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ANALYTICAL SUBCELLULAR FRACTIONATION
OF CULTIVATED MOUSE RESIDENT
PERITONEAL MACROPHAGES*

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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 character-
ized 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 inflam-
matory 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 cooper-
ation 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)† 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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† Abbreviations used in this paper: MRP macrophage, mouse resident peritoneal macrophage; PBS, phos-
phate-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 $4 \times 10^5$ cells/cm$^2$. After incubation for 90 min at $37^\circ$C in the presence of 10% CO$_2$ 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 Na$_2$HPO$_4$, and 1.5 mM KH$_2$PO$_4$). 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 suspended at $4^\circ$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 supernate was 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 $4^\circ$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-acetylgalcosaminidase, acid β-glucuronidase, acid α-mannosidase, α-glucosidase, sulphotase 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 | Reference |
|-------------------------|-----------------------|-------------------------|----------------------------|----------------|-------------------|-----------|
| Mannosyltransferase     | GDP*-[U-14C]glucose  | Tris-HCl, 80 mM, pH 7.6  | 2 mM EDTA, 15 mM MgCl₂, 1 mM ATP, 2.5 mM DTT | 15             | 14C in lipid extract | 19        |
|                         | (5.6A0 mg/ml)         |                         |                            |                |                   |           |
|                         | Dolichyl phosphate    |                         | 0.4% Triton X-100           |                |                   |           |
|                         | (44 mg/ml)            |                         |                            |                |                   |           |
|                         | 0.4% Triton X-100     |                         |                            |                |                   |           |
|                         | Sodium phosphate, pH 6.8 |                       | 0.2 mM NaCN, 0.2 mM NaN₃, 0.2 M NaCl | 25             | Reduced cytochrome c | 20        |
| NADH cytochrome c reductase | NADPH (75 µM) | Sodium phosphate, pH 6.8 | 30 mM nicotinamide | 25             | Reduced cytochrome c | 20        |
| NADPH cytochrome c reductase | NADPH (75 µM) | Sodium phosphate, pH 6.8 | 30 mM nicotinamide | 25             | Reduced cytochrome c | 20        |
| NAD glycohydrolase      | (Carboxyl-14C)cytochrome | Mesyl KOH, 50 mM, pH 6.5 | 1 mM EDTA, 0.1% Triton X-100 | 37             | 14C in the medium | 21        |
| Catalase                | H₂O₂ (1.3 mM)         | Imidazole-HCl, 25 mM, pH 7.4 | 1 mg/ml albumin, 0.5% Triton X-100 | 25             | Residual H₂O₂ | 22        |
| Alkaline phosphodiesterase | p-nitrophenyl thymidine | Glycine-KOH, 0.1 M, pH 9.6 | 2 mM Zn acetate, 0.1% Triton X-100 | 37             | p-Nitrophenol | 20        |

* Guanosine diphosphate.
† Dithiothreitol.
§ 2-[N-morpholino]ethane sulfonic acid.

### 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. × 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. 3. Effect of temperature on the time course of mannosyltransferase reaction. Cytoplasmic extracts (14 μg protein per assay) were incubated under the conditions given in Table I, at 10°C (○), 15°C (●), and 25°C (▲) 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 μg protein and 1.3 μg 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 μl 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
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, 83% (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, 98% (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%.

The 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 μg/mg protein (mean ± 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 μg 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-acetylgalcosaminyltransferase (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 a-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, a-mannosidase, b-glucuronidase, and N-acetyl-b-glucosaminidase, showed the characteristics of acid a-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 a-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 |
|--------------------------|----------------------|------------------------------------|-------------------------|
| Manosyltransferase       | 1.171 (4)*           | 1.150 (3)*                         | -21                     |
| Sulphatase C             | 1.177 (4)            | 1.153 (2)                          | -24                     |
| NADPH cytochrome c reductase | 1.159 (3)         | 1.136 (1)                          | -23                     |
| NADH cytochrome c reductase | 1.168 (4)         | 1.156 (3)                          | -12                     |
| Alkaline phosphodiesterase | 1.138 (3)          | 1.133 (2)                          | -5                      |
| Cytochrome c 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 \( \approx 1.1 \) U and \( \approx 1 \mu m \) 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-acetylgalactosamine 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 ≈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-acetylgalactosaminytransferase 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-sensitive 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 digitonin-sensitive elements. 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
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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 C 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 (bc1) 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$_2$O$_2$ in macrophages and confine the catalase-dependent disposal of H$_2$O$_2$ 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 particle-
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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References

