Characterization of Subcellular Components in Synchronized Hepatoma Cells as a Function of the Cell Cycle

By Joël QUINTART, Jacques BARTHOLEYNS and Pierre BAUDHUIN
Laboratoire de Chimie Physiologique, Université de Louvain and International Institute of Cellular and Molecular Pathology, Brussels B-1200, Belgium

(Received 2 March 1979)

The specific activity and subcellular distribution of marker enzymes for the main subcellular components were analysed in homogenates of synchronized hepatoma cells (Morris 7288c), obtained by selective detachment at mitosis combined with a metaphase block with Colcemid. Markers for lysosomes, mitochondrial outer membrane, plasma membrane and cytosol are synthesized throughout the cycle at the same rate as the bulk of cellular protein. Larger variations are observed for a Golgi marker; after a decrease around mitosis, the specific activity of galactosyltransferase increases steadily from middle G₁-phase on, and at the end of G₂-phase it is nearly twice that observed at the beginning of G₁-phase. Our results show that synthesis of cytochrome oxidase may occur preferentially in G₂-phase. Large modifications of the density distribution of lysosomes are observed during the cell cycle; the median equilibrium density of lysosomal markers decreases in G₁-phase, and some increase in soluble activity occurs at the same time. Reverse changes occur progressively during S- and G₂-phases. At mitosis, Golgi galactosyltransferase shows a more dispersed distribution, and modifications in the density distribution of endoplasmic-reticulum NADPH-cytochrome *c* reductase are observed. The latter can be most easily explained by a detachment of ribosomes from endoplasmic-reticulum membranes. No significant modifications occur in mitochondrial and plasma-membrane markers.

Cellular growth during the cell cycle has been the object of numerous studies [for recent reviews, see Mitchison (1971), Prescott (1976) and Baserga (1976)], but the information on the evolution of populations of cell organelles during the cycle is fragmentary or, in the case of morphological studies (Erlandson & de Harven, 1971), mostly qualitative. Although cell-fractionation data on lysosomes in differentiated or confluent cells in culture have been published [see, for example, Tulkens *et al.* (1974), Warburton & Wynn (1976) and Lopez-Saura *et al.* (1978)], no study has been specifically devoted to establishing if properties of lysosomes are modified along the cell cycle. Such modifications may be important in view of the potential use of lysosomotropic drugs (de Duve *et al.*, 1974; Trouet, 1978) for the control of proliferation of tumoral cells. Furthermore, modifications of the distribution of lysosomes in mitotic cells have been repeatedly described (Robbins & Gonatas, 1964; Erlandson & de Harven, 1971), and even the intervention of these particles in mitosis initiation has been proposed (Allison, 1969).

The present paper describes a study of marker enzymes in synchronized populations of hepatoma tissue-culture (HTC) cells with particular emphasis on lysosomes. For the latter particles, the aim was to

establish whether the modifications of the endocytotic process during the cell cycle (Quintart & Baudhuin, 1976; Quintart *et al.*, 1976) and during mitosis (Berlin *et al.*, 1978) lead to alterations of their physical properties. Preliminary reports on these studies have already appeared (Quintart *et al.*, 1975; Quintart & Baudhuin, 1977).

Materials and Methods

Cell culture and synchronization

Hepatoma tissue-culture (HTC) cells, generously given by Dr. B. L. M. Hogan (Department of Biochemistry, University of Sussex, Falmer, Brighton, U.K.), were derived from Morris hepatoma 7288c. They were cultivated in suspension at 37°C as described by Samuels & Tomkins (1970) in medium 213, supplemented with 10% (v/v) foetal-calf serum and have, during exponential growth, an average generation time of 24 h. The number and size of cells were determined with a Celloscope counter model 302 (Linson Instruments, A.B. Lars Ljungberg and Co., Stockholm, Sweden). Mitotic indices and thymidine incorporation were measured as previously described (Bartholeyns *et al.*, 1979).

