INTRACELLULAR DISTRIBUTION OF A PRIMATE-SPECIFIC ESTERASE IN CULTURED CELLS AND TISSUES

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INTRODUCTION

The carboxylesterases are a most complex group of esterases, and there is little knowledge of their natural substrates or their biological function (Beckman and Hauge, 1966; Myers, 1960). Studies in our laboratory on a primate-specific carboxylesterase isoenzyme have led to its purification (Munjal and Rose, 1972; Therrien et al., 1971) and characterization (Bartholomew et al., 1971; Bartholomew et al., 1972; Munjal and Rose, 1973; Vladutiu and Rose, 1973). Changes in the esterase activity in cultured cells have also been studied during mycoplasmal infection (Rose et al., 1972). SV40 transformation (Bartholomew et al., 1969), and in cell hybridization (Bartholomew et al., 1971). Briefly, the primate esterase is antigenically distinct, having been detected only in cells and tissue of human and monkey origin. It migrates cathodally during zonal electrophoresis. It uses two-carbon chain esters optimally as substrates and is inhibited by 10^{-4} M diisopropylfluorophosphate but not by eserine. Its molecular weight is approximately 136,000. The activity of the esterase is greatly reduced in SV40-transformed cells. This report deals with localization of the esterase by subcellular fractionation using immunological methods for the assay of the esterase activity.

MATERIALS AND METHODS

Cells

The FL human amnion cell line, derived from human amnion tissue (Fogh and Lund, 1957), was used in this study. These cells were originally obtained from Microbi-

ological Associates, Bethesda, Md. The cells were cultured in Eagle's basal medium (BME) (Grand Island Biological Co., Grand Island, N. Y.) with 10% fetal calf serum (North American Biologicals, Inc., Rockville, Md.) in large roller bottles 475 mm × 110 mm with a growth surface area of 1,320 cm² (New Brunswick Scientific Co., Inc., New Brunswick, N. J.). A suitable cell pellet for fractionation (2–4 g wet weight) was obtained from approximately 10 roller bottle cultures grown for 5 days at 37°C.

Tissues

Normal rhesus monkey kidney and liver tissues and Buffalo strain rat liver were taken immediately after exsanguination. A single human kidney specimen was obtained within 2 h after death. It was found that an 8-g sample of tissue was optimal for fractionation. The tissue specimens were immediately submerged in tris-buffered 0.25 M sucrose, pH 7.0, at 4°C, finely minced, and washed repeatedly in cold sucrose solution to remove whole blood.

Cell Homogenization

A washed pellet of FL cells was resuspended in 0.25 M sucrose buffer (1:1 wt/vol) and homogenized in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 750 g for 5 min. The resulting pellet was resuspended and homogenized two more times. The final pellet combined with both supernates constituted the whole homogenate.

Tissue Homogenization

The minced tissue was homogenized by hand in a ground glass TenBroeck tissue homogenizer (VWR Scientific, Buffalo, N. Y.) in 0.25 M tris-buffered sucrose, pH 7.0, (1:3 wt/vol), until all visible pieces of tissue were dispersed into a homogeneous suspension.

Fractionation

The fractionation procedure employed a combination of differential centrifugation and density gradient centrifugation to prepare heavy mitochondrial, lysosomal, microsomal, and soluble fractions for further analysis. All centrifugations up to 17,300 g were carried out in a Sorvall RC2-B centrifuge using the SS-34 and GSA rotors (Ivan Sorvall, Inc., Newtown, Conn.). For higher speed centrifugations a Spinco Model L centrifuge was used with a no. 40 rotor or an SW-39L rotor for the density gradient centrifugation (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). An outline of the procedure for cell fractionation is depicted in Fig. 1.

