

ANALYTICAL FRACTIONATION OF HOMOGENATES FROM CULTURED RAT EMBRYO FIBROBLASTS

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ABSTRACT

Homogenates of cultured rat embryo fibroblasts have been assayed for acid phosphatase, *N*-acetyl- β -glucosaminidase, cathepsin D, acid deoxyribonuclease, cytochrome oxidase, NADH cytochrome *c* reductase, 5'-nucleotidase, inosine diphosphatase, acid pyrophosphatase, neutral pyrophosphatase, esterase, catalase, cholesterol, and RNA. The validity of the assay conditions was checked. Neutral pyrophosphatase is a readily soluble enzyme. Acid hydrolases, except acid pyrophosphatase, are particle-bound enzymes, which exhibit a high degree of structural latency. They are activated and solubilized in a parallel fashion by mechanical treatments and tensio-active agents. Catalase is also particle-bound and latent; activating conditions stronger than those for hydrolases are required to activate the enzyme. Acid pyrophosphatase, 5'-nucleotidase and inosine diphosphatase are firmly particle-bound, but not latent; they are not easily solubilized.

In differential and isopycnic centrifugation, the latent hydrolases, cytochrome oxidase and catalase dissociate largely from each other; this suggests the occurrence of lysosomes and peroxisome-like structures besides mitochondria. The distribution patterns of 5'-nucleotidase and cholesterol are largely similar; digitonin influences their equilibrium density to the same extent; these two constituents are thought to be related to the plasma membrane. Inosine diphosphatase and acid pyrophosphatase are also partially associated with the plasma membrane, although some part of these enzymic activities probably belongs to other structures. NADH cytochrome *c* reductase is associated partly with the endoplasmic reticulum, partly with mitochondria.

Cultured mammalian cells represent a choice material for many physiopathological studies. They are easily submitted to various treatments under well-controlled conditions; the model is a relatively simple one, mainly because interactions between different cell types are abolished.

Characterization of the subcellular organelles of cultured cells by fractionation techniques has already been initiated in several laboratories. Efforts along this line have been devoted mostly to the isolation and subsequent analysis of their plasma

membranes (4, 12, 13, 16, 26, 32, 44, 45, 59, 60, 64). Lysosomes were similarly studied but to a lesser extent (31, 38, 39, 43, 51, 61). In no instance, however, has a systematic analytical fractionation been carried out.

The lysosomal functions are presently being investigated on cultured rat embryo fibroblasts in our laboratory, and preliminary reports on these studies have already appeared (53-56). A better knowledge of the biochemical and physical characters of the subcellular components of these cells

was a prerequisite in this work. This paper presents the results obtained by quantitative fractionation of cultured fibroblasts. An analytical approach has been followed throughout (19) in order to survey the properties of the various populations of subcellular components.

MATERIALS AND METHODS

Cell Culture

Primary cultures of fibroblasts were obtained by trypsinization of eviscerated 17-day old embryos (0.25% trypsin in Ca^{++} - and Mg^{++} -free Hanks' solution). The cultures were initiated by 10^5 cells/cm² of growing surface. After 7 days, cells were detached with 0.1% trypsin in PBS (phosphate-buffered saline, NaCl, 0.15 M; KCl, 2.7 mM; $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$, 3 mM; pH 7.4), and subcultures were started at a density of about 5×10^4 cells/cm². Confluency was reached in 4–7 days; the density was then about 2.5×10^6 cells/cm², or approximately 90 μg of cell protein/cm².

Tightly stoppered 1-liter Roux flasks were used for most experiments. They contained 100 ml of culture medium (ca 0.5 ml/cm² of growing surface). Eagle's minimum essential medium (25) was used with the following modifications: amino-acids were replaced by 0.5% lactalbumin hydrolysate; glucose and glutamine were, respectively, 25 mM and 0.2 mM; the initial concentration of sodium bicarbonate was 9 mM; small additions of a 0.6 M solution were made when necessary to maintain the pH around 7.2. Medium was supplemented with 10% calf serum. Sterilization was performed on Seitz^R filter pads (type EKS I, Bad-Kreuznach, Germany) or on Millipore^R membrane filters (type GS, 0.22 μm , Millipore Corp., Bedford, Mass.). Penicillin (100 IU/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) were routinely added to the culture media.

Cells at the second and third subculture were harvested soon after confluency. The appearance of these cells under the electron microscope is illustrated in Fig. 1. Nuclei display convoluted profiles, with chromatin present in a peripheral layer and scattered throughout the nuclear matrix. In the cytoplasm, the rough endoplasmic reticulum presents dilated cisternae, limited by membranes heavily loaded with ribosomes and polyribosomes. The Golgi apparatus (not shown) is well developed and restricted to a perinuclear zone. Dense bodies are numerous and display a polymorphic content. Many small vesicles are observed, especially in the peripheral areas of the cell; some of them are related to the pericellular membrane. In essence, the appearance of the cells is similar to that described for type II cells, derived from human embryo lung in culture, by Franks and Cooper (29). These fibroblast-like cells were dominant under our conditions of culture; no other cell type, including muscle cells, could be clearly recognized.

The cell sheet was washed twice with PBS and

detached from glass with 0.5 mM EDTA in the same medium. The use of trypsin was avoided because of possible deleterious effects on membranes and interference with protease assays. The cells were gently suspended, spun down at 800 rpm for 10 min, washed twice in ice-cold 0.25 M sucrose and resuspended in the same medium. All subsequent operations were carried out close to 0°C.

Cell Fractionation

The harvest from about eight Roux bottles was homogenized in 7 ml of 0.25 M sucrose containing 1 mM EDTA at pH 7.4 (sucrose-EDTA) by six strokes of the tight pestle of a Dounce homogenizer (Kontes Glass Co., Vineland, N. J.). Homogenization was checked by phase-contrast microscopy. EDTA was found necessary to prevent agglutination of subcellular particles.

Fractionation by differential centrifugation was carried out in several ways. In some experiments, the method of de Duve et al. (23) was followed exactly. In short, four particulate fractions (N, M, L, P) and a final supernate (S) were separated by successive centrifugations at increasing speeds and times. The angular velocities (rpm) and the time integral of the squared angular velocities $W = \int_0^t \omega^2 dt$ ($\text{rad}^2 \text{ s}^{-1}$) were respectively; 1,700 and 1.9×10^7 , 12,500 and 3×10^8 , 25,000 and 2.5×10^9 , 40,000 and 3×10^{10} . Centrifugations were carried out in the rotor no. 40 (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) except for the runs at 1,700 rpm, which were performed in an I.E.C. centrifuge (Damon Corp. Needham Heights, Mass., model PR-J, head no. 253, meniscus and bottom of the column fluid at, respectively, 13.5 and 21.5 cm from the axis). All pellets were washed twice in sucrose-EDTA and the supernates were combined for sedimenting the next fraction. In other experiments, three particulate fractions were obtained: N, ML, and P. The post-N fraction supernate (cytoplasmic extract) was then centrifuged directly at 25,000 rpm ($W = 2.5 \times 10^9 \text{ rad}^2 \text{ s}^{-1}$) and the ML fraction was equivalent to the sum of the M and L fractions. To study the influence of digitonin on the buoyancy of microsomal constituents, the post-N fraction supernate was divided into M', LP and S fractions by centrifuging successively at 12,500 rpm for a longer time ($W = 5.9 \times 10^8 \text{ rad}^2 \text{ s}^{-1}$) and at 40,000 rpm as usual ($W = 3 \times 10^{10} \text{ rad}^2 \text{ s}^{-1}$).