1. Cohn, Z. A., and E. Wiener. 1963. The particulate hydrolases of macrophages. I. Comparative enzymology, isolation, and properties. J. Exp. Med. 118:991.
2. Cohn, Z. A., and E. Wiener. 1963. The particulate hydrolases of macrophages. II. Biochemical and morphological response to particle ingestion. J. Exp. Med. 118:1009.
3. Nachman, R. L., B. Ferris, and J. G. Hirsch. 1971. Macrophage plasma membranes. I. Isolation and studies on protein components. J. Exp. Med. 133:785.
4. Werb, Z., and Z. A. Cohn. 1971. Cholesterol metabolism in the macrophage. I. The regulation of cholesterol exchange. J. Exp. Med. 134:1545.
5. Edelson, P. J., and Z. A. Cohn. 1976. 5'-Nucleotidase activity of mouse peritoneal macrophages. II. Cellular distribution and effects of endocytosis. J. Exp. Med. 144:1596.
6. Edelson, P. J., and C. Erbs. 1978. Plasma membrane localization and metabolism of alkaline phosphodiesterase I in mouse peritoneal macrophages. J. Exp. Med. 147:77.
7. Artman, M., and J. Seeley. 1979. Nicotinamide adenine dinucleotide splitting enzyme: a plasma membrane protein of murine macrophages. Arch. Biochem. Biophys. 195:121.
8. Edelson, P. J. 1980. Macrophage ecto-enzymes: their identification, metabolism, and control. In Mononuclear Phagocytes: Functional Aspects. R. Van Furth, editor. Martinus Nijhoff Publishers. The Hague. 665–681.
9. Pearlstein, E., S. R. Dienstman, and V. Defendi. 1978. Identification of macrophage external membrane proteins and their possible role in cell adhesion. J. Cell Biol. 79:263.
10. Yin, H. L., C. Bianco, and Z. A. Cohn. 1980. The iodination and turnover of macrophage plasma membrane polypeptides. In Mononuclear Phagocytes: Functional Aspects. R. Van Furth, editor. Martinus Nijhoff Publishers, The Hague. 649–662.
11. Silverstein, S. C., R. M. Steinman, and Z. A. Cohn. 1977. Endocytosis. Annu. Rev. Biochem. 46:669.
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12. Van Furth, R., editor. 1980. Mononuclear Phagocytes. Functional Aspects. Martinus
Nijhoff Publishers, The Hague. part I:1-885 and part II:893–1931.

13. Canonico, P. G., H. Beaufay, and M. Nysens-Jadin. 1978. Analytical fractionation of
mouse peritoneal macrophages: physical and biochemical properties of subcellular organ-
elles from resident (unstimulated) and cultivated cells. J. Reticuloendothel. Soc. 24:115.

14. Lowrie, D. B., P. W. Andrew, and T. J. Peters. 1979. Analytical subcellular fractionation
of alveolar macrophages from normal and BCG-vaccinated rabbits with particular reference
to heterogeneity of hydrolyase-containing granules. Biochem. J. 178:761.

15. Andrew, P. W., D. B. Douglas, P. S. Jackett, and T. J. Peters. 1980. Analytical subcellular
fractionation of rabbit alveolar macrophages with particular reference to the subcellular
localisation of pyridine nucleotide-dependent superoxide-generating systems and superox-
ide dismutase. Biochim. Biophys. Acta. 611:61.

16. Kröner, E. E., H. Fischer, and E. Ferber. 1982. Subcellular fractionation of bone marrow-
derived macrophages: localization of phospholipase A1 and A2 and acyl-CoA:1-acylglycero-
3-phosphorylcholine-O-acyltransferase. Z. Naturforsch. 37c:314.

17. Beaufay, H., and A. Amar-Costesec. 1976. Cell fractionation techniques. Methods Membr.
Biol. 61.

18. de Duve, C., B. C. Pressman, R. Gianetto, R. Wattiaux, and F. Appelmans. 1955. Tissue
fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue.
Biochem. J. 60:504.