Testing of Fractions

Samples of five fractions including the whole homogenate, the heavy mitochondrial, lysosomal, microsomal, and soluble components were assayed for cathodal esterase activity and acid phosphatase activity. The lysosomal pellet was examined by electron microscopy for purity.
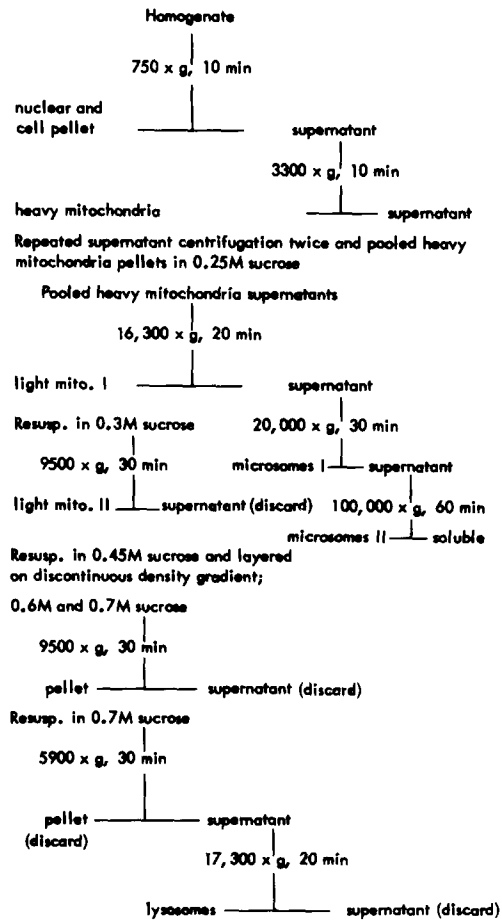

FIGURE 1   Subcellular fractionation procedure.

Esterase Activity Assay

A quantitative estimation of esterase activity was determined by the radial immunodiffusion technique of Mancini et al. (1964). Glass slides (80 × 10 mm) were layered with 13 ml of a 1% agarose solution prepared in equal volumes of triple distilled water and phosphate-buffered saline (PBS), pH 7.2 and containing 1% of a standard antiesterase rabbit serum. The antiserum had been prepared as previously described using an ammonium sulfate-precipitated fraction of human urine as a source of esterase for immunization (Therrien et al., 1971). Wells were cut in the antibody-containing gel, 4 mm in diameter, 10 mm apart, and each containing 20 μl of test sample. Dilutions of a kidney extract, used as a standard source of esterase (Vladutiu and Rose, 1973), were placed in the first three wells of each plate. The plates were incubated for 24 h at room temperature in a moisture chamber and then washed for 24 h in PBS at 4°C. The plates were then stained, without drying, for esterase activity using indoxyl acetate as a substrate. Based on the diameters of the stained immune precipitate rings, a curve for the standard kidney samples on each plate was plotted and the number of esterase units for each test was determined for the curve. Arbitrarily, the greatest migration distance of the concentrated standard was set at 100 U. Specific esterase activity was determined by dividing units by protein concentration and was termed relative since the units obtained were related to an arbitrary kidney standard.

Acid Phosphatase Activity

A colorimetric method was utilized for the determination of acid phosphatase activity (Sigma Chemical Co., St. Louis, Mo.) using p-nitrophenyl phosphate as a substrate. A calibration curve was prepared using p-nitrophenol. One Sigma unit of acid phosphatase liberated 1 μM p-nitrophenol/h (1 μM = 0.1391 mg). Units were determined on this basis. The specific activity was determined by dividing the number of units in each sample by the protein concentration of the sample.

Electron Microscopy

Electron microscope examination of the lysosomal pellet provided a criterion for the assessment of purity. The electron microscope study was kindly performed by Doctor G. Andres (Department of Pathology, State University of New York, Buffalo, N. Y.).

RESULTS

Subcellular Fractionation

FL human amnion cells and rhesus monkey liver were studied extensively for the localization of primate-specific esterase. Due to the inavailability of fresh human tissue (taken within 1 h after death), a comparison with human liver was not

possible. However, the single fresh human kidney specimen was utilized along with a rhesus monkey kidney sample and such a comparison was made.