For synchronizing the cells, a method derived from Martin *et al.* (1969) and Sellers & Granner (1974)

was used. It rests on selective detachment at mitosis, combined with a metaphase block with 100 nM-Colcemid [(S)-6,7-dihydro-1,2,3,10-tetramethoxy-7-(methylamino)benzo[a]heptalen-9(5H)-one]. The cells suspended in 300 ml of culture medium (1.5×10^5 cells/ml) were allowed to adhere on a 500 cm² surface of glass (5-litre flasks; Arthur Thomas, Philadelphia, PA, U.S.A.). In this first step, use of CaCl₂ to increase cell adhesiveness, as described in the original method, was avoided, since it was found to induce some cell aggregation, decreasing the yield of synchronized cells. After 12–24 h, the medium was replaced by a fresh medium containing 100 nM-Colcemid and 2.4 mM-CaCl₂. Mitotic cells were harvested after 5 h by gentle shaking and decantation. They were washed twice with culture medium without Colcemid and further cultured in suspension. Zero time corresponds to the resuspension of the detached cells in fresh medium.

Homogenization and fractionation procedures

After two washings at 0°C with 0.25 M-sucrose/3 mM-imidazole, pH 7.4, cells (2×10^8 – 5×10^8 for asynchronous cultures, 5×10^7 for synchronized cells) were homogenized by 32 strokes of the tight pestle of a Dounce-type homogenizer (Kontes Glass Co., Vineland, NJ, U.S.A.); this allowed sufficient cell breakage and limited damage to cell organelles. Fractionation by differential centrifugation was performed as described by de Duve *et al.* (1955) and adapted by Lopez-Saura *et al.* (1978) in 0.25 M-sucrose in 3 mM-imidazole, pH 7.4. For density-equilibration studies, the automatic E40 titanium-rotor assembly of Beaufay & Amar-Costesec (1976) was used under the same conditions as described previously (Bartholeyns *et al.*, 1979). For flotation experiments, a dense sucrose solution was added to the 0.25 M-sucrose homogenate, to obtain a final density of 1.27 g/cm³. The linear sucrose gradient extended in this case from 1.06 to 1.24 g/cm³.

Biochemical assays

Assay conditions for marker enzymes were the same as those used by Bartholeyns *et al.* (1979). Additional enzyme determinations were performed as described in the following references: adenylate kinase (EC 2.7.4.3), Peeters-Joris *et al.* (1975); cathepsin B (EC 3.4.22.1), Barrett (1976); monoamine oxidase (EC 1.4.3.4), Wurtman & Axelrod (1963); acid α -glucosidase (EC 3.2.1.20), acid α -mannosidase (EC 3.2.1.24) and acid α -L-fucosidase (EC 3.2.1.51), Libert *et al.* (1976). Proteins, DNA and RNA were determined as described by, respectively, Lowry *et al.* (1951), Burton (1956) and Fleck & Munro (1962).

For the determination of NMN adenyllyltransferase (EC 2.7.7.1), samples were incubated for 30 min at 25°C in a medium containing 4 mM-NMN, 4 mM-

ATP, 0.2 M-nicotinamide and 10 mM-MgCl₂ in 50 mM-glycylglycine buffer, pH 7.4, with 1 mg of bovine serum albumin/ml. The reaction was stopped with 0.5 ml of 0.1 M-HCl at 0°C. A portion (0.2 ml) of the reaction medium was then added to 2.6 ml of a mixture containing 38.5 μ M-cytochrome *c* and 0.66 M-ethanol in 50 μ M-glycylglycine buffer, pH 8.7. After addition of 60 units of yeast alcohol dehydrogenase and 3 μ mol of KCN, the cyclic reaction of Glock & McLean (1955) was started with 0.1 ml of stock rat liver microsomal fraction diluted 1/12.5 (w/v), used as source of NADPH-cytochrome *c* reductase. The liver microsomal fraction was prepared in 1.8 mM-CaCl₂/0.25 M-sucrose, washed once with 1 M-NaCl, thereafter with 0.25 M-sucrose, and stored at –25°C.

