THE ISOLATION OF LYSOSOMES FROM EHRLICH ASCITES TUMOR CELLS FOLLOWING PRETREATMENT OF MICE WITH TRITON WR-1339

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ABSTRACT

A method is described for obtaining highly purified lysosomes from Ehrlich ascites tumo cells grown in mice injected with Triton WR-1339. The isolated particles show a high specific activity for aryl sulfatase, representing an 80–90-fold purification over the homogenate, and a 15–18% yield of the total enzyme activity. Mitochondrial and microsomal marker enzymes are present in negligible amounts (0.2% of the activity of the homogenate). The biochemical evidence for a rather high degree of homogeneity of the fraction is supported by the electron microscopic examination of the purified lysosomes. The intracellular localizations of N-acetyl-β-glucosaminidase, NADH-cytochrome c reductase and NADPH-cytochrome c reductase in Ehrlich ascites cells are also reported, the first two being present in highest concentration in the combined mitochondrial-lysosomal fraction and the third in the microsomal fraction.

INTRODUCTION

Recently the remarkable stability of Ehrlich ascites tumor lysosomes has been reported, and it has been postulated that the composition of the membranes of these organelles may be responsible for their stability (1). Similarly the lysosomes of HEp–2 cells have been found by Thacore and Wolff (2) to be highly resistant to hypotonic media. Although there is evidence for variation in lysosome fragility based upon the tissue of origin (3–5), it is of considerable interest that Wagner and Roth (6) have also reported an increased stability of the lysosomes of certain hepatomas as compared with normal liver. On the basis of the limited data available, it has been suggested (1) that rapidly dividing cells may in general contain lysosomes with particularly stable membranes.

In order to obtain further evidence bearing on this suggestion and to study the chemical composi-

tion of the membranes of tumor cell lysosomes, it is desirable that these organelles be prepared in a highly purified state. In this report, the preparation of such a purified subcellular fraction from Ehrlich ascites cells is described. The procedure developed takes advantage of the effect of Triton WR–1339 on the density of lysosomes when this detergent is injected into the tumor-bearing mice. The criteria used for assessing the purity of the isolated particles are biochemical and morphological.

MATERIALS AND METHODS

Treatment of Animals

Ehrlich ascites tumor cells were propagated, harvested, and washed as described earlier (1). Triton WR–1339 (Ruger Chemical Company, Irvington-

on-Hudson, N.Y.) was injected intraperitoneally into the tumor-bearing mice at a dosage of 75 mg/100 g body weight 48–60 hr before harvesting the cells.

Fractionation of Cells

The washed and packed cells were homogenized and fractionated into the four major subcellular fractions [nuclear, mitochondrial-lysosomal (M + L), microsomal, and soluble fractions] according to methods described previously (1). The M + L fraction was further subfractionated on a discontinuous sucrose gradient. The M + L pellet, as obtained after the differential centrifugation, was gently suspended in sucrose solution ρ = 1.20 with one or two strokes of a teflon pestle in a Duall homogenizer (Kontes Glass Co., Vineland, N.J.). This suspension was usually prepared in 1:1 ratio, i.e., the M + L pellet from 1 ml of packed cells being suspended in 1 ml of the sucrose solution. The suspension was then transferred to the bottom of centrifuge tubes. On top of this solution two additional sucrose solutions, one having a density of 1.13 and the other of 1.05, were layered carefully, with the use of the Buchler Polystaltic Pump (Buchler Instruments, Inc., Fort Lee, N. J.). In most of these experiments the SW 25.1 rotor was used in the Spinco Model L–2 ultracentrifuge (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.); in a few experiments the SW 41 rotor was employed. With either rotor, the ratio of the sucrose solutions was kept constant, i.e., 1:2:2.5, extending from the top to the bottom of the centrifuge tube. The centrifugation in the SW 25.1 rotor was carried out at 25,000 rpm for 2.5 hr, the time being calculated from the moment the top speed had been reached. Deceleration of the rotor was allowed to take place with the brakes on, until the speed had slowed down to 10,000 rpm, at which time the brake was disengaged. Fractions (see Results) were collected manually from the top of the tubes with a glass syringe, and the volumes of the fractions were determined. The pellet present at the bottom of the centrifuge tube was suspended in 0.25 M sucrose to yield a 1:1 suspension in sucrose with respect to the original packed cell equivalent. (When the SW 41 rotor was used, centrifugation was carried out at 41,000 rpm for 50 min).

A second sucrose gradient (SW 41 rotor) was carried out on the lysosome-rich fraction (see Results) obtained from the above gradient. The discontinuous gradient consisted of the following volumes and densities of sucrose solutions: 3 ml of ρ = 1.17, 2 ml each of ρ = 1.14, 1.12 and 1.10, 1.5 ml of ρ = 1.08, and 1 ml of ρ = 1.05. Centrifugation was performed at 41,000 rpm for 2 hr. Fractions (see Results) were collected manually with a glass syringe. Since the volumes of the bands at the interfaces of

the sucrose solutions were rather small, they were determined by transferring the fraction into tared, small test tubes, weighing the solution and determining the density with an Abbe refractometer (Bausch & Lomb Inc., Rochester, N.Y.).