The fractionation techniques employed in this study were particularly designed to prepare pure lysosomes. Consequently, low yields were expected. Acid phosphatase was used as a marker enzyme for the lysosomal fraction.

Fig. 2 is an electron micrograph of a lysosomal pellet isolated by differential and density gradient centrifugation. Dense, spherical, granule-containing bodies may be seen with single limiting membranes. Many of them are primary lysosomes with no evidence of ingested material. Several other secondary lysosomes may be seen containing fragments of ingested particles. Still others appear to be shells of lysosomes devoid of their granules. There are also a few strands of endoplasmic reticulum in the field.

Acid Phosphatase

Table I shows the combined distribution of acid phosphatase in the subcellular fractions of FL human amnion and rhesus monkey liver cells. The recovery of acid phosphatase in the fractions studied was not high. 22% of the total acid phosphatase in the FL cell homogenate and 38% of the acid phosphatase in the monkey liver homogenate were recovered in the four fractions tested. In preliminary tests, it was found that much of the total activity (25-40%) in both species was lost during the sequential separation of lysosomes from mitochondria. The FL cell lysosomal pellet represented only about 0.08% of the total protein in the homogenate and contained 0.2% of the total acid phosphatase. The distribution was similar in monkey liver lysosomes which contained 0.06% of the total protein and 0.27% of the total acid phosphatase.

The ratio of the specific activities of the various fractions to the specific activity of the whole homogenate was significantly greater in the lysosomal fractions of both species.

Esterase

Table II shows the relative specific activities of cathodal esterase in the subcellular fractions of FL amnion and monkey liver cells. The total esterase recovered in the four fractions studied was 74.4% from FL amnion cells and 59.7% from monkey liver. These percentages were higher than the

corresponding yields of acid phosphatase. The nuclear pellet of both species contained 17-27% of the total esterase activity. The lysosomal pellet of both species had the highest specific esterase activity of all fractions, and far higher than that of the homogenate. The esterase content of the lysosomal pellet, however, represented only 2.5% of the total esterase activity in FL amnion cells and 1.7% of the total activity in monkey liver cells.

Comparison of Esterase and Acid Phosphatase

Since acid phosphatase is considered to be primarily a lysosomal enzyme, its presence in other fractions was taken to indicate contamination of those fractions with lysosomes. If acid phosphatase is found in the soluble fraction, for instance, it is assumed that a certain portion of the lysosomes lysed during the fractionation, releasing acid phosphatase. A ratio of the percent total acid phosphatase in the soluble fraction to the percent in the lysosomes shows 95.5 times more acid phosphatase in the soluble fraction of monkey liver and 72.6 times more acid phosphatase in the soluble fraction of FL cells. However, in comparison, the proportion of total esterase in the soluble fraction to the proportion in the lysosomes was only 22.9 and 24.2 for monkey and FL, respectively. If one assumes that the esterase is predominantly lysosomal, then when the lysosomes broke, releasing acid phosphatase, esterase was not released to the same degree. It may have remained more tightly bound to the lysosomal membrane.

We also compared the specific acid phosphatase activities in the subcellular fractions of different species and of tissues from different origins. Rat gave the highest recovery, 13.3, which was twice as high as the recovery from human cells and more than twice as high as the recovery from monkey. A comparison among tissues showed that human and monkey kidney lysosomes had a greater enrichment in acid phosphatase content than did monkey liver lysosomes.

