The Use of Conventional and Zonal Centrifugation to Study the Life Cycle of Mammalian Cells

PHOSPHOLIPID AND MACROMOLECULAR SYNTHESIS IN NEOPLASTIC MAST CELLS

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1. Conventional gradient centrifugation has been used to separate cells according to their position in the cell cycle, and to obtain synchronously growing cells. Analysis of prelabelled cells by gradient centrifugation confirms that phospholipid, protein and RNA synthesis is continuous throughout the cell cycle and shows that the rate of synthesis begins to increase already during the G₁ phase. The pattern of phospholipid degradation follows that of synthesis. 2. The limitations of conventional gradient centrifugation have been overcome by use of a zonal rotor. Analysis of prelabelled cells confirms the results obtained by conventional centrifugation and in addition shows that the rates of phospholipid, protein and RNA synthesis decrease during the G₂ phase. The mean cell volume and the net amount of phospholipid, protein and RNA, unlike that of DNA, are found to increase continuously throughout the intermitotic period. 3. These results show that the synthesis of macromolecules, and probably that of membranes also, is controlled by a mechanism other than that of gene dosage.

We have recently studied the synthesis of phospholipids in neoplastic mast cells synchronized by treatment with excess of thymidine (Bergeron, Warmley & Pasternak, 1969, 1970). Chemical synchronization methods are open to criticism (e.g. Petersen, Tobey & Anderson, 1969) inapplicable to physical methods, and we have accordingly sought for such a method in order to confirm our previous results. One of the most effective physical methods is mitotic selection (Terasima & Tolmach, 1963; Petersen *et al.* 1969), but this is not suitable for cells, such as P815Y or HC neoplastic mast cells, which do not attach to glass. An alternative is to separate cells according to their age by gradient centrifugation (Sinclair & Bishop, 1965) and we have now been able to use this technique (Bergeron *et al.* 1969), not only to analyse biochemical events (Morris, Cramer & Reno, 1967), but also to obtain synchronously growing cells (Warmley, Bergeron & Pasternak, 1969). Confirmation of this aspect of our work (Ayad, Fox & Winstanley, 1969; Fox & Pardee, 1970; Schindler, Ramseier, Schaer & Grieder, 1970) encouraged us to try to overcome two limitations of such 'conventional' centrifugation, namely the rather poor separation of cells towards the bottom of the gradient and the relatively low yield of cells. Centrifugation in a zonal rotor has proved success-

ful for this and has enabled us to confirm and extend our previous results (Bergeron *et al.* 1970) on the timing of phospholipid and macromolecular synthesis in cultured cells.

EXPERIMENTAL

Chemicals. [1-¹⁴C]Glucosamine hydrochloride (CFA 422, sp. radioactivity 56mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were as described by Bergeron *et al.* (1970) and Pasternak & Bergeron (1970).

Cell culture. P815Y and HC neoplastic mast cells, kindly given by Dr G. A. Fischer and Dr Margaret Fox, were cultured and their number was estimated as described by Pasternak & Bergeron (1970). A Coulter model A counter was calibrated with puffball spores (3.5 μ m diam.), polystyrene divinylbenzene latex beads (12 μ m diam.), paper-mulberry pollen (13.5 μ m diam.), and silver-birch pollen (22.9 μ m diam.) (Coulter Electronics Ltd., Dunstable, Beds., U.K.) and the mean cell volume was calculated from the size-distribution curve by the group-deviation method described by Stanley (1963); the mean cell volume during exponential growth was approx. 1200 μ m³. The medium referred to is Fischer's medium (Pasternak & Bergeron, 1970); growth medium had serum added. When larger quantities of cells were required, a single batch of up to 2 litres was grown in spinner culture (Paul, 1965).