Enzyme Assays

Aryl sulfatase (aryl-sulfate sulfohydrolase, EC 3.1.6.1) and succinate-cytochrome c reductase (succinate: cytochrome c oxidoreductase, EC 1.3.99.1) were assayed as described previously (1). N-acetyl-β-glucosaminidase (β-2-acetamido-2-deoxy-D-glucoside acetamido deoxyglucohydrolase, EC 3.3.1.30) was assayed by an adaptation of the method of Weissmann et al. (7). The incubation mixture (0.2 ml) consisted of 0.05 M Na citrate-HCl buffer, pH 4.3, 0.005 M substrate, p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma Chemical Co., St. Louis, Mo.), and 0.02–0.1 ml of enzyme preparation, depending on the activity expected to be present. The incubation was performed at 37° for 30 min, the reaction being stopped by the addition of 2 ml of 0.05 M Na borate buffer, pH 10.4. The solution was deproteinized by shaking vigorously with 1 ml of a 1-pentanol-chloroform mixture (1:5, v/v). After centrifugation at 3000 rpm for 15 min in the International PR–2 centrifuge, (International Equipment Co., Needham Heights, Mass.) the clear upper layer was separated with a Pasteur pipette and the absorbance of the solution was determined at 400 nm in the Beckman DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) with cuvettes of 1 cm light path. One unit of the enzyme is defined as the amount of enzyme that hydrolyzes 1 μmole of substrate per min.

NADH-cytochrome c reductase (EC 1.6.11.3) was assayed by an adaptation of the method of Kamat and Wallach (8) for NADH diaphorase. The incubation mixture (2.0 ml) consisted of 0.05 M phosphate buffer, pH 7.4, 2 $\times$ 10^{-4} M KCN, 1 mg of cytochrome c, and an amount of enzyme which gave a satisfactory rate of reduction of cytochrome c in the presence of NADH. The mixture was first allowed to equilibrate at room temperature for about 5 min and then was checked at 37° in the Gilford recording spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio) at 550 nm for any reduction of the cytochrome c possibly due to the presence of endogenous NADH. NADH (P-L Laboratories, Milwaukee, Wis.) was then added to give a final concentration of 10^{-4} M, and the rate of reduction (linear part of the curve) of cytochrome c was measured against a blank containing all of the components of the reaction mixture except the enzyme. NADPH-cytochrome c reductase was measured in the same manner, except that the reaction was started with the addition of 10^{-4} M NADPH (P-L Laboratories,

Milwaukee, Wis.). One unit of each enzyme is defined as the amount of enzyme which reduces 1 nmole of cytochrome *c* per min.

Proteins were determined according to the method of Miller (9), with bovine serum albumin (Armour) as the standard. The sucrose used in the gradients was the density gradient grade sucrose from Mann Research Laboratories, Inc., New York, N.Y. Doubly distilled water which was deionized prior to the second distillation was used throughout.

Electron Microscopy

Samples for electron microscopy were fixed in cold 2% osmium tetroxide in Palade's buffer, pH 7.4 (10). Fractions collected from gradients were pelleted before fixation. Fixed material was dehydrated in graded alcohols and propylene oxide, and embedded in Epon 812 (11). Sections were cut on an LKB Ultrotome (LKB-Produkter Ab Stockholm-Brommal, Sweden), stained with lead citrate (12), and examined in a Hitachi HU-11B (Hitachi, Ltd., Tokyo, Japan) electron microscope.

RESULTS

Intracellular Distribution Patterns of N-Acetyl-β-Glucosaminidase and NADH- and NADPH-Cytochrome c Reductases

For the biochemical assessment of the purity of the lysosomes the presence or absence of five marker enzymes was used: succinate-cytochrome *c* reductase, aryl sulfatase, *N*-acetyl-β-glucosaminidase, NADH- and NADPH-cytochrome *c* reductases. Succinate-cytochrome *c* reductase and aryl sulfatase have been shown to be adequate markers for mitochondria and lysosomes, respectively, in Ehrlich ascites tumor cells (1). The intracellular

distribution pattern of the other three enzymes is shown in Fig. 1. It is evident that the glucosaminidase and NADH-cytochrome *c* reductase show the highest relative specific activity in the fraction (M + L) previously shown to contain the mitochrondrial and lysosomal enzymes (1). On the other hand, NADPH-cytochrome *c* reductase shows the highest enrichment in the microsomal fraction. Accordingly, NADH-cytochrome *c* reductase may be considered primarily a mitochondrial marker, whereas NADPH-cytochrome *c* reductase may be considered a microsomal marker. Bock et al. (13) consider NADH-cytochrome *c* reductase as a marker for both fractions, the mitochondrial and the microsomal, since they obtained a similar relative specific activity for this enzyme in the two fractions in Ehrlich ascites tumor cells, while de Duve et al. (14) have reported a reversed localization of the two enzymes in rat liver.

