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Lysosomes
placenta
peroxisomes

Subcellular Constituents of Human Placenta. I. Isolation and Characterization of Lysosomes from Term Tissue

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Extract

Human term placental lysosomes have been isolated and their quantitative density distribution has been established by biochemical and morphologic analysis. Histologic examination of placental villi after passage through the tissue press reveals minimal contamination due to platelets and leukocytes. Fluorescence microscopy with acridine orange demonstrates lysosomes in the syncytium. Differential centrifugation yields two lysosome populations. One segregates with endoplasmic reticulum, whereas the other is similar to lysosomes isolated from other tissues. The classic lysosomes have a peak density of 1.195 g/ml and are heterogeneous with respect to size and distribution within the density gradient. Acid phosphatase is differently distributed than the other acid hydrolases and is not a suitable marker for placental lysosomes. The enzyme cleaving glucose 6-phosphate is broadly distributed and not necessarily identical with the glucose 6-phosphatase (EC. 3.1.3.9) of other tissues. Peroxisomes are absent from term placenta.

Speculation

Placental lysosomes are considered to play a role in fetal-maternal transport, cellular differentiation, and remodeling. The organelle may be affected by sequential fluxes in steroidal sex hormones. Present knowledge of the characteristics of placental lysosomes and their role in these physiologic processes is limited. Isolation and characterization of lysosomes from placentas of different gestational ages will provide answers to these questions.

Introduction

The isolation and characterization of lysosomes from various tissues is well described; liver [2, 26, 31, 33], skeletal muscle [10], kidney [36], lymphoid tissue [8], polymorphonuclear leukocytes [11], and platelets [14] have been the most widely used sources. Contractor [12] demonstrated the existence of lysosomes in human placenta but did not provide extensive quantitative characterization. Subsequently, Schultz and Jacques [35] partially characterized lysosomes isolated from rat chorioallantoic placentas. In this paper we describe the isolation of human term placental lysosomes and their quantitative subcellular distribution as established by biochemical and morphologic analysis.

The unique physiology of the placenta offers an interesting environment for lysosomes. The tissue has a limited life cycle, undergoes extensive cellular modifi-

cation, participates in fetal-maternal transport, and is influenced by sequential fluxes of steroidal sex hormones. It is claimed that lysosomes are critically involved with each of these events. The latter topic is of special interest to investigators who seek to elucidate mechanisms which affect the lysosome and possibly regulate tissue function through lysosomal enzyme activities [17]. Szego *et al.* [40] explored the *in vivo* effect of sex hormones on lysosomes from rat preputial glands. Using 17β-estradiol, diethylstilbesterol, and testosterone, these authors found a labilizing effect which they attributed specifically to the steroid structure. Hempel *et al.* [22] have reported that an as yet unknown substance from 3rd trimester pregnancy sera stabilizes isolated polymorphonuclear leukocyte lysosomes.

Changes in hormone levels during the life cycle of the placenta may provide experimental models for the investigation of the involvement of lysosomes in these physiologic events.

Materials and Methods

Materials

Sucrose, density gradient grade, and crystalline bovine albumin, *fraction V,* were bought from Schwarz-Mann [44]. Disodium β-glycerophosphate (*grade I*), *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, phenolphthalein glucuronic acid dipotassium salt, cytochrome *c* (horse heart *type III*), succinic acid, monosodium glucose 6-phosphate, *p*-nitrocatechol sulfate, and *p*-nitrophenol were obtained from Sigma Chemical Company [45]. Benzylamine and 2,4-diaminophenol dihydrochloride were purchased from Eastman Kodak [46]. Levor IV was procured from Technicon Corporation [47]. All other chemicals were of reagent grade and were acquired through regular supply channels.

Preparative Procedures

Homogenization. Placenta from uncomplicated term vaginal deliveries was obtained at the time of delivery and immersed in cold 0.29 M sucrose solution. Placentas were fractionated within 2 hours after delivery. One-inch cubes of placenta were transferred to a tissue press with 1.3-mm grid, finely minced with scissors, and washed with cold 0.29 M sucrose solution until the filtrate showed a pale pink color. The washed tissue was pressed through the grid and collected. All steps were carried out in a cold room at 4°.

Thirty-milliliter aliquots of 1:2 (w/w) suspension of placental tissue in 0.29 M sucrose solution were transferred to a smooth-walled Teflon and glass homogenizer [48] with a clearance of 0.27 mm. The pestle was driven by an electric motor [49] at 1,725 rpm. Each aliquot was subjected to 10 cycles (one cycle consisting of one stroke up and one stroke down) while the vessel was cooled in crushed ice. Homogenized tissue was pooled and stirred until a second homogenization was carried out in the same way in another Teflon and glass homogenizer with a clearance of 0.20 mm. The final pooled homogenate was filtered through four layers of common cheesecloth to remove large pieces of connective tissue.

Differential centrifugation. The homogenate was centrifuged following the scheme outlined in Figure 1. *Fraction* $P_{2.1}$ was diluted with 0.29 M sucrose solution to a final volume of 90 ml, and 30-ml aliquots were centrifuged to a final force of 22×10^7 rad^2/sec. The top 26 ml of each aliquot were withdrawn and designated as *fraction* $P_{2.1g}$. The bottom 4 ml (*fraction* $P_{2.1p}$) were set aside for analysis, as was an aliquot of *fraction* $P_{2.1g}$.

Isopycnic centrifugation. A linear, continuous sucrose gradient, limit concentrations from 20–60%, was constructed in the Sorvall SZ 14 zonal rotor. The volume of the gradient, including a 100-ml cushion of 60% sucrose solution, was 1,250 ml. *Fraction* $P_{2.1g}$ was introduced into the axial core of the rotor at the light end of the gradient and the rotor was accelerated to 15,000 rpm for a period of 12 hr at 4°. The rotor was decelerated with the rate controller and 55 fractions of 20 ml each were collected with a fraction

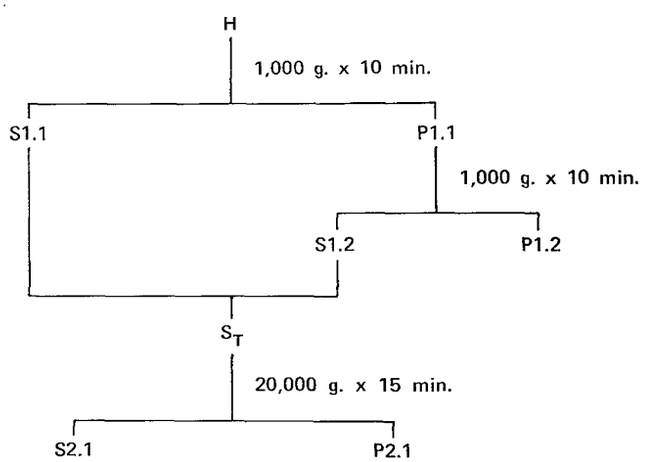


Fig. 1. Scheme of differential centrifugation. All steps are carried out in a Sorvall RC 2-B centrifuge [58] with the GSA rotor at 4°. Centrifugal forces and times are shown.

collector, examined for enzyme content, and subjected to morphologic studies.

Biochemical analysis. Enzyme and protein assays were performed on aliquots diluted appropriately with aqueous 0.01% Triton X-100. Final sucrose concentrations of diluted aliquots range from 0.145 to 0.875 M. All aliquots were frozen at least once before assay except when latency studies were performed. Latency was determined by incubating organelles in 0.29 M sucrose at 4° for 60 min in the presence and the absence of 0.01% Triton X-100. After incubation the samples were centrifuged at 50,000 × *g* for 60 min and the supernatant portion was assayed. Latent enzyme activity is the difference between total activity (Triton released) and free activity. When necessary, samples were centrifuged at 600 × *g* for 5 min to remove large tissue debris. Where possible, automated or semi-automated adaptations of enzyme assays were used in conjunction with the Technicon AutoAnalyzer [50]. Buffers for enzyme assays contained 0.1% (w/v) Triton X-100. Methods employed in enzyme assays are presented in Table I together with references and modifications.