Precursor incorporation. (a) For the analysis of prelabelled cells, exponentially growing cells were collected

by centrifugation and resuspended (1×10^7 – 3×10^7 cells/ml) in 10–30 ml of warm growth medium, radioactive compound was added (1 – $5 \mu\text{Ci}$ of ^3H -labelled compound/ml; 0.5 – $1 \mu\text{Ci}$ of ^{14}C -labelled compound/ml), followed by incubation at 37°C for 30 min. (b) To measure incorporation during synchronous growth, samples (2×10^5 – 5×10^5 cells/ml) were similarly exposed to radioactive precursor ($1 \mu\text{Ci}$ of ^3H - or ^{14}C -labelled compound/ml). Extent of incorporation into phospholipids and macromolecules was determined by filtering and washing with trichloroacetic acid as described by Pasternak & Bergeron (1970).

Radioautography. [^3H]Thymidine-labelled cells were washed with medium and resuspended in 0.3 ml of phosphate-buffered saline (Krebs & Eggleston, 1940). Drops of cell suspension were added to microscope slides and, after standing for 5–10 min, the cells were fixed by immersion in Solvent Methanol (microscopical reagent; BDH Chemicals Ltd., Poole, Dorset, U.K.) for 5–10 min and dried in air. The slides were dipped in Kodak Nuclear Track Emulsion NTB2 at 43°C , dried in a stream of warm air for 2 h and stored at 4°C . After exposure periods of up to 21 days the slides were developed in Kodak D-19b developer for 4 min at 16°C , followed by staining with Harris's haematoxylin (BDH Chemicals Ltd.). A minimum of 500 cells and 20 fields per slide were examined, cells with less than five grains being scored as background. Results are expressed as the percentage of cells that are labelled.

Conventional gradient centrifugation. Centrifugation was carried out at 4°C in an MSE Mistral 6 L centrifuge with a swing-out rotor (no. 62301). A linear gradient (40 ml) of 5–10% (w/v) Ficoll (Sigma Chemical Co., St Louis, Mo., U.S.A.) dissolved in medium was generated with an apparatus (Salo & Kouns, 1965) lent by Dr M. R. Lunt. (a) For the analysis of prelabelled cells, the suspension (see above) (5×10^7 – 10×10^7 cells) was washed twice with cold medium containing the corresponding unlabelled compounds, resuspended in 1 ml of medium, layered on to the gradient and stirred slightly to minimize streamer formation (Brakke, 1955). Centrifugation was for 5–7 min (depending on cell load) at $80g_{av.}$. Fractions (1 ml) collected by puncturing the tube at the bottom were analysed for cell number, mean cell volume

and radioactivity incorporated. (b) To obtain synchronously growing cells, the operation was carried out aseptically at room temperature. Cell load was increased to about 2×10^8 cells and centrifugation time was decreased to 3–5 min. After collection of fractions, those from the chosen regions were diluted fivefold with warm medium, the cells collected by centrifugation, resuspended (approx. 1×10^5 cells/ml) in warm growth medium and incubated at 37°C ; samples were removed every 2 h and analysed for cell number, radioactivity incorporated after a 30 min pulse and mitotic index (Tobey, Petersen, Anderson & Puck, 1966).

Zonal centrifugation. All operations were at 4°C in an MSE type A zonal rotor, run in an MSE Mistral 6 L centrifuge, kindly made available by the Department of Forestry, University of Oxford. A gradient (1 litre) of 2–10% (w/v) Ficoll dissolved in 50 mM-tris-HCl buffer, pH 7.4, containing NaCl (0.9%), linear with respect to volume, was generated with an MSE fixed-profile gradient former. It was pumped in at 50 ml/min while the rotor was spinning at 300 rev./min and was followed by an underlay of 15% (w/v) sucrose. Exponentially growing cells (5×10^8 – 10×10^8 cells) labelled with radioactive precursor and washed as described above were suspended in 25 ml of chilled 1% (w/v) Ficoll and introduced into the centre of the rotor followed by an overlay of 80 ml of the tris-buffered saline (so that the centre of the sample band reached a distance of about 2–3 cm from the rotor centre). Centrifugation was carried out for 12–15 min, depending on cell load, at 500 rev./min. The rotor speed was then decreased to 300 rev./min and 25 ml fractions were collected by pumping more underlay at 70 ml/min to the outer edge of the rotor. Fractions were analysed for cell number, mean cell volume, extent of radioactivity incorporated and for phospholipid, DNA, RNA and protein content.