N-acetyl-β-glucosaminidase in the tumor cells, when assayed under isotonic conditions, i.e. in 0.25 M sucrose, for 10 min, displays the well established latency of lysosomal enzymes. The free activity is approximately 15% of the total, i.e. the activity measured in the presence of 0.2% v/v Triton X-100. Kinetic studies on the fully activated enzyme (i.e., in the presence of Triton X-100) showed that the reaction is linear with time of incubation, at least up to 1 hr. Under the conditions of the assay described in Materials and Methods, there is a linear relationship between the protein concentration of the enzyme preparation and the amount of product formed in the range between 0.1 and 1.0 mg of protein.

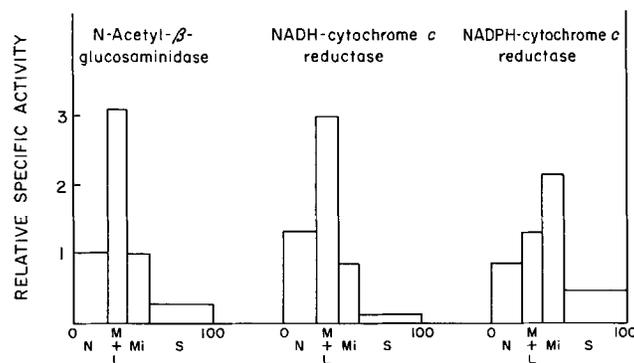NADH-cytochrome *c* reductase showed linearity

Figure 1 Subcellular distribution of *N*-acetyl-β-glucosaminidase, NADH-cytochrome *c* reductase, and NADPH-cytochrome *c* reductase in Ehrlich ascites tumor cells. Relative specific activity = percentage of total activity/percentage of total protein in the particular fraction. On the abscissa the fractions are represented by their relative protein content. *N* = nuclear fraction; *M* + *L* = mitochondrial + lysosomal fraction; *Mi* = microsomal fraction; and *S* = supernatant.

with enzyme concentration in the range between 10 and 100 μg of protein, while NADPH-cytochrome *c* reductase was linear between 200 and 600 μg of protein.

The method described for the subfractionation of the M + L fraction is based on that of Trouet (15), which was originally developed for the purification of rat liver lysosomes and which has been used with great success for the large scale isolation of various subcellular particles of rat liver (16). Fig. 2 shows the pattern obtained after the flotation centrifugation of an M + L fraction of Ehrlich ascites tumor cells from animals previously treated with Triton WR-1339. At the bottom of the centrifuge tube a gelatinous pellet is formed, and at the interface between the middle and lower sucrose solutions a thick layer of particles accumulates. As a result of these separations the sucrose solution in which the M + L fraction has originally been suspended (ρ = 1.20) becomes almost clear, and so is in most instances the middle layer (ρ = 1.13). At the interface between the uppermost and middle sucrose solutions, there accumulates another very light layer of particles, which is cloudy in appearance. It should be noted that a very similar pattern is obtained when the same procedure is used with an M + L fraction obtained from mice which have not been treated with Triton WR-1339. The main differences are in the size of the pellet, and in the width and composition of the top layer of particles.

Table I shows the data obtained in an experiment typical of many gradient experiments performed to fractionate the M + L preparations
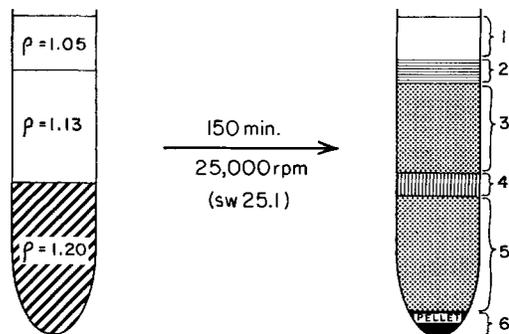

FIGURE 2 Purification of lysosomes from an *M* + *L* fraction by discontinuous gradient centrifugation. Preparation of the gradient and conditions of centrifugation are described under Materials and Methods. The numbers on the right side of the tube correspond to the numbers of the fractions collected by a glass syringe.

from control cells and from cells of animals injected with Triton WR-1339. As already indicated, fraction 2, which represents the purified lysosomes, presumably of a lighter density than normal lysosomes due to the accumulation of Triton (17), is present in the gradient obtained from normal cells as well. This finding is not unexpected, since it has been reported that lysosomes from these tumor cells have a median density of 1.155 (1). 5 per cent of the total lysosomal enzyme activity applied to the gradient could well be associated with particles of density of about 1.099, the density of the interface where the lighter lysosomes accumulate. As can be seen from Table I, this low-density lysosome fraction is practically free of any other marker enzymes. On treatment of the tumor-bearing mice with Triton WR-1339, however, the fraction becomes greatly enriched in lysosomal enzymes (aryl sulfatase increases from 5.4 to 41.3% and the glucosaminidase from 4.8 to 28.5%). Changes in protein content and in mitochondrial and microsomal enzymatic activities are relatively minor.

