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The Heterogeneous Distribution of Acid Hydrolases within a Homogeneous Population of Cultured Mammalian Cells

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1. Chinese-hamster ovary fibroblasts were cultured to provide a homogeneous cell population. Homogenates obtained from these cells were fractionated by centrifugation techniques and the resulting fractions were analysed for protein and for enzymes representative of certain subcellular particles. 2. Unlike those in rat liver homogenates, the mitochondrial and lysosomal populations proved impossible to separate by differential centrifugation owing to the similarity of their sedimentation properties. Their resolution was possible by using isopycnic centrifugation in a continuous sucrose density gradient. 3. The mitochondrial population equilibrated at a density of $1.17\,g\cdot cm^{-3}$ as in rat liver homogenates. However, the lysosomal population equilibrated at a lower rather than a higher density position than the mitochondria and the probable reasons for this are discussed. 4. The lysosomal population subdivided into two groups characterized by differences in acid hydrolase content and equilibrium densities. The fraction with a density of $1.15\,g\cdot cm^{-3}$ contained the majority of arylsulphatases A and B, of cathepsin and of β-acetylglucosaminidase activities, whereas that with a density of $1.09\,g\cdot cm^{-3}$ contained the majority of the acid phosphatase and acid ribonuclease activities. The probable division of the lysosomal population of a single cell into a number of distinguishable sub-groups is suggested.

The complexity of the lysosomal system has become increasingly apparent since its initial establishment as an integral component of the cell (de Duve *et al.*, 1955). Lysosomes contain a large number of acid hydrolases, and because of their different specificities virtually all high-molecular-weight compounds found in the organism can be digested. It is not clear, however, whether these hydrolase activities are randomly distributed throughout the system as a whole, or whether further specialization causes the segregation of specific hydrolases into an individual lysosome, giving it a characteristic digestive function. Such a specialization of lysosomal function could vary in degree from the extreme case of a single enzyme in each lysosomal body, to the case of each lysosomal body containing a number of related enzyme activities designed for a specific purpose, e.g. membrane or nucleic acid digestion. The existence of such a system would imply an ability of 'recognition' between individual vacuoles, both to introduce a substrate to its appropriate enzyme and to prevent the fusion of different lysosomal populations, causing a reversal of the situation to a random array of enzymes.

The original concept of lysosomes was a homogeneous population of particles (de Duve *et al.*, 1955). However, systematic differences observed in the distribution pattern of a number of acid hydrolases after cell fractionation of a variety of tissue sources have caused some doubt as to the validity of this concept (see, e.g., Beaufay *et al.*, 1959; Bowers & de Duve, 1967*a*; Vaes & Jacques, 1965). To a large extent, this observed heterogeneous distribution of lysosomal enzymes may be explained by the cellular heterogeneity of the tissue rather than by the heterogeneity of the lysosomes in a single cell. The organs and tissues commonly used for lysosomal studies invariably contain more than one population of cells and it is unlikely that all such populations are identical in their acid hydrolase content. For example, the liver consists of at least two cell populations, which have been shown to contain different proportions of acid hydrolases (Wattiaux *et al.*, 1956). Therefore, in a study using a whole liver homogenate, even if the lysosomes from each cell type were enzymically homogeneous, their mixture would appear heterogeneous if the centrifugal properties of the two populations were not identical. Such intercellular acid hydrolase heterogeneity has been demonstrated in several tissue types (Vaes, 1965; Bowers & de Duve, 1967*b*).

A detailed study of lysosomal heterogeneity requires the elimination of the ambiguities connected with the presence of more than one cell type. Such

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conditions may be achieved through the use of tissue-culture technique, by using a line of mammalian cells previously cultured for a sufficient length of time to render them a homogeneous population. The present investigations concern the application of fractionation techniques, previously used to study the lysosomal population within tissue homogenates, to a line of cultured mammalian cells in order to elucidate the degree of lysosomal heterogeneity.