Table I. Biochemical methods

Enzyme[1]	Substrate	Buffer	Modifications	Enzyme activity, units	Reference
Protein			AutoAnalyzer, Lowry method	mg/ml	[26]
Acid phosphatase (EC. 3.13.2)	0.1 M disodium β-glycerophosphate	0.1 M Na-acetate, pH 5.0	AutoAnalyzer, color complex with 1% 2,4-diaminophenol dihydrochloride, 20% sodium sulfite, and 0.05% aqueous Levor IV at 75°	μmoles/mg protein/hr	[26]
β-Acetyglucosaminase (EC. 3.2.1.30)	7.2 mM *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide	0.1 M Na-citrate and 0.1 M NaCl, pH 4.2	AutoAnalyzer	μmoles/mg protein/hr	[9]
Arylsulfatase (EC. 3.1.6.1	10.5 mM (*p*-nitrocatechol sulfate)	0.05 M Na-acetate, pH 5.0	Incubated 1 hr at 37°	μmoles/mg proteins/hr	[9]
β-Glucuronidase (EC. 3.2.1.31)	2.5 mM phenolphthalein glucuronic acid	0.3 M Na-acetate, pH 4.6	Incubated 1 hr at 37°; larger volumes than in original method	μmoles/mg protein/hr	[29]
Monoamine oxidase (EC. 1.4.3.4)	10 mM benzylamine hydrochloride	0.2 M phosphate, pH 7.6	Incubated 1 hr at 37°	ΔA_{250}/mg protein/hr	[41]
Succinate cytochrome *c* reductase (EC. 1.6.99.3)	3 mM succinate	0.05 M phosphate, pH 7.6	Gilford recording spectrophotometer, 25°	ΔA_{550}/mg	[37]
Glucose 6-phosphate hydrolyzing enzyme[2]	66 mM monosodium glucose 6-phosphate	0.33 Na-cacodylate, pH 6.5, 20 mM L(+)-Na-tartrate[3]	Incubated 1 hr at 37° measure inorganic phosphate as for acid phosphatase	μmoles/mg protein/hr	[27] [24]
Catalase (EC. 1.11.16)	7.5 mM HO	10 mM imidazole-HCl-1% Triton X-100, pH 7.2	Incubated 10 min at 0°	$\log \frac{C_i}{C_f} \frac{H_2O_2}{H_2O_2}$[4]	[5]

[1] Names and numbers of enzymes follow the Recommendations (1964) of the International Union of Biochemistry on the Nomenclature and Classification of Enzymes [30].

[2] This designation has been chosen in view of the unresolved issue about the identity of the placental enzyme with glucose 6-phosphatase from other tissue sources.

[3] Na-tartrate was used as the acid phosphatase inhibitor.

[4] C_i: initial concentration; C_f: final concentration.

Morphologic analysis. Light microscopy. After passage through the tissue press, samples of whole placenta were examined histologically. Hematoxylin and eosin stains were used to evaluate the contribution of various cell lines to the homogenate.

Fluorescence microscopy. Fragments of whole placentas are incubated for 30 min in cell culture *medium 199* [51], containing 0.01% acridine orange and 10% fetal calf serum. The tissue was then washed free of dye, suspended in fresh culture medium, and incubated for 4 hr at 37° in an atmosphere of 5% CO_2. After incubation, the tissue was washed in phosphate-buffered saline, pH 7.4, containing 0.2% glucose, pelleted at 15,000 rpm, embedded, frozen in liquid nitrogen, and 12-μm sections were cut with a cryostat. The sections were mounted in 0.1 M phosphate buffer, pH 6.1, and examined with a Leitz fluorescent microscope using a UG 1 exciter filter and a K 430 barrier filter [52]. Organelle fractions were suspended in 50% sucrose containing 50 μg/ml acridine orange, mounted under a coverslip, and viewed with the fluorescence microscope under the same filter system as before.

Electron microscopy. Samples for electron microscopy were fixed with 1.5% glutaraldehyde in 0.05 M cacodylate buffer of pH 7.4. After 2 hr of fixation, the aliquots were collected by filtration on a 25-mm Millipore filter of 0.01 μm pore size. The filter was washed with 0.05 M cacodylate buffer and stored in the same buffer to await processing. This technique is a modification of the method of Baudhuin *et al.* [6] adapted to use a Millipore microsyringe filter holder [53] together with a 25-mm filter. Filtration was accomplished at a pressure of 40 psi nitrogen. Fixed specimens were then processed in this order: postfixation in 1% osmium tetroxide, staining with uranyl acetate, and progressive dehydration in alcohol ranging in concentration from 50% to 100%. The samples were embedded in Epon and sections were cut with an LKB microtome [54]. Electron microscopy is performed with a Siemens Elmiskope *model 1A* [55] at 75 kV.

Results

Cellular Morphology: Light and Fluorescence Microscopy

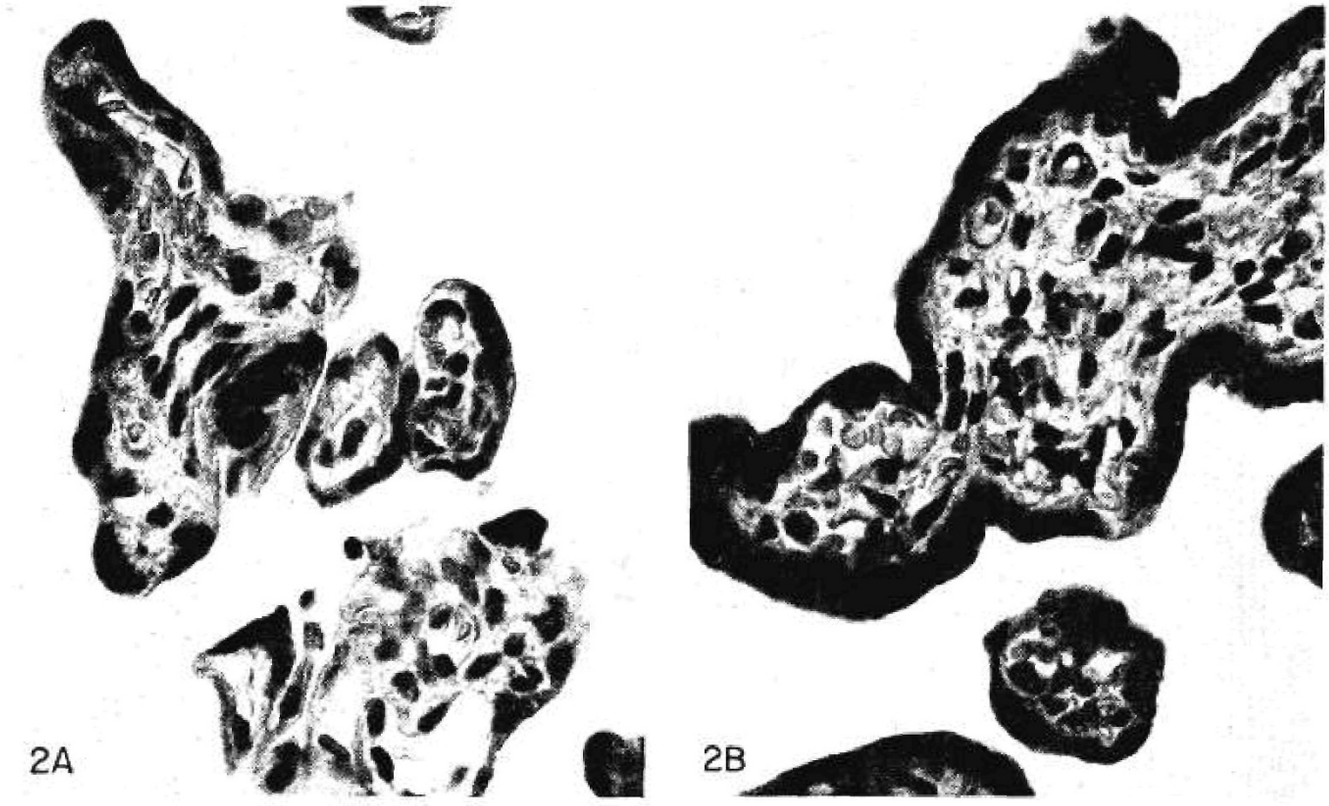
Isolated human term placenta is a complex tissue composed of several intrinsic cell types, contaminating peripheral blood cells, and maternal decidua. Any effort to isolate cell organelles from placenta must take into account the heterogeneity of the cell population which is sampled and attempt to estimate the contribution of individual cell lines. Hematoxylin and eosin-stained placental tissue after the tissue press stage but before homogenization is shown in Figure 2. Most of the large vasculature has been removed; organized thrombi which would contribute platelets do not appear to be prominent. A high power view of a single villus in cross-section illustrates the lack of cellularity in the stroma as compared with the syncytium. The results of incubating placental tissue in the presence of acridine orange are shown in Figure 3. Nuclei and cytoplasm fluoresce pale green whereas lysosomes selectively concentrate the dye and show intense orange fluorescence [1]. Orange fluorescence is widespread in the syncytium and high power views demonstrate that the fluorescence is associated with granules. Localized collections of lysosomes are seen which demonstrate granularity at high power. Invading phagocytes appear to contribute minimally to the syncytial activity. In paranuclear regions, pale orange streaks are sometimes observed. This is possibly the result of nucleic acid staining but is different in intensity and form from lysosomal fluorescence.