Analysis by density equilibration in a sucrose gradient was performed in a special zonal rotor whose principle and advantages have already been described (8, 11, 35). We have introduced successively (a) 10 ml of sample in sucrose-EDTA; (b) 32 ml of a sucrose gradient, the density of which increased linearly from 1.10 to 1.25; (c) 6 ml of sucrose solution of 1.34 density, acting as a cushion. All solutions contained 1 mM EDTA. The gradient was centrifuged for 3 h at 35,000 rpm ($W = 1.45 \times 10^{11} \text{ rad}^2 \text{ s}^{-1}$); under these conditions, most subcellular particles are brought to their equilibrium position (1). Finally, the content of the rotor was divided into 13–15

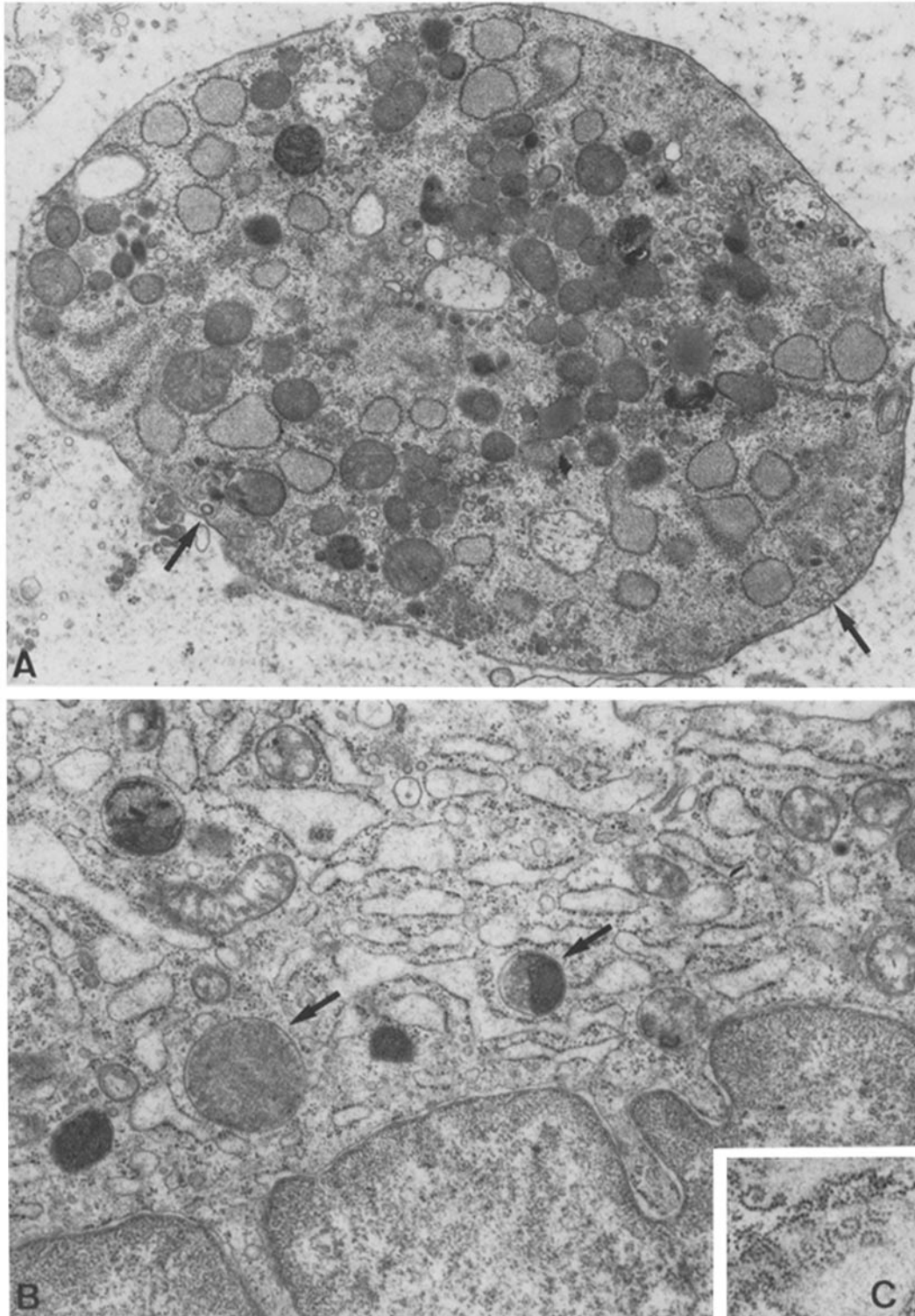


FIGURE 1 Ultrastructural aspects of cultured rat embryo fibroblasts. Cells were collected and examined as previously described (56). **A.** General view of a peripheral part of the cytoplasm. Cisternae of the endoplasmic reticulum are dilated by an electron-dense material and are heavily coated with ribosomes. Many small smooth vesicles are observed, some of which are in continuity with the plasma membrane (arrows). Mitochondria, dense bodies, and lipid droplets are present ($\times 13,000$). **B.** Alternating smooth and rough regions of the endoplasmic reticulum are clearly visible. Dense bodies (arrows) show a well-defined membrane surrounding a pleomorphic material. The convoluted aspect of the nucleus profile is illustrated ($\times 23,000$). **C.** Polysomal arrangement of ribosomes, probably associated with a membrane of the endoplasmic reticulum seen in an oblique section ($\times 30,000$). Courtesy of Dr. F. Van Hooft.

fractions whose weights and densities were determined (9, 35).

The results are expressed as histograms of the density distribution of the constituents (9). In order to average the data of several experiments, the distributions were standardized by dividing the 1.07–1.27 density interval in 15 virtual fractions of equal increment of density. The percentage amount of constituent corresponding to each interval was computed, following published methods (35), from primary plots of the concentration and of the density in the actual fractions versus their cumulated volumes. Two additional fractions are shown on each side of the histogram; they represent, respectively, the material recovered below 1.07 and above 1.27.

Biochemical Assays

Enzymes were assayed by published methods, with minor modifications for increasing their sensitivity. The references and a survey of the methods used are given in Table I. For Mg^{++} -dependent enzymes, the cation is in large excess over the amount of EDTA added with the sucrose solutions in which the particles are suspended. Activities are expressed in standard milliunits (nanomoles of substrate degraded per minute under assay conditions), except for the following enzymes. Cytochrome oxidase and catalase activities are expressed in the units defined by Cooperstein and Lazarow (17) and by Baudhuin et al. (6), respectively. 1 U of cathepsin has been taken as the enzyme activity releasing per minute an amount of TCA-soluble oligopeptides equivalent to 1 mg of bovine serum albumin in the Lowry assay (36).

Unless otherwise stated, the activities given refer to the *total activity*, which is that measured when the structural barriers slowing down the reactions catalysed by particle-bound enzymes have been suppressed. In most cases, the structural latency was released by adding Triton X-100 in the assay mixture at 0.1% final concentration. Complete activation of catalase was achieved by pretreating the sample with 0.5% Triton X-100, the detergent being thereafter diluted five times in the assay mixture. For cytochrome oxidase, the sample was treated in 0.2 ml of digitonin at 0.25% before starting the reaction by addition of 3 ml of solution containing the substrate and the buffer. In this way, the inhibitory effect of the detergent at the concentration which suppresses the latency was avoided. In some cases, activities, referred to then as *free activities*, were measured under conditions that preserve the structural-latency of enzymes: addition of sucrose at 0.25 M concentration to the assay mixture and shortening of the incubation time to a maximum of 10 min. The pH was increased to 5 for determination of free cathepsin. The part of the total activity which is sedimented by centrifuging 30 min at 40,000 rpm (rotor 40) will be referred to as the *sedimentable activity*.

Ribonucleic acid was measured according to Schneider (46), care being taken to wash out the sucrose

completely (63). Proteins were determined by the method of Lowry et al. (36). Cholesterol was assayed by gas chromatography, with stigmasterol as an internal standard (10).

Materials

All chemicals were of analytical grade. Most were purchased from E. Merck A. G. (Darmstadt, West Germany), Sigma Chemical Co. (St. Louis, Mo.), and Koch-Light Laboratories Ltd. (Colnbrook, Buckinghamshire, England); Triton X-100 is the product of Rohm and Haas Co. (Philadelphia, Pa.); lactalbumin hydrolysate was supplied by ICN Nutritional Biochemicals Div., International Chemical and Nuclear Corp. (Cleveland, Ohio); vitamins for Eagle's minimal essential medium were obtained as a 100 times concentrated solution from either Laboratoires Eurobio, Paris, France, or Flow Laboratories, Irvine, Scotland; trypsin was the TC powder trypsin 1:250 of Difco Laboratories, Detroit, Mich.; calf-serum was either obtained from blood collected at a local slaughter-house and processed in our laboratory, or purchased from Laboratoires Eurobio or Flow Laboratories. Pregnant rats were of the Wistar strain, bred in the Animal House of the University of Louvain.