A comparison of esterase activity in the subcellular fractions of various tissues of human and monkey origin showed that the monkey liver lysosomal fraction had the lowest specific activity compared to the lysosomal fractions of human and monkey kidney and FL cells. The ratios of the esterase enrichment in the lysosomal fraction over
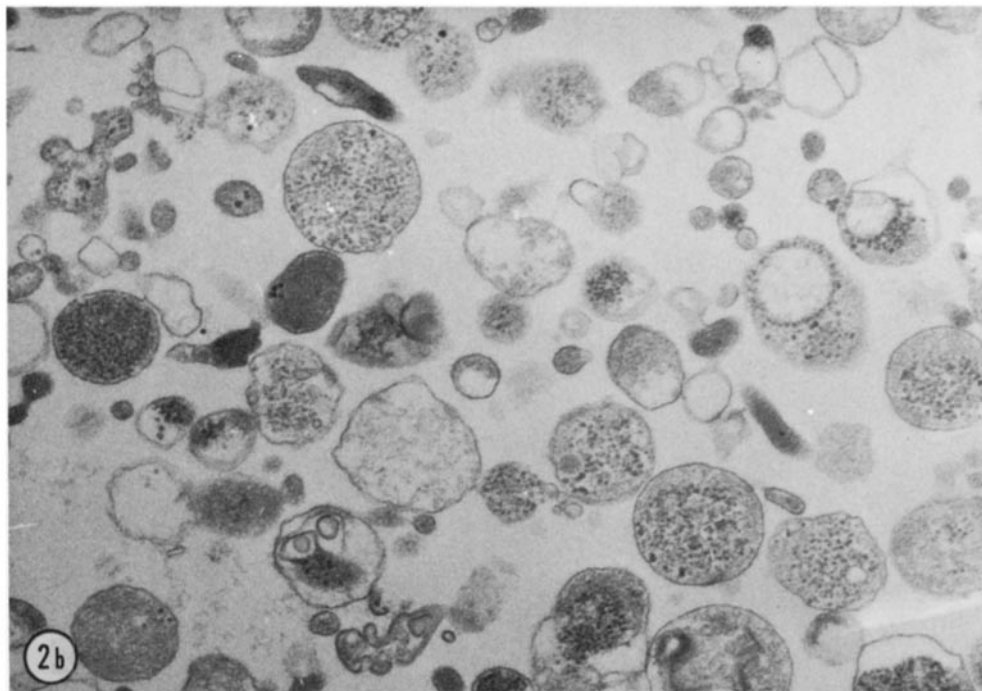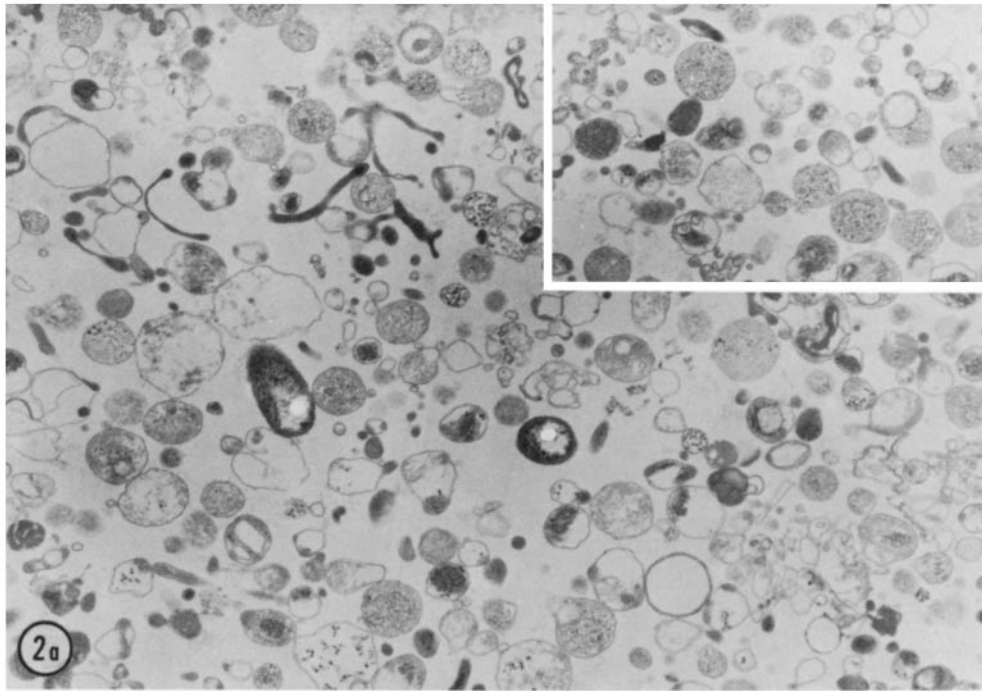
FIGURE 2 *a* Electron micrograph of a section of lysosomal pellet isolated from FL amnion cells. Photographed at × 12,000.