On the basis of increased specific activities, fraction No. 2 of the sucrose gradient of the Triton preparation represents, on the average, a 20-fold purification for aryl sulfatase over the starting material (M + L) (specific activity: M + L fraction, 0.042 unit/mg protein; fraction No. 2, 0.950 unit/mg protein), and about an 80- to 90-fold purification above the original homogenate (specific activity: 0.011 unit/mg protein). N-acetyl-β-glucosaminidase does not follow closely the purification pattern of the sulfatase, a larger fraction of the former enzyme being found in the original sucrose layer or in the pellet. The increase of the glucosaminidase in fraction No. 2 ranges only between 12- and 17-fold (specific activity: M + L fraction, 0.045 unit/mg protein; fraction No. 2, 0.715 unit/mg protein). This increase in purity represents about 50-fold purification over the homogenate (specific activity, 0.014 unit/mg protein). It should be noted that fraction No. 2 of the normal cells also represents an enrichment in lysosomal enzyme activity, but not more than a 3-fold purification with respect to the M + L fraction. The total yield of aryl sulfatase in fraction No. 2 is between 15 and 18% of the content of the homogenate, but only 7-8% in the case of N-acetyl-β-glucosaminidase.

Succinate-cytochrome *c* reductase and NADH-cytochrome *c* reductase show a 2-fold increase in specific activity in fraction No. 4 of the gradient

TABLE I

Separation of Lysosomes from M + L Fractions Obtained from Ehrlich Ascites Tumor Cells

Fraction number	Proteins % of total*		Aryl sulfatase % of total*		N-acetyl-β-glucosaminidase % of total*		Succinate-cytochrome c reductase % of total*		NADH-cytochrome c reductase % of total*		NADPH-cytochrome c reductase % of total*	
	N	T	N	T	N	T	N	T	N	T	N	T
1	0.2	0.2	0.2	0.6	0.1	1.1	0	0	—	—	0	0
2	0.8	2.1	5.4	41.3	4.8	28.5	0.1	0.7	0.7	0.9	0.6	1.0
3	1.5	2.1	5.8	10.7	4.2	8.5	0.4	3.4	2.7	3.6	2.1	1.8
4	17.5	22.4	37.9	28.7	27.9	29.4	45.3	56.2	25.3	37.8	14.7	17.4
5	42.8	49.4	31.7	14.6	38.7	23.4	27.3	33.2	55.2	49.1	64.2	65.2
6	37.1	23.6	18.9	3.9	24.2	8.5	27.1	6.6	16.0	8.5	18.4	14.4

Density gradient centrifugation of an M + L fraction of Ehrlich ascites tumor cells obtained from mice previously injected with Triton WR–1339, and from control cells, was performed as described under Materials and Methods. The percentages of each cellular constituent recovered in the M + L fractions from cells of the normal (N) and Triton-injected (T) mice were as follows: proteins — N 11.6, T 9.4; aryl sulfatase — N 58.5, T 43.6; N-acetyl-β-glucosaminidase — N 40.5, T 28.5; succinate-cytochrome c reductase-N 63.6, T 43.4; NADH-cytochrome c reductase — N 26.1, T 19.0; NADPH-cytochrome c reductase — N 22.0, T 20.4. The amount of M + L fractions, in terms of protein, which was placed in each tube was: N 57.0 mg, T 44.9 mg. These amounts correspond to the M + L fraction derived from about 6 ml of packed cells. The numbers of the fractions correspond to those described in the legend to Fig. 2.

* % of total refers to the percentage of the amount of protein and total units of enzymes recovered from the gradient. The overall recoveries from the amounts applied to the gradient were: proteins, 98%; succinate-cytochrome c reductase, 89%; aryl sulfatase, 98%; N-acetyl-β-glucosaminidase, 99%; NADH-cytochrome c reductase, 102%; NADPH-cytochrome c reductase, 89%.

(as compared with the M + L fraction), and a 4-fold purification over the whole homogenate. The activity of these two enzymes, as well as of NADPH-cytochrome c reductase, which is considered here as a microsomal marker, is insignificant in the purified lysosomal fraction and represents only 0.2% of the activities of the total homogenate. There was no meaningful increase in specific activity of NADPH-cytochrome c reductase throughout the gradient. On one occasion RNA was also determined in the various fractions of the gradient. Fraction No. 2 contained 0.7% of the RNA of the M + L fraction, a result indicating very little contamination by the rough endoplasmic reticulum.

The particulate nature of the two lysosomal enzymes in fraction No. 2 has been demonstrated in the following way. A desired volume of the fraction was diluted with 0.2 M sucrose to give a final concentration of sucrose between 0.25 and 0.4 M. The diluted suspension was centrifuged at 25,000 rpm for 25 min in the Spinco No. 40 rotor, the supernatant was separated with the aid of a

Pasteur pipette, and the pellet was carefully resuspended in 0.25 M sucrose in a ratio 1:1 with respect to the packed cell volume. Since the resuspended pellet contained approximately 90% of the enzymatic activities present in fraction No. 2 the lysosomal enzymes in this fraction are still particle-bound. A number of tests of the latency of fraction No. 2 preparations have given variable results. Further studies are required to elucidate the reason for this variability.