Differential Centrifugation

The results of differential centrifugation are shown in Figure 4. Acid phosphatase, β-acetylglucosaminase, β-glucuronidase, and arylsulfatase are used as lysosomal marker. Monoamine oxidase and succinate cytochrome *c* reductase serve as mitochondrial markers. The catalytic activity conventionally ascribed to glucose 6-phosphatase (EC. 3.1.3.9) was to serve as marker for microsomes. However, data accumulated in the course of the present study leave doubt about the identity of these phosphate hydrolyases and we shall, henceforth, refer in more general terms to the enzyme of human term placenta as glucose 6-phosphate hydrolyzing enzyme. After homogenization and low speed centrifugation there is similar distribution of protein and enzymes between the pellet ($P_{1.2}$) and the combined supernatant fractions (S_T). The efficiency of disrupting the cells and extracting the organelles is 75%.

The second differential centrifugation develops a compact cytoplasmic pellet ($P_{2.1}$) consisting largely of mitochondria and lysosomes, whereas microsomes remain in the supernatant phase ($S_{2.1}$). Lysosomes are almost equally distributed between the two fractions with minor variations depending on the enzyme assayed.

The large amount of acid hydrolases in the $S_{2.1}$ fraction prompted us to explore its subcellular origin



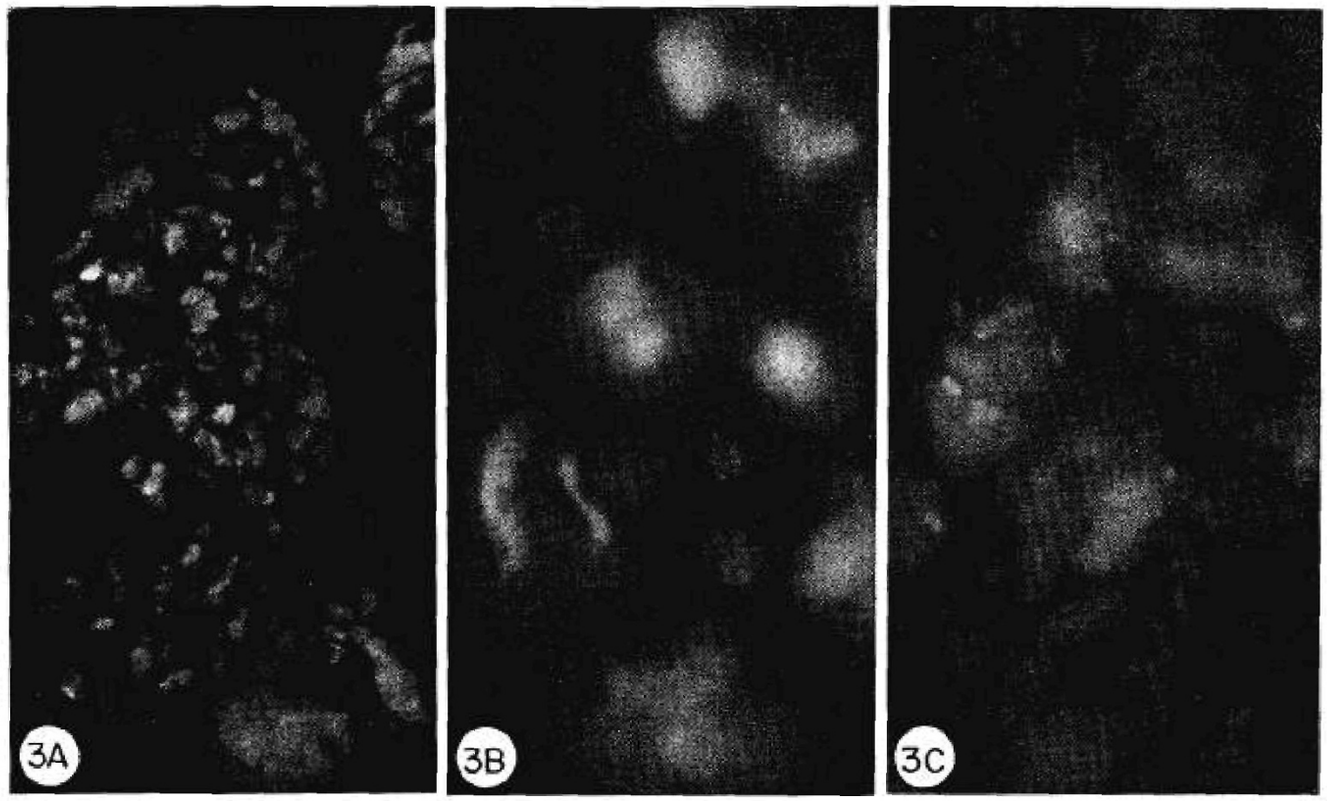


Fig. 2. Placental villi after the tissue press; hematoxylin and eosin stain. Section *A*, × 312. Section *B*, × 675.
Fig. 3. Fragments of placental villi incubated with 0.01% acridine orange (*cf. Materials and Methods*). *A:* widespread distribution of lysosomes, × 312; *B:* granular appearance of lysosomes, × 540; *C:* clusters of lysosomes in the cytoplasm, × 1200.

rather than dismiss it as soluble enzyme. The supernatant fraction, $S_{2.1}$, was layered over a 50% sucrose cushion and centrifuged for 12 hr at 27,000 rpm in the Beckman SW 27 rotor [56]. Of the acid hydrolase activity, 50–85%, varying with the specific markers, is associated with the particulate interface fraction. An electron micrograph of this interface is shown in Figure 5. It contains abundant smooth vesicles and no classic lysosome profiles. Ninety percent of the glucose 6-phosphate hydrolyzing activity also sediments with the particulate matter. Comparable acid hydrolase assays with and without Triton X-100 show a 60% increase in activity in the presence of detergent, which indicates considerable latency. Washing of the fraction with 0.29 M sucrose solution causes only 10–20% of the total sedimentable activity to transfer to the soluble fraction. This indicates that the enzyme activity is sedimentable, latent, and not extensively removed by washing.

Hydrolysis of glucose 6-phosphate, assayed in the presence of the acid phosphatase inhibitor L(+)-tartrate, is found predominantly in the supernatant fraction, $S_{2.1}$. Monoamine oxidase and succinate cytochrome *c* reductase are mainly in the pellet. Two mitochondrial markers, an outer and an inner membrane enzyme, are used to determine the extent of the organelle distintegration during the isolation process [32, 43]. Some succinate cytochrome *c* reductase activity remains in the $S_{2.1}$ fraction.

