

ANALYTICAL SUBCELLULAR FRACTIONATION OF CULTIVATED MOUSE RESIDENT PERITONEAL MACROPHAGES*

BY C. DARTE‡ AND H. BEAUFAY

*From the Laboratoire de Chimie Physiologique, Université Catholique de Louvain, and International
Institute of Cellular and Molecular Pathology, Brussels, Belgium*

The understanding of several key functions of macrophages at the subcellular level still calls for a better knowledge of the enzyme and centrifugal characteristics of their subcellular components. Previous cell fractionation studies on macrophages have often been focused on a particular organelle: lysosomes have been early identified by Cohn and Wiener (1, 2); plasma membranes have been prepared taking the specific activity of 5'-nucleotidase as a putative index of their purification (3); and various plasma membrane constituents including lipids, enzymes, and receptors have been characterized using isolated membranes (3, 4) or intact cells (5–11). Nevertheless, the present knowledge of the subcellular biochemistry of macrophages and of the experimental conditions that make it possible to separate their different cell components is sparse and strongly contrasts with the abundant information on their functions in inflammatory and host-defense processes (see reference 12 for a review). This gap may become a limiting factor in future studies on functions that require intimate cooperation between several subcellular entities.

A more comprehensive and analytical approach to the subcellular biochemistry of macrophages has been attempted in recent years (13–16). In this work, we have determined the distribution patterns of a wide spectrum of enzymes in differential and isopycnic centrifugation, starting from cytoplasmic extracts of mouse resident peritoneal (MRP)¹ macrophages cultivated for 3 d. Density equilibration experiments were also carried out on cytoplasmic extracts treated with digitonin or with sodium pyrophosphate, and on cytoplasmic extracts derived from cells cultivated for 24 h in the presence of Triton WR-1339. Our results establish enzyme and centrifugation properties of the cytoplasmic components in cultivated MRP macrophages that have been used to investigate subcellular aspects of the synthesis of prostaglandin E₂ (Darte and Beaufay, manuscript in preparation).

Materials and Methods

Harvest and Fractionation of MRP Macrophages. Unstimulated macrophages were obtained from NMRI mice by peritoneal washing with 5 ml Dulbecco's modified Eagle's medium

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‡ To whom requests for reprints should be addressed at Université Catholique de Louvain, Laboratoire de Chimie Physiologique, UCL 75.39, Avenue Hippocrate 75, B-1200 Brussels, Belgium.

¹ Abbreviations used in this paper: MRP macrophage, mouse resident peritoneal macrophage; PBS, phosphate-buffered saline.

supplemented with 15% inactivated fetal calf serum, 200 U/ml penicillin, 0.1 mg/ml streptomycin, and 6 U/ml heparin (medium A). Peritoneal exudate cells suspended in 15 ml medium A were transferred into Falcon petri dishes (Becton, Dickinson & Co., Grenoble, France) at a density of 4×10^5 cells/cm². After incubation for 90 min at 37°C in the presence of 10% CO₂ the culture medium was removed and nonadherent cells were discarded by vigorously washing the dishes with three changes of phosphate-buffered saline (PBS) solution (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, and 1.5 mM KH₂PO₄). Adherent cells were reincubated in 15 ml of fresh medium A, without heparin, for 72 h (cultivated MRP macrophages), or for a shorter time when specified. Afterwards, the medium was poured off and the cell monolayer was washed again with three changes of ice-cold PBS solution.

Cells were then harvested from the petri dishes by being scraped with a rubber spatula, suspended in 2.5 ml ice-cold PBS solution, and recovered by centrifugation at 2,000 rpm for 10 min in the JS-4.2 rotor of the J-6 Beckman centrifuge (Beckman Instruments, Inc., Palo Alto, CA). The resulting pellets were resuspended at 4°C in 1 ml suspension medium that contained 0.25 M sucrose, 1 mM EGTA, and 3 mM imidazole-HCl buffer, pH 7.4. Cells were disrupted by five successive slow passages through a syringe needle (16 × 0.5 mm bore). After centrifugation for 3 min at 1,400 rpm the supernate was removed and saved, and the pellet was resuspended in 1 ml suspension medium for another series of five passages through the needle. The whole operation was repeated until a total number of 30 passages was reached. The supernates were pooled and denoted "cytoplasmic extract"; the pellet was resuspended and denoted "nuclear fraction." The cytoplasmic extract was fractionated quantitatively either by differential centrifugation or by density equilibration in a linear gradient of sucrose, according to the principles and methods reviewed by Beaufay and Amar-Costesec (17).

Fractionation by differential centrifugation was carried out at 4°C, in the n° 40 rotor of the Spinco centrifuge (Beckman Instruments, Inc., Spinco Div.), using the suspension medium described above to wash and resuspend the pellets. The flow diagram included centrifugation of the cytoplasmic extract for 3 min at 12,500 rpm and washing in 6 ml medium to give the M fraction, centrifugation of the combined supernates for 6.75 min at 25,000 rpm and washing in 6 ml medium to give the L fraction, centrifugation of the resulting combined supernates (≈20 ml) for 30 min at 40,000 rpm and washing in 10 ml medium to give the P (microsomes) and S (supernate) fractions. This experimental procedure is similar to that originally adopted in studies on the liver (18).

Isopycnic centrifugation was carried out in a linear gradient of sucrose, using the E-40 rotor described elsewhere (18) under the exact conditions adopted previously by Canonico et al. (13). Cytoplasmic extracts (10 ml) were laid over: (a) a gradient of sucrose (32 ml) extending linearly with respect to volume from 1.10 to 1.25 density units, and (b) a layer of sucrose solution (6 ml) with a density of 1.35. After centrifugation for 160 min at 40,000 rpm, the gradient was quantitatively recovered in 12 fractions of nearly identical volume. Weight, average density, and protein and enzyme activities were determined in each gradient fraction. Density distributions were computed, normalized, and averaged as described in reference 17. In some experiments (see legends to Figs. 6 and 7) the cytoplasmic extract was supplemented with digitonin (70 μg/mg protein), or with sodium pyrophosphate (15 mM, pH 8) before the run. In other experiments the cytoplasmic extracts that were loaded onto the gradient derived from MRP cells cultivated for 24 h in medium A, without heparin, supplemented with 0.2 mg Triton WR-1339/ml.

Enzyme and Biochemical Assays. The methods described previously (13) have been used to assay lactate dehydrogenase, malate dehydrogenase, cytochrome *c* oxidase, *N*-acetylglucosaminyltransferase, galactosyltransferase, acid α -galactosidase, acid β -*N*-acetylglucosaminidase, acid β -glucuronidase, acid α -mannosidase, α -glucosidase, sulphatase C, and 5'-nucleotidase. The conditions used to assay other enzyme activities are summarized in Table I. They have been adapted from previously published methods, with minor modifications to increase their sensitivity or to adapt the assay for the kinetics of the macrophage enzyme. Protein was determined according to Böhlen et al. (23); cholesterol was determined by gas chromatography as described earlier (20).