These biochemical findings appear to be supported by the electron microscopic observations on fraction No. 2. It should be pointed out first that the M + L fraction is very heterogeneous, containing, in addition to mitochondria and lysosomes, vesicular profiles, free ribosomes, and amorphous material. Fig. 3 a represents fraction No. 2 from control cells, and Fig. 3 b fraction No. 2 from cells obtained from mice previously injected with Triton WR–1339. In Fig. 3 a (control), electron-translucent vesicles and membranes with some amorphous material are spread over the whole field, and very few intact structures which
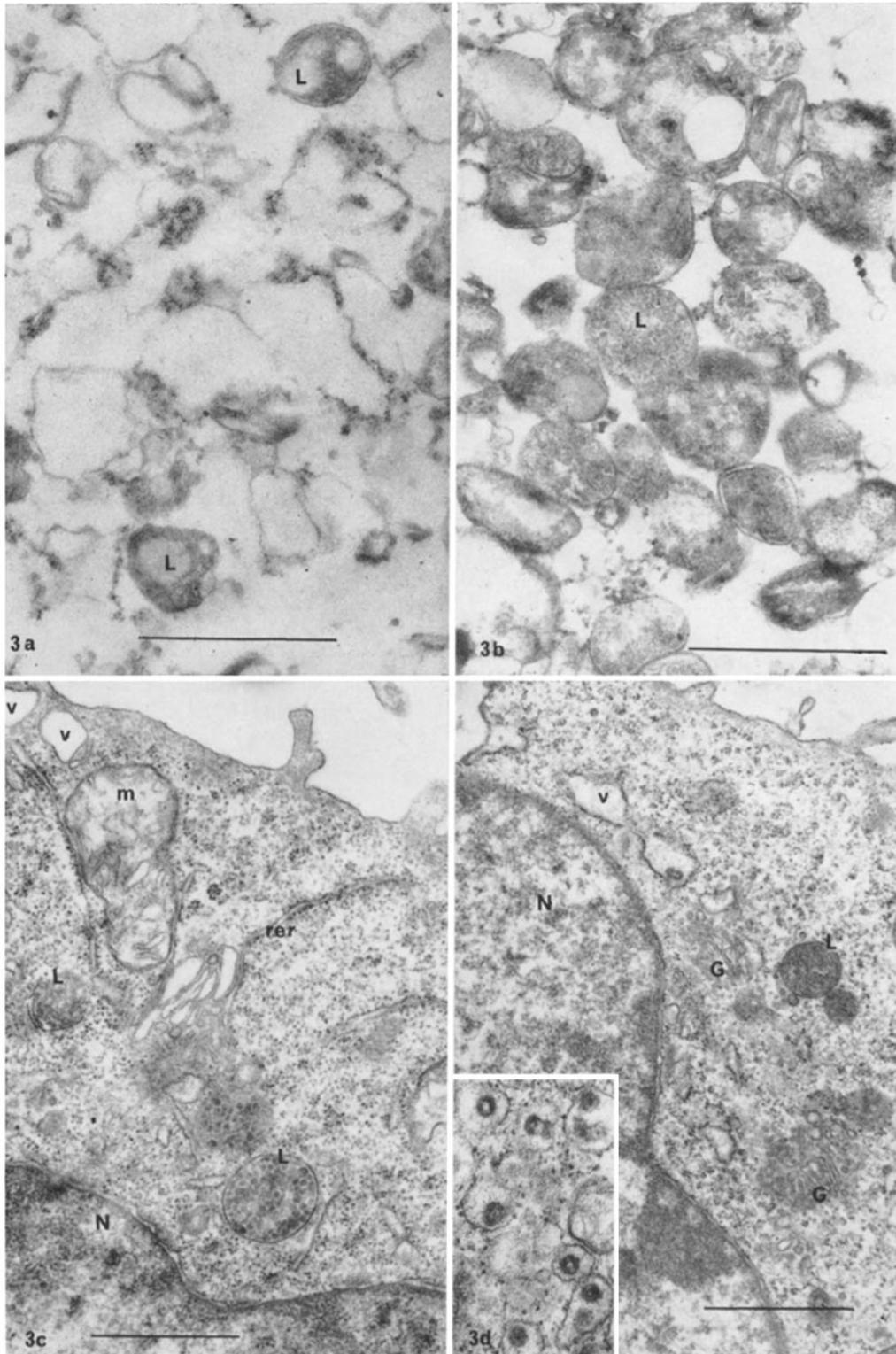
FIGURES 3 a and 3 b Electron micrographs. Fraction No. 2 obtained after centrifugation of M + L fractions on a discontinuous sucrose gradient (see Materials and Methods). (3 a) normal ascites cells and (3 b) ascites cells from Triton-injected mice. Fig. 3 b contains a larger proportion of lysosomes (L) and less membranous material than Fig. 3 a. Scale = 1 μ. × 29,000.

