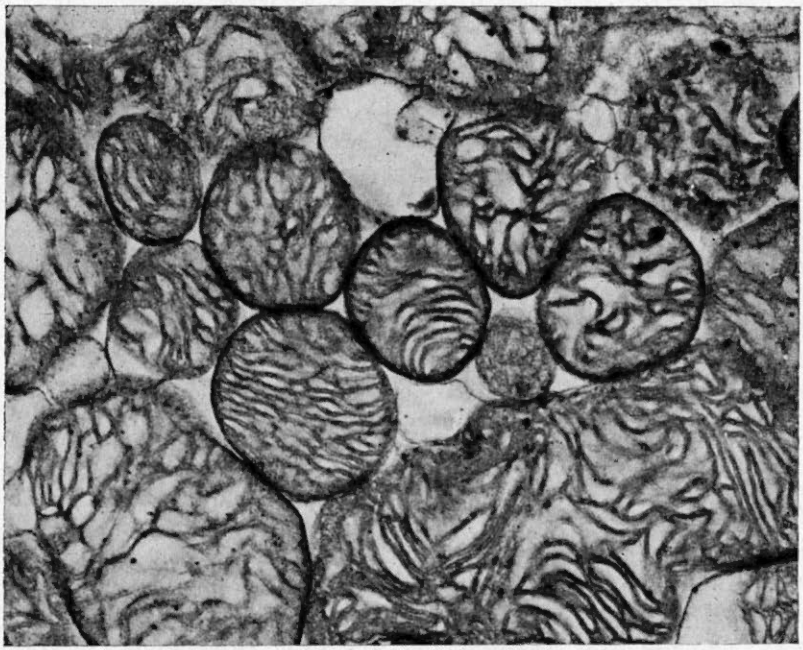

Fig. 3. Purified mitochondrial fraction (x 25 200).

TABLE 2

LIPOPROTEIN LIPASE ACTIVITY IN MICROSOMAL FRACTIONS ISOLATED FROM RAT HEART HOMOGENATES *

Microsomal fraction	*Total activity*		*Specific activity*
	nmoles glycerol/ hour, g fresh tissue	*% of total activity*	*nmoles glycerol/ hour, mg protein*
First	1912 ± 428	71.1 ± 2.5	3320 ± 571
Second	767 ± 163	28.9 ± 2.5	1707 ± 327

* Average values from four experiments ± standard deviation.

TABLE 3

CHEMICAL COMPOSITION OF THE MICROSOMAL FRACTIONS ISOLATED FROM RAT HEART *

	First fraction	*Second fraction*
P-PL (μg/g fresh tittue)	84.8 ± 3.4	15.2 ± 3.4
P-RNA (μg/g fresh tittue)	64.9 ± 6.1	35.1 ± 6.2
$\frac{\text{P-RNA}}{\text{N}}$	40.9 ± 3.6	52.9 ± 18.8
μg N/g fresh tissue	145.7 ± 23.5	58.5 ± 3.3

* Average values from five experiments ± standard deviation.

is around the same in both fractions (Table 3). As far as N is concerned, again a higher value is found in the first fraction. Electron micrographs show that the first fraction is very rich in smooth vesicles (membranes) and poor in free or bound ribosomes (Fig. 4). The second fraction contains smaller vesicles and is richer in ribosomes, specially in the free-state (Fig. 5).

These results suggest that LPL activity is bound to the cardiac cell membranes. It has been shown that enzymes of the microsomal fractions in various tissues are usually associated to the membranes (Moulé, 1968). However, in the case of rat heart it seem desirable to attempt preparations of a purer membranous fraction to determine whether LPL activity is only located there.