Several enzymes showed structure-linked latency. Unless otherwise specified, the values given refer to activities determined in the presence of Triton X-100 at a high enough concentration

TABLE I
Assay Conditions for Enzymes

Enzyme	Substrates	Buffer	Additions	Temperature °C	Compound measured	References
Mannosyltransferase	GDP*-[U- ¹⁴ C]mannose (5.6.10 ⁻³ mM) Dolichyl phosphate (44 μg/ml)	Tris-HCl, 80 mM, pH 7.6	2 mM EDTA 11.5 mM MgCl ₂ 1 mM ATP 2.5 mM DTT‡ 0.4% Triton X-100	15	¹⁴ C in lipid extract	19
NADH cytochrome <i>c</i> reductase	NADH (75 μM) Cytochrome <i>c</i> (5.2 μM)	Sodium phosphate, 0.36 M, pH 6.8	30 mM nicotinamide 0.2 mM NaCN	25	Reduced cytochrome <i>c</i>	20
NADPH cytochrome <i>c</i> reductase	NADPH (75 μM) Cytochrome <i>c</i> (5.2 μM)	Sodium phosphate, 0.36 M, pH 7.7	30 mM nicotinamide 0.2 mM NaCN 0.38 M NaCl 0.1% Triton X-100	25	Reduced cytochrome <i>c</i>	20
NAD glycohydrolase	[Carbonyl- ¹⁴ C]nicotin- amide adenine dinu- cleotide (1.5 mM)	MES§-KOH, 50 mM, pH 6.5	1 mM EDTA 0.1% Triton X-100	37	¹⁴ C in the medium passed through alumina columns	21
Catalase	H ₂ O ₂ (1.3 mM)	Imidazole-HCl, 25 mM, pH 7.4	1 mg/ml albumin 0.5% Triton X-100	25	Residual H ₂ O ₂	22
Alkaline phosphodies- terase I	<i>p</i> -Nitrophenol-thymi- dine 5'-phosphate (1.5 mM)	Glycine-KOH, 0.1 M, pH 9.6	2 mM Zn acetate 0.1% Triton X-100	37	<i>p</i> -Nitrophenol	20

* Guanosine diphosphate.

‡ Dithiothreitol.

§ 2-[*N*-morpholino]ethane sulfonic acid.

to remove the diffusion barrier of the surrounding membrane (total activities). When the enzyme latency was examined (Fig. 4), assays were also performed in the absence of the detergent, in a medium that contained 0.25 M sucrose to avoid osmotic imbalance between the vesicle content and the surroundings (free activities). Absolute values of enzyme activities are given in international units, i.e., micromoles of substrate acted upon per minute under the assay conditions. However, units were as defined by Cooperstein and Lazarow (24) for cytochrome *c* oxidase, and by Baudhuin et al. (22) for catalase, because these reactions follow first-order kinetics.

Electron Microscopy. Cells cultivated for 48 h were fixed in the Falcon petri dishes for 1 h at room temperature in 1% glutaraldehyde and postfixed in 1% osmium tetroxide at 4°C for 1 h, both fixatives having been made in 0.1 M cacodylate buffer, pH 7.4. They were then dehydrated and embedded in Spurr (25). Thin sections were stained with lead citrate and uranyl acetate, and examined on a Philips EM 301 electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ).

Materials. Radiochemicals were purchased from The Radiochemical Centre Ltd., Amersham, United Kingdom (UK). Digitonin was from Merck AG, Darmstadt, Federal Republic of Germany. Substrates for fluorimetric assays were obtained from Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, UK. Dulbecco's modified Eagle's medium and fetal calf serum were from Gibco Bio-Cult Ltd., Paisley, Scotland. Biofluor was a product of New England Nuclear, Boston, MA. Spurr was a product of Agar Aids, Stansted, Essex, UK. All other analytical grade reagents were purchased from J. T. Baker Chemicals B.V., Deventer, The Netherlands.

Results

Morphology. Fig. 1 illustrates some structural features of the cells after 48 h cultivation. In addition to mitochondria, stacks of smooth cisternae characteristic of Golgi complexes, and large cisternae of the rough endoplasmic reticulum, the cytoplasm contains many small smooth vesicular elements, and membrane-bounded organelles loaded with electron-dense polymorphous material, extremely variable in

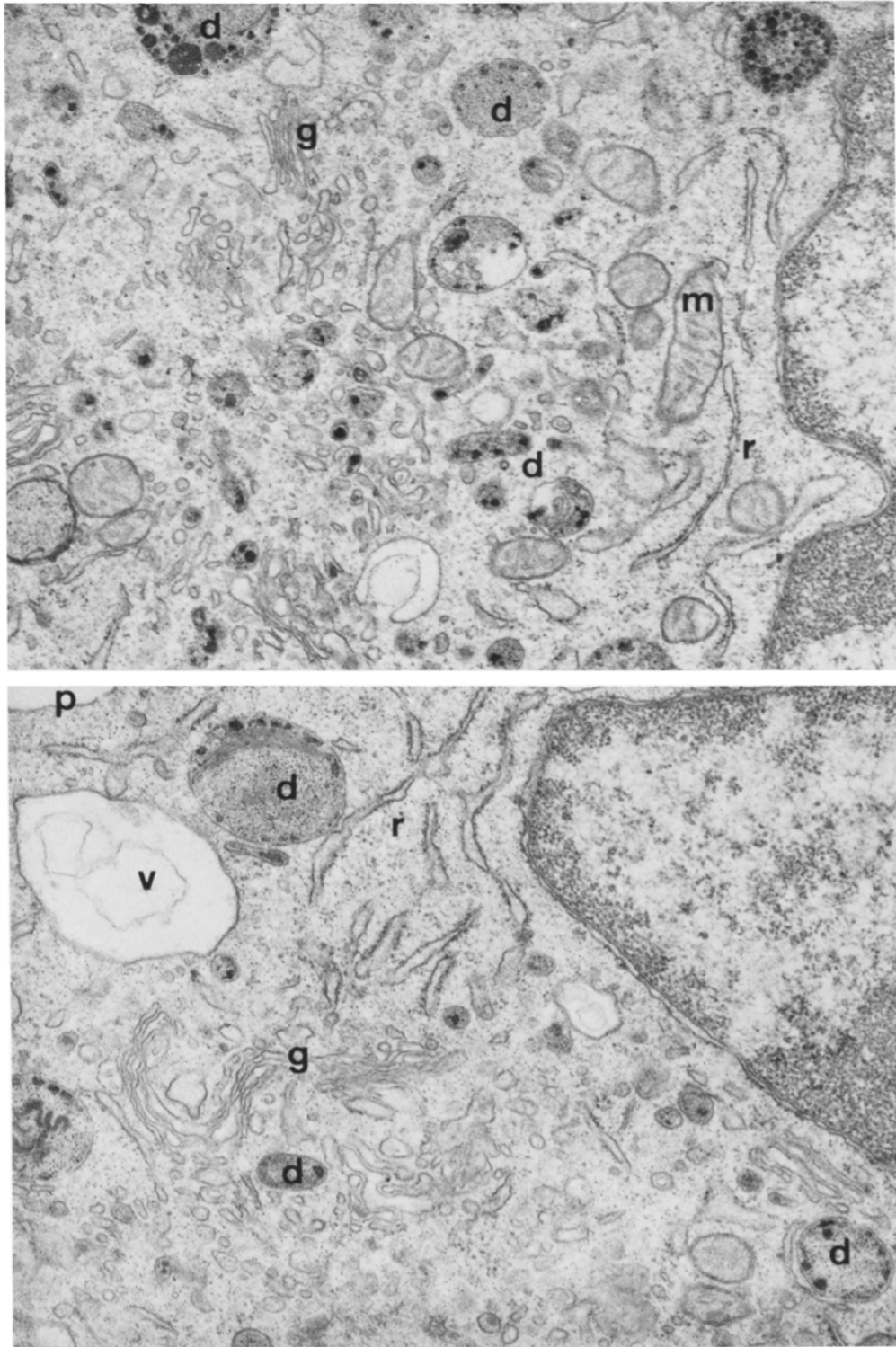


FIG. 1. Electron micrographs of MRP macrophages cultivated for 48 h. These micrographs illustrate the morphology of mitochondria (m), dense bodies (d), Golgi complexes (g), and rough endoplasmic reticulum (r) in these cells. The large vacuole (v) is near the cell periphery (p). Dense bodies vary widely in sizes and vesicular contents. $\times 30,000$.

size and studded with ferritin particles. Ferritin also occurs in the cytoplasmic sap. Some large empty vesicles are seen occasionally and become more prominent during aging of the cells. A number of these vesicles are located near the cell periphery and probably correspond to invaginations of the plasma membrane.