19. Ravoet, A.-M., A. Amar-Costesec, D. Godelaine, and H. Beaufay. 1981. Quantitative assay
and subcellular distribution of enzymes acting on dolichyl phosphate in rat liver. J. Cell
Biol. 91:679.

20. Beaufay, H., A. Amar-Costesec, E. Feytmans, D. Thines-Sempoux, M. Wibo, M. Robbi,
and J. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes
from rat liver. I. Biochemical methods. J. Cell Biol. 61:188.

21. Nakazawa, K., K. Ueda, T. Honjo, K. Yoshihara, Y. Nishizuka, and O. Hayashi. 1968.
Nicotinamide adenine dinucleotide glycohydrolases and polyadenosine diphosphate ribose
synthesis in rat liver. Biochem. Biophys. Res. Commun. 32:143.

22. Baudhuin, P., H. Beaufay, Y. Rahman-Li, O. Z. Selliger, R. Wattiaux, P. Jacques, and
C. de Duve. 1964. Intracellular distribution of monoamine oxidase, aspartate aminotrans-
ferase, alanine aminotransferase, α-amino acid oxidase and catalase in rat-liver tissue.
Biochem. J. 92:179.

23. Böhlen, P., S. Stein, W. Dairman, and S. Udenfriend. 1973. Fluorometric assay of proteins
in the nanogram range. Arch. Biochem. Biophys. 155:213.

24. Cooperstein, S. J., and A. Lazarow. 1951. A microspectrophotometric method for the
determination of cytochrome oxidase. J. Biol. Chem. 189:665.

25. Spurr, A. R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy.
J. Ultrastruct. Res. 26:31.

26. Phillips, A. H., and R. G. Langdon. 1962. Hepatic triphosphoridine nucleotide-cytochrome
C reductase: isolation, characterisation and kinetic studies. J. Biol. Chem. 237:2652.

27. de Duve, C. 1965. The separation and characterization of subcellular particles. In The
Harvey Lectures, Series 59. Academic Press Inc., New York. 49–87.

28. Amar-Costesec, A., M. Wibo, D. Thines-Sempoux, H. Beaufay, and J. Berthet. 1974.
Analytical study of microsomes and isolated subcellular membranes from rat liver. IV.
Biochemical, physical, and morphological modifications of microsomal components in-
duced by digitonin, EDTA, and pyrophosphate. J. Cell Biol. 62:717.

29. Sachs, H. 1958. The effect of pyrophosphate on the amino acid incorporating system of rat
liver microsomes. J. Biol. Chem. 233:690.

30. Straus, W. 1954. Isolation and biochemical properties of droplets from the cells of rat
kidney. J. Biol. Chem. 207:745.
31. Straus, W. 1956. Concentration of acid phosphatase, ribonuclease, deoxyribonuclease, β-glucuronidase, and cathepsin in "droplets" isolated from the kidney cells of normal rats. J. Biophys. Biochem. Cytol. 2:513.

32. Wattiaux, R., M. Wibo, and P. Baudhuin. 1963. Influence of the injection of Triton WR-1339 on the properties of rat-liver lysosomes. In CIBA Foundation Symposium: Lysosomes. A. V. S. de Reuck and M. P. Cameron, editors. Churchill, London. 176-196.

33. Henning, R., and H. Plattner. 1975. Formation of Triton WR-1339-filled rat liver lysosomes. I. Properties and intracellular distribution of [3H]Triton WR-1339. Exp. Cell Res. 94:363.

34. Plattner, H., R. Henning, and B. Brauser. 1975. Formation of Triton WR-1339-filled rat liver lysosomes. II. Involvement of autophagy and of pre-existing lysosomes. Exp. Cell Res. 94:377.

35. Nichols, B. A., and D. F. Bainton. 1975. Ultrastructure and cytochemistry of mononuclear phagocytes. In Mononuclear Phagocytes in Immunity, Infection and Pathology. R. Van Furth, editor. Blackwell Scientific Publications, Oxford, United Kingdom. 17-55.

36. Thines-Sempoux, D., A. Amar-Costesc, H. Beaufay, and J. Berthet. 1969. The association of cholesterol, 5'-nucleotidase, and alkaline phosphodiesterase I with a distinct group of microsomal particles. J. Cell Biol. 43:189.