Materials

All chemicals for assay were of analytical grade. They were purchased from E. Merck A.G. (Darmstadt, Germany), Sigma Chemical Co. (St. Louis, MO, U.S.A.), Calbiochem (San Diego, CA, U.S.A.) and Koch-Light Laboratories Ltd. (Colnbrook, Bucks., U.K.). Glucose 6-phosphate dehydrogenase was a product from Sigma, and hexokinase was from Boehringer (Mannheim, Germany). Radioactive reagents were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.) and from NEN (Dreieichenhain, Germany). Cell-culture components and Colcemid were products from Gibco (Paisley, Scotland, U.K.).

Results

Fractionation of asynchronous cultures

Since partial biochemical characterization of asynchronous HTC cells by fractionation has already been reported from this laboratory (Lopez-Saura *et al.*, 1978), description of fractionation data will be restricted to the additional markers included in the present work. Enzyme-distribution patterns after fractionation by differential centrifugation of homogenates from asynchronously growing cells are summarized in Fig. 1. The nuclear (N) fraction contained the bulk of the NMN adenyllyltransferase. Monoamine oxidase, essentially found in M- and L-fractions, sedimented more in the P-fraction when compared with cytochrome oxidase. All the acid hydrolases studied showed a similar fractionation pattern, with a maximum in the L-fraction. For galactosyltransferase, mostly concentrated in the P-fraction, the amount found in the L-fraction was smaller than for alkaline phosphodiesterase I. More than 95% of the phosphoglucomutase activity appeared in the final supernatant. Finally, the bulk of adenylate kinase was found in the soluble fraction, but a minor part of the enzyme was sedimentable, with a small peak in the M- and L-fractions.

For determination of equilibrium density, whole homogenates were used rather than post-nuclear

Fig. 1. Fractionation of asynchronous cell populations by differential centrifugation

On the ordinate the specific activity of fractions is expressed relative to that of the homogenate (=1); the abscissa represents the relative protein content of the fractions, cumulatively from left to right, in the order in which fractions were isolated. Abbreviations used: N, nuclear fraction; M, heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, supernatant. Recoveries (sum of the activity of all fractions with respect to the activity of the homogenate), ranged between 82.0 and 108.0% for all markers. The numbers of experiments are given in parentheses.

supernatants (Lopez-Saura *et al.*, 1978). Preliminary isolation of a nuclear fraction would complicate seriously the interpretation of data for mitotic cells. Moreover, this avoids selection in the populations of subcellular organelles; the heaviest organelles would be discarded by the isolation of a nuclear fraction. Results of density-equilibration experiments are summarized in Fig. 2. Distributions of acid hydrolases were very similar and lysosomes appeared thus rather homogeneous in their enzymic content. Their distribution patterns were different from mitochondrial markers. Catalase was partly soluble, and the

sedimentable enzyme was less disperse than lysosomal hydrolases. Galactosyltransferase presented a slightly lower median equilibrium density than did alkaline phosphodiesterase I, although both distributions were considerably overlapping. Distribution of monoamine oxidase was similar to that of cytochrome oxidase, but slightly broader. Compared with DNA, NMN adenylyltransferase was somewhat displaced toward lighter densities. Phosphoglucosmutase and the small peak in the catalase distribution showed the displacement observed under our centrifugation conditions for two soluble proteins with mol.wts. of

Fig. 2. Density equilibration of homogenates prepared from asynchronous suspension-cultured (thick lines) or layer-cultured (thin lines) cells

The abscissa is a density scale that is divided in 15 fractions of equal density increments from 1.07 to 1.27 g/cm³. Outside this range, fractions were combined. The ordinate represents the frequency (activity/unit density increment); the area of each histogram is equal to unity after normalization. Standardization of the histograms was performed as described by Bowers & de Duve (1967). Recoveries from the homogenate ranged between 87.2 and 111.2%.

70000 and 200000 respectively. Since the synchronization technique relies on cell attachment to glass, fractionation was also performed on homogenates from layer-cultured cells. Except for lysosomes from layer-cultured cells, which equilibrated at lower density than those of cells in suspension, other markers were not appreciably altered by culture conditions.