Chemical determinations. To extract phospholipids, cell pellets were treated with chloroform-methanol (2:1, v/v) (Folch, Lees & Sloane-Stanley, 1957) and phospholipid P was determined in the lower layer by the method of Bartlett (1959). For measurement of DNA and protein, cell suspensions were treated with an equal volume of 10% (w/v) trichloroacetic acid and the resulting pre-

Fig. 1. Analysis of prelabelled cells by conventional gradient centrifugation. Cells (1×10^8 in 10 ml) exposed to $10 \mu\text{Ci}$ of [^3H]thymidine were separated and analysed as described in the Experimental section. ●, Cell number; ■, mean cell volume; ○, ^3H -labelled DNA; □, % of cells labelled with [^3H]thymidine, determined radioautographically.

precipitate was washed with 5% (w/v) trichloroacetic acid. DNA was measured by treating the precipitate with 2 ml of 5% (w/v) trichloroacetic acid at 100°C for 15 min to extract nucleic acids (Schneider, 1945), and deoxyribose in the resulting supernatant was assayed either by the method of Ceriotti (1952) or by that of Burton (1956). Protein was determined with the Folin reagent (Lowry, Rosebrough, Farr & Randall, 1951) in a solution obtained by boiling the washed precipitate with 2 ml of 2M-NaOH for 60 min. For RNA determination, cell suspensions were incubated with an equal volume of 0.6M-NaOH at 37°C for 2 h (Schmidt & Thannhauser, 1945), after which an equal volume of 1M-HClO₄ was added; ribose was

determined in the supernatant by the orcinol reaction (Hurlbert, Schmitz, Brumm & Potter, 1954).

RESULTS

Conventional gradient centrifugation and its use to obtain synchronously growing cells. The use of conventional gradient centrifugation to study cells at different stages of their life cycle assumes that the mean cell volume increases as cells progress from the G₁ phase to the G₂ phase. Fig. 1 shows a steady increase in mean cell volume and therefore

Fig. 2. Growth and [³H]thymidine incorporation by synchronous cells. Cells (1.5×10^8) were separated by conventional gradient centrifugation and fractions from the upper region (18.7–19.0 cm from rotor centre) and lower region (22.0–22.3 cm from rotor centre) were treated and incubated at 37°C as described in the Experimental section. Samples (1 ml) were removed at intervals, exposed to $2 \mu\text{Ci}$ of [³H]thymidine for 30 min and radioactivity incorporated and cell number were determined as described in the Experimental section. (a) Upper region; (b) lower region. ●, Cell number; ○, ³H-labelled DNA.

makes it unlikely that cell mass changes independently of volume (Petersen *et al.* 1969). Two criteria have been used to demonstrate that cells at the top of the gradient are indeed in the G_1 phase, those in the middle in the S phase and those at the bottom in the G_2 phase.

First, the capacity to synthesize DNA, judged by the incorporation of thymidine, is low at the top and increases towards the bottom, reaching a maximum just below the cell peak (Fig. 1). This is the result obtained by Morris *et al.* (1967). HC cells behave in a similar manner. The resolution at the G_2 -phase

region is poor (Bergeron *et al.* 1969); it deteriorates progressively from top to bottom of the gradient as a result of wall effects and other factors (Anderson, 1955). These are minimized in a zonal rotor (Anderson, 1966) and a satisfactory resolution, despite the low percentage of G_2 -phase cells in an exponential population (Bergeron *et al.* 1970), is achieved (Fig. 4).