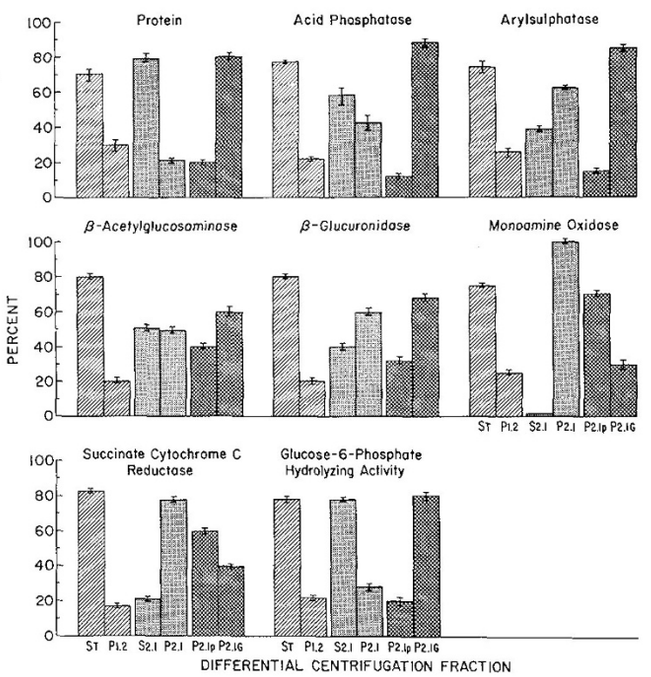


Fig. 4. Percentage of distribution of protein, lysosomal acid hydrolases, mitochondrial, and microsomal markers during differential centrifugation. *Fractions* $P_{1.2} + S_T$, $P_{2.1} + S_{2.1}$, and $P_{2.1p} + P_{2.1g}$ total 100% and represent the three phases of differential centrifugation.

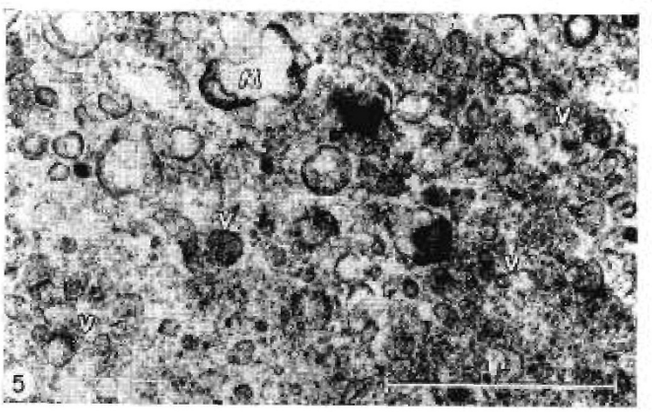


Fig. 5. Electron micrograph of *fraction* $S_{2.1}$ concentrated on a 50% sucrose cushion. Material is from the interface after centrifugation at 27,000 rpm for 12 hr. Note that smooth vesicles (*V*) are the predominant form and that lysosomes of classic morphology are absent. × 60,000. *M:* mitochondria

Catalase is present in crude homogenates of human term placenta but can be removed in the nuclear pellet together with contaminating erythrocytes. Approximately 3% of the total activity enters the isopycnic gradient and is limited largely to the region of soluble protein.

Before isopycnic centrifugation, depletion of *fraction* $P_{2.1}$ of mitochondria is accomplished by integrator controlled differential centrifugation. Sixty percent of the mitochondria sediment, whereas 70–80% of the lysosomes remain in the supernatant portion.

A summary of specific activities and recoveries during differential centrifugation is presented in Table II. Figure 6 is an electronmicrograph of *fraction* $P_{2.1g}$ showing an abundance of smooth membrane profiles and lysosomes, as well as mitochondria.

Isopycnic Centrifugation. The results of isopycnic centrifugation are illustrated in Figure 7. The protein distribution is trimodal with the first peak at *tube 23,* the second peak at *tube 31,* and the third peak in the region of soluble material in the gradient. β-Acetylglucosaminase shows a sharp peak at *tube 23* and a small portion as soluble enzyme. Monoamine oxidase is distributed in a bimodal fashion with peaks coinciding with *fractions 23* and *35.* The mitochondria introduced into the density gradient are those derived from

Table II. Specific activities in differential centrifugation[1]

Enzyme	Fraction							
	H	S_t	$P_{1.2}$	$S_{2.1}$	$P_{2.1}$	$P_{2.1p}$	$P_{2.1g}$	Recovery
Acid phosphatase	1.58 ± 0.110	1.67 ± 0.190	1.27 ± 0.240	1.32 ± 0.210	3.4 ± 0.400	3.62 ± 0.340	2.76 ± 0.410	95.0 ± 2.0
Arylsulfatase	0.063 ± 0.015	0.050 ± 0.015	0.038 ± 0.012	0.021 ± 0.013	0.178 ± 0.060	0.224 ± 0.080	0.196 ± 0.070	91.4 ± 1.8
β-Glucuronidase	0.166 ± 0.080	0.165 ± 0.067	0.107 ± 0.043	0.082 ± 0.024	0.511 ± 0.080	0.677 ± 0.104	0.550 ± 0.094	95.0 ± 1.4
β-Acetyl glucosaminase	1.97 ± 0.560	1.94 ± 0.240	1.29 ± 0.110	1.28 ± 0.160	5.07 ± 0.240	6.90 ± 0.280	4.20 ± 0.240	96.1 ± 2.3
Monoamine oxidase	0.219 ± 0.030	0.197 ± 0.017	0.169 ± 0.013	0.03 ± 0.010	0.965 ± 0.100	3.12 ± 0.310	0.308 ± 0.026	92.0 ± 1.6
Succinate cytochrome *c* reductase	0.069 ± 0.003	0.053 ± 0.006	0.033 ± 0.002	0.015 ± 0.003	0.207 ± 0.050	0.552 ± 0.080	0.058 ± 0.014	82.4 ± 1.8
Glucose 6-phosphate hydrolyzing enzyme	0.325 ± 0.040	0.337 ± 0.010	0.153 ± 0.080	0.260 ± 0.030	0.431 ± 0.060	0.403 ± 0.042	0.638 ± 0.058	89.0 ± 2.1

[1] Results are expressed as described in *Methods* per milligram of protein per unit time. These values represent the averages of seven experiments.

$P_{2.1g}$ and therefore are not representative of the entire population.

Extensive organelle damage during the fractionation procedure would give rise to artifactual mitochondrial subpopulations of altered densities. To evaluate this hypothesis, we determined the density distribution of monoamine oxidase, a marker enzyme for the outer membrane of mitochondria, and of succinate cytochrome *c* reductase, a marker enzyme for the inner membrane of mitochondria.

The density distribution of the two enzymes is compared in Figure 8. The enzymes exhibit parallel density distributions, which suggests that the organelles are intact and of bimodal density. However, morphologic studies do not corroborate these findings. Rather, they provide evidence that the organelle underwent extensive morphologic changes. Figures 9 and 10 are electronmicrographs of fractions from the peak regions and Figure 11 shows the electronmicrograph of a fraction from the gradient light end where there is succinate cytochrome *c* reductase activity. The micrographs of the first two fractions show swollen and distorted mitochondria. Outer and inner membrane appear to exist, but there is general dissolution of matrix and no internal fine structure is detectable. The electronmicrograph of the third fraction shows only membrane profiles but no discernible mitochondria. Obviously

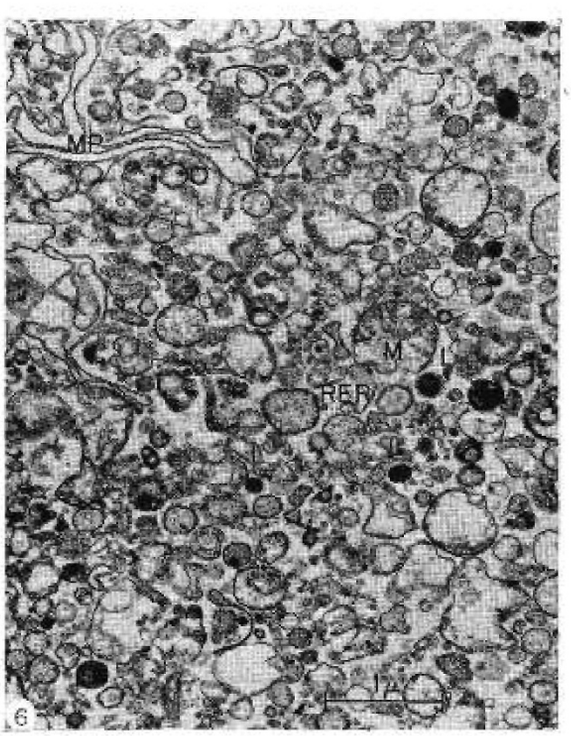


Fig. 6. Fraction $P_{2.1g}$ which is placed into the zonal rotor. Notice the prominent membrane profiles (*MP*). Some swollen and partially disrupted mitochondria (*M*) are present as well as smooth vesicles (*V*), rough endoplasmic reticulum (*RER*), and lysosomes (*L*) of different size and electron density. × 30,000.