Characterization of synchronized cells and evolution of specific activity of marker enzymes during the cell cycle

The initial mitotic index ($87.7 \pm 4.2\%$, mean $\pm$ S.D. of seven experiments) decreased rapidly after removal of Colcemid and then remained to less than 1% until 16 h after the beginning of the experiment. At this time it increased again progressively with a maximum

value of 11%. This low value showed that desynchronization was occurring in HTC cells as usually observed in mammalian cells. However, it should be pointed out that easily recognizable mitotic figures last only for about $\frac{1}{2}$ h and that our samplings were separated by 4 h periods. When 100 nM-Colcemid was again introduced in the medium 18 $\frac{1}{2}$ h after mitosis to accumulate cells in metaphase, a mitotic index of 64% can be observed, showing that the bulk of the cell population was still committed to division.

Evolution of the specific activity of acid hydrolases was characterized by variations smaller than 10% over the cycle (Fig. 3); this was also the case for phosphoglucumutase, NMN adenylyltransferase and NADPH-cytochrome *c* reductase (results not shown). In contrast, the largest variation was observed for

Fig. 3. Specific activity of marker enzymes in synchronized populations
Numbers in parentheses refer to the numbers of

galactosyltransferase, which roughly doubled its specific activity at the end of the cycle. Cytochrome *c* oxidase also showed a significant variation, its specific activity being minimal during the S-phase. Monoamine oxidase, although behaving in a similar manner to cytochrome oxidase in fractionation experiments, had nearly constant specific activity. Catalase showed a significant decrease in specific activity after mitosis. Measurements of cell volume and protein mass (results not shown) indicated that the concentration of protein in the cells was practically constant during the cycle. As a consequence, variations of specific activity during the cycle provide a good approximation of changes in the cellular concentration of enzymes.

Fractionation of mitotic cells

Compared with asynchronous cultures, density equilibration of a mitotic-cell homogenate (Fig. 4)

experiments; vertical bars represent S.D. values. The additional curve on the upper-left graph corresponds to the average thymidine incorporation (seven experiments). For each enzyme, specific activity is expressed with respect to its average over a cycle.

Fig. 4. Density equilibration of mitotic-cell homogenate
Results are presented as in Fig. 2. Recoveries from the homogenate were between 74.6 and 108.2%.

shows the following differences. A lower median density and larger dispersion characterized both DNA and NMN adenylyltransferase. Chromosome breakage or agglutination of cytoplasmic material to chromosomes is the most likely explanation for the broad distribution of DNA. Dissociation was observed between NMN adenylyltransferase and DNA in the low-density region. A significant part of RNA was only slightly displaced by the centrifugation and remained in the region of low density; the heavy component of the distribution observed in asynchronous cultures was absent in mitotic cells. Particulate NADPH-cytochrome *c* reductase equilibrated at a lower density and was more dissociated from RNA in mitotic cells. Galactosyltransferase presented a larger dispersion, and the median density was slightly increased; moreover, the distribution of this marker at mitosis was not very different from that of NADPH-cytochrome *c* reductase. The distribution of *N*-acetyl- β -glucosaminidase was intermediary between those observed for cells cultured in layer and in suspension. It is noteworthy that the amount of soluble catalase was decreased at mitosis. Finally, cytochrome *c* oxidase and alkaline phosphodiesterase I distributions were not significantly modified.

Fractionation at different stages of the cell cycle

Only small changes in the density distribution of cytochrome oxidase and alkaline phosphodiesterase I were observed during the cell cycle. For galactosyl-

transferase, the modifications described above for mitotic cells were no longer observed 10 and 14 h after mitosis; the median equilibrium density decreased slightly and the distribution became more homogeneous (Fig. 5). Distribution of NADPH-cytochrome *c* reductase was shifted progressively towards the high-density region at 10 and 18 h after mitosis (Fig. 5); soluble activity was slightly less at the end of the cycle. The light component of RNA distribution disappeared progressively after mitosis, whereas more RNA was found in the region of high density. Evolution of DNA distribution may be described as a progressive shift towards higher equilibration densities.