Experimental

Cell culture

Chinese-hamster clone A ovary fibroblasts (Munro *et al.*, 1964), initially obtained from the Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester, U.K., were grown in monolayer culture. The medium utilized was F12 (Ham, 1965) supplemented with 10% foetal calf serum (Flow Laboratories, Irvine, Ayrshire, U.K.) together with sodium penicillin B.P. (20000 units/litre) (I.C.I. Ltd., Alderley, Cheshire, U.K.), streptomycin sulphate B.P. (50mg/litre) (Glaxo, Ulverston, Lancs., U.K.) and amphotericin B (5mg/litre) (Squibb, Liverpool, U.K.). Cultures were incubated in 5% CO_2 in air at 37°C. Cells in their exponential growth phase were detached from the surface of the culture bottles by treatment with 0.025% (w/v) trypsin [Sigma (London) Chemical Co., London S.W.6, U.K.] in a salt solution designed to minimize cell damage (Nias & Lajtha, 1965). After removal of trypsin solution by centrifugation, the cells were washed and resuspended in 0.25M-sucrose.

Cell homogenization

A Tri-R Stir-R (Tri-R Instruments, Jamaica, N.Y., U.S.A.) mechanical homogenizer, consisting of a glass tube (S30) of 3ml capacity and a pestle (S20) with a diameter of 7.82mm and a clearance of 10–13μm rotating at a speed of 2000rev./min, was used. Thirty up-and-down strokes were applied to freshly isolated cell suspensions, which were cooled in ice throughout.

Fractionation of homogenates

(*a*) *Differential centrifugation.* Homogenates derived from approx. 12×10^7 cells were fractionated by using a method based on the fractionation of rat liver homogenates (de Duve *et al.*, 1955). However, the original scheme, outlined in Table 1, was slightly modified by the omission of a second homogenization step applied to the nuclear pellet and by decreasing the number of pellet washes.

Each pellet isolated, comprising nuclear (N), heavy mitochondrial (M), light mitochondrial (L) and microsomal (P) fractions, was ultimately resuspended in 0.25M-sucrose–1mM-EDTA, to give a final volume of 2.5ml. A portion of the original homogenate was, after dilution with 0.25M-sucrose–1mM-EDTA, termed the H fraction. The supernatant from isolation of the P fraction was termed the S fraction.

(*b*) *Density-gradient isopycnic centrifugation.* A linear sucrose density gradient was produced by the method of Ayad *et al.* (1968) by using a three-channel peristaltic pump. The gradient (22ml vol.) extended between densities of $1.278\,g\cdot cm^{-3}$ [75% (w/v) sucrose] and $1.037\,g\cdot cm^{-3}$ [10% (w/v) sucrose] and rested on a cushion (3ml) of a solution of density $1.297\,g\cdot cm^{-3}$ [80% (w/v) sucrose].

A whole homogenate, derived from approx. 12×10^7 cells suspended in 2ml of 0.25M-sucrose, was layered on to the gradient. Centrifugation was at 4°C in the SW25 rotor of a Spinco model L ultracentrifuge at 25000rev./min (63600$g_{av.}$) for 3h. Extension of the centrifugation period to 5h caused no change in the distribution patterns obtained, showing that isopycnic conditions had been reached after the 3h period.

After centrifugation, each tube was divided into approx. 13 fractions (each 2ml) starting from the top of the gradient. This was done by slowly pumping a

Table 1. *Differential centrifugation scheme used for the fractionation of homogenates of Chinese-hamster fibroblasts*

For further details of fractions see the Experimental section.

Fraction	Centrifuge	Tube volume (ml)	Speed (rev./min)	Time	Total force (g-min)
N	MSE Magnum (8-place head)	10	1700	10min	6000
M	Spinco L2 (40 rotor)	10	12500	3min 2s	33000
L	Spinco L2 (40 rotor)	10	25000	6min 42s	250000
P	Spinco L2 (40 rotor)	10	35000	40min	3000000

dense (85%, w/v) sucrose solution through a needle piercing the base of the tube, thus displacing the whole of the gradient upwards out of the top of the tube.