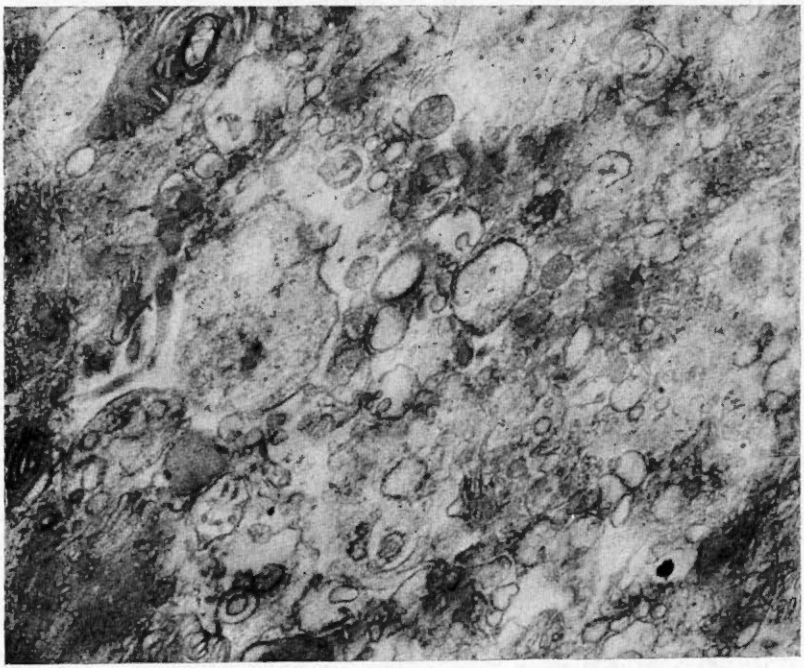

Fig. 4. First microsomal fraction (x 25 200).

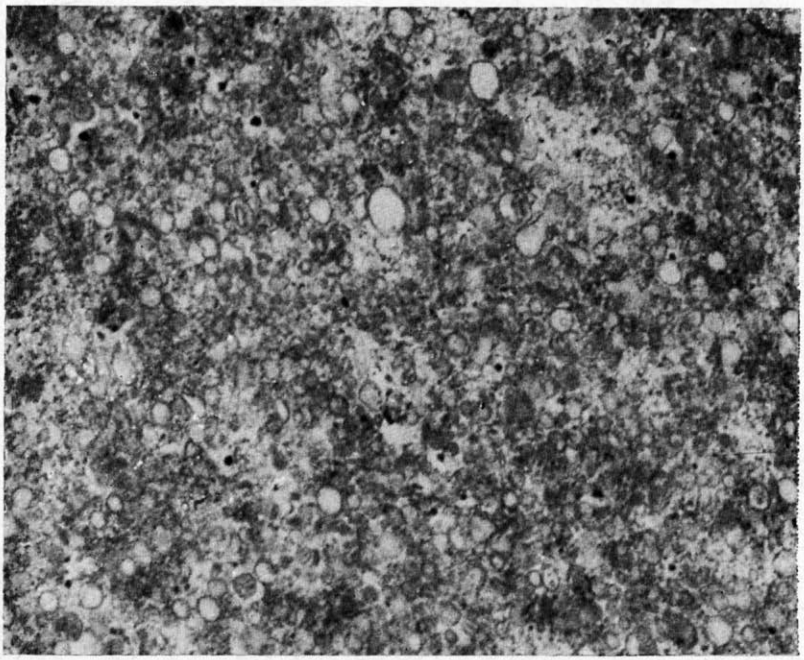

Fig. 5. Second microsomal fraction (x 28 300).

The absolute values of the enzymatic activity are lower than those of the original fractions (Tables 1 and 4). This decrease is probably due to a partial inactivation of the enzyme during the 20 hours of centrifugation or to the separation of an activator carried out in the other fractions.

In agreement with the data in Table 4, most of the total activity was found in the first microsomal fraction, and within each fraction, it is totally localized in the membranes. The second microsomal fraction is poor in membranes and has only around 15% of the total activity. Due to the contamination of this pellet by the pellicle it was not surprising to find most of the activity in the pellet.

TABLE 4

DISTRIBUTION OF LPL ACTIVITY IN THE MICROSOMAL SUBFRACTIONS FROM RAT HEART

Microsomal fraction	*Microsomal subfraction* *	*Total activity mμ Molesglyceroy/hr/g fresh tissue*	*% of total activity*	*Specific activity mμ Moles glyceroy/hr/mg protein*
First	Membranes	428	85	696
	Unsedimented fraction	0.0	0.0	0
	Pellet	0.0	0.0	0
Second	Membranes	17.1	3.4	891
	Unsedimented fraction	0.0	0.0	0
	Pellet	58.5	11.6	863

* Each one of the membranous fractions and the pellets were resuspended in 0.025 M NH_4OH to make up a 1.0 ml. volume. The incubation mixture included a 1.65M sucrose concentration.

The unsedimented fractions were added directly to the incubation mixtures, which included NH_4OH to give the same final concentration in all the tubes.

The rest of the experimental conditions were as described in the text.