FIGURE 2 *b* Inset from Fig. 2 *a* demonstrates membrane-bound lysosomal structures. × 120,000.

TABLE I

Distribution of Acid Phosphatase and Protein in Subcellular Fractions of FL Human Amnion Cells and Rhesus Monkey Liver

Fraction	FL Cells			Monkey Liver		
	Specific activity	R	Total phosphatase	Specific activity	R	Total phosphatase
			%			%
Homogenate	0.79 ± 0.07	1	100	0.37 ± 0.01	1	100
Mitochondria	1.81 ± 0.32	2.30	6.54 ± 1.74	0.54 ± 0.08	1.46	8.66 ± 2.24
Microsomes	1.26 ± 0.08	1.60	1.50 ± 0.35	0.76 ± 0.13	2.06	3.15 ± 1.08
Soluble	1.05 ± 0.36	1.33	13.80 ± 0.94	0.41 ± 0.06	1.11	25.80 ± 0.67
Lysosomes	5.44 ± 0.46	6.90	0.19 ± 0.06	1.66 ± 0.13	4.48	0.27 ± 0.07

Specific activities are expressed as units per hour per milligram protein (±SE) where $1 \, U = 1 \, \mu M$ p-nitrophenol liberated/h/mg protein. R is the ratio of specific activity of the respective fraction to the mean specific activity of the homogenate.

TABLE II

Distribution of Cathodal Esterase in Subcellular Fractions of FL Human Amnion Cells and Rhesus Monkey Liver

Fraction	FL Cells			Monkey Liver		
	Relative specific activity	R	Total esterase	Relative specific activity	R	Total esterase
			%			%
Homogenate	0.47 ± 0.05	1	100	0.38 ± 0.04	1	100
Mitochondria	1.04 ± 0.20	2.22	6.95 ± 0.94	0.58 ± 0.05	1.53	11.44 ± 2.16
Microsomes	1.01 ± 0.23	2.14	4.34 ± 1.05	0.82 ± 0.21	2.16	6.41 ± 2.21
Soluble	1.62 ± 0.63	3.44	60.60	1.29 ± 0.46	3.40	40.10 ± 3.64
Lysosomes	12.86 ± 2.07	27.40	2.49 ± 0.05	8.45 ± 0.86	22.20	1.75 ± 0.60

Relative specific activities are expressed as units per milligram protein where relative units were determined by quantitative immunoelectrophoresis.

the homogenate were all quite close, with FL cells having the highest.

DISCUSSION

Localization of a primate-specific esterase by combined differential and density gradient centrifugation showed a subcellular distribution, as measured by its relative specific activity, similar to acid phosphatase. There was a parallel increase in the specific activity of esterase and acid phosphatase in the lysosomal fraction compared with the whole homogenate of both rhesus monkey liver and FL amnion cells. The purity of the lysosomal fraction was assessed not only by the increase in acid phosphatase specific activity but also by electron microscope examination of the pellet. Electron-dense organelles, limited by a single unit membrane, appeared to have the characteristics of lysosomes.