RESULTS

Enzyme Kinetics

A preliminary kinetic study was performed on the various enzyme reactions, in order to establish optimal assay conditions. Fig. 2 shows the effects of pH at the substrate concentrations given in Table I. Acid phosphatase, cathepsin, *N*-acetyl- β -glucosaminidase, deoxyribonuclease, and pyrophosphatase (in presence of 2 mM EDTA) are most active at pH 5 or below. The other enzymes require a neutral or slightly alkaline pH for maximum activity. Esterase was assayed only at pH 7.4, since the substrate readily decomposes at acid or alkaline pH. Except for cytochrome oxidase and catalase, which obey first-order kinetics, the K_m values were determined, and the concentrations of substrate selected provide the reaction with at least 75% of the maximum velocity. Calibration experiments according to amount of tissue and to time of incubation are presented in Fig. 3, which shows the ranges of linearity.

Addition of $MgCl_2$ enhanced considerably the activities of 5'-nucleotidase, inosine diphosphatase, and neutral pyrophosphatase. The cation was present in the assay mixtures at concentrations three to four times the minimum required to gain full activity. Sucrose was found to inhibit *N*-ace-

TABLE I
Assay Conditions for Enzymes

E.C.*	Name	Substrate	Concentration	pH of assay	Buffer	Addition	Temp °C	Compound measured	References
3.1.3.2	Acid phosphatase	Sodium β -glycerophosphate	50 mM	5.0	0.1 M Sodium acetate	—	37	Inorganic phosphate	23
3.2.1.30	<i>N</i> -Acetyl- β -glucosaminidase	<i>p</i> -Nitrophenyl-2-Acetamido- β -D-glucopyranoside	3 mM	4.5	0.1 M Sodium citrate	—	37	<i>p</i> -Nitrophenol	47
3.4.4.23	Cathepsin D	Acid denatured haemoglobin	2%	3.6†	0.1 M Sodium acetate	—	37	TCA-soluble oligopeptides	30, 58
3.1.4.6	DNAse II	Heat denatured highly polymerised DNA	0.06%	5.0	0.1 M Sodium acetate	0.2 M KCl	37	PCA-soluble oligonucleotides	23
1.11.1.6	Catalase	H ₂ O ₂	1.3 mM	7.4	25 mM Imidazole HCl	—	25	Residual H ₂ O ₂	6
3.1.3.5	5'-Nucleotidase	Sodium 5'-AMP	1 mM	8.5	50 mM Tris HCl	20 mM MgCl ₂	37	Inorganic phosphate	62
3.6.1.6	Inosine diphosphatase	Sodium IDP	5 mM	7.0	50 mM Tris HCl	20 mM MgCl ₂	37	Inorganic phosphate	62
3.6.1.-	Acid pyrophosphatase	Sodium pyrophosphate	2 mM	5.0	50 mM Sodium acetate	2 mM EDTA	25	Inorganic phosphate	41
3.6.1.-	Neutral pyrophosphatase	Sodium pyrophosphate	1 mM	7.5	50 mM Tris HCl	20 mM MgCl ₂	37	Inorganic phosphate	50
1.9.3.1	Cytochrome oxidase	Reduced cytochrome <i>c</i>	34 μ M	7.4	30 mM Sodium phosphate	—	25	Residual reduced cytochrome <i>c</i>	17, 35
1.6.2.1	NADH cytochrome <i>c</i> reductase	Oxidized cytochrome <i>c</i>	43 μ M	7.5	40 mM Sodium phosphate	—	25	Reduced cytochrome <i>c</i>	23
3.1.1.2	Esterase	<i>o</i> -Nitrophenyl-acetate	4.7 mM	7.4	50 mM Sodium phosphate	—	25	<i>o</i> -Nitrophenol	14

* Report of the commission of enzymes of the International Union of Biochemistry (1961), Pergamon Press, Oxford.
† Free cathepsin was assayed at pH 5; total cathepsin was assayed at pH 5 only to determine free/total activities ratio.

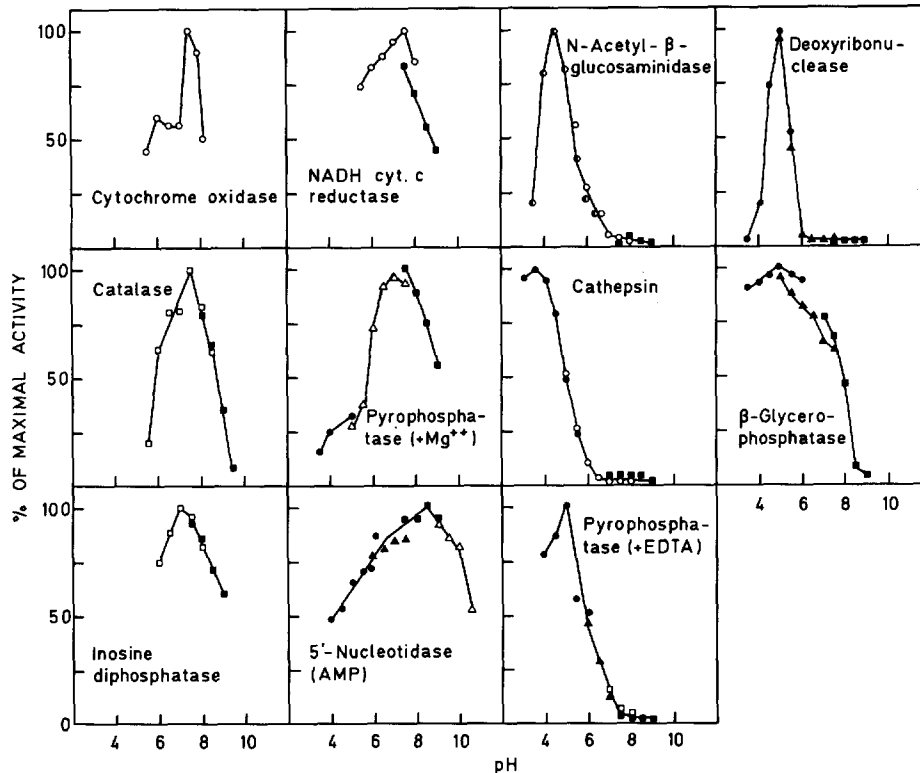


FIGURE 2 pH activity curves of enzymes in homogenates of cultivated fibroblasts. Incubations were carried out in the following buffers: acetate (●), citrate (◐), cacodylate (▲), phosphate (○), imidazole-HCl (◻), Tris-HCl (■), glycine-NaOH (△), each at 50 mM concentration. Other conditions of the assays are given in Table I.

tyl- β -glucosaminidase to an extent requiring suitable corrections when the concentration exceeded 0.1 M. The fibroblast enzymes acting on phosphoric monoester bonds or on phosphoric anhydride bonds were further characterized by inhibitors. Tartrate (10 mM D-L-sodium potassium tartrate) inhibited acid phosphatase as observed in other tissues (5, 40). Sodium fluoride (1 mM) inhibited acid phosphatase, 5'-nucleotidase and neutral pyrophosphatase. Inosine diphosphatase and acid pyrophosphatase were unaffected by either treatment, but the latter was strongly inhibited by ammonium molybdate, 1 mM (41).

Specific activities of fibroblast enzymes are compared, in Table II, to values reported for their homologues in rat liver homogenates. Inosine diphosphatase, esterase, catalase, and the two pyrophosphatases are much less active in cultivated fibroblasts; the other enzyme activities are of the same order of magnitude in the two materials. We have also found that glucose-6-

phosphate is slowly hydrolyzed by fibroblast homogenates. However, we are most likely dealing here with an unspecific phosphatase, not with the specific glucose-6-phosphatase described in liver and kidney, for the fibroblast enzyme has an optimum at pH 5 and is not inactivated by preincubation without substrate at pH 5 and 37°C (22).