Determination of densities of gradient fractions

The refractive index of each fraction was determined with an Abbe refractometer. The density and sucrose concentrations were calculated from data given by Weast & Selby (1964).

Biochemical assay of fractions obtained by differential or density-gradient centrifugation

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (Sigma) as a standard. Fumarase (EC 4.2.1.2) was assayed spectrophotometrically by the method of Racker (1950) with L-malic acid as substrate. The assay used for cytochrome oxidase (EC 1.9.3.1) was that described by Applemans *et al.* (1955), with cytochrome *c* (Sigma; horse heart) as substrate. Both these assays were carried out at 37°C, the change in the extinction of the incubation mixture being measured on an Optica recording spectrophotometer. Cathepsin D (EC 3.4.4.23) was assayed by the method of Barrett (1967) at pH3.0, 45°C, for 1h, except that the total reaction volume was decreased to 0.6ml. The substrate was native haemoglobin (8%, w/v) prepared from ox blood by the method of Barrett (1970). The reaction was terminated with 3ml of 3% (w/v) trichloroacetic acid and the extinction was related to that of bovine serum albumin in water. The E_{280} of the supernatant was linear with enzyme concentration up to an extinction of 0.3. Arylsulphatases A and B (EC 3.1.6.1) were assayed by a modification of the procedure of Dodgson *et al.* (1955) in which the sample enzyme was incubated in a total volume of 0.6ml containing 10mM-nitrocatechol sulphate (prepared by the method of Roy, 1953) and 50mM-sodium acetate buffer, pH5.0, for 1h. The reaction was terminated with 2.4ml of 0.2M-NaOH and nitrocatechol was determined from the E_{515}. Acid phosphatase (EC 3.1.3.2) was assayed by the method of Gianetto & de Duve (1955) with sodium β-glycerophosphate (BDH Chemicals Ltd., Poole, Dorset, U.K.) except that the total reaction volume was decreased to 0.4ml. The reaction was stopped with 1.25M-$HClO_4$ and P_i was determined in the supernatant by the method of Atkinson (1971). Supernatant (1ml) was added to 2ml of a mixture containing 0.5% (w/v) Lubrol W (I.C.I. Ltd.) and 0.5% (w/v) ammonium molybdate in 0.45M-H_2SO_4. After 10min at room temperature, P_i was determined from the E_{390}. β-Glucuronidase (EC 3.2.1.31) was assayed by the method of Gianetto & de Duve (1955) with phenolphthalein glucuronide as substrate, except that the total reaction mixture was decreased in volume to 0.6ml. β-Acetylglucosaminidase (EC 3.2.1.30) was assayed by the method of Bowers *et al.* (1967), with incubation for 1h and with 4.5mM-*p*-nitrophenyl 2-acetamido-2-deoxy-D-glucoside (Sigma) as substrate. Acid ribonuclease (EC 2.7.7.16) was assayed by a modification of the method of Romeo *et al.* (1966) with yeast RNA (BDH) as substrate, previously dialysed against water to remove nucleotides. Sample enzyme was incubated in a total volume of 0.6ml containing 0.15% (w/v) RNA in 0.1M-sodium acetate buffer, pH5.0, for 1h. After termination of the reaction with 0.1ml of cold 0.75%

Table 2. *Distribution of protein and enzyme activities in the nuclear (N), heavy mitochondrial (M), light mitochondrial (L), microsomal (P) and supernatant (S) fractions of Chinese-hamster fibroblasts isolated by differential centrifugation*

Enzyme activity in each fraction, measured as described in the Experimental section, was calculated as a percentage of the total activity in all the fractions. The percentage total recovery compared with the whole homogenate is also given. Results are given as means±S.D. for six experiments.