Enzyme Kinetics. The conditions described in Table I for the assay of enzymes were established after kinetic studies, taking into account the influence of pH, temperature, substrate concentration, ionic composition, addition of detergent, and other factors on the velocity of each enzyme-catalyzed reaction. In many cases these studies have demonstrated much similarity to the properties of the homologous enzyme reaction in rat liver.

The NADPH cytochrome *c* reductase activity in cultivated MRP macrophages is markedly influenced by the ionic strength of the reaction medium and reaches a maximum at 0.4 M (Fig. 2). In agreement with observations made by Phillips and Langdon (26) on the purified rat liver enzyme, a similar effect is seen with the NADPH cytochrome *c* reductase of rat liver microsomes, except that the activity curve levels off at 0.4 M. Mannosyltransferase was readily inactivated upon incubation at 25°C or above (Fig. 3) and, as for the rat liver enzyme (19), production of dolichyl phosphate mannose evolved linearly only at a low enough temperature (10–15°C). Interestingly, catalase is fairly active in MRP macrophages and the enzyme occurs to a large extent in a latent form (Fig. 4). Without detergent in the reaction medium (free activity) catalase activity is <20% of the activity measured in detergent-disrupted particles, which indicates that the enzyme occurs within membrane-bounded organelles. Moreover, digitonin also reveals the full activity of catalase (Fig. 4), but this result requires a much higher concentration of digitonin than full activation of acid β -*N*-acetylglucosaminidase and other lysosomal enzymes (13). The digitonin-induced increase in free activity is concomitant with the release of catalase in the suspension medium (not shown). The differential susceptibility to digitonin of latent catalase relative to latent acid hydrolases is reminiscent of the distinct behavior of lysosomes and peroxisomes in rat liver (27).

Table II gives the specific activities of the enzymes assayed in our experiments.

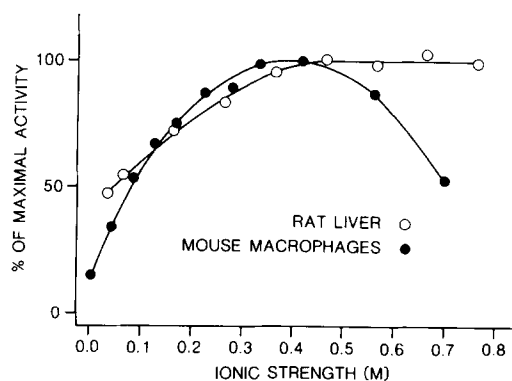


FIG. 2. Effect of ionic strength on NADPH cytochrome *c* reductase activity. Enzyme activities were assayed in liver microsomes and in cytoplasmic extracts obtained from cultivated MRP macrophages. The assay conditions were as described in Table I, except that the concentration of phosphate and sodium chloride was varied in order to adjust the ionic strength at the values given on the abscissa. Maximal activities, expressed in nanomoles cytochrome *c* reduced per minute \times milligram protein were 32 in macrophages, and 168 in liver microsomes.

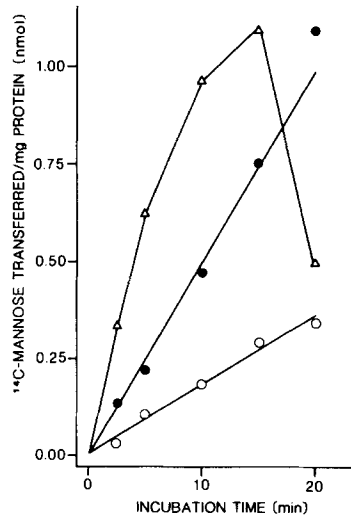


FIG. 3. Effect of temperature on the time course of mannosyltransferase reaction. Cytoplasmic extracts (14 μ g protein per assay) were incubated under the conditions given in Table I, at 10°C (○), 15°C (●), and 25°C (Δ) for various times, before extraction of glycosylated lipids and measurement of their radioactivity.

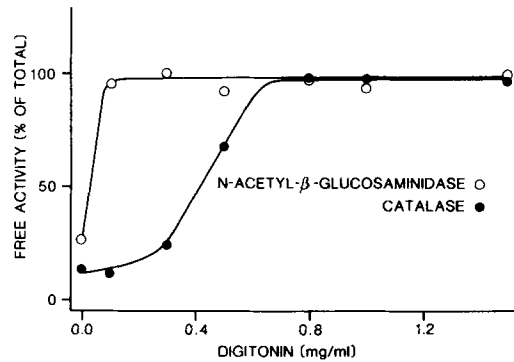


FIG. 4. Latency of catalase and *N*-acetyl- β -glucosaminidase, and influence of digitonin. Cytoplasmic extracts (66 μ g protein and 1.3 μ g cholesterol) were maintained in 0.2 ml suspension medium that contained digitonin at the concentration given on the abscissa scale for 30 s at 25°C. Free and total activities of catalase and *N*-acetyl- β -glucosaminidase were determined (see Table I and Materials and Methods), using 100 and 20 μ l of the digitonin-treated material, respectively. Total activities were 10.4 mU/mg protein for catalase and 90 mU/mg protein for *N*-acetyl- β -glucosaminidase.

These values are similar to those reported earlier (13), except for galactosyltransferase and *N*-acetylglucosaminyltransferase, which were about fourfold higher in our preparations. The differences perhaps result from changes in the medium used for cultivation of the cells.

Fractionation by Differential Centrifugation. The sedimentation profiles of enzymes after fractionation of macrophage homogenates by differential centrifugation are shown in Fig. 5. Fractions N, M, L, and P correspond to material of decreasing average sedimentation rate; S is the final supernate (see Materials and Methods). This latter fraction contained most of the lactate dehydrogenase (Fig. 5 r) and half of the malate dehydrogenase (f) activity. The other enzymes were mainly recovered in

TABLE II
*Enzyme Activities in Cytoplasmic Extracts Obtained from MRP Macrophages
 Cultivated for 72 h*

Constituents	mU/mg protein*
Lactate dehydrogenase	1,062 ± 366 (7)
Cytochrome oxidase	13.0 ± 4.7 (8)
Malate dehydrogenase	875 ± 311 (6)
Acid α -mannosidase	17.0 ± 10.5 (7)
Acid <i>N</i> -acetyl- β -glucosaminidase	94 ± 29 (6)
Acid α -galactosidase	6.7 ± 2.7 (6)
Acid β -glucuronidase	1.94 ± 0.54 (5)
Catalase	10.2 ± 4.2 (5)
Galactosyltransferase	0.39 ± 0.22 (9)
<i>N</i> -Acetylglucosaminyltransferase	0.017 ± 0.008(11)
5'-Nucleotidase	68.8 ± 11.2 (9)
Alkaline phosphodiesterase I	10.5 ± 1.2 (3)
NAD-glycohydrolase	510 ± 110 (11)
α -Glucosidase	1.53 ± 0.39 (10)
NADH cytochrome <i>c</i> reductase	144 ± 40 (7)
NADPH cytochrome <i>c</i> reductase	31.5 ± 5.7 (5)
Sulphatase C	0.58 ± 0.20 (11)
Mannosyltransferase	0.069 ± 0.014(6)