Structural Latency and Association of Enzymes with Subcellular Components

When homogenates of fibroblasts are centrifuged for 30 min at 40,000 rpm (Beckman/Spinco rotor no. 40), the neutral pyrophosphatase, about one-third of the esterase activity and one-third of the RNA are recovered in the supernate. The other constituents assayed in this work are found in the pellet to a very large extent. Repeated freezing and thawing or addition of digitonin released 75% of *N*-acetyl- β -glucosaminidase, acid phosphatase, cathepsin, and acid deoxyribonucle-

ase in soluble form, whereas inosine diphosphatase, 5'-nucleotidase, and acid pyrophosphatase remained essentially particle bound; some release of esterase was noticed (Fig. 4).

In fresh homogenates, acid deoxyribonuclease, cathepsin, *N*-acetyl- β -glucosaminidase, acid phosphatase, and catalase were latent to the extent of at least 75% of their total activity. The latency was partially or completely abolished by repeated freezing and thawing and by addition of digitonin or Triton X-100 (Fig. 5). The activation curves of acid phosphatase, acid deoxyribonuclease, *N*-acetyl- β -glucosaminidase, and cathepsin are hardly distinguishable from one another, but they dissociate clearly from the activation curve of catalase. The latter enzyme is more resistant to activating means, especially to digitonin. Full activation of acid phosphatase and *N*-acetyl- β -glucosaminidase is achieved as soon as digitonin is stoichiometrically in excess over the amount of cholesterol present in the preparation; at that digitonin concentration the latency of catalase is still unaffected.

The activities of 5'-nucleotidase and neutral pyrophosphatase were not modified by repeated freezing and thawing or by addition of detergents. Inosine diphosphatase was only slightly activated (less than 15%) by these treatments, which were checked to cause considerable activation of hepatic inosine diphosphatase as previously described (42).

Fractionation by Differential Centrifugation

The distributions obtained after fractionation of fibroblast homogenates by differential centrifugation into N, ML, P, and S fractions are presented in Fig. 6. As expected from the data of Fig. 4, neutral pyrophosphatase is recovered essentially in the S fraction. The low activity found in the N fraction demonstrates that very few cells were left unbroken by the homogenization. Another typical pattern is given by cytochrome oxidase, *N*-acetyl- β -glucosaminidase, acid phosphatase, and deoxyribonuclease, which are largely recovered in the large granule fraction (ML), the remainder being

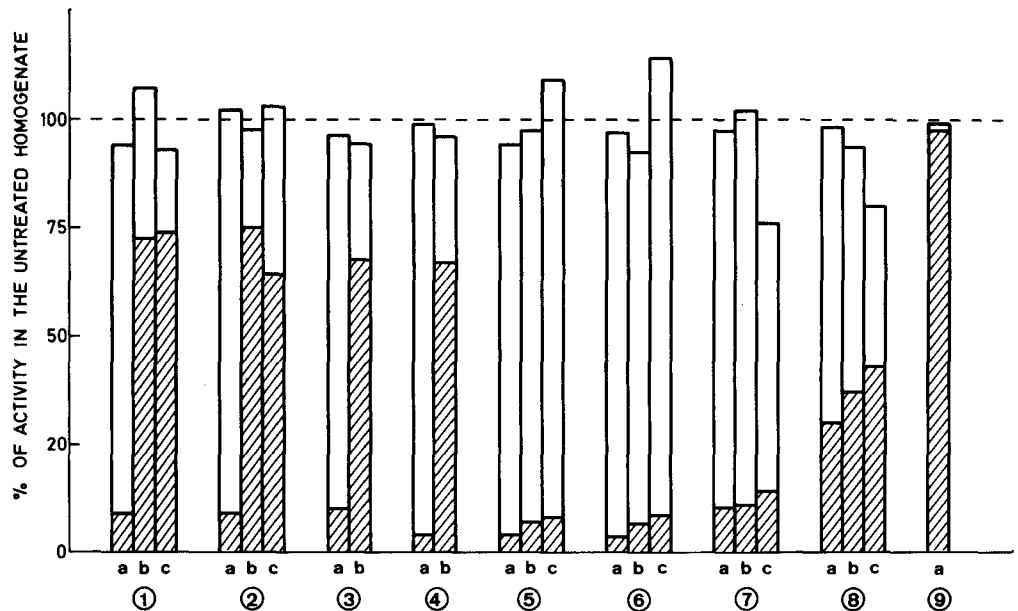


FIGURE 3 Kinetics of fibroblast enzymes. Fibroblast homogenates were incubated under the conditions given in Table I. \blacktriangle , times of incubation were varied with the following constant amounts of cell protein: acid phosphatase, 85 μ g; cathepsin, 166 μ g; *N*-acetyl- β -glucosaminidase, 7.5 μ g; 5'-nucleotidase, 120 μ g; inosine diphosphatase, 275 μ g; catalase, 36 μ g. \bullet , tissue concentrations were varied, with incubation time kept at 30 min for acid phosphatase, cathepsin, *N*-acetyl- β -glucosaminidase, and acid deoxyribonuclease; 20 min for 5'-nucleotidase, inosine diphosphatase, acid and neutral pyrophosphatases; 10 min for catalase. NADH cytochrome *c* reductase, cytochrome oxidase, and esterase were followed spectrophotometrically for 10 min.

TABLE II
Comparison of the specific content in enzymes and chemical constituents in homogenates of fibroblasts and of rat liver

Enzyme or constituent	Fibroblasts	Liver	
		Activity or content	Reference
Acid phosphatase	46.1 ± 7.2 (12)	30.2	23
<i>N</i> -Acetyl-β-glucosaminidase	80.7 ± 12.3 (21)	34.5	47
Cathepsin	18.6 ± 7.2 (18)		
DNAse	14.2 ± 6.7 (5)	6.55	23
Catalase	20.6 ± 6.3 (12)	354.5	6
5'-Nucleotidase	73.2 ± 7.5 (10)	51.6	2
Inosine diphosphatase	39.3 ± 15.7 (12)	456.6	2
Acid pyrophosphatase	16.3 ± 5.8 (9)	177	49
Neutral pyrophosphatase	23.6 ± 9.1 (6)	107	50
Cytochrome oxidase	61.2 ± 18.7 (14)	153.0	23
NADH cytochrome <i>c</i> reductase	115 ± 18 (5)	456	2
Esterase	162 ± 45 (4)	1173	2
RNA	87.1 ± 7.2 (6)	39.13	2
Cholesterol	38.4 ± 3.1 (8)	12.69	2

Results are given as means ±SD in milliunits (enzymes) or in micrograms (chemical constituents) per milligram protein; figures in parentheses refer to number of experiments.

mostly divided between the N and P fractions in a way particular for each enzyme. The activities in fraction N are not attributable only to unbroken cells, for they largely exceed that of neutral pyrophosphatase. Finally, NADH cytochrome *c* reductase, acid pyrophosphatase, inosine diphosphatase, 5'-nucleotidase, and catalase attain the highest specific activity in the microsomal (P) fraction. However, although somewhat variable, the amounts of these enzymes sedimented with the large granules are nearly equal to those found in the microsomes.

In the experiment reported in Fig. 7, the large granule fraction has been resolved into an M and an L fraction. Cytochrome oxidase now shows a distribution pattern distinct from those of acid phosphatase, *N*-acetyl-β-glucosaminidase, and cathepsin: the three acid hydrolases have a somewhat higher specific activity in L than in M, whereas cytochrome oxidase is more concentrated in the M fraction. The dissociation is, however, much less marked than it is in liver tissue (23). The difference between the M and L fractions is more pronounced for the enzymes that attain their highest specific activity in the microsomes. In this group, to which cholesterol may be added, catalase differs clearly from the other enzymes by having a sevenfold higher specific activity in the L than in the M fraction. RNA is mainly associated with fractions

S (34%) and P (29%), and shows the highest specific content in fraction P. An appreciable amount of RNA sediments with the N fraction. Esterase exhibits the broadest distribution pattern, with a small peak in the microsomes and a low activity in fraction N.