	Distribution [% of (N+M+L+P+S)]					Recovery (%)
	N	M	L	P	S	
Protein	10.8±1.9	7.8±0.9	8.7±0.4	9.6±0.5	62.6±3.7	103.4±3.7
Acid phosphatase	5.4±0.7	6.2±0.5	19.3±0.5	21.4±1.8	47.7±1.6	101.3±5.4
Arylsulphatases A and B	5.4±0.7	11.2±1.0	37.7±0.7	17.0±0.5	28.7±1.1	99.2±4.2
β-Acetylglucosaminidase	4.0±0.7	12.8±0.5	31.6±1.1	13.5±0.9	38.3±1.2	98.6±9.1
Acid ribonuclease	7.5±2.0	8.7±0.5	27.9±1.8	14.7±3.5	41.2±3.7	99.6±5.1
β-Glucuronidase	9.2±0.4	17.6±1.8	20.3±2.3	26.9±3.2	26.1±0.9	93.5±1.2
Cathepsin D	3.0±0.3	12.1±1.8	32.0±4.1	9.0±1.1	44.1±5.8	90.9±8.8
Fumarase	5.2±1.1	17.9±2.0	37.3±3.2	9.9±1.6	29.7±3.6	95.3±3.4
Cytochrome oxidase	10.3	22.8	39.4	9.9	18.2	89