FIGURES 3 c and 3 d Electron micrographs of ascites tumor cells from (3 c) untreated mice and (3 d) Triton-injected mice. These cells contain a number of vacuoles (v) and little rough endoplasmic reticulum (rer). G = Golgi apparatus; N = nucleus, m = mitochondrion. Scale = 1 μ. × 22,000. Inset: virus-like particles seen in a number of cells from both groups. × 33,500.

are identifiable as lysosomes can be seen. It is possible that the electron-translucent vesicles are mainly damaged lysosomes, or that they may be the vacuoles seen in the intact cell (Fig. 3 c). The latter structures are probably part of the vacuolar apparatus of the cell, and therefore an integral part of the system of uptake, transport and excretion in the cell. On the other hand, in Fig. 3 b (Triton-injected) the most prevalent structures are lysosomes, with only some of the membranous and amorphous material contaminating the M + L fraction. It is interesting to note that, in distinction to the Triton-filled lysosomes from rat liver described by Wattiaux, Wibo and Baudhuin (17), these lysosomes do not appear to be as distended and greatly enlarged above their normal size. However, since they do have an altered density, they are likely to be secondary lysosomes. An electron micrograph of a cell from an animal injected with Triton WR-1339 is shown in Fig. 3 d, which may be compared with the control cell (Fig. 3 c). The virus-like particles are shown (Fig. 3 d, inset) because of their usual presence in electron micrographs of our cells. Also, although we have not made a systematic study of the point, it appears that the Golgi apparatus is more prominent in the cells from detergent-treated mice than from controls.

Ribosome-bearing microsomal vesicles are absent from both preparations (Figs 3 a and 3 b), which is in accordance with the very low amount of RNA found in these fractions. However, both the intact cell and the microsomal fraction contain little rough endoplasmic reticulum, and the ribosomes are mainly free or as polysomes.

Attempts were made to achieve further purification of the lysosomes by fractionating the particles from fraction No. 2 in a second discontinuous sucrose gradient. In experiments of this type, a pellet of the particulate material of fraction No. 2 was obtained from the diluted suspension as described above. The pellet was suspended in 0.6 ml of 0.25 M sucrose, and 0.5 ml was layered on top of the gradient (see Materials and Methods). The remainder of the suspension was used for enzyme assays and for protein determinations. After centrifugation at 41,000 rpm for 120 min in the SW-41 rotor, four distinct layers separated at the interfaces of the various sucrose solutions. Fig. 4 shows the pattern obtained, and Table II, the results for enzymatic activities and protein content in the separated fractions. From the table it can be seen
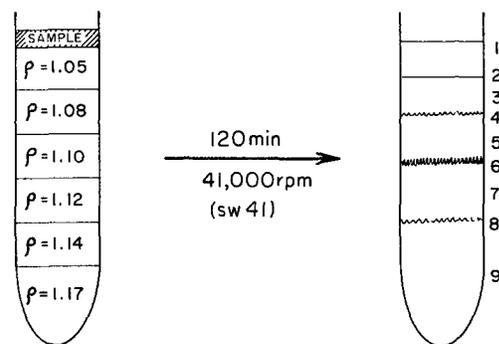

FIGURE 4 Subfractionation of lysosomes by discontinuous sucrose gradient centrifugation. Preparation of the gradient and conditions of centrifugation are described under Materials and Methods. The numbers on the right side of the tube correspond to the numbers of the fractions collected.

that the band (No. 6) at the interface between the sucrose solutions of density 1.10 and 1.12 contains the highest percentage of both lysosomal enzymes, and of proteins as well. In this band, the specific activity remained unchanged with respect to the specific activity of the material applied to the gradient, a result indicating that no further purification of the bulk of the particles was achieved. The high specific activity and total aryl sulfatase content of fraction No. 1 are probably due to the solubilized enzyme. Fraction No. 2, which was a distinct band at the interface of sucrose solutions of density 1.05 and 1.08, contains only a very small portion of the total protein content of the gradient, and shows a very high specific activity for aryl sulfatase. We do not have a good explanation for this high specific activity, since the electron micrographs (Fig. 5 a) revealed mostly amorphous material (perhaps derived from the contents of lysosomes) as well as membranous material, with only very occasional intact particles. Since lysosomes filled with Triton WR-1339 are probably more fragile than normal lysosomes, the former may have incurred some damage when the original fraction No. 2 was being handled for preparation for its further fractionation on the second gradient.

The distribution of total activities, as well as of the incidence of the highest specific activities for N-acetyl-β-glucosaminidase, in this second gradient does not follow exactly that of aryl sulfatase. In the major band for proteins and for aryl sulfatase (No. 6), the specific activity of the glucosaminidase is practically the same as that of the

TABLE II

Results of Subfractionation of Lysosomes by Discontinuous Sucrose Gradient Centrifugation

Fraction number[*]	Proteins		Aryl sulfatase		N-Acetyl-β-glucosaminidase	
	% of total	mg/ml	% of total	Units/mg protein	% of total	Units/mg protein
1	9.7	0.038	17.6	1.539	8.2	0.500
2	2.4	0.045	4.6	1.633	3.4	0.844
3	4.3	0.036	2.4	0.483	7.6	1.055
4	3.8	0.059	2.9	0.654	4.0	0.627
5	7.5	0.025	6.4	0.736	11.0	0.880
6	26.2	0.214	34.9	1.128	32.9	0.747
7	24.4	0.095	18.3	0.637	18.5	0.452
8	6.8	0.119	7.8	0.974	8.9	0.773
9	14.9	0.018	4.8	0.277	5.4	0.215