Fractionation by Density Equilibration

The results obtained by density equilibration in linear sucrose gradients are shown in Table III and Fig. 8. Cytoplasmic extracts, free of nuclei and unbroken cells, were used rather than complete homogenates in these experiments, in order to avoid artifacts caused by the presence of nuclei. It can be seen from the data presented above that, except for RNA, no more than 10% of each constituent is discarded with the N fraction. Several patterns of density distribution can be distinguished. (a) Profiles of *N*-acetyl-β-glucosaminidase, cathepsin, acid phosphatase, and deoxyribonuclease are very similar and extend asymmetrically over the whole length of abscissa. Their modes coincide with the 1.203–1.217 fraction; the median densities are comprised between 1.199 and 1.206, except for acid phosphatase (1.189) which exhibits a second small but reproducible mode near the density 1.16. (b) Catalase presents a sharper profile, with a mode in the 1.177–1.190 fraction and a median density within these limits.

(c) The distribution pattern of cytochrome oxidase is the sharpest and peaks at a lower density, in the 1.150–1.177 fractions. NADH cytochrome *c* reductase shows a peak in the same range of density, but is much more broadly spread through the gradient, and occurs in fractions virtually free of cytochrome oxidase activity. (d) 5'-nucleotidase and inosine diphosphatase show similar, highly skewed profiles, with a mode in the 1.123–1.137 fraction and extending asymmetrically over the whole density range. Cholesterol and acid pyrophosphatase behave similarly, except that their

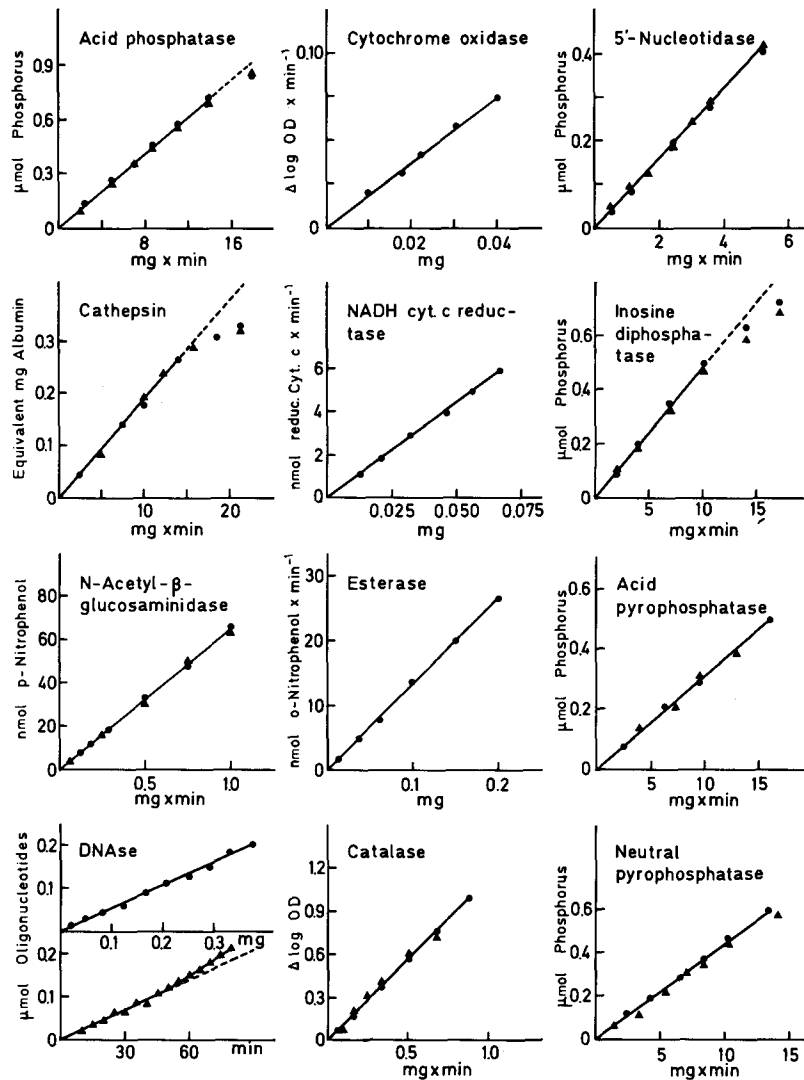


FIGURE 4 Solubilization of enzymes of the homogenate (1: acid phosphatase; 2: *N*-acetyl- β -glucosaminidase; 3: acid deoxyribonuclease; 4: cathepsin; 5: 5'-nucleotidase; 6: acid pyrophosphatase; 7: inosine diphosphatase; 8: esterase; 9: neutral pyrophosphatase) by various treatments: (a) control homogenate; (b) homogenate frozen and thawed five times; (c) homogenate treated with digitonin at a final concentration of 0.02% (0.92 mg of cell protein \times ml⁻¹; molar digitonin/cholesterol ratio: 1.93). After treatment, fractions of homogenates were centrifuged at 40,000 rpm for 30 min (rotor no. 40 Beckman/Spinco). Enzymes were assayed under "total" conditions in resuspended pellets, supernates, and original homogenates; recoveries ranged between 92 and 104%. The figure shows the sedimentable (white blocks) and soluble (shaded blocks) activities as percentage of the total activity of the untreated homogenate.

modes appear in the next denser fraction. In addition, the distribution pattern of acid pyrophosphatase shows a shoulder on its dense side, which increases somewhat the median density accordingly. Other distributions are more complex. Esterase is spread over the whole gradient; its activity in the low density region is largely due to soluble enzyme (25–35%, see Figs. 5 and 7). The same comment applies to protein which attains the highest concentration at densities around 1.14. RNA is rather broadly, but symmetrically distributed. It probably represents the sum of the density distribution of RNA-coated vesicles and of the sedimentation pattern of free ribosomes which are still remote from their equilibrium position.

Effect of Digitonin on the Density Distribution of Microsomal Constituents

It has been reported that digitonin, at concentrations too low to disrupt most subcellular particles, causes a distinct increase in the equilibrium density of cholesterol-rich membranes from rat liver, particularly the plasma membrane and the microsomal components related to the plasma

membrane (3, 52). In view of this finding, the influence of digitonin on the density distribution of microsomal constituents from cultured fibroblasts was investigated with the hope that different patterns of behavior might be revealed in this way. Two enlarged microsomal fractions (*LP* fractions, see Materials and Methods) were prepared. One was washed twice with 0.25 M sucrose; the other was washed once with sucrose-EDTA, resuspended in sucrose-EDTA supplemented with 0.03% digitonin, and recentrifuged as usual. The concentration of microsomes was such that the molar ratio digitonin/cholesterol was close to 1. It can be seen from Table IV that the two preparations had similar biochemical compositions.

The density distributions observed with the two microsomal preparations are presented and compared in Fig. 9. Considering first the untreated preparation, it appears that, except for protein and RNA, the median densities and the profiles resemble those obtained with cytoplasmic extracts. Inosine diphosphatase, however, and, to a lesser extent, acid pyrophosphatase, exhibit an important tailing towards high densities. The distribution of protein is largely similar to that of