37. Amar-Costesc, A., and H. Beaufay. 1977. The association of NAD glycohydrolase with the plasma membrane in rat liver. Arch. Int. Physiol. Biochim. 85:949.

38. Smith, G. D., and T. J. Peters. 1980. Analytical subcellular fractionation of rat liver with special reference to the localisation of putative plasma membrane marker enzymes. Eur. J. Biochem. 104:305.

39. Amar-Costesc, A. 1981. Analytical study of microsomes and isolated subcellular membranes from rat liver. VII. The distribution of protein-bound sialic acid. J. Cell Biol. 89:62.

40. Wibo, M., D. Thines-Sempoux, A. Amar-Costesc, H. Beaufay, and D. Godelaine. 1981. Analytical study of microsomes and isolated subcellular membranes from rat liver. VIII. Subfractionation of preparations enriched with plasma membranes, outer mitochondrial membranes, or Golgi complex membranes. J. Cell Biol. 89:456.

41. Tulkens, P., H. Beaufay, and A. Trouet. 1974. Analytical fractionation of homogenates from cultured rat embryo fibroblasts. J. Cell Biol. 63:303.

42. Lopez-Saura, P., A. Trouet, and P. Tulkens. 1978. Analytical fractionation of cultured hepatoma cells (HTC cells). Biochem. Biophys. Acta. 543:30.

43. Zenebergh, A., Y.-J. Schneider, M.-C. Loni, and A. Trouet. 1981. Enzymatic characterization and analytical fractionation of L1210 cells. Biochimie. 63:411.

44. Schachter, H., and S. Roseman. 1980. Mammalian glycosyltransferases. Their role in the synthesis and function of complex carbohydrates and glycolipids. In The Biochemistry of Glycoproteins and Proteoglycans. W. J. Lennarz, editor. Plenum Press, New York. 85-160.

45. Verbert, A., R. Cacan, and J. Montreuil. 1976. Ectogalactosyltransferase. Presence of enzyme and acceptors on the rat lymphocyte cell surface. Eur. J. Biochem. 70:49.

46. Pierce, M., I. A. Turley, and S. Roth. 1980. Cell surface glycosyltransferase activities. Int. Rev. Cytol. 65:1.

47. Beaufay, H., A. Amar-Costesc, D. Thines-Sempoux, M. Wibo, M. Robbi, and J. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. III. Subfractionation of the microsomal fraction by isopycnic and differential centrifugation in density gradients. J. Cell Biol. 61:213.

48. Sottocasa, G. L., B. Kuylenstierna, L. Ernster, and B. Bergstrand. 1967. An electron-transport system associated with the outer membrane of liver mitochondria. A biochemical and morphological study. J. Cell Biol. 32:415.

49. Beaufay, H., A. Amar-Costesc, and C. de Duve. 1981. The topology of enzymes in microsomes from liver. In Mitochondria and Microsomes. C. P. Lee, G. Schatz, and G. Dallner, editors. Addison-Wesley Publishing Company, Reading, MA. 629-653.
50. Hanover, J. A., and W. J. Lennarz. 1981. Transmembrane assembly of membrane and secretory glycoproteins. *Arch. Biochem. Biophys.* 211:1.

51. Bainton, D. F. 1980. Changes in peroxidase distribution within organelles of blood monocytes and peritoneal macrophages after surface adherence in vitro and in vivo. *In Mononuclear Phagocytes. Functional Aspects.* R. Van Furth, editor. Martinus Nyhoff Publishers, The Hague. 61–86.

52. Stossel, T. P., R. J. Mason, T. D. Pollard, and M. Vaughan. 1972. Isolation and properties of phagocytic vesicles. II. Alveolar macrophages. *J. Clin. Invest.* 51:604.

53. de Duve, C. 1964. Principles of tissue fractionation. *J. Theor. Biol.* 6:33.

54. Schnyder, J., and M. Baggioiini. 1978. The secretion of lysosomal hydrolases by stimulated and nonstimulated macrophages. *J. Exp. Med.* 148:435.

55. Wyllie, J. C. 1979. The subcellular distribution of iron in rabbit alveolar macrophages. *Biochem. Biophys. Res. Commun.* 89:1307.