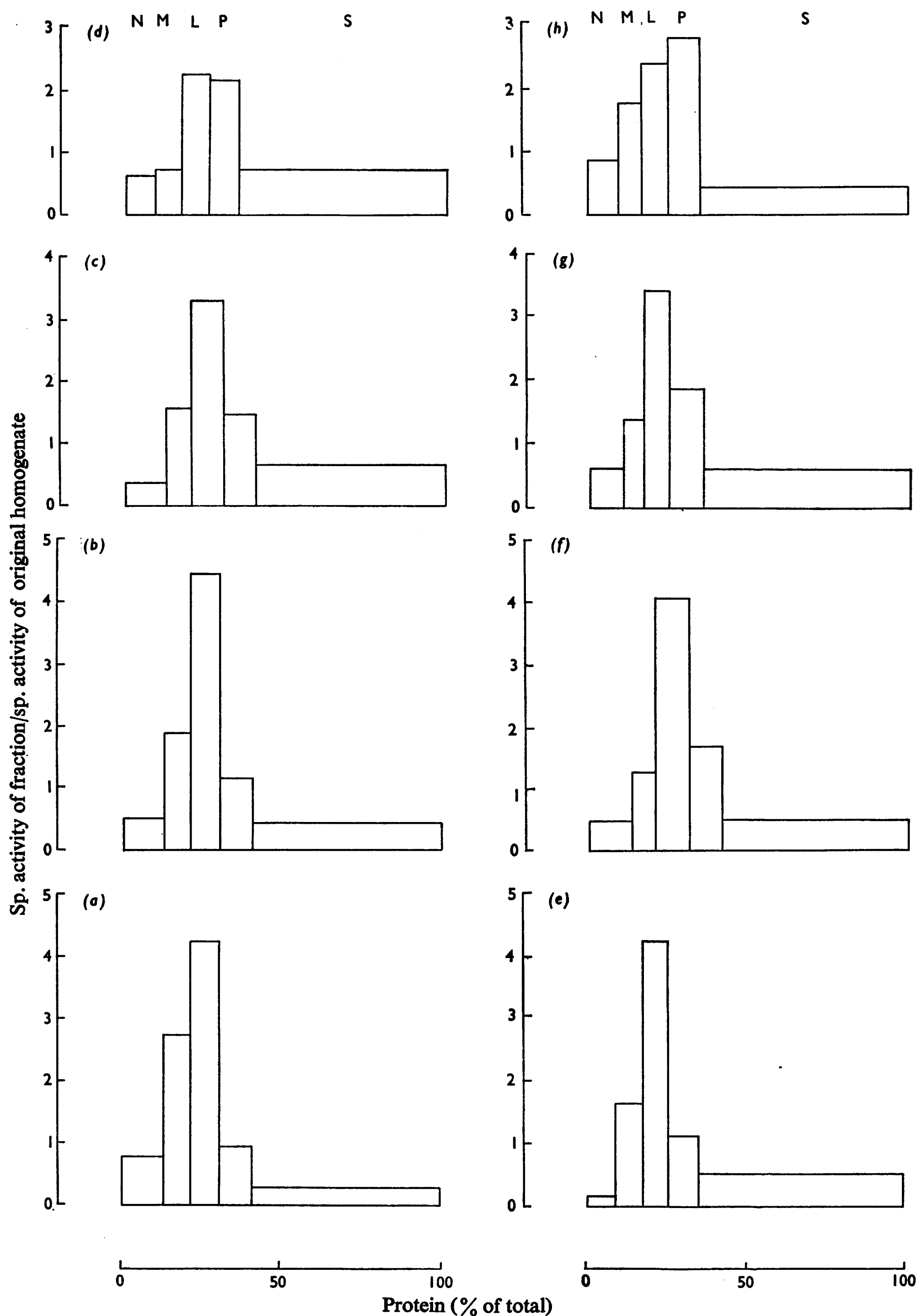

Fig. 1. *Distribution of cytochrome oxidase (a), fumarase (b), β-acetylglucosaminidase (c), acid phosphatase (d), cathepsin (e), arylsulphatases A and B (f) acid ribonuclease (g) and β-glucuronidase (h) in the nuclear (N), heavy mitochondrial (M), light mitochondrial (L), microsomal (P) and supernatant (S) fractions isolated by differential centrifugation from Chinese-hamster fibroblast homogenates*

Details are given in the Experimental section.

(w/v) uranyl acetate in 25% (v/v) $HClO_4$, supernatant breakdown products were estimated from their E_{260}.

All the above assays were performed in the presence of 0.1% (w/v) Triton X-100 to permit the measurement of total activities.

Results

Differential centrifugation

Table 2 shows the distributions obtained for mitochondrial and lysosomal enzymes after fractionation of Chinese-hamster fibroblast homogenates by differential centrifugation. A marked difference is apparent between these results and those obtained by fractionation of rat liver homogenates by the same scheme (de Duve *et al.*, 1955). In the fibroblasts a far higher proportion of enzyme activity is present in the supernatant fraction. The protein present in this fraction is greater than 60% of the total cell protein, compared with less than 40% in the liver, resulting in less protein constituting the other fractions. Whether these increased supernatant values reflect the true composition of the cell or are merely the result of rupture of subcellular structure is questionable.