Tumor cells from mice previously injected with Triton WR–1339 were used to prepare an M + L fraction, and then purified lysosomes as in Fig. 2. The specific activity of the pelleted fraction No. 2 from the first gradient was for aryl sulfatase 1.200 and for N-acetyl-β-glucosaminidase 0.825 unit/mg of protein. This fraction (0.493 mg protein) was then subjected to a further fractionation in a discontinuous sucrose gradient as described in the text and under Materials and Methods. The recoveries for protein were 108%, for aryl sulfatase, 96% and for the glucosaminidase, 86%, of the amount applied to the gradient.

[*] Numbers of fractions refer to the numbers shown on Fig. 4.

material applied to the gradient. It is interesting to note that this enzyme shows the highest specific activity in Fraction No. 3 below the uppermost interface, in the sucrose layer of a density of 1.08.

Electron micrographs of three fractions, 2, 6, and 8, show that the first one (No. 2) differs significantly morphologically from the other two, in that it contains mainly membranous material (Fig. 5). The other two fractions (Nos. 6 and 8) are very similar in their appearance (Figs 5 b and 5 c) and are almost identical to fraction No. 2 from the first gradient (Fig. 3 b).

Three separate experiments of the type described in Table II were performed on the fraction No. 2 obtained from gradient experiments of the type reported in Table I. Data similar to those shown in Table II were obtained in all experiments.

DISCUSSION

The isolation of lysosomes in sufficient yield and purity to carry out analyses of the chemical composition of their membranes is complicated by the heterogeneity of these subcellular organelles in regard to their size and functional state at a given time. The difficulty was overcome to some extent by the discovery of Wattiaux, Wibo, and Baud-

huin (17) that animals which are injected either intravenously or intraperitoneally with Triton WR–1339 accumulate this detergent in the lysosomes of liver (17), kidney (18), some hepatomas (19), and rat yolk sac cells (20). Since detergent-filled lysosomes are lighter than normal, their separation from other subcellular particles by isopycnic centrifugation is greatly facilitated.

Trouet (15) has suggested that the detergent is concentrated in the lysosomes of liver in the form of a serum α-lipoprotein complex. The actual formation of such complexes in animals injected with Triton WR–1339 has been shown by Scanu, and Oriente (21). In the experiments described in the present report, the tumor cells were directly exposed to the detergent in the mouse peritoneal cavity. Miller, and Janicki (22, 23) have shown a selective cytotoxic effect of Triton WR–1339 on cell cultures of malignant origin even in media of low protein content. This may simply be an effect on the plasma membrane, but it also may indicate the direct uptake of the detergent, thus exerting a higher toxicity on the tumor cells.

Although the presence of Triton WR–1339 in the tumor lysosomes has not been directly demonstrated through the use of labeled detergent, previous studies on the uptake of the detergent by
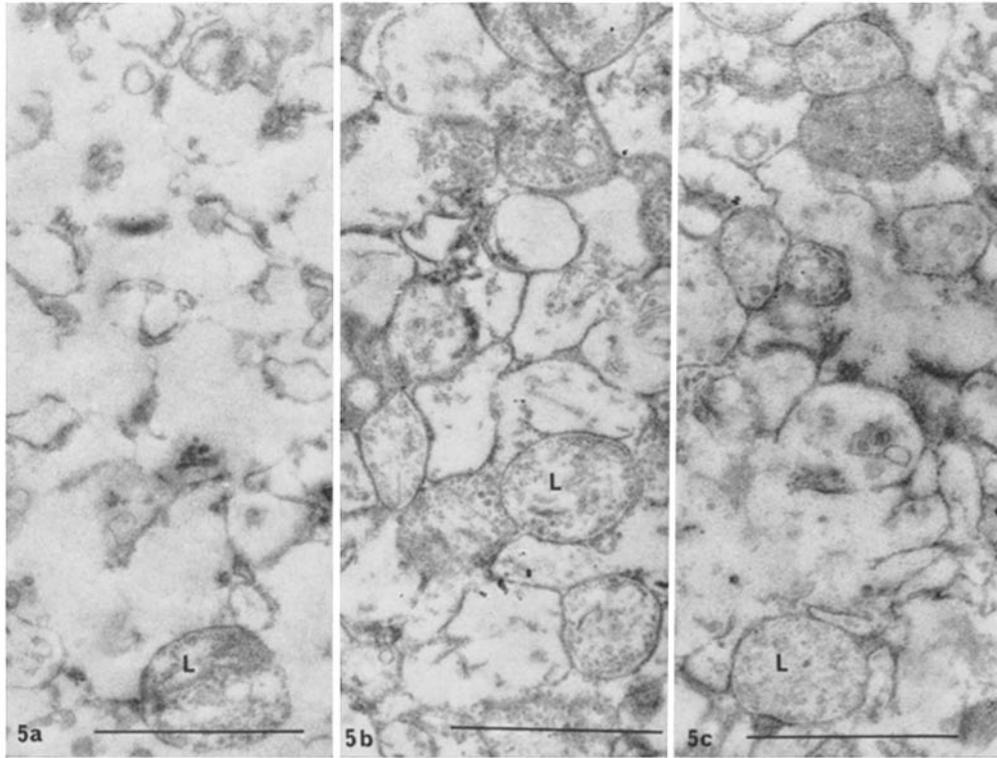
FIGURE 5 Electron micrographs of fractions obtained by an additional sucrose gradient centrifugation (see Materials and Methods) of the lysosome fraction from ascites cells of Triton-injected mice. Fraction No. 2 from the second density gradient (Fig. 5 a) contains a large proportion of membranous material, as well as some amorphous material, and few intact lysosomes. Fraction No. 6 (Fig. 5 b) and fraction No. 8 (Fig. 5 c) contain mainly lysosomes and resemble the original preparation shown in Fig. 3 b. L = lysosome. Scale = 1 μ. × 28,000.