* Values are means \pm SD; the number of experiments is given in parentheses.

the various pellets. Although their distributions overlap considerably, several characteristic behaviors are easily distinguished. Acid hydrolases (a-d) were mainly found in the M fraction in which they attain their highest specific activity; they also occurred in the L fraction and for a small part in the final supernate. Cytochrome *c* oxidase (e) and the particle-bound malate dehydrogenase (f) were similarly recovered in the M and L fractions, but with almost the same specific activity in either fraction. The distribution of malate dehydrogenase between particles and suspension medium is in agreement with its behavior in isopycnic centrifugation (Canonico et al. [13] and Fig. 7). Catalase (g) differs from all the other enzymes by a high yield in the L fraction, and a high specific activity in fractions L and P. All the other enzymes (h-q) may be regarded as microsomal because their specific activity peaks in the P (microsome) fraction. However, their yield in this fraction is not greater than in the M and L fractions. Apparently, the cell structures that house these enzymes were only partly broken down into microsomal elements upon homogenization, or a significant part of the microsomal elements sedimented as aggregates. Attempts to improve the resolution in differential centrifugation by varying the salt composition of the suspension medium, or by using other homogenization means, were unsuccessful. Minor differences between the microsomal enzymes are noted on the sedimentation profiles. α -Glucosidase, mannosyltransferase, sulphatase C, NADPH cytochrome *c* reductase, and NADH cytochrome *c* reductase (h-l) had the same specific activity in N and M fractions. In contrast, galactosyltransferase and *N*-acetyl- β -glucosaminyltransferase (p, q) were nearly undetected in the N fraction, and alkaline phosphodiesterase I, 5'-nucleotidase, and NAD glycohydrolase (m-o) behaved intermediately.

Fractionation by Isopycnic Centrifugation. The density distribution of enzymes in a

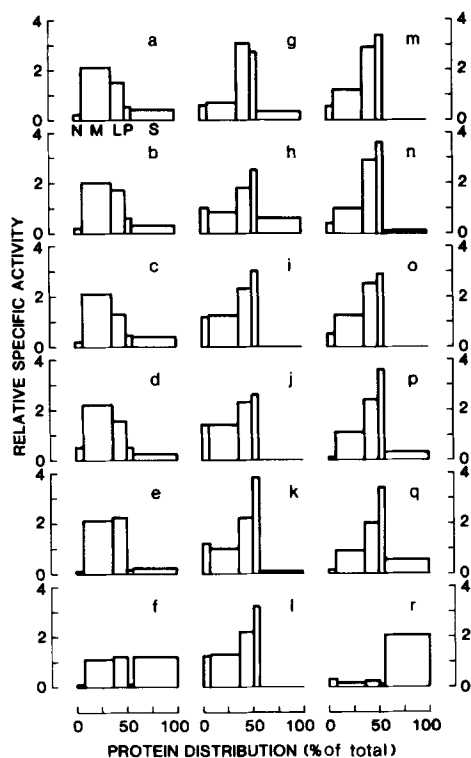


FIG. 5. Distribution of constituents after fractionation of cultivated MRP macrophages by differential centrifugation. The method used for fractionation by differential centrifugation is outlined in Materials and Methods. For each enzyme, graphs were constructed as described in reference 17 from the average results of two experiments. Fractions are plotted from left to right in the following order: N, M, L, P, and final supernate (S). Each fraction is represented separately on the ordinate by the relative specific activity of the enzyme, i.e., the percentage amount of total recovered activity divided by the percentage amount of total recovered protein. On the abscissa, the protein content of fractions is represented cumulatively. Enzymes, and their average recoveries from the homogenate, were: acid α -galactosidase, 94% (a); acid α -mannosidase, 83% (b); acid β -glucuronidase, 94% (c); acid *N*-acetyl- β -glucosaminidase, 86% (d); cytochrome *c* oxidase, 100% (e); malate dehydrogenase, 93% (f); catalase, 87% (g); α -glucosidase, 92% (h); mannosyltransferase, 99% (i); sulphatase C, 90% (j); NADPH cytochrome *c* reductase, 91% (k); NADH cytochrome *c* reductase, 78% (l); alkaline phosphodiesterase I, 88% (m); 5'-nucleotidase, 89% (n); NAD glycohydrolase, 89% (o); galactosyltransferase, 81% (p); *N*-acetylglucosaminyltransferase, 110% (q); and lactate dehydrogenase, 88% (r). Recovery of protein was 101%.

sucrose gradient has been determined by equilibrium centrifugation of untreated cytoplasmic extracts, and of cytoplasmic extracts supplemented with digitonin that was added in slight excess over their cholesterol content. This content was 19.1 ± 2.7 $\mu\text{g}/\text{mg}$ protein (mean \pm SD of six values), which would need ≈ 61 μg digitonin/mg protein in 1:1 stoichiometry. Under these conditions, formation of the digitonin-cholesterol complex within cholesterol-rich membranes increases their equilibrium density, leaving other membranes unaltered in their physical characteristics (28). The results of these experiments are presented in Figs. 6 and 7.

In Fig. 6, six typical enzymes, each behaving in a different manner, have been selected. The density distributions after addition of digitonin are superimposed on the density profiles obtained with the untreated preparations. NAD glycohydrolase was

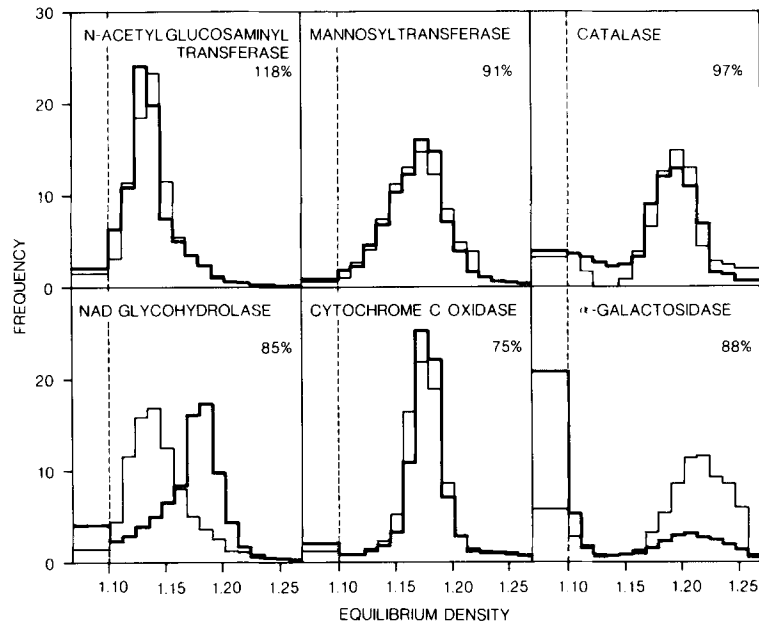


FIG. 6. Typical density distributions of enzymes. Cytoplasmic extracts were divided in two portions. One portion was subjected to isopycnic centrifugation as described in Materials and Methods. The other portion was immediately assayed for protein, supplemented with $70 \mu\text{g}$ digitonin per milligram protein, and centrifuged under exactly the same conditions as the first portion. Results are presented as described in reference 17. Frequency is the fractional amount of enzyme activity in the gradient fraction divided by the density increment across that fraction. Fractional amounts of activity (activity in the fraction/activity in the gradient) are thus represented by the areas under the successive density increments. Graphs show the average results of three experiments and include the density distributions found without (thin line) and after (thick line) addition of digitonin. The represented portion of density distributions extends from 1.10 to 1.27 density units. On the left-hand side of the demarcation line at 1.10, blocks, arbitrarily constructed through 0.03 density units, represent the material recovered in the sample layer. Percentage values give the average recovery of constituents in the gradient fractions relative to the cytoplasmic extract.