The chemical composition of the membranes and pellets is shown in Tables 5 and 6. As expected, the highest values of P-PL per gram of fresh tissue were found in the membranes of the first microsomal fraction. However, the membranes of the second microsomal fraction seem to have a higher P-PL/N ratio than those from the first microsomal fraction. Both membrane fractions contain RNA and again the second-fraction vessicles seem to have a higher P-RNA/N ratio. However, it was interesting to find the highest amount of P-RNA/g of fresh tissue localized in the first-fraction membranes. The clear supernatant subfractions were studied and they contained phospholipids (first fraction: 1.575 μg P/g fresh tissue, 156 μg P/mg N; second fraction: 1.28 μg P/g fresh tissue, 58 μg P/mg N), as well as RNA (first fraction: 0.30 μg P/g fresh tissue, 30 μg P/mg N; second fraction:

0.43 μg P/g fresh tissue, 20 μg P/mg N). Most of the phospholipid phosphorus contained in the whole microsomal fraction is obtained in the membranes, as well as a definite amount of RNA (Tables 5 and 6).

TABLE 5

CHEMICAL COMPOSITION OF THE FIRST MICROSOMAL SUBFRACTION

	membranes	*Pellets*
P-PL (μg/g fresh tissue)	8.8 ± 1.1	0.37 ± 0.13
P-RNA (μg/g fresh tissue)	1.95 ± 0.35	0.38 ± 0.17
P-PL/N	121.6 ± 3.8	60.3 ± 21.0
P-RNA/N	27.0 ± 0.95	66.1 ± 37.0

TABLE 6

CHEMICAL COMPOSITION OF THE SECOND MICROSOMAL SUBFRACTION

	Membranes	*Pellets*
P-PL (μg/g fresh tissue)	0.8 ± 0.2	0.46 ± 0.13
P-RNA (μg/g fresh tissue)	0.20 ± 0.06	0.53 ± 0.12
P-PL/N	192 ± 0.3	38.3 ± 6.2
P-RNA/N	50.3 ± 8.4	44.1 ± 4.5

The electron micrograph of the first subfraction showed practically pure preparation of smooth membranes (Fig. 6). The pellet of the first microsomal fraction was small whereas that of the second microsomal fraction was larger with a thin pelliole at the top that stickes to the wall of the tube all the way down and even covers this second pellet.

Using the membrane subfractions as the source of LPL the optimum pH was found to be 9.45 as previously described by Calva *et al.* (1962) for myocardial acetone powder extract and human serum lipoproteins Sf 20-400. The enzymatic activity was inhibited 65% by 5×10^{-5} M protamine and 80% by 0.4 M NaCl.

The clear supernatant subfraction of the first and second microsomal fractions were centrifuged down for 20 more

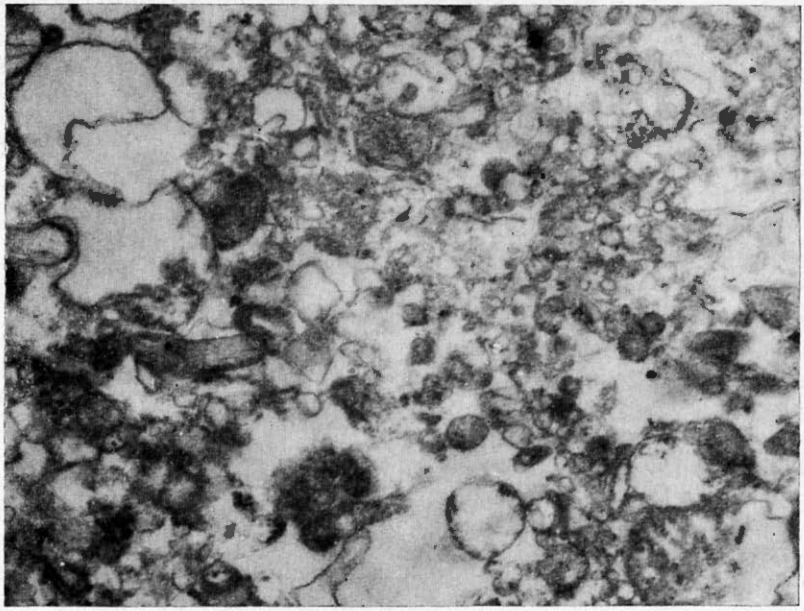

Fig. 6. Membranes of the first microsomal subfraction (x 25 200).

hours at 105,000 g. New pellets were obtained showing LPL activity and chemical characteristics similar to the second microsomal fractions. This observation agree with that of Siekevitz (1962), who still obtained particles from the heart "final supernatant" after 14 hours of high speed centrifugation. In the "true final supernatant" again no LPL activity could be detected.