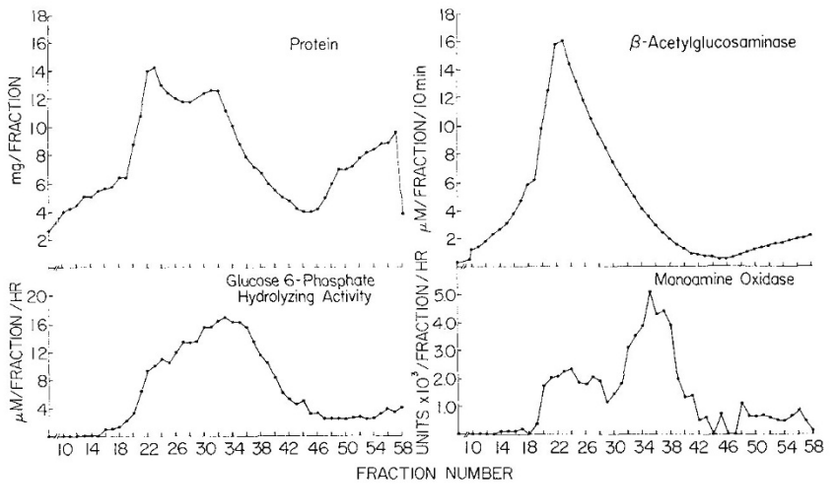


Fig. 7. Distribution of protein, β-acetylglucosaminase, glucose 6-phosphate hydrolyzing enzyme, and monoamine oxidase. Isopycnic centrifugation with an SZ 14 rotor.

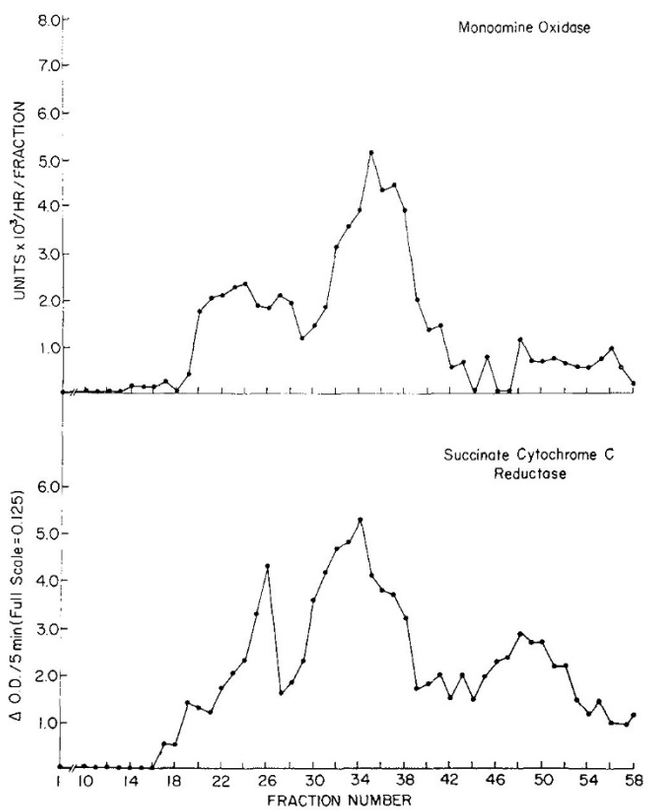


Fig. 8. Comparative distribution of the two mitochondrial marker enzymes after isopycnic centrifugation.

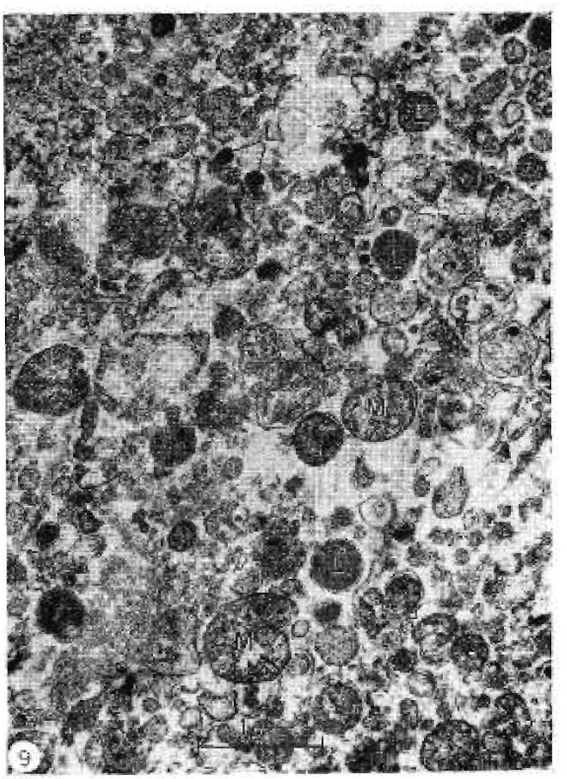


Fig. 9. Fraction 23 from the region of the peak for lysosomes (*L*) and the first peak for mitochondria. Mitochondria (*M*) appear to be swollen. × 30,000.

structural changes did occur and led to altered densities of the mitochondria which, in turn, gave rise to the spurious bimodal distribution of the organelle. This may be because the term placenta has reached the final state of its physiologic life cycle and because extensive autophagy is under way and responsible for the unusual distribution of mitochondria and their uncommon morphologic appearance. Damage to some extent may also be ascribed to unfavorable conditions

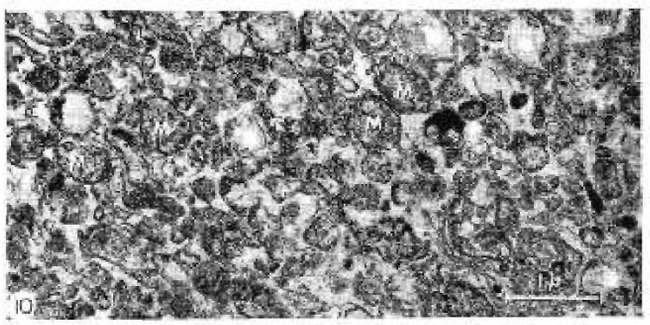


Fig. 10. Fraction 31 from the region of the second peak for mitochondria. There are intact mitochondria (*M*) with rather indistinct matrices suggestive of disruption to a certain degree. × 30,000.

during fractionation processes. Klimek *et al.* [25] have shown that mitochondria isolated from human term placenta in the absence of 1.0% bovine serum albumin display altered structure and decreased oxidative phosphorylation ability. Boime *et al.* [7] observed this effect in rat liver and concluded that binding of free fatty acids by albumin protected mitochondria from damage. The same condition may prevail here.

The homogeneity of the lysosomes may be evaluated biochemically by determining the density distribution of acid hydrolases (Fig. 12). β-Glucuronidase, arylsulfatase, and β-acetylglucosaminase are similarly distributed. Acid phosphatase, however, exhibits a different and considerably broader distribution pattern. The

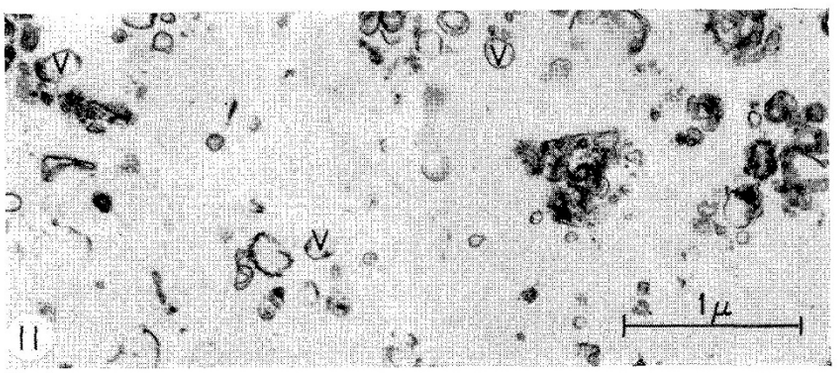


Fig. 11. Electron micrograph of *fraction 49* which shows no intact mitochondria. Enzyme assays, however, demonstrate the presence of considerable quantities of succinate cytochrome *c* reductase. Smooth membrane vesicles (*V*) are the predominant morphologic form. × 30,000.