markedly shifted by digitonin, the median equilibrium density rising from 1.140 to 1.173. Acid α -galactosidase was almost completely released by digitonin and was found in the sample layer, in agreement with the high sensitivity of the lysosomal membrane to digitonin reported above (Fig. 4). The other enzymes retained their normal density distribution in the presence of digitonin. They differ from one another by the shape and the average density of their distribution profiles. *N*-acetylglucosaminyltransferase shows a rather narrow peak at 1.13–1.14 density units. Its density profile is quite similar to that of NAD glycohydrolase in the untreated preparations but not after addition of digitonin. Mannosyltransferase and cytochrome *c* oxidase have almost the same modal density (≈ 1.17), but the transferase profile is much broader. Catalase peaks at a slightly higher density (1.20), between mannosyltransferase and acid α -galactosidase (1.22 in untreated preparations).

In Fig. 7 it is seen that the other enzymes behave as one of the typical enzymes presented in Fig. 6. Graphs are paired; in each pair the upper histograms derive from untreated cytoplasmic extracts and the lower histograms from digitonin-treated preparations. Solid lines were redrawn from Fig. 6 and give the density profiles of the reference enzymes; hatched (untreated preparations) and stippled (digitonin-treated

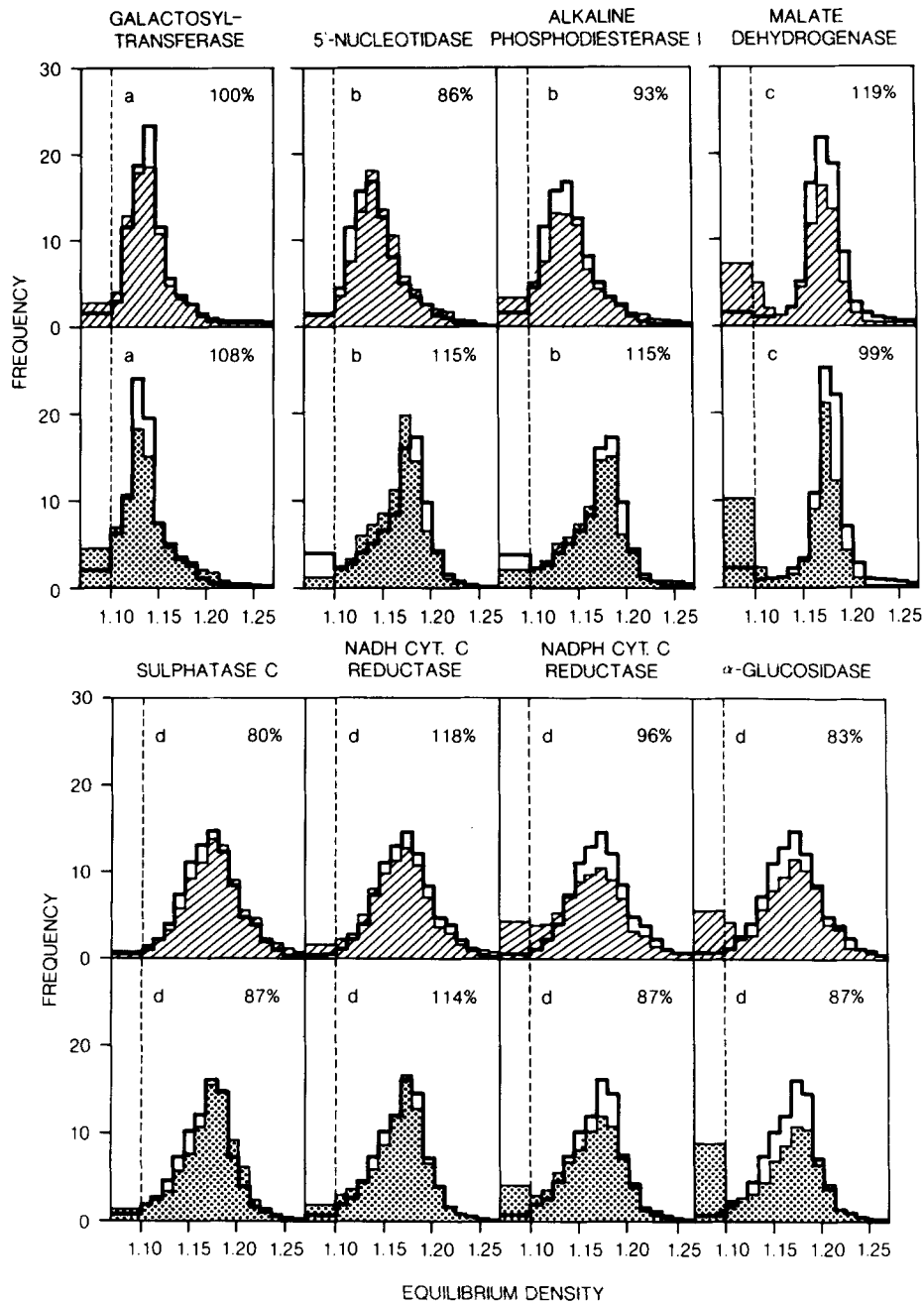


FIG. 7. Density distribution of macrophage enzymes compared with that of the typical enzymes. These additional enzyme distributions were obtained in the experiments presented in Fig. 6. For the sake of comparison between enzymes, the density distributions yielded by untreated cytoplasmic extracts and by the digitonin-treated preparations are plotted in separate graphs arranged in pairs: upper plots derive from the untreated preparations and lower plots from the digitonin-treated preparations. The reference enzymes are *N*-acetyl- β -glucosaminidase (a), NAD glycohydrolase (b), cytochrome *c* oxidase (c), and mannosyltransferase (d) and appear as a solid line. Plots of the other enzymes specified above each pair of graphs are superimposed on those of the reference enzymes and shown by crosshatched or stippled areas, depending on whether they stem from untreated or digitonin-treated preparations, respectively. Otherwise, the presentation is as in Fig. 6, and percentage values give the average recoveries in the gradient fractions.

preparations) profiles give the density distributions of the enzymes specified on top of the graphs. Except for a slightly greater amount in the sample layer the density distribution of galactosyltransferase coincides with that of *N*-acetylglucosaminyltransferase (Fig. 7a). Alkaline phosphodiesterase I and 5'-nucleotidase behaved like NAD glycohydrolase (b) and shifted to a higher equilibrium density after treatment with digitonin. The shift of 5'-nucleotidase seems however slightly less. Malate dehydrogenase differs from cytochrome *c* oxidase (c) only by the amount of enzyme left in the sample layer. Finally a number of enzymes, e.g., sulphatase C and NADH cytochrome *c* reductase, closely follow mannosyltransferase (d). Although a greater fraction of the enzyme activity remained in the sample layer, NADPH cytochrome *c* reductase and α -glucosidase may also be included in this group. None of the enzymes assayed in the density gradient fractions gave a density distribution similar to that of catalase. Several acid hydrolases, α -mannosidase, β -glucuronidase, and *N*-acetyl- β -glucosaminidase, showed the characteristics of acid α -galactosidase: a broad density profile centered around 1.22 density units in the untreated cytoplasmic extracts and a prominent activity in the sample layer after treatment with digitonin. These distributions are not shown, because they were as expected from previous studies from this laboratory (13).