Larger variations were observed for the density distribution of lysosomal markers (Fig. 6). In early G₁-phase, the median equilibrium density of lysosomal markers decreased appreciably after 5 h and then increased progressively. The amplitude of the variation contrasted with the observations for markers of the other structures. Independent measurements of soluble *N*-acetyl- β -glucosaminidase after centrifugation of homogenates at 40000 rev./min for 30 min ($W = 3 \times 10^{10}$ rad²/s) showed an increase from 5% at mitosis to 19% 5 h after mitosis. Since it is difficult to differentiate between soluble and particle-bound activity in the experiments shown in Fig. 6, density equilibration by flotation of particles was also attempted. In such experiments (results not shown), a lighter component in the distribution of acid hydrolases, completely dissociated from soluble

Fig. 5. Density equilibration of synchronized-cell homogenates: distribution of galactosyltransferase, NADPH-cytochrome *c* reductase, RNA and DNA

Results are presented as in Fig. 2. Thin lines give the distribution found in mitotic cells (Fig. 4) for comparison. Times refer to the homogenization of cells after mitosis. For NADPH-cytochrome *c* reductase, a broken line has been drawn at a density of 1.15 g/cm³ for identification of the dense and light components. Recoveries were $113.6 \pm 19.1\%$, 102.4 ± 10.1 , 112.3 and $75.4 \pm 16.2\%$, respectively, for galactosyltransferase, NADPH-cytochrome *c* reductase, RNA and DNA.

Fig. 6. Density equilibration of synchronized cell homogenates: distribution of lysosomal marker enzymes. Results are presented as in Fig. 5. Recoveries from homogenates were respectively $97.5 \pm 17.2\%$ for cathepsin B, $102.6 \pm 11.2\%$ for acid phosphatase and $100.4 \pm 6.7\%$ for *N*-acetyl- β -glucosaminidase.

phosphoglucumutase, could be observed during the G_1 -phase.

Discussion

Asynchronous cultures

Density distributions are in good agreement with those of Lopez-Saura *et al.* (1978), if it is taken into account, as already mentioned, that whole homogenates rather than post-nuclear supernatants were used in the present study. For monoamine oxidase, fractionation by differential centrifugation shows a distribution similar to that reported for rat liver (Amar-Costesec *et al.*, 1974; Beaufay *et al.*, 1974). Adenylate kinase, which is associated mainly with the mitochondrial intermembrane space in rat liver (Peeters-Joris *et al.*, 1975), is largely (approx. 75%) found in the final supernatant of HTC-cell homogenates; this reflects either the presence of a soluble isoenzyme more abundant than in rat liver (Criss, 1970) or the leaking of adenylate kinase resulting from partial disruption of the mitochondrial outer membrane, which co-sedimented, nevertheless, with the mitochondrial inner membrane. As in rat liver

(Hogeboom & Schneider, 1952), NMN adenylate transferase is associated mainly with nuclei.

Synchronized cell populations

After a high degree of synchronization, desynchronization occurs during the cell cycle. It should thus be kept in mind that this implies a poor resolution at the end of the cycle, and especially for G_2 -phase. Such results are in agreement with those obtained by Martin *et al.* (1969) on synchronized HTC cells.

Lysosomes. As reported for synchronized HeLa cells (Churchill & Studzinski, 1970; Berg *et al.*, 1975), lysosomal hydrolases in HTC cells are synthesized concomitantly with the bulk of cellular protein. In other cell types, however, variations of the specific activity of lysosomal enzymes through the cell cycle have been described (Bosmann & Bernacki, 1970; Chatterjee *et al.*, 1975; Thilly *et al.*, 1975). The shift observed in the distribution of acid hydrolases during the cell cycle must be correlated with some changes of the physical characteristics of lysosomes. An increased fragility is presumably responsible for the increase in soluble activity after homogenization; this is probably a consequence of an increase in size

(Baudhuin *et al.*, 1979). An increase in the proportion of a lysosomal component of low density (lipids or hydration water) could explain the shift observed in modal equilibrium density.