hepatomas (19) and by normal tissues (17, 18, 20) make it very likely that the uptake has occurred in a similar manner in the Ehrlich cells. On the basis of altered equilibration density with no change in the sedimentation velocity of Triton-treated rat liver lysosomes, Wattiaux has concluded (24) that the uptake of Triton must be accompanied by an increased hydration of the matrix of the organelles. Our findings support his conclusions, since the equilibration density of the Ehrlich ascites lysosomes, 1.113, as determined in the second gradient, is even lower than the density reported for Triton WR-1339, namely, 1.135 (24), and is the same as that found for detergent used in our laboratory, namely, 1.113.

It seems appropriate, therefore, to assume that intraperitoneally-injected Triton WR-1339 has been taken up by Ehrlich ascites tumor cells and

has led to the formation of lysosomes of lowered density. Although such a response would be similar to that of liver cells of detergent-injected rats, the morphological appearance of the light lysosomes of tumor cells is quite different from that of light lysosomes of rat liver (16). Whereas most of the particles that accumulated in fraction No. 2 of the first gradient (Fig. 3) appear to be of the same size and similar electron-opacity as the lysosomes in the intact control cells, liver lysosomes isolated by a similar procedure from rats injected with Triton WR-1339 are very much enlarged with the electron-opaque material pushed to the sides of the particles. The difference in the response of the lysosomes to Triton WR-1339 could be due to a difference in the composition and architecture of the membranes of these particles. This hypothesis receives some support from previous observations

(1) showing that Ehrlich ascites tumor lysosomes are more resistant to hypotonic media and to thermal activation than rat liver lysosomes. On the other hand, it is also possible that endocytosis is much more intensive in the tumor cells than in the liver cells, the formation of the endocytic vacuoles being faster than in liver cells, thereby yielding smaller vacuoles. Thus, it could well be that the size and membrane characteristics of the secondary lysosomes in Ehrlich ascites tumor cells are determined mainly by the primary lysosomes. These considerations might also explain the very high level of purification of the particles in a single step, and the relatively high yield of total enzyme activity in these purified lysosomes. In liver the highest purification obtained was about 50-fold, with an 8–10% yield of the total acid phosphatase activity (16), while the purified lysosomes reported herein represent an 80–90-fold purification with a 15–18% yield of the total aryl sulfatase activity. On the other hand, the results are not as good when measured with N-acetyl-β-glucosaminidase and point to a rather significant heterogeneity of the enzymatic composition of the particles. Therefore, comparisons based on different marker enzymes do not seem to be warranted.

Ehrlich ascites tumor cells (25), like the cells of most rapidly proliferating tumors (26), have a poorly organized endoplasmic reticulum, with few vesicles, but a large number of polysomes and free ribosomes. This fact may also contribute to the ease with which a highly purified lysosomal fraction can be obtained, since contamination of the fraction by the endoplasmic reticulum is minimized for

tumor cells, especially when compared to rat liver. The application of Trouet's method (15) for the isolation of lysosomes from a homogeneous cell population such as our strain of Ehrlich ascites tumor cells (1) should be of value in further studies of the mechanism of the functioning of the vacuolar apparatus of the cell, and of the possible relationship of the chemical composition of the membrane to certain characteristics of these particles. As compared to liver lysosomes, the tumor particles, when obtained after Triton WR-1339 pretreatment, seem to retain more of their original character, as judged by their electron microscopic appearance and by the less marked drop in their density (from 1.155 to 1.113; liver lysosomes show a drop in density from 1.21 to 1.117). If the postulation mentioned earlier regarding the relative sizes of endocytic vacuoles and primary lysosomes is correct, secondary lysosomes of the tumor cells should have membranes with a relatively high percentage of primary lysosome membrane and, therefore, be especially suitable for chemical studies of lysosomal membranes. These remarks are, of course, based upon the difficulty of preparing primary lysosomes, with the possible exception of those in leukocytes.

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