Density Perturbation by Treatment with Pyrophosphate. Membrane-bound ribosomes are readily detached from the rough microsomes by washing in sucrose solutions that contain sodium pyrophosphate (29). Stripped rough vesicles equilibrate at a lower density than the unstripped microsomes in a sucrose gradient, and this property has been used in analytical studies on rat liver microsomes to distinguish enzymes associated with microsomes derived from the endoplasmic reticulum from other microsomal elements (28). Experiments in which the density distribution of several enzymes was established using pyrophosphate-treated cytoplasmic extracts of cultivated MRP macrophages are summarized in Fig. 8 and Table III. Whereas cytochrome *c* oxidase, acid α -galactosidase, and alkaline phosphodiesterase I were not perceptibly altered, mannosyltransferase, sulphatase C, and NADPH cytochrome *c* reductase acquired a lower equilibrium density as a result of the treatment by pyrophosphate (Table III). The three latter enzymes behaved similarly, in the manner shown for mannosyltransferase in Fig. 8. The density distribution of NADH cytochrome *c* reductase was also shifted to lower values, but in a manner that suggests the presence of this enzyme activity in pyrophosphate-sensitive and pyrophosphate-insensitive components (Fig. 8).

Density Distribution of Enzymes in Cytoplasmic Extracts Derived from MRP Cells Cultivated in the Presence of Triton WR-1339. Endocytic activity of cells influences specifically the centrifugation properties of lysosomes and provides a useful tool in studies on their constituents and functions. This influence, early observed in rat kidney cells after intraperitoneal injection of egg white (30, 31), has been widely documented by Wattiaux et al. (32), who demonstrated that the equilibrium density of rat liver lysosomes decreases without perceptible change in the physical properties of other organelles after intravenous injection of Triton WR-1339.

In preliminary experiments we sought conditions for cultivation of cells in the presence of Triton WR-1339, and adopted a concentration of 0.2 mg/ml in the medium. Judging from the number of nonadherent cells, Triton WR-1339 became harmful at a higher concentration. In addition, cells were cultivated for 24 h instead

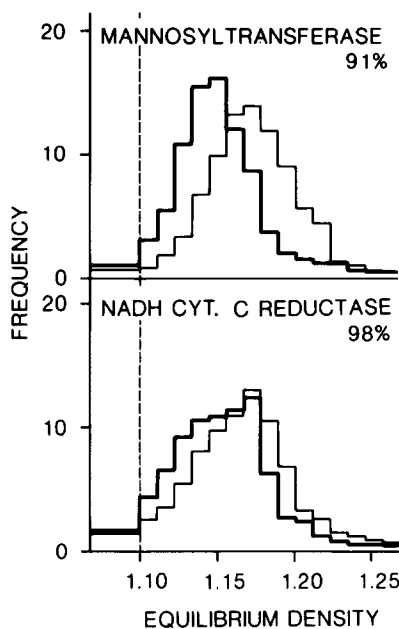


FIG. 8. Effect of treatment with pyrophosphate on the equilibrium density of mannosyltransferase and NADH cytochrome *c* reductase. Cytoplasmic extracts were supplemented with sodium pyrophosphate, pH 8, 15 mM final concentration. They were then subjected to equilibrium centrifugation in a sucrose gradient exactly as described in Fig. 6. The solid lines represent the average results of three experiments. For comparison the average distributions (four experiments) obtained with untreated cytoplasmic extracts are shown by the thin lines. Otherwise, the presentation of results is as described in Fig. 6 and the percentage values give the recoveries of enzyme activity in the gradient fractions. The distributions of enzymes not shown on this figure were also determined and used to compute the median densities presented in Table III.

TABLE III
Modification of Median Equilibrium Density of Enzymes Resulting from Treatment of Cytoplasmic Extracts by Pyrophosphate.

Enzymes	Control preparations	Pyrophosphate-treated preparations	Difference from control
			$\times 10^3$
Mannosyltransferase	1.171 (4)*	1.150 (3)*	-21
Sulphatase C	1.177 (4)	1.153 (2)	-24
NADPH cytochrome <i>c</i> reductase	1.159 (3)	1.136 (1)	-23
NADH cytochrome <i>c</i> reductase	1.168 (4)	1.156 (3)	-12
Alkaline phosphodiesterase I	1.138 (3)	1.133 (2)	-5
Cytochrome <i>c</i> oxidase	1.177 (5)	1.174 (3)	-3
Acid α -galactosidase	1.204 (1)	1.206 (1)	+2

* Median density values were computed according to reference 17 from density distributions obtained in the experiments described in Fig. 8. They take into account the enzyme activity recovered in the sample layer. However, none of the differences merely reflects an increase of that activity. The number of experiments is given in parentheses.

of 3 d, because the latency of *N*-acetyl- β -glucosaminidase markedly decreased at later time points.

Fig. 9. illustrates the behavior of some enzymes in isopycnic centrifugation. The

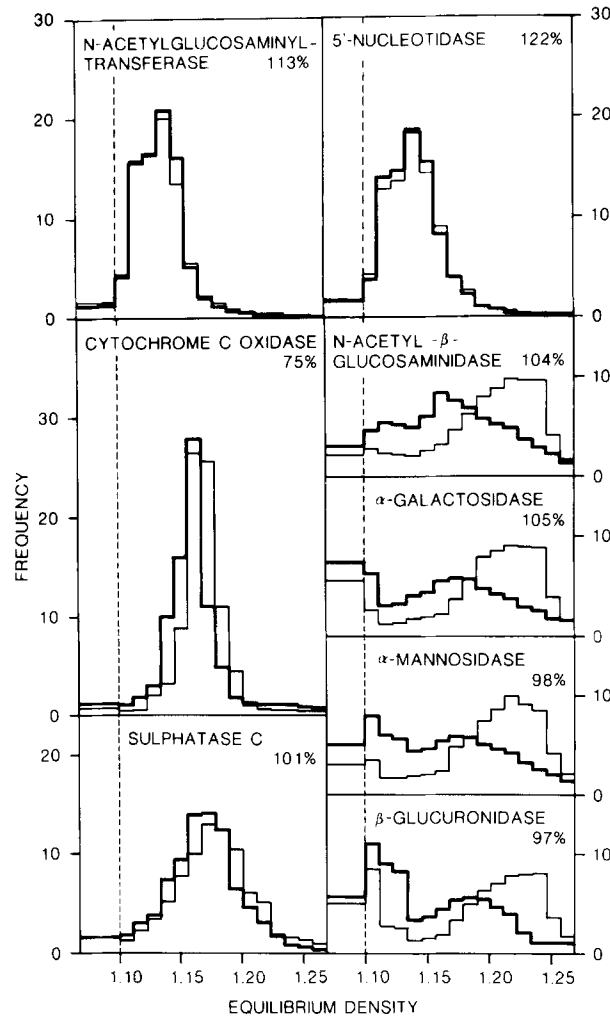


FIG. 9. Density distribution of enzymes in cytoplasmic extracts derived from MRP cells cultivated for 24 h in the presence of Triton WR-1339. Adherent cells freshly harvested were cultivated for 24 h as described in Materials and Methods, either in the usual medium, or in that medium supplemented with 0.2 mg Triton WR-1339/ml. Cells were homogenized and cytoplasmic extracts were centrifuged in a linear gradient of sucrose (see Materials and Methods). Density profiles correspond to cells cultivated in the presence (solid line) and in the absence (thin line) of Triton WR-1339. Otherwise, results are presented exactly as described in Fig. 6 and percentage values give the average recoveries in the gradient fractions.

density distributions derived from MRP cells cultivated for 24 h in the absence of Triton WR-1339 did not differ from those obtained in cells cultivated for 3 d. In preparations derived from cells cultivated in the presence of Triton WR-1339, acid hydrolases equilibrated at a much lower density, and showed a more complex pattern with a peak of variable importance behind the sample layer. In contrast, cytochrome *c* oxidase, sulphatase C, NADH cytochrome *c* reductase (not shown), and NADPH cytochrome *c* reductase (not shown) were only moderately shifted, and *N*-acetylglucosaminyltransferase and 5'-nucleotidase retained their normal distribution.