These results are presented graphically in Fig. 1. Very little separation was produced between mitochondrial marker enzymes and certain of the lysosomal acid hydrolases. A comparison of these results with those obtained for rat liver shows that the chief difference between the two cell types lies in the distribution of cytochrome oxidase and fumarase activities. Whereas in the liver cells these indicate a mitochondrial population present in the M fraction, the same population in the fibroblasts is apparently sedimenting at a much higher value and hence is appearing in the L fraction. This signifies a difference in sedimentation coefficient between the mitochondria of the two cell types, reflecting some possible structural difference between them. Similarly, the distributions obtained for acid hydrolase activities are also shifted towards higher centrifugal fields. The difference between individual acid hydrolase distributions are more pronounced in the fibroblast fractionation than in rat liver fractionation and the greatest variation occurs between the distribution patterns of β-glucuronidase and cathepsin.

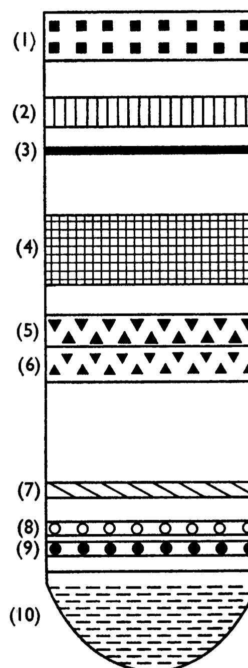

Fig. 2. *Appearance of bands after centrifugation of a Chinese-hamster fibroblast whole homogenate at 63600g for 3h in a Spinco SW 25 rotor*

The bands are, from top to bottom: (1) a whitish layer representing the original location of the homogenate; (2) a faint white cloudy band, possibly representing a microsomal population; (3) a sharp thin band of unknown composition; (4) a pale-yellow cloudy zone, subsequently shown to contain the lysosomal population; (5) a very dense off-white band, in which mitochondria were later shown to be localized; (6) a faint cloudy area, which may simply be the tailing-off of the mitochondria; (7) a very granular white band with an upper boundary so sharp as to form a skin, representing whole cells; (8) and (9) granular bands, separated from each other, one of which may represent nuclei; (10) the gradient cushion of 80% sucrose.