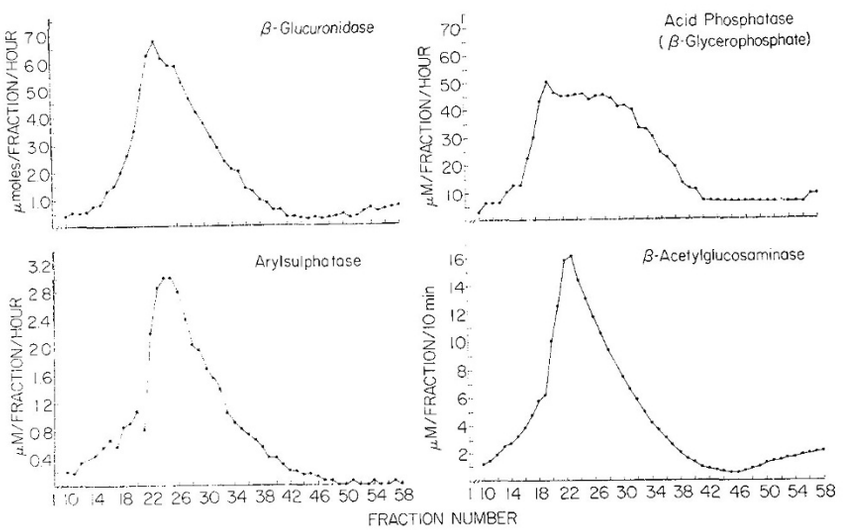


Fig. 12. Distribution pattern of the four lysosomal acid hydrolases after isopycnic centrifugation.

distribution curves of the first three hydrolases are skewed toward the light end of the gradient. Although the density distribution of these enzymes is heterogeneous in itself, the identical distribution patterns of the three enzymes suggest lysosomal homogeneity on biochemical grounds. Maximal concentration of each enzyme is observed at a density of 1.195 g/ml.

Hydrolytic activity directed toward glucose 6-phosphate shows a peak at 1.59 g/ml and is also distributed in a rather heterogeneous manner (Fig. 7). The microsomes applied to the zonal rotor consist of the larger vesicles which are collected in the 300,000 × g/min pellet ($P_{2.1}$). Morphologic analysis reveals that the rough endoplasmic reticulum is present in fractions of the higher density region of the gradient where there is no glucose 6-phosphate hydrolyzing activity (Fig. 13). The microsomes at the light end of the gradient, where this activity is found, consist of small vesicles of smooth membrane.

Morphologic studies on fractions from different regions of the lysosome distribution demonstrate a spectrum of structures varying from homogeneous dense bodies to small less dense vesicles similar to those of the interface material of *fraction* $S_{2.1}$. *Tube 13* (Fig. 14) contains large, multivesiculated, dense structures which we interpret to be autophagic vacuoles. Toward the peak of the acid hydrolase distribution there are more uniform dense bodies typical of the classic lyso-

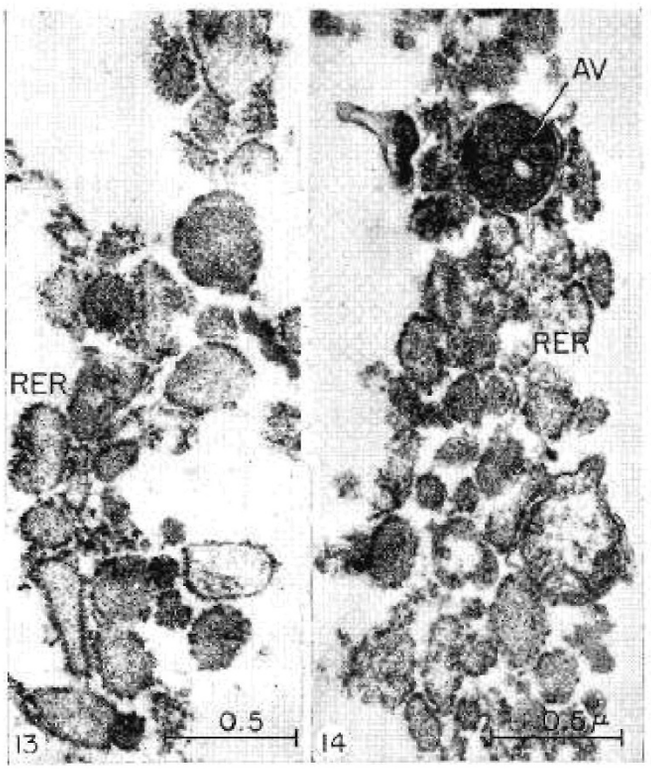


Fig. 13 (left). Electron micrograph of *fraction 9* showing a predominance of rough endoplasmic reticulum (*RER*). × 60,000. *Fig. 14 (right).* Electron micrograph of *fraction 13*. Rough endoplasmic reticulum (*RER*) and a large body, thought to be an autophagic vacuole (*AV*), are present. × 60,000.

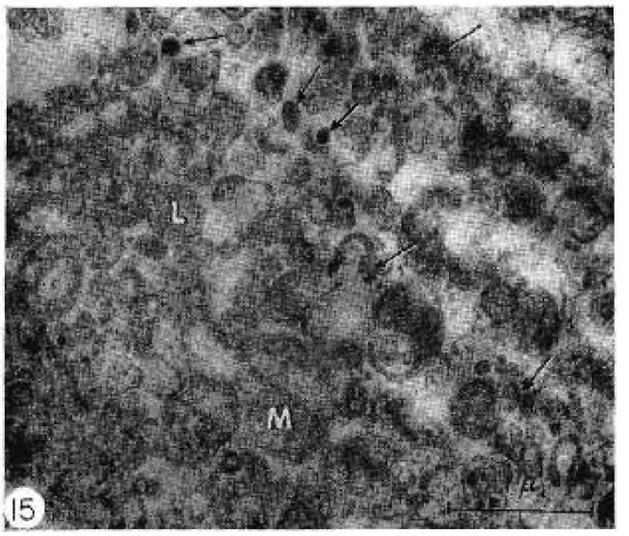


Fig. 15. Electron micrograph of *fraction 27*, represented in the skewed portion of the acid hydrolase distribution. Large lysosomes (*L*) are rare, an occasional mitochondrion (*M*) is present. Note the small dense bodies (*arrows*) which may be members of a lysosome subpopulation of smaller size. × 30,000.

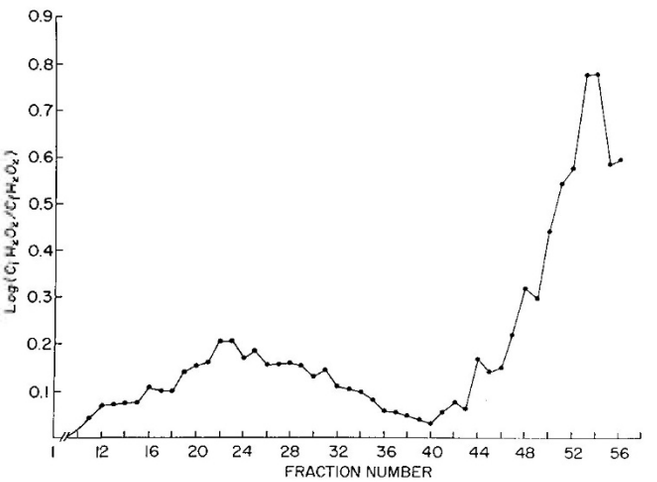


Fig. 16. Distribution of catalase in the gradient. Activity is expressed as the logarithm to the base of 10 of the ratio of the initial concentration of substrate *versus* the final concentration of substrate.