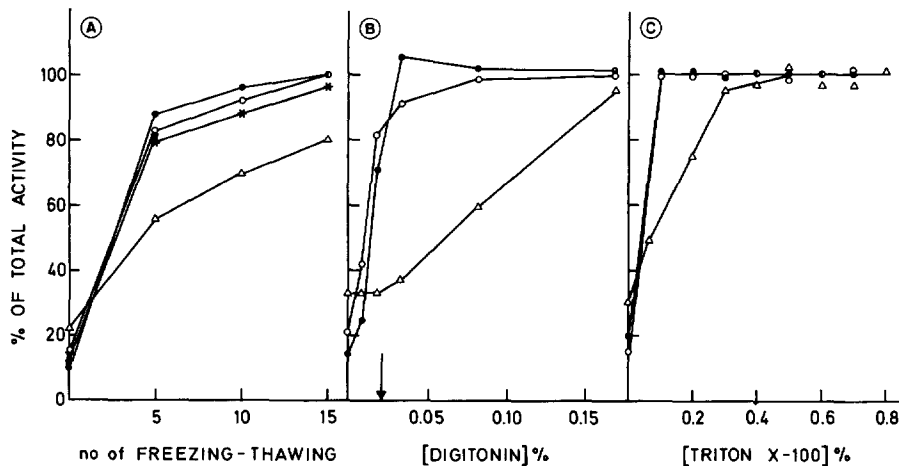


FIGURE 5 Suppression of structural latency of acid phosphatase (●), *N*-acetyl- β -glucosaminidase (○), cathepsin (*), acid deoxyribonuclease (▼) and catalase (Δ) by various treatments. Graphs show free activities in percent of total activities, both measured after the treatment (A and B). The treatments never modified the total activities significantly. A, homogenate (2.4 mg of protein/ml) repeatedly frozen in dry ice-acetone mixture and thawed in water at 10°C. B, homogenate (1.7 mg of protein/ml) supplemented with digitonin at various concentrations. The point of stoichiometric equivalence between digitonin and cholesterol of homogenate is indicated by the arrow. C, homogenates (500 μ g of protein/ml for phosphatase; 100 μ g/ml for *N*-acetyl- β -glucosaminidase and catalase) were treated with Triton X-100 at the concentrations presented on the abscissa. The reaction was started thereafter by addition of 4 vol of buffered substrate. Assays were performed under "free activity" conditions; activities are expressed in percentage of the activity observed in the same homogenate under "total activity" conditions.

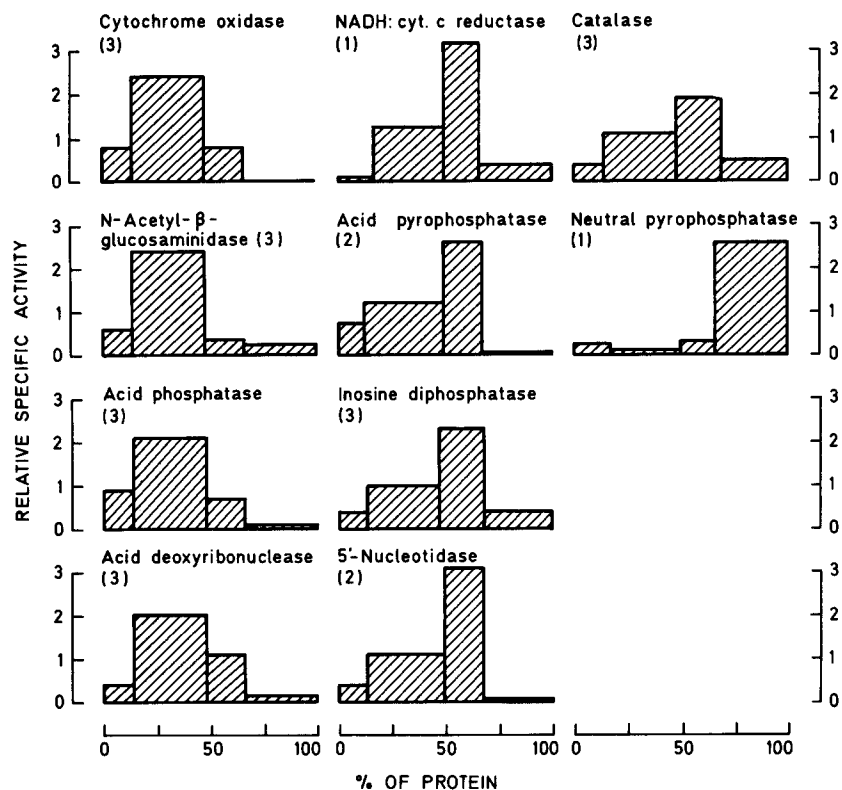


FIGURE 6 Distribution patterns of enzymes after fractionation by differential centrifugation. Fibroblast homogenates were divided into four fractions: N, ML, P, and S. These are represented by blocks ordered according to the same sequence on the abscissa where they span a length proportional to their protein content. The ordinate (heights of the blocks) gives the relative specific activities (percentage of activity over percentage of protein) of the fractions. Graphs represent the mean result of several experiments; the actual number is given between parentheses for each enzyme. Percentages relate to the sum of N, ML, P, and S fractions. Averaged recoveries from the homogenates ranged between 90 and 114%.

NADH cytochrome *c* reductase. The distribution of the microsomal RNA is symmetrical with a mode in the fractions 1.163–1.177. Digitonin treatment causes marked changes in distribution patterns, characterized by an increased median (Table IV) and modal density and a smaller dispersion of density for both 5'-nucleotidase and cholesterol. The shift of these profiles is such that they dissociate from the normal ones by 70% of their surface area. In contrast, the distributions of NADH cytochrome *c* reductase and RNA are not significantly influenced. Acid pyrophosphatase, inosine diphosphatase, and protein exhibit an intermediate behavior. The two enzymes show a modal shift comparable to that of 5'-nucleotidase and cholesterol, but the profiles are slightly asymmetrical, as if the shift concerned only part of the activities.

DISCUSSION

Homogenates of cultured rat embryo fibroblasts have been analyzed by quantitative fractionation techniques. The distribution patterns obtained have led to the biochemical characterization of several subcellular components. In the following discussion, these are identified with a given component of the intact cell. Obviously some of these identifications remain tentative, awaiting a correlated morphological and biochemical study.

Mitochondria

Mitochondria, detected by their specific marker cytochrome oxidase (24), are recovered largely with the large granules after differential centrifugation, and after equilibration in a sucrose gradi-

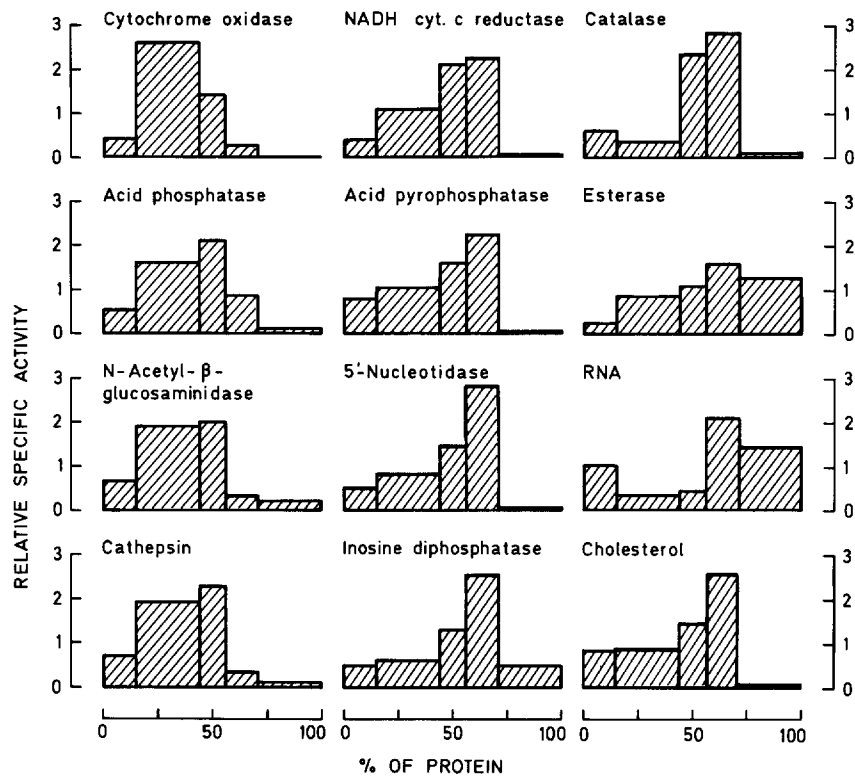


FIGURE 7 Distribution patterns of enzymes and constituents after fractionation by differential centrifugation. Fibroblast homogenate was divided into five fractions: N, M, L, P, and S. Same mode of representation as in Fig. 6. Recoveries from the homogenate ranged between 84 and 118%.