Isopycnic density-gradient centrifugation

Fig. 2 illustrates the various bands formed during the isopycnic density-gradient centrifugation of a Chinese-hamster fibroblast whole homogenate.

Fig. 3 represents the distribution of protein and of lysosomal and mitochondrial enzyme activities. A previous identical centrifugation of intact, unhomogenized cells had shown the entire protein and enzyme activity to be located at a density of $1.26\,g\cdot cm^{-3}$. A comparison with the present distribution patterns obtained therefore suggests that the peak present in fraction 12, at a density of $1.26\,g\cdot cm^{-3}$, represents intact cells. A large peak of fumarase activity, containing 25–35% of the total activity, is present in fraction 8 at a density of $1.17\,g\cdot cm^{-3}$, suggesting that most of the mitochondria are located around this point. Most of the protein appears to be in the supernatant, and some 6% of the total protein constitutes the mitochondrial fraction, giving a specific activity of approx. 5 relative to the specific

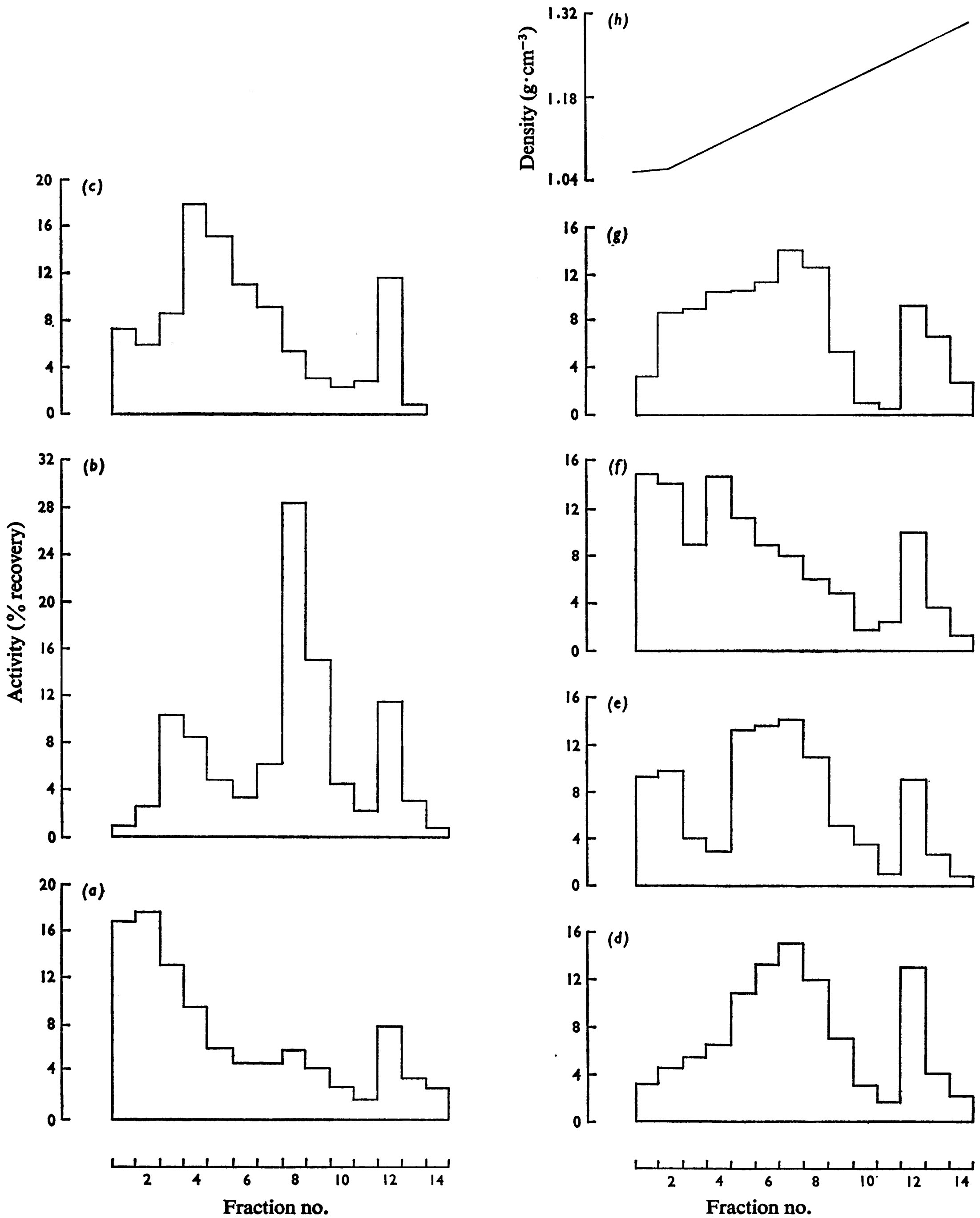

Fig. 3. *Distribution pattern of enzymes and protein after centrifugation of a Chinese-hamster fibroblast whole homogenate in a sucrose density gradient at 63600g for 3h in a Spinco SW25 rotor*

For details see the Experimental section. (*a*) Protein; (*b*) fumarase; (*c*) acid phosphatase; (*d*) aryl sulphatases A and B; (*e*) cathepsin D; (*f*) acid ribonuclease; (*g*) β-acetylglucosaminidase; (*h*) density.

activity of the original homogenate. The acid hydrolase activities measured form peaks at a region of the gradient less dense than that occupied by fumarase, suggesting that the lysosomal population has a median density lower than that of the mitochondria. The acid hydrolases appear to be sequestered into two different populations, each occupying a distinct region of the gradient. Acid phosphatase and acid ribonuclease activities exhibit nearly identical distributions, with a peak at a density of $1.09\,g\cdot cm^{-3}$. An equivalent similarity exists between arylsulphatases A and B and cathepsin, with a peak at a density of $1.15\,g\cdot cm^{-3}$. β-Acetylglucosaminidase activity, although having a peak coincident with those of arylsulphatases A and B and cathepsin, also exhibits a distinct shoulder at the same position as the peak of acid phosphatase and acid ribonuclease activity.

Discussion

The differential-centrifugation scheme of de Duve *et al.* (1955), although capable of separating lysosomal and mitochondrial populations of rat liver homogenates, is apparently ineffective in producing a similar resolution in Chinese-hamster fibroblast homogenates. There is virtually no distinction between the distribution patterns of representative marker enzymes, each possessing its highest specific activity relative to the specific activity of the original homogenate in the L fraction. However, isopycnic density-gradient centrifugation appears to be more successful in producing a segregation between the organelles of the cultured fibroblasts.

The equilibrium density of mitochondria, as represented by fumarase, is $1.17\,g\cdot cm^{-3}$ in a continuous sucrose gradient. Although lower than that of liver mitochondria, which have an equilibrium density of $1.19\,g\cdot cm^{-3}$ (Beaufay & Berthet, 1963), it is similar to values obtained for spleen (Bowers & de Duve, 1967*a*), skeletal muscle (Stagni & de Bernard, 1968) and Ehrlich ascites-tumour cells (Horvat & Touster, 1967).