Table III. Relative specific activities at various preparative stages and gradient recoveries[1]

	H	$P_{2,1}$	$P_{2,1g}$	Gradient	
				Peak	Recoveries, %
Lysosomes					
Acid phosphatase	1.00	2.21	2.28	3.00	101.6
Arylsulfatase	1.00	2.82	3.11	4.17	84.6
β-Glucuronidase	1.00	3.07	3.68	8.56	95.0
β-Acetylglucosaminase	1.00	2.46	2.20	4.27	94.0
Mitochondria					
Monoamine oxidase	1.00	4.40	1.40	2.63	89
Succinate cytochrome *c* reductase	1.00	3.00	0.84	5.30	86
Microsomes					
Glucose 6-phosphate hydrolyzing enzyme	1.00	1.23	1.96	4.6	119

[1] Results are the averages of seven experiments.

some (Fig. 9). In the less dense area of the lysosome distribution there are small vesicles of medium density (Fig. 15) which are similar to those seen in Figure 10.

The catalase activity is primarily confined to the low density region of the gradient where soluble protein is found (Fig. 16). Electron micrographs of this region do not show structures resembling peroxisomes, nor are there any peroxisomes in the dense regions of the gradient where they might be expected. The small amount of activity which parallels the protein distribution is probably the result of adsorption or trapping of soluble material; it accounts for less than 1% of the total activity. Table III presents a summary of the specific activities throughout the purification.

Discussion

The fractionation of subcellular constituents from human term placenta confirms earlier observations [12] that particles may be isolated which contain acid hydrolases, demonstrate latency, fluoresce with acridine orange, and thus deserve to be designated lysosomes. Routine histologic studies show minimal contamination of placental villi by platelets and leukocytes after the tissue press stage. Vital staining using acridine orange indicates that the syncytiotrophoblast is a major source of lysosomes.

The existence of two groups of latent acid hydrolyases is clearly indicated by the differential centrifugation experiments. The first group corresponds to the L and M fraction of de Duve [15]. This population contains 50–60% of the acid hydrolases, demonstrates latency, and on electron microscopy, shows many classic lysosome profiles (Fig. 6). The second group found in the 300,000 × g/min supernatant fraction is also particulate and demonstrates latency as well. Electron microscopy of this fraction (Fig. 5) reveals abundant profiles of smooth membrane and the absence of the classic lysosome as it is defined morphologically.

Baccino *et al.* [4] have demonstrated that soluble acid hydrolases may be adsorbed to sedimenting material with variable affinities. If extensive lysosome damage occurs during the isolation procedure, then the acid hydrolases of *fraction* $S_{2.1}$ may be due to adsorbed soluble enzyme. To test this hypothesis, we attempted to elute the acid hydrolases using 0.29 M sucrose. According to Baccino [3], this is an effective medium for solubilization of adsorbed enzyme. Only 20% of the acid hydrolases were eluted and there was no differential solubility among the various enzymes. The demonstration of latency for the $S_{2.1}$ acid hydrolases is added evidence against adsorption. Finally, to find up to 50% of the postnuclear enzymes in the supernatant *fraction* $S_{2.1}$ would require rather extensive organelle damage during the fractionation. On the basis of these characteristics, we conclude that the presence of sedimentable hydrolases, capable of exhibiting latency, is real and representative of a lysosomal subpopulation.

Canonico and Bird [10] have discussed the theory that lysosomes in skeletal muscle may exist as part of a sarcotubular-lysosomal system and cite the work of Pearce [28] as histologic evidence for this theory. In view of the fact that one-half of the hydrolases is located in *fraction* $S_{2.1}$, placental lysosomes may well exist in close association with endoplasmic reticulum [24]. Holzman *et al.* [23] have emphasized the close relation between Golgi-associated endoplasmic reticulum and lysosomes in normal and chromatolytic neurons and have postulated that the functional correlate of this association is the autophagic and cell remodeling process. Term placenta may be a similar case in which the syncytiotrophoblast is a degenerating cell in which autophagy is a predominant process and Golgi-associated endoplasmic reticulum and lysosomes are closely related.

The lysosome population isolated in the isopycnic gradient is heterogeneous with respect to density. Arylsulfatase, β-glucuronidase, and β-acetylglucosaminase are represented by peaks at the identical density of

1.195 g/ml, which is the same density as that given by Schultz and Jacques [35] for rat placental lysosomes. Acid phosphatase is distributed over a broad range located between the peaks for the other acid hydrolases and hydrolytic activity toward glucose 6-phosphate. Morphologic analysis revealed a spectrum of lysosomes ranging from the fractions of high density to the region of less density. The density distributions of arylsulfatase, β-acetylglucosaminase, and β-glucuronidase are skewed toward the light end of the gradient, which suggests biochemical continuity with the microsomes or a second unresolved lysosome population of lower density. They may also be due to the association of the same enzyme activity with different subcellular particles. Di Pietro and Zengerle [18] describe three "biochemically distinct" placental acid phosphatases, only one of which is attributed to lysosomes. We do not have definitive evidence at this time to specifically assign a nonlysosomal origin to the acid hydrolase activities which sediment separately from the organelles of the peak region, but conclude that the placental acid phosphatase is more widely and differently distributed than the other three acid hydrolases. Additional marker enzymes for lysosomes and improved gradient resolution may be useful in definding this heterogeneity.

The purity of placental lysosomes in this effort does not approach that of rat liver lysosomes isolated by the method of Leighton *et al.* [26] or Stahn *et al.* [38]. Of the total lysosomal activity, 20–25% is lost in the nuclear pellet and 50% is found to be associated with smooth endoplasmic reticulum. The balance in activity may be located in digestive vacuoles which contain nonlysosomal protein, thus further reducing the ultimate degree of purity. Studies with placentas of different gestational ages may prove more fruitful in yielding lysosomes of higher purity.

The broad distribution of hydrolytic activity toward glucose 6-phosphate and its partial overlap with acid phosphatase activity caused us to question the validity of this enzyme as a microsomal marker in placenta [27]. Schultz [34], who worked with rat placentas, has previously raised this issue. In a review of placental enzymes, Hagerman [19] included glucose 6-phosphatase without comment as to subcellular localization. Earlier work by Villee [42] postulated that the enzyme was present in 1st trimester placentas but absent at term. In a subsequent study by Hagerman *et al.* [21], glucose 6-phosphatase activity was attributed to nonspecific alkaline phosphatases associated with microsomal fractions. Stetten [39] reported that the microsomal glucose 6-phosphatase (EC. 3.1.3.9) of liver displays several activties: hydrolytic cleaveage of inorganic pyrophosphate, hydrolysis of glucose 6-phosphate, and synthetic pyrophosphate phosphotransferase activity. To date, none of these activities has been conclusively demonstrated in term placenta. Preliminary investigations in our laboratory did not reveal pyrophosphate phosphotransferase activity.

Peroxisomes are not constituents of human term placenta. Catalase present is almost entirely confined to the nuclear fraction and is likely to be associated with intact erythrocytes found there. Only small quantities of the enzyme are in the region of the gradient where soluble protein is found. We had reported previously the absence of D-amino and L-α-hydroxy acid oxidases from homogenates of human term placenta [13]. Schultz and Jacques [35] have demonstrated the absence of amino acid oxidase, catalase, and urate oxidase from fractions isolated from rat chorioallantoic placenta and also concluded that peroxisomes are absent from that tissue.

Conclusion

Lysosomes exist in human term placenta. The syncytium is the major source of the organelle. Of the placental lysosomes, 50% are found with the endoplasmic reticulum, whereas the remainder may be isolated by isopycnic ultracentrifugation at a peak density of 1.195 g/ml at which the classic lysosome is expected to sediment. The special relation between lysosomes and endoplasmic reticulum may be exaggerated in term placenta because of inherent properties of the terminal tissue. Time-dependent studies to characterize lysosomes of different gestational ages may establish more clearly the presence and role of lysosomes of different densities. Acid phosphatase, because of its lack of specificity, is not a suitable lysosomal marker for placenta. The glucose 6-phosphate hydrolyzing enzyme of human term placenta is not necessarily identical with glucose 6-phosphatase from other tissues. Peroxisomes, as evidenced by the lack of amino acid oxidase and catalase activities, are absent from human term placenta.

References and Notes

1. Allison, A.: The role of lysosomes in the action of drugs and hormones. Advan. Chemother., *3:* 253 (1968).
2. Applemans, F., Wattiaux, R., and de Duve, C.: Tissue fractionation studies. 5. The association of acid phosphatase with a special class of cytoplasmic granules in rat liver. Biochem. J., *59:* 438 (1955).