DeDuve (1971) previously demonstrated that lysosomes were mixed with mitochondria in the light mitochondrial fraction. He recognized a major problem in separating the two organelles. The effectiveness of the method of Sawant et al. (1964) in separating lysosomes from mitochondria is noteworthy. Using the Waring blender for homogenization (Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.), Sawant et al. (1964) obtained a 10–15% yield of total acid phosphatase activity in the lysosomal pellet with a specific acid phosphatase activity increase of nearly 70 times the activity of the homogenate. However, most investigators report increases in specific activity of 10–27 with a yield of lysosomal acid phosphatase of less than 7% (Beaufay, 1972). The yield of acid phosphatase obtained in the present study of FL amnion and monkey liver homogenates was only 0.2–0.3% of the total acid

phosphatase in the homogenate, but the increase of specific activity in the lysosomes was 4.5 to 7-fold.

There are several possible explanations for the lower yield and specific acid phosphatase activity in our studies. First, the method of homogenization may influence the yield and specific activity of enzymes. When we used the blender method described by Sawant et al. (1964) for the homogenization of monkey liver, a very poor recovery of acid phosphatase activity in the lysosomal fraction was obtained when compared to rat liver using the same method. However, homogenization of monkey liver with the TenBroeck homogenizer was more efficient in preserving lysosomes as measured by acid phosphatase specific activity. A difference in the sensitivity of primate lysosomes, compared with rat lysosomes, to homogenization by blending may therefore be the explanation. The increment in specific acid phosphatase activity in the rat lysosomes was 13.3 which was within the acceptable range of 10–27 previously reported (Beaufay, 1972). Therefore, the use of Sawant's homogenization procedure in our laboratory gave comparable results in the purification of rat liver lysosomes but not monkey liver lysosomes. Secondly, in monkey liver homogenates, 30% of the acid phosphatase was lost in the first nuclear and cell debris pellet and 22% was lost in the corresponding FL cell pellet which may further account for the overall low yield.

In the general consideration of all fractions there is higher total esterase than acid phosphatase activity in the soluble fraction of both FL cells and monkey liver cells. Bowers and DeDuve (1967) obtained similar results in fractionation studies of rat spleen. In rat spleen an esterase appeared to be partly lysosomal but with much greater levels in the soluble fraction than was found with most other lysosomal enzymes. These authors agreed with the proposal of Shibko and Tappel (1964) and Barrow and Holt (1971) that the esterase may be loosely attached to the surface of lysosomes and be translocated onto the microsomes upon homogenization. At any rate, esterase would be released more readily than the intralysosomal hydrolases, and therefore be more abundant in the soluble fraction. At the same time, the esterase that remained on the lysosomes would not display latency and appear to be more concentrated upon analysis than the latent hydrolases, as is also suggested by our data.

Lysosomes are believed to be involved in the control of cell division. A rearrangement and loss of lysosomes has been found to precede cell division. Also, treatment of cells in such a way as to make their lysosomal membranes permeable will induce division. Conversely, stabilization of lysosomal membranes with certain agents retards cell division (Allison and Mallucci, 1964 a).

The findings of Shibko and Tappel (1964) and Barrow and Holt (1971), associating an esterase on the membrane of rat lysosomes with the possibility of its functioning in membrane permeability, bring these studies together with those of Allison and Mallucci (1964 b) concerning the effects of lysosome permeability on cell division. The evidence, of course, is not conclusive but it does stimulate speculations which could lead to a determination of the biological function of the esterase in cell division.

SUMMARY

A cathodally migrating esterase isoenzyme has been localized in cultured FL human amnion cells and monkey liver tissue by subcellular fractionation using differential and density gradient centrifugation methods.

A ratio of the percent of total enzyme activity in the soluble fraction to the lysosomal fraction suggested that a greater proportion of esterase than acid phosphatase remained bound to the lysosomes.

A comparison of different human and monkey tissue fractions demonstrated the highest relative specific acid phosphatase and esterase activities to be lysosomal. The lysosomal activity of both enzymes was particularly high in kidney lysosomes of both man and monkey.

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