The apparent position of the lysosomal populations at equilibrium densities lower than that of the mitochondria, i.e. at 1.15 and $1.09\,g\cdot cm^{-3}$, represents a situation at considerable variance with that in rat liver, whose lysosomes have an equilibrium density of $1.22\,g\cdot cm^{-3}$ (Beaufay & Berthet, 1963). However, previous observations made on other cell and tissue types suggest that such a result may be appropriate for this type of cell. A lysosomal equilibrium density of $1.155\,g\cdot cm^{-3}$ was obtained for Ehrlich ascites-tumour cells (Horvat & Touster, 1967) and similar low values have been found in skeletal muscle (Stagni & de Bernard, 1968) and heart muscle (Romeo *et al.*, 1966). Two possible explanations may account for these 'light' lysosomes. The density of the organelle may be a function merely of the material acquired within it, in which case the large amounts of undegraded material, accumulated within the lysosomes of liver and similar phagocytic tissue, would be expected to impart a high equilibrium density. Such a system reaches a maximum in the macrophages, which are very actively phagocytosing cells and have lysosomes of density $1.26\,g\cdot cm^{-3}$ (Cohn & Wiener, 1963). On the contrary, the lysosomes of cells undergoing little phagocytosis, e.g. cultured cells and muscle cells, may be expected to contain very little material. This postulate is supported by the work of Bowers & de Duve (1967*a*,*b*) who, differentiating between lysosomal populations of densities 1.15 and $1.19\,g\cdot cm^{-3}$ from spleen, showed the former to be of lymphocytic cell origin and the latter to be derived from phagocytic cells. Work by Rahman & Cerny (1969) on the liver cells of young and suckling rats revealed, in addition to the normal population of lysosomes, a population of lower density not present in the cells of adult rats. The sensitivity to puromycin of these less-dense acid hydrolases gave rise to the suggestion that these might represent newly synthesized enzymes in primary lysosomes budding off from the Golgi apparatus. Thus the knowledge that Chinese-hamster fibroblast lysosomes must undergo frequent regeneration owing to the high rate of cell division, and are also likely to have very little phagocytic function under normal conditions, owing to the nature of the medium, may explain their low equilibrium density.

Alternatively, the value of lysosomal density may be determined by the composition of the organellar membrane itself, in which case a low density compared with liver may be the result of a higher lipid/protein ratio. This could be expected to confer greater stability on the particles by establishing a membrane more resistant to osmotic and other effects. Preliminary experiments (West, 1971) have shown that Chinese-hamster fibroblast lysosomes, compared with rat liver lysosomes, possess a greater resistance to lysis induced by such conditions as incubation at 37°C in buffer at pH 5.0.

It appears from the results obtained that particles bearing acid hydrolases can be divided into two groups according to a specific parameter, in this case their median density. Those equilibrating at a density of $1.15\,g\cdot cm^{-3}$ apparently contain the majority of arylsulphatases A and B and cathepsin D activity, with a high percentage of β-acetylglucosaminidase activity. Those with an equilibrium density of $1.09\,g\cdot cm^{-3}$ contain the majority of the acid phosphatase and acid ribonuclease activities, with a minor percentage of the β-acetylglucosaminidase activity. There is thus the possibility of the existence of two distinct populations of lysosomes.

The possibility that one of these two populations, namely that of lower density, is of microsomal origin

rather than lysosomal appears unlikely. Attempts have been made to locate a number of predominantly microsomal enzymes within this cell line, including arylsulphatase C, glucose 6-phosphatase, NADPH–cytochrome *c* reductase and NAD nucleosidase, and all have proved undetectable. Microscopic evidence (West, 1971) also suggests that the microsomal content of the fibroblasts is extremely low, although a high concentration of free ribosomes is present. These presumably may be expected to provide for the high synthetic rate required by the rapidly dividing cells. Although the peak of fumarase activity detected at a density of approx. $1.08\,g\cdot cm^{-3}$ could be construed as microsomal in view of its bimodal distribution in rat liver (de Duve *et al.*, 1962), the lack of supernatant fumarase activity suggests that this peak may equally be the result of soluble activity adsorbed on other membrane structures. The evidence therefore tends to refute the possibility of one of the two peaks of acid hydrolase activity having an origin other than lysosomal.

The possibility is that heterogeneity of the lysosomes in a single cell, uncomplicated by cellular heterogeneity, can indeed exist, in this case dividing the populations into two subgroups. It must be emphasized that these variations in enzyme distribution are detectable only as a result of variations in the density of the host particles and it is quite probable that other forms of heterogeneity may exist.

Further elucidation of lysosomal heterogeneity is now required, particularly to determine any functional significance.

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