TABLE III
Median Densities and Recoveries of Constituents after Equilibration of Cytoplasmic Extracts in Sucrose- H_2O Gradient

Constituent	Median density of equilibration	Recovery in the gradient	No. of experiments
		%	
Inosine diphosphatase	1.137 ± 0.010	90.7 ± 5.1	4
Cholesterol	1.144 ± 0.005	102.0 ± 6.1	2
5'-Nucleotidase	1.144 ± 0.008	105.2 ± 3.2	3
Acid pyrophosphatase	1.157 ± 0.008	100.7 ± 6.8	2
Esterase	1.152 ± 0.011	98.7 ± 0.9	2
RNA	1.156	108.2	1
Cytochrome oxidase	1.161 ± 0.006	90.2 ± 10.4	5
NADH cytochrome c reductase	1.166 ± 0.003	87.1 ± 9.3	3
Catalase	1.184 ± 0.002	90.9 ± 6.2	3
Acid phosphatase	1.189 ± 0.006	98.1 ± 4.2	3
Cathepsin	1.198 ± 0.001	94.4 ± 7.2	3
Acid deoxyribonuclease	1.205	97.3	1
N-Acetyl-β-glucosaminidase	1.206 ± 0.007	95.3 ± 9.7	5
Protein	1.150 ± 0.002	99.7 ± 9.2	5

Values listed are means ± SD.

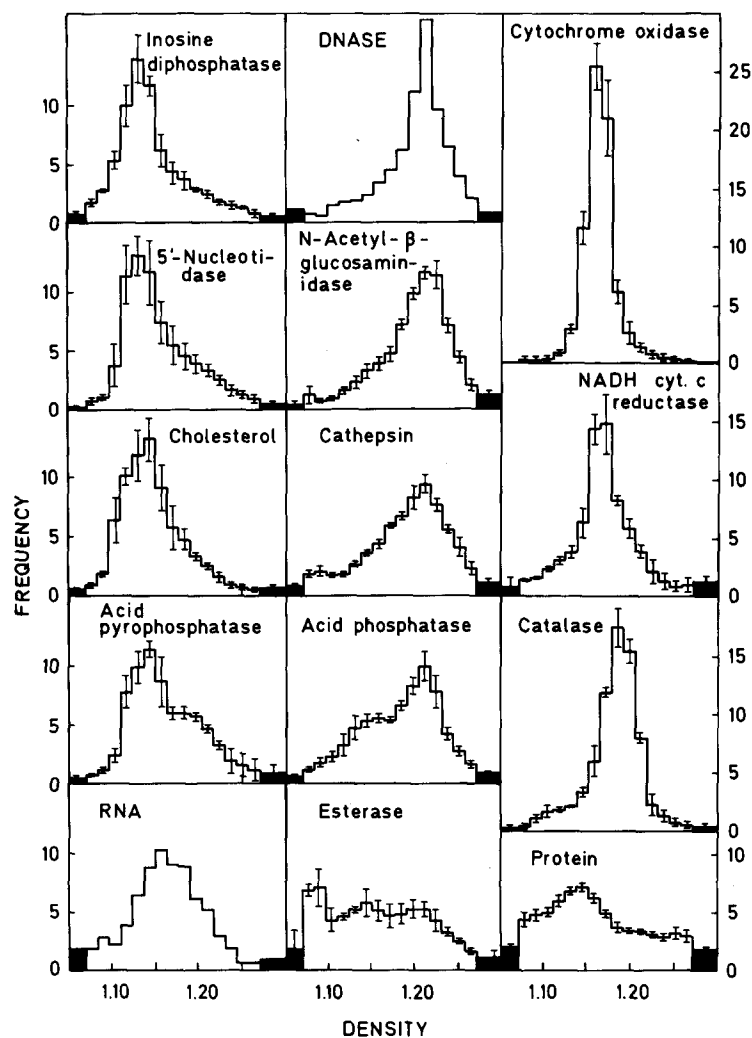


FIGURE 8 Distribution patterns of enzymes and chemical constituents after fractionation by density equilibration in linear sucrose gradient. Cytoplasmic extracts were equilibrated as described under Materials and Methods. Results are plotted in the form of normalized histograms (11, 35). The abscissa is the density scale divided in 15 equal sections of density increment $\Delta\rho = 0.013$, over the span 1.070-1.270. The frequency given in ordinate (\pm standard deviation) is $\Delta Q/(\Sigma Q \cdot \Delta\rho)$, where ΔQ is the amount of constituent present within the section, and ΣQ the sum of the amounts found on all the subfractions. The surface area of each section of the diagram gives the fractional amount of constituent present within the section. Solid blocks on each side of the distribution profile represent material recovered below 1.07 and above 1.27; they are arbitrarily constructed over the density spans 1.05-1.07 and 1.27-1.30. The total area of each histogram is then equal to 1. Complementary data are presented in Table III.

ent, show a sharp peak at a density of about 1.17, a value somewhat lower than that observed for liver mitochondria (9, 35) but comparable to that reported for the mitochondria of several other tissues, including spleen (15), skeletal muscle (48),

Ehrlich ascites tumour cells (34), and Chinese hamster ovary fibroblasts (38). The mitochondria seem to contain some NADH cytochrome *c* reductase activity but not all of it, since after fractionation by differential centrifugation or den-

TABLE IV
Effect of Digitonin on Properties of Fibroblast Microsomes

	Content of homogenate (LP fraction)		Median density of equilibra- tion		Dissociation of patterns
	Untreated fraction	Digitonin- treated fraction	Untreated fraction	Digitonin- treated fraction	
	%	%			%
Protein	21.1	21.3	1.163	1.178	22.0
5'-Nucleotidase	54.6	51.3	1.140	1.190	70.2
Cholesterol	49.2	53.2	1.141	1.192	69.4
Acid pyrophosphatase	49.5	43.4	1.145	1.189	53.7
Inosine diphosphatase	62.5	57.5	1.151	1.181	38.5
Ribonucleic acid	25.2	20.1	1.163	1.165	8.4
NADH cytochrome <i>c</i> reductase	40.3	41.0	1.162	1.161	4.4
<i>N</i> -Acetyl- β -glucosaminidase	23.0	15.5	—	—	—

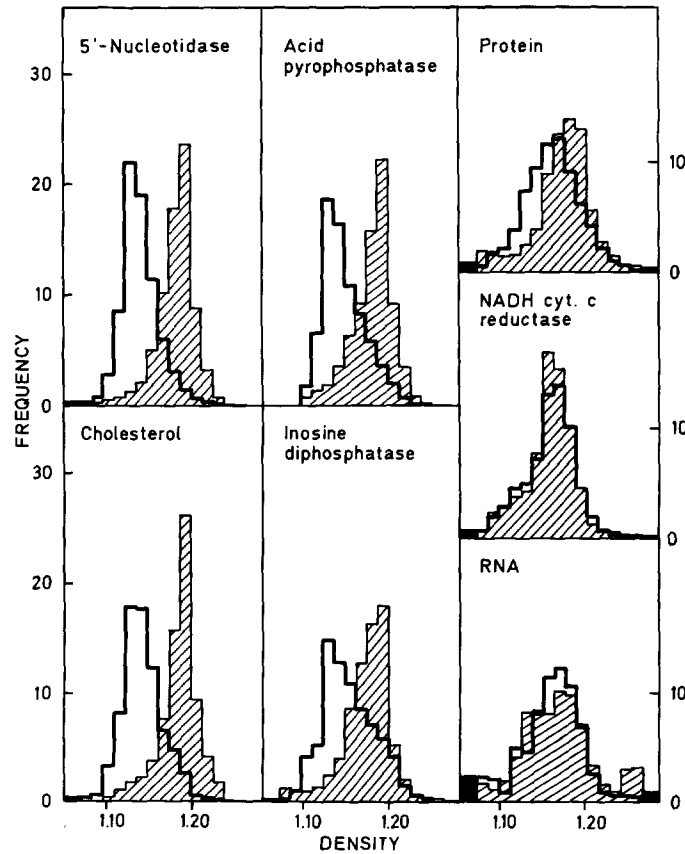


FIGURE 9 Influence of digitonin on the density distribution of microsomal constituents in a sucrose gradient. Enlarged microsomal fractions (LP) were equilibrated in a linear sucrose gradient. Results are presented as described in Fig. 8. Density distributions were obtained from untreated microsomes (thick line) and from microsomes treated with digitonin as described in the text (shading). Complementary data are presented in Table IV.

sity equilibration a substantial reductase activity is found in fractions possessing little or no cytochrome oxidase activity.