Discussion

Several characteristic centrifugation behaviors, each shown by two or more enzyme activities except for catalase, have been demonstrated in cytoplasmic extracts of cultivated MRP macrophages. This leads us to classify the particle-bound enzymes into six groups, and to tentatively relate each group with a particular species of subcellular organelle.

Lysosomes. Acid hydrolases are the first in the order of sedimentation velocity (Fig. 5). Our results confirm their structure-linked latency, the exquisite susceptibility of their surrounding membrane to digitonin, and their high average equilibrium density in sucrose gradient (13). In addition, their equilibrium density is much lower when the cells have been cultivated for 1 d in the presence of Triton WR-1339, unlike that of other enzymes, which is either unaltered or only moderately altered. We have no direct proof that this change results from endocytosis and accumulation of Triton WR-1339 within the acid hydrolase-containing vesicles. Nevertheless, this interpretation is the most likely. It is consistent with the distinctive influence of this compound on the density distribution of acid hydrolases, and with the magnitude of the shift, which is similar to that occurring in liver lysosomes after injection of Triton WR-1339 in rats (32). In the latter case it has been unambiguously shown that accumulation of Triton WR-1339 is the major factor responsible for the density perturbation of lysosomes (32–34).

In consideration of their latency and centrifugation properties, α -mannosidase, α -galactosidase, β -glucuronidase, and *N*-acetyl- β -glucosaminidase certainly identify the lysosomes of cultivated MRP macrophages. Their relatively fast rate of sedimentation is consistent with the large size of many electron-dense bodies present in the cytoplasm. Our results also reveal some differences among the density profiles of acid hydrolases (Fig. 9). This heterogeneity is in keeping with previous reports from this (13) and other laboratories (14, 35), in which its possible significance has been examined. Despite these differences between individual enzymes, all the acid hydrolases distinguish themselves sharply from the other enzymes included in this study.

Mitochondria. Cytochrome *c* oxidase and the sedimentable part of malate dehydrogenase follow each other closely under all conditions, including differential centrifugation and isopycnic centrifugation in the presence or absence of digitonin. This strengthens the contention that they faithfully reflect the distribution of mitochondria (13). In particular, the sedimentation patterns in differential centrifugation are as expected for particles of density ≈ 1.1 U and ≈ 1 μ m in size, in 0.25 M sucrose.

Plasma Membrane. In the present study we have succeeded in dissociating largely the glycosyltransferases that elongate glycoproteins by sequential addition of *N*-acetylglucosamine and galactose units (terminal glycosyltransferases), from 5'-nucleotidase, alkaline phosphodiesterase I, and NAD glycohydrolase. Unlike the terminal glycosyltransferases, the three latter enzymes acquire a higher equilibrium density in the presence of digitonin. In this respect they are undistinguishable from their homologues in rat liver microsomes (28, 36–38) and plasma membrane preparations (39, 40). In liver cells they characterize a subcellular entity that includes the plasma membrane and possibly endomembranes related with the plasma membrane. This entity is sharply distinct from the endoplasmic reticulum and the Golgi complex elements that house several glycosyltransferases (40). The conclusion reached from studies on liver seems to a large extent applicable to cultivated MRP macrophages.

It agrees with the inactivation of 5'-nucleotidase (5), alkaline phosphodiesterase I (6), and NAD glycohydrolase (7) upon incubation of various types of macrophages with the diazonium salt of sulfanilic acid, or with proteolytic enzymes. It is most significant that enzymes localized at the cell periphery by nonpenetrating probes behave in the same characteristic way in fractionation experiments. The digitonin shift of 5'-nucleotidase or alkaline phosphodiesterase I has also been demonstrated on subcellular preparations derived from other cells (41-43), including rabbit alveolar macrophages (14).

The existence of a small intracellular pool of 5'-nucleotidase, alkaline phosphodiesterase I, and NAD glycohydrolase, associated with secretory vesicles or with vesicles derived from the cell periphery by endocytosis, is not ruled out by our results. Experiments using nonpenetrating probes indicate that such a pool may account for $\approx 20\%$ of 5'-nucleotidase and alkaline phosphodiesterase I, and that it becomes greater after the cells have ingested latex beads (5, 6). However, the presence of Triton WR-1339 in the cultivation medium had no perceptible effect on the 5'-nucleotidase activity and on its density distribution (Fig. 9). Apparently, the size of the putative endocytic pool of 5'-nucleotidase is only a small part of the total activity, or the contents of endocytic vesicles after Triton WR-1339 do not differ enough from the normal contents to change the density distribution of 5'-nucleotidase.

Golgi Complex. In contrast to 5'-nucleotidase and its companion enzymes, the terminal glycosyltransferase activities are insensitive to digitonin. As a consequence, after addition of digitonin their distribution profile in isopycnic centrifugation is centered around a density value significantly lower than that of any other enzyme considered in this work. Hence, they characterize a distinct subcellular species, which we believe to stem from the Golgi complex. A number of morphological and biochemical data convincingly document the role of the Golgi complex in synthesis of the terminal trisaccharide units (AcNeu-Gal-GlcNAc) of complex glycoproteins (see reference 44 for a review).

However, the extent to which terminal glycosyltransferases may be constitutive enzymes of other cell membranes is still controverted. Subcellular fractionation studies from this laboratory favor their specific association with the Golgi apparatus of rat liver cells (28, 40), compared with probing spleen lymphocytes (45) and other cells (46) for ectoenzyme terminal glycosyltransferases that assign some activity to the cell periphery. In our density equilibration experiments, galactosyltransferase and *N*-acetylglucosaminyltransferase still partly overlap the plasma membrane-associated enzymes in the gradient after addition of digitonin (Figs. 6, 7). Nevertheless, our data demonstrate that the terminal glycosyltransferases are undetectable in the digitonin-insensitive elements. Having obtained evidence that the fragments of plasma membrane react with digitonin (see above), we are led to conclude that the terminal glycosyltransferases are absent from the pericellular membrane in cultivated MRP macrophages. In contrast, we cannot rule out unambiguously the presence of a small part of 5'-nucleotidase, alkaline phosphodiesterase I, or NAD glycohydrolase activities in digitonin-insensitive elements perhaps derived from the Golgi apparatus. 5'-Nucleotidase is somewhat less shifted by digitonin than is NAD glycohydrolase, which might be meaningful in this respect.