3. BACCINO, F. M., RITA, G. A., AND ZURETTI, M. F.: Studies on the structure-bound sedimentability of some rat liver lysosome hydrolases. Biochem. J., *122:* 363 (1971).
4. BACCINO, F. M., AND ZURETTI, M. F.: On the structure linked sedimentability of rat liver β-*N*-acetylglucosaminidase. Biochim. Biophys. Acta, *235:* 353 (1971).
5. BAUDHUIN, P., BEAUFAY, H., RAHMAN-LI, Y., SELLINGER, O. Z., WATTIAUX, R., JACQUES, P., AND DE DUVE, C.: Tissue fractionation studies. 17. Intracellular distribution of monoamine oxidase, aspartate amino transferase, alanine amino transferase, D-amino acid oxidase, and catalase in rat liver tissue. Biochem. J., *92:* 179 (1964).
6. BAUDHUIN, P., ERRAR, P., AND BERTHET, J.: Electron microscopic examination of subcellular fractions. I. Preparation of representative samples from suspensions of particles. J. Cell Biol., *32:* 181 (1967).
7. BOIME, I., SMITH, E. E., AND HUNTER, F. E., JR.: The role of fatty acids in mitochondrial changes during liver ischemia. Arch. Biochem. Biophys., *139:* 425 (1970).
8. BOWERS, W. E., FINKENSTAEDT, J. T., AND DE DUVE, C.: Lysosomes in lymphoid tissue. I. Measurement of hydrolytic activities in whole homogenates. J. Cell Biol., *32:* 325 (1967).
9. BRADLEY, D. W., AND TAPPEL, A. L.: Automated multiple enzyme analysis systems. Anal. Biochem., *33:* 400 (1970).
10. CANONICO, P. G., AND BIRD, J. W. C.: Lysosomes in skeletal muscle tissue. J. Cell Biol., *45:* 321 (1970).
11. COHN, Z. A., AND HIRSCH, J.: The isolation and properties of the specific cytoplasmic granules of rabbit polymorphonuclear leukocytes. J. Exp. Med., *112:* 938 (1960).
12. CONTRACTOR, S. F.: Lysosomes in human placenta. Nature, *223:* 1274 (1968).
13. CORASH, L., AND GROSS, E.: Isolation and characterization of lysosomes from human term placenta. Fed. Proc., *31:* 254 (1972).
14. DAY, H. J., HOLMSEN, G., AND HOVIG, T.: Subcellular particles of human platelets. Scand. J. Haematol., *3:* Suppl. 7 (1969).
15. DE DUVE, C., PRESSMAN, B. C., GIANETTO, R., WATTIAUX, R., AND APPLEMANS, F.: Tissue fractionation studies. 6. Intracellular distribution of enzymes in rat-liver tissue. Biochem. J., *60:* 604 (1955).
16. DE DUVE, C., AND WATTIAUX, R.: Functions of lysosomes. Ann. Rev. Physiol., *28:* 435 (1966).
17. DE DUVE, C., WATTIAUX, R., AND WIBO, M.: Effects of fat soluble compounds on lysosomes in vitro. Biochem. Pharmacol., *9:* 97 (1962).
18. DI PIETRO, D. L., AND ZENGERLE, F. S.: Separation and properties of three acid phosphatases from human placenta. J. Biol. Chem., *242:* 3391 (1967).
19. HAGERMAN, D. D.: Enzymatic capabilities of the placenta. Fed. Proc., *23:* 785 (1964).
20. HAGERMAN, D. D.: Enzymology of the placenta. In: A. Klopper and E. Diczfalusky: Foetus and Placenta, p. 434 (Blackwell Scientific Publications, Oxford, England, 1969).
21. HAGERMAN, D. D., ROUX, J., AND VILLEE, C. A.: Studies of the mechanism of fructose production by human placenta. J. Physiol., *146:* 98 (1959).
22. HEMPEL, H., FERNANDEZ, L. A., AND PERSELLIN, R. H.: Effect of pregnancy sera on isolated lysosomes. Nature, *225:* 955 (1970).
23. HOLZMAN, E., NOVIKOFF, A. B., AND VILLAVERDE, H.: Lysosomes and GERL in normal and chromatolytic neurons of rat ganglion nodosum. J. Cell Biol., *33:* 419 (1967).
24. KAULEN, H. D., HENNING, R., AND STOFFEL, W.: Comparison of some enzymes of the lysosomal and the plasma membrane of the rat liver cell. Hoppe-Seyler's Z. Physiol. Chem., *351:* 1555 (1970).
25. KLIMEK, J., ALEKSANDROWICS, Z., WRZOLKOWA, T., AND ZELEWSKI, L.: The oxidative phosphorylation and electron microscopic structure of mitochondria isolated from human term placenta. Biochem. Med., *6:* 426 (1972).
26. LEIGHTON, D., POOLE, B., BEAUFAY, H., BAUDHUIN, P., COFFEY, J. W., FOWLER, S., AND DE DUVE, C.: Large-scale separation of peroxisomes, mitochondria, and lysosomes from the livers of rats injected with Triton WR-1339. J. Cell Biol., *37:* 482 (1968).
27. NORDLIE, R. C., AND ARION, W. J.: Methods Enzymol., *9:* 619 (1966).
28. PEARCE, C. W.: The sarcolemma and sarcotubular systems in normal and dystrophic muscle. Res. Muscular Dystrophy Proc. 34th Symp., *146* (1965).
29. PLAPP, B. V., AND COLE, R. D.: Purification and characterization of bovine liver β-glucuronidase. Arch. Biochem. Biophys., *116:* 193 (1966).
30. Recommendations of the International Union of Biochemistry on the Nomenclature and classification of enzymes. *In:* M. Florkin: Comprehensive Biochemistry, Vol. 13 (Elsevier Publishing Company, Amsterdam, The Netherlands, 1965).
31. SAWANT, P. L., SHIBKO, S., KUMTA, U. S., AND TAPPEL, A. L.: Isolation of rat liver lysosomes and their general properties. Biochim. Biophys. Acta, *85:* 82 (1964).
32. SCHNAITMAN, C., AND GREENWALT, J. W.: Enzymatic properties of the inner and outer membranes of rat liver mitochondria. J. Cell Biol., *38:* 158 (1968).
33. Schuel, H., Schuel, R., and Unaker, N. J.: Separation of rat liver lysosomes and mitochondria in the A-XII zonal centrifuge. Anal. Biochem., *25:* 146 (1968).
34. SCHULTZ, R. L.: Effects of ovariectomy and hypervitaminosis A on lysosomes of the rat conceptus. Teratology, *2:* 283 (1969).
35. SCHULTZ, R. L., AND JACQUES, P.: Characteristics of lysosomes in rat placental cells. Arch. Biochem. Biophys., *144:* 292 (1971).
36. SHIBKO, S., AND TAPPEL, A. L.: Rat kidney lysosomes: Isolation and properties. Biochem. J., *95:* 731 (1965).
37. SOTTOCASA, G. L., KUYLENSTIERNA, B., ERNESTER, L., AND BERGSTRAND, A.: An electron transport system associated with the outer membrane of liver mitochondria. J. Cell Biol., *32:* 415 (1969).
38. STAHN, R., MAIER, K. P., AND HANNIG, K.: A new method for the preparation of rat liver lysosomes. J. Cell Biol., *46:* 576 (1970).
39. STETTEN, M.: Metabolism of inorganic pyrophosphate. J. Biol. Chem., *240:* 2248 (1965).
40. SZEGO, C. M., SEELER, B. J., STEADMAN, R. A., HILL, D. F., KIMURA, A. K., AND ROBERTS, J. H.: The lysosomal membrane complex. Focal point of primary steroid hormone action. Biochem. J., *123:* 523 (1971).
41. TABOR, C. W., TABOR, H. AND ROSENTHAL, S. M.: Purification of amine oxidase from beef plasma. J. Biol. Chem., *208:* 645 (1954).
42. VILLEE, C. A.: Regulation of blood glucose in the human fetus. J. Appl. Physiol., *5:* 437 (1953).

43. WERNER, D., AND NEUPERT, E.: Functional and biochemical heterogeneity of the inner membrane of rat liver mitochondria. Eur. J. Biochem., *25:* 379 (1971).
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