Ribosomes and endoplasmic reticulum. RNA distribution results both from the equilibration of ribosomes attached to membrane vesicles of the endoplasmic reticulum and from the sedimentation of free ribosomes, which in our fractionation system do not reach their equilibrium density. At mitosis, it is clear that RNA is more concentrated in the middle part of the gradient than at the other stages of the cell cycle. Since polyribosomes should migrate farther than individual ribosomes, our observations are entirely consistent with a decrease in the number of polyribosomes (Erlandson & de Harven, 1971).

The shift of NADPH-cytochrome *c* reductase to lower density at mitosis can be explained by detachment of ribosomes from endoplasmic-reticulum membranes rather than by changes in the membranes associated to this marker. In rat liver, it has been shown that ribosomal load affects the equilibration density of the endoplasmic-reticulum membranes (Wibo *et al.*, 1971). In our opinion, the decrease in equilibrium density of NADPH-cytochrome *c* reductase indicates a diminution of the abundance of rough endoplasmic reticulum at mitosis. If it is remembered that the decrease in ribosome binding to mRNA at mitosis was previously reported by Salb & Marcus (1965) and that, on the other hand, prior binding of ribosomes to mRNA is needed for their association to the endoplasmic reticulum, according to Blobel & Dobberstein (1975), binding of ribosomes to membranes should hence be decreased at mitosis. Our fractionation data are in agreement with this conclusion.

Golgi complex. Large functional variations of the Golgi complex during the cell cycle have already been suggested by morphological observations (Erlandson & de Harven, 1971); nevertheless, to the best of our knowledge, variation in the specific activity of a Golgi marker enzyme has not been reported before. Moreover, its distribution pattern is modified during the cell cycle. It is noteworthy that density-equilibration patterns of markers for endoplasmic reticulum and Golgi complex are the most similar at mitosis.

Other subcellular components. As already observed in other cell types (Warmley *et al.*, 1970; Bosmann, 1971), the evolution of the specific activity of enzymes associated to different mitochondrial compartments presented differences during the cycle; in HTC cells this applies to monoamine oxidase and cytochrome oxidase. This suggests a control of their synthesis by different genomes (Schatz & Mason, 1974) with non-concomitant expression (Bosmann, 1971; Prescott, 1976). Finally, a small decrease in the median equilibrium density of alkaline phosphodiesterase I occurs concomitant with the decrease in density of

lysosomes observed during the cell cycle. This enzyme can be considered as a plasma-membrane marker according to Sauvage *et al.* (1975), who have observed a relative specific activity of 22 in highly purified plasma-membrane preparation from HTC cells.

Conclusions

Our present study shows several biochemical modifications of subcellular structures as a function of the cell cycle. First, changes in distribution patterns of RNA and NADPH-cytochrome *c* reductase after density equilibration suggest a detachment of ribosomes from the endoplasmic-reticulum membranes during mitosis. Secondly, modifications of distribution and specific activity of galactosyltransferase represent the biochemical counterpart of the variations reported for the Golgi complex (Erlandson & de Harven, 1971). Finally, it is also tempting to explain the variation of the lysosomal equilibrium density by the modifications of endocytosis observed during the cell cycle and documented elsewhere (Quintart & Baudhuin, 1976; Berlin *et al.*, 1978). The decrease in equilibrium density should in this case be related to a greater abundance of phagolysosomes, resulting of the fusion of lysosomes with a larger number of endocytotic vesicles arising during the second half of the G₁-phase; morphometrical studies have shown modifications of both average volume and fractional volume of lysosomes (Baudhuin *et al.*, 1979).

We thank Professor C. de Duve for many critical comments and his continued interest in this work. We are also indebted to Professor A. Trouet and Professor F. Van Hoof for stimulating discussions of this study. The excellent technical assistance of Mrs. J. Dubois-Vandendriesche, Miss N. Delfasse, Mrs. M.-J. Ledrut-Damanet and Mr. B. Thiry is gratefully acknowledged. This work was supported by grants from the Belgian C.G.E.R. (Caisse Générale d'Epargne et de Retraite) and F.R.F.C. (Fonds de la Recherche Fondamentale Collective). J. Q. was a Fellow of I.R.S.I.A. (Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture).

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