Terminal glycosyltransferases differ from the homologous enzymes in liver, in that the latter are moderately shifted by digitonin (28, 40). The reason for this difference

is still unclear. In liver, isolated elements of the Golgi apparatus were altered in a complex manner in their morphology by digitonin; in particular, very low density lipoprotein particles became less apparent in number or electron density (40). The slight digitonin shift of Golgi elements in liver may result from a change in the vesicular contents, rather than merely from binding of digitonin to the surrounding membrane. The contents of Golgi vesicles are probably quite different in hepatocytes and macrophages.

Endoplasmic Reticulum. The density distribution of α -glucosidase and sulphatase led Canonico et al. (13) to hypothesize that these enzymes are constituents of the endoplasmic reticulum in cultivated MRP macrophages. This conclusion now rests upon stronger experimental evidence. These enzymes belong to a particular group that, in addition, includes mannosyltransferase, NADPH cytochrome *c* reductase, and NADH cytochrome *c* reductase. In the liver, these latter enzymes appear to be exclusive constituents of the endoplasmic reticulum membrane (19, 28, 40, 47), except for the presence of NADH cytochrome *c* (*b₅*) reductase in the outer membrane of mitochondria (48). In our macrophages, this group dissociates from the other microsomal enzymes by having a relatively high equilibrium density in sucrose gradient, which reduces after pyrophosphate is added to the suspension medium (Fig. 8 and Table III). This group is also characterized by a lack of sensitivity to digitonin (Fig. 7). These properties are similar to those reported for the microsomes derived from the endoplasmic reticulum in liver (28, 47, 49). The shift caused by pyrophosphate treatment may be a clue for the proposed identification. This agent removes membrane-bound ribosomes from the rough microsomes (28, 29), and lowers their equilibrium density accordingly (28). Possibly, the removal of bound ribosomes is also responsible for the pyrophosphate shift of several enzymes in cultivated MRP macrophages. The presence of mannosyltransferase within the group is another clue for its association with the endoplasmic reticulum. Mannosyltransferase is involved in core glycosylation of proteins through the pathway of lipid intermediates. Evidence that core glycosylation is an early event in the biosynthesis of *N*-glycosylated proteins, and is most often a cotranslational event that consequently must occur in the rough endoplasmic reticulum membrane, has grown continuously over the last years (see reference 50 for a review).

Catalase-bearing Organelles. Finally, catalase is unique in many respects, including distribution in differential centrifugation (Fig. 5), structure-linked latency resulting from a membrane barrier much more resistant to digitonin than the lysosomal membrane (Fig. 4), and density distribution centered around an average density slightly greater than that of mannosyltransferase (Fig. 6). These distinguishing properties make it likely that cultivated MRP macrophages contain a species of organelle that may be related to peroxisomes. Interestingly, cytochemical studies on resident macrophages from the rabbit peritoneal cavity have revealed catalase reaction products in small membrane-bounded organelles, and failed to demonstrate these products in the rough endoplasmic reticulum or perinuclear cisternae (51). In cell-free preparations derived from rabbit alveolar macrophages, catalase is also present in a latent form much less sensitive to digitonin than is the latent activity of acid phosphatase (52). These findings pertain to the metabolism of H_2O_2 in macrophages and confine the catalase-dependent disposal of H_2O_2 to a specific compartment of the

cytoplasm, particularly if the nonsedimentable activity stems from catalase-containing organelles altered in the course of cell disruption.

Conclusion. In subcellular fractionation experiments it is necessary to rely on reference enzymes that reflect the distribution of the various subcellular entities. Such enzymes should be homogeneously distributed through a single organelle or membrane species (17, 53). Although they do not demonstrate such qualities unequivocally, our results fairly qualify some enzymes as references in studies of MRP macrophages. In view of the enzyme heterogeneity of lysosomes, it would be wise to rely on several acid hydrolases and perhaps to consider enzymes that have not been studied in this work (14). Cytochrome *c* oxidase seems to be a reliable reference for the distribution of mitochondria. For the plasma membrane and related subcellular elements, NAD glycohydrolase could be preferred to 5'-nucleotidase because it is somewhat more shifted by digitonin. Either of the two terminal glycosyltransferases marks the Golgi apparatus, or at least some of its membrane components. For the endoplasmic reticulum, mannosyltransferase and sulphatase C, which in no case dissociate from one another, should be preferred to α -glucosidase, which is partly soluble and readily released into the suspension medium (13), and to NADH cytochrome *c* reductase, which might also occur in other membranes (Fig. 8). Catalase is still the only reference available for its housing structure.

The behavior of the proposed reference enzymes in our experiments emphasizes the extent to which populations of subcellular organelles derived from cultivated MRP macrophages are polydisperse with respect to size and equilibrium density in sucrose gradients. Overlap is most troublesome in fractionation by differential centrifugation. Not only does the microsome fraction consist of elements derived from the plasma membrane, Golgi complex and endoplasmic reticulum, and of peroxisome-like organelles, but these elements are recovered with nearly the same yield in M, L, and P fractions. The meaning of distributions obtained after subcellular fractionation of macrophages by differential centrifugation (4, 7, 16, 54, 55) must be reconsidered accordingly. Analytical equilibrium centrifugation in a sucrose gradient is a more efficient approach, especially when means that modify the density of distinct subcellular entities in different ways are used.

Summary

Resident peritoneal macrophages of the mouse, cultivated for 3 d, have been studied by quantitative subcellular fractionation using differential centrifugation and density equilibration in linear gradients of sucrose. Density equilibration experiments were carried out on untreated cytoplasmic extracts, on cytoplasmic extracts treated with digitonin or sodium pyrophosphate, and on cytoplasmic extracts derived from cells cultivated for 24 h in the presence of Triton WR-1339. The enzyme distributions obtained distinguished six typical behaviors characteristic of distinct subcellular entities. Acid α -galactosidase and other acid hydrolases displayed the highest average velocity of sedimentation and equilibrium density. Culturing in a medium that contained Triton WR-1339 markedly decreased their density, most likely as a result of Triton WR-1339 accumulation within lysosomes. Cytochrome *c* oxidase and the sedimentable activity of malate dehydrogenase showed a narrow density distribution centered around 1.17, very similar under all the experimental situations; their rate of sedimentation fell within the range expected for mitochondria. Catalase was particulate-

bound and exhibited structure-linked latency (80%); it was released in soluble and fully active form by digitonin, but this required a much higher concentration than in the case of lysosomal enzymes. Differences relative to all the other enzymes studied suggest the existence of a particular species of organelles, distinctly smaller than mitochondria, and possibly related to peroxisomes. Many enzymes were microsomal in the sense that the specific activities, but not the yields, were greater in microsomes than in other fractions obtained by differential centrifugation. These enzymes were distinguished in three groups by their properties in density equilibration experiments. NAD glycohydrolase, alkaline phosphodiesterase I, and 5'-nucleotidase had low equilibrium densities but became noticeably more dense after addition of digitonin. The other microsomal enzymes were not shifted by digitonin, in particular *N*-acetylglucosaminyltransferase and galactosyltransferase, which otherwise equilibrated at the same position in the gradient. We assign the digitonin-sensitive enzymes to plasma membranes and possibly to related endomembranes of the cells, and the two glycosyltransferases to elements derived from the Golgi apparatus. Finally, α -glucosidase, sulphatase C, NADH cytochrome *c* reductase, NADPH cytochrome *c* reductase, and mannosyltransferase, equilibrated at a relatively high density but were shifted to lower density values after addition of sodium pyrophosphate. These properties support their association with elements derived from the endoplasmic reticulum.

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