Lysosomes

Fibroblast homogenates were found to contain four hydrolases that are: active at acid pH, largely latent in fresh preparations, unmasked together by repeated freezing and thawing and by addition of detergents, associated with particles of fairly high sedimentation coefficient and of high equilibrium density in sucrose gradient, and released to a large extent in soluble form by treatments that suppress latency. These properties correspond to those described previously for lysosomal enzymes from rat liver (9, 23) and to the biochemical concept of lysosomes as it has been proposed by de Duve (18). It may be taken that they characterize also the lysosomes of cultured fibroblasts. High sensitivity to disruption by digitonin represents another similarity between fibroblast and liver (47) lysosomes and suggests that cholesterol is a constituent of their membranes. As in many other tissues (5, 40), the lysosomal acid phosphatase of fibroblasts is inhibited by fluoride and tartrate.

The lysosomes dissociate clearly from catalase in differential centrifugation, from mitochondria in density equilibration, and from other components in both methods. Our data, however, are not incompatible with a localization of some esterase activity in lysosomes.

Whatever the separation method applied, the distribution profiles of the lysosomal enzymes are never quite identical. The small differences between cathepsin, *N*-acetyl- β -glucosaminidase, and acid deoxyribonuclease reflect probably some biochemical heterogeneity within the lysosomal population of cultured fibroblasts. Another possible source of partial dissociation is the interference by enzymes associated with other particles in assays of lysosomal enzymes. In this respect, too much significance should not be attributed to the excess of acid phosphatase at the density of 1.15, where other enzymes capable of hydrolyzing β -glycerophosphate may equilibrate.

Catalase-Bearing

Particles (Peroxisomes ?)

Catalase is essentially associated with subcellular particles in fibroblast homogenates. The high degree of latency exhibited by fresh preparations (70-80%) and the manner in which it can be

suppressed indicate that this association is not merely the consequence of an adsorption at the surface of subcellular particles. The greater resistance of the catalase-bearing particles to activating agents, particularly digitonin, differentiates them clearly from lysosomes. The results of differential centrifugation show convincingly that we are not dealing with mitochondria, and the density distributions obtained by equilibration in sucrose gradient distinguish the catalase-bearing particles from other microsomal constituents.

In liver and kidney, catalase has been shown to be a typical constituent of peroxisomes (21). Even though the structural latency of fibroblast catalase and its relative resistance to activation by digitonin are reminiscent of properties reported for rat liver peroxisomes, identification of the catalase-bearing organelles of fibroblasts with peroxisomes would be premature. Indeed, liver (9) and kidney (7) peroxisomes sediment more rapidly and equilibrate at a higher density. Furthermore, they have been found to contain several hydrogen peroxide-producing oxidases which could not be detected in the present case.

Plasma Membrane and

Related Structures

Cholesterol, 5'-nucleotidase, inosine diphosphatase, and acid pyrophosphatase share several properties. All are associated with sedimentable components of the homogenate; they are purified in the microsomal fraction but differ from typical microsomal components in contributing considerable amounts to the heavier N, M, and L fractions. They show similar patterns of distribution in the sucrose gradient, although slight differences are observed, especially after digitonin treatment. The linkage of the enzymes to subcellular structures is not suppressed by treatments that release lysosomal enzymes into the suspension medium.

Fragments of plasma membrane or related structures are likely to be the cytological entity bearing the bulk of 5'-nucleotidase in cultured rat fibroblasts. This enzyme exhibits most properties of the hepatic plasma membrane-bound 5'-nucleotidase: activation by Mg^{++} , inhibition by F^- ions and resistance to tartrate (27), absence of structure-linked latency, association with subcellular components of low density, density shift following addition of digitonin (3, 20, 52). The characteristic "nucleo-microsomal" distribution of the liver enzyme has not been obtained with fibroblast

homogenates. But this is easily explainable, since the result of mechanical disruption of the cell membrane by homogenization may differ, depending on whether we are dealing with an organized tissue or with a suspension of cells. The close parallel between the distributions of 5'-nucleotidase and cholesterol indicates that, as in the liver, most of the latter constituent is associated with the pericellular membrane and related structures of cultured fibroblasts. Judging from the digitonin shift, these components represent a sizeable fraction of the microsomal protein. They are likely to bear also the larger part of the acid pyrophosphatase activity. Some of this activity, however, may be linked to another subcellular component equilibrating at a higher density (Fig. 8), less sensitive to the treatment with digitonin (Fig. 9) and still to be identified.

The distribution of inosine diphosphatase follows closely that of 5'-nucleotidase in differential and isopycnic centrifugation. Only partial dissociation is achieved by digitonin treatment. It is therefore likely that inosine diphosphatase belongs largely to plasma membrane. The presence of an enzyme splitting the pyrophosphate bond of inosine diphosphate in the plasma membrane of cultured fibroblasts may be related to that of an unspecific nucleoside diphosphatase, assayed with ADP, in the plasma membrane of rat liver (62), chick embryo fibroblasts (44), and Ehrlich ascites cells (59). In liver, nucleoside diphosphatase activity is mainly due to an enzyme acting preferentially on IDP, UDP, and GDP and associated with microsomal elements derived from the endoplasmic reticulum (11, 28) which, in contrast to the ADPase of plasma membrane, is strongly activated and released in soluble form by sodium deoxycholate (42). The inosine diphosphatase of fibroblast homogenates lacks these properties, thereby resembling the enzyme localized in rat liver plasma membranes. The absence of a specific nucleoside diphosphatase in the endoplasmic reticulum would account for the lower specific activity of inosine diphosphatase found in fibroblast homogenates (Table II). The partial dissociation achieved between 5'-nucleotidase and inosine diphosphatase by digitonin may reflect a biochemical heterogeneity of the plasma membrane, or the association of some of the inosine diphosphatase activity with another type of subcellular component.

Endoplasmic Reticulum and Other Components

The morphology of the cells, illustrated in Fig. 1, leaves little doubt that in the cytoplasmic fractions of cultured fibroblasts the bulk of the RNA is contributed by the RNA of ribosomes. In the case of the S fraction, this has been confirmed by the observation that most of the RNA sedimented by a centrifugation of $W = 1.2 \times 10^{11} \text{ rad}^2 \text{ s}^{-1}$ (40,000 rpm for 2 h, rotor 40, Beckman/Spinco Centrifuge). In the M, L, and P fractions, RNA must be, to a large extent, ribosomal RNA associated with the vesicles derived from the rough endoplasmic reticulum which is very expanded in these cells. After density equilibration of LP fractions, the distribution of RNA thus reflects, in a somewhat crude fashion, the density distribution of ribosome coated vesicles. Since the density distribution of RNA is not shifted after the treatment of LP fractions with digitonin, the endoplasmic reticulum distinguishes itself clearly from the plasma membrane in fibroblast homogenates, as it does in the case of liver (3, 52).

The enzyme responsible for the nonmitochondrial activity of NADH cytochrome *c* reductase is the only one which may be tentatively attributed to elements derived from the endoplasmic reticulum. It is associated with microsomal entities that show no digitonin shift, and are therefore largely different from those bearing the 5'-nucleotidase and the bulk of cholesterol. However, the dual localization of NADH cytochrome *c* reductase makes this activity of little use as an enzymic marker of the endoplasmic reticulum membranes.

Esterase does not seem to belong to a single subcellular component. In liver, the microsomal activity prevails (2, 57) and belongs to elements derived from the endoplasmic reticulum (11). This localization, however, is disputed by cytochemical data which display reaction products of esterase activity in cytoplasm, in the endoplasmic reticulum, and in lysosomal bodies (33, 37). In spleen, the enzyme assayed with *o*-nitrophenyl acetate has been attributed partially to lysosomes (14). In fibroblast homogenates, esterase activity occurs in the final supernate and in subcellular particles which cover a wide range of size and density. This may reflect the adsorption of a soluble enzyme on various subcellular components.

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