DISCUSSION

Data found in the literature concerning the intracellular localization of lipoprotein lipase activity in the heart are in general agreement with our results.

However, the general methods of differential centrifugation used in such works to separate the cell fractions were applied without any further purification and without taking into account the special technical problems inherent to heart tissue.

Gartner and Vahouney (1966), as well as Alousi and Mallov (1964) found LPL activity distributed among the different fractions; the maximal total activity was associated with the nuclear fraction and the maximal specific activity located in the microsomal fraction. These observations were confirmed by our data when a crude separation of the homogenate was prepared (Table 1). Moreover, Schnatz *et al.* (1963) using 11-28 hours post-mortem human hearts found the activity mostly associated with the "heavy particles". These findings are not surprising if one accepts that in the type of preparations the so called nuclear fraction is heavily contaminated by intact and partially broken cells. When a purer preparation of nuclei is obtained (Fig. 2), LPL activity was no longer demostrable in it.

The activity shown in the mitochondrial fractions is also probably due to a contamination by the membranous fraction, since none could be detected when

purer mitochondria were prepared (Fig. 3).

Finally, some workers found LPL activity in the so called cell sap. It is apparent from our data, which confirm the observations reported by Siekevitz (1962) that very small particles still remain in suspension after a routinary separation of the myocardial microsomal fraction. In our experiments no LPL activity was found in the supernatants.

To our knowledge the present work is the first attempt to fractionate further the microsomal fraction from rat heart by taking advantage of the differences in density between the various subcellular components. It proved to be an adecuate procedure to localize an enzymatic activity in such a heterogenous fraction.

Lipoprotein lipase activity was found associated to the rat heart membranous fraction and this localization may be of great importance to the heart, if one considers certain known facts of myocardial metabolism. Accumulating evidence indicates that free fatty acids serve as oxidizable substrates for the heart and, hence as a source of energy (Craig, 1963; Crass and Meng, 1966; Borensztajn and Robinson, 1970) and that the activity of LPL of the rat heart rises notably when the animals are starved for periods of 10-24 hr (Borensztajn *et al.*, 1970). Furthermore, the needs for such substrates seem to vary in relation to changes in heart work and in carbohydrate utilization by this organ (Allousi and Mallov, 1964). Lipoprotein lipase hydrolyses triglycerides to fatty acids and glycerol when they are associated to serum lipoproteins (Korn, 1957; Calva *et al.*, 1962; Bier, 1970; Fielding, 1970). It has been shown that the rat myocardium has the ability to remove circulating dietary chylomicron triglycerides and that the enhancement of LPL activity by heparin facilitates the utilization of free fatty acids by the heart (Crass and Meng, 1966). Borensztajn and Robinson (1970) suggests that correlation between triglycerides fatty acids uptake and LPL activity should only be sought in that portion of the total tissue activity which is capable of being released from the intact organ by heparin, and that only the heparin-releasable enzyme is concerned with triglycerides fatty acids uptake. On the other hand, when several hormones and dietary modifications induced changes in the utilization of fatty acids in the heart, concomitant changes in cardiac LPL activity were reported (Allousi and Mallov, 1964). This was suggested also for rat adiposites by Patten (1970). These observations underline the involvement of LPL activity in the general metabolism of the heart as well as point out that the enzyme should be located at a cell- environment interfase (Allousi and Mallov, 1964).

According to our data the enzyme is located at a interfase, since the vacuolar system has been suggested to represent such a cell environmental interfase (De Robertis *et al.*, 1965).

It is of interest to recall that LPL is not totally released by heparin from the heart (Brady and Higgins, 1967; Robinson and Jennings, 1965; Borensztajn *et al.*, 1970) as it is from other tissues (Korn, 1955); thus indicating that the enzyme is either at a site not easily reached by the extraction medium or it is bound in a stronger form that in other tissues. Neither of these posibilities can actually be completely ruled out because, even though the enzyme is associated to a structure that should be accesible to an extraction medium, nothing is know about how, within the membrane, is bound the enzyme.

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