Secondly, cells separated by centrifugation grow synchronously. Thus cells from the upper region of the gradient have the growth characteristics expected of G_1 -phase cells (e.g. ~10 h lag before cell

Fig. 3. Phospholipid turnover in prelabelled cells separated by conventional gradient centrifugation. Cells (6×10^7 in 10 ml) were exposed to $100 \mu\text{Ci}$ of [$M\epsilon$ - ^3H]choline and $10 \mu\text{Ci}$ of [^{14}C]thymidine and separated as described in the Experimental section. Fractions from all parts of the gradient were diluted fivefold with warm medium, centrifuged, resuspended (approx. 1×10^5 cells/ml) in fresh growth medium containing 20 mM-choline, and incubated at 37°C . Samples were removed for radioactive assay as described. Fractions: (a) 17.7–18.2 cm from rotor centre; (b) 18.5–18.8 cm; (c) 19.2 cm; (d) 19.6 cm; (e) 20.1 cm; (f) 20.6 cm; (g) 21.1 cm; (h) 21.6 cm; (i) 22.1 cm; (j) 22.5 cm. ■, ^3H -labelled phospholipid; ○, ^{14}C -labelled DNA. The percentage of stable ^3H -labelled phospholipid in each fraction is indicated.

division), whereas those from the lower region behave in a manner expected of G_2 -phase cells (a 2 h lag before cell division) (Fig. 2). In each case the generation time (15 h) is the same as that of cells taken after the whole gradient has been mixed; mixed cells show an exponential growth curve typical of unsynchronized cells. Measurement of mitotic index confirms the synchrony, being maximal during the period of cell division, when thymidine incorporation (Fig. 2) is at a minimum. These results, which have been extended to P388 cells (Ayad *et al.* 1969) and independently confirmed (Schindler *et al.* 1970), show that conventional gradient centrifugation does not subject such cells to adverse physiological effects (cf. Petersen *et al.* 1969).

Incorporation of choline by synchronized cells follows a pattern similar to that of thymidine in the same way as in cells synchronized chemically (Bergeron *et al.* 1969, 1970). Although this result substantiates the thymidine method for synchronizing cells, the rather sharp decrease in rate of incorporation during the G_2 phase (Bergeron *et al.* 1969, 1970) is surprising in view of the results obtained with prelabelled cells (Bergeron *et al.* 1969; Fig. 4). Perhaps the dramatic decrease during the G_2 phase in growing cells is due to contamination with mitotic cells, in which all biosynthetic activity is at a minimum (Robbins & Scharff, 1966). Certainly the phases of the cell cycle are more clearly defined in cells isolated directly from a gradient.

Analysis of prelabelled cells by conventional

gradient centrifugation. The rates of synthesis of phospholipid, RNA and protein show a more gradual change during the cell cycle than does that of DNA (Bergeron *et al.* 1969; Warmley *et al.* 1969). Thus the rates of proline, uridine and glucosamine incorporation increase two- to three-fold between the G₁ phase and the S-G₂ phase, those of choline and inositol incorporation increase three- to four-fold, and thymidine incorporation increases more than sixfold. Essentially similar results for proline and choline have been obtained with HC cells. The distribution of label in the gradient after a 15 min pulse of uridine or choline is identical with that obtained with cells incubated for five generations (60–70 h) in the presence of labelled compound, and coupled with the fact that net phospholipid phosphorus and protein show the same pattern, is indicative of a gradual and continuous increase in the content of phospholipid, RNA and protein throughout the whole of the intermitotic period.

The biphasic pattern of phospholipid turnover (Pasternak, 1969; Pasternak & Bergeron, 1970) is seen at all stages of the cell cycle (Fig. 3); the rate of turnover falls to that of DNA, used as a control, by 40–50 h. The amount of 'stable' phospholipid remaining after this time varies from 15 to 35% of the total, being lowest in S-phase cells, when incorporation is maximal, and highest in G₁-phase cells, when incorporation is minimal. This is the result obtained by Bergeron *et al.* (1970) with cells synchronized by treatment with excess of thymi-

dine, and makes it unlikely that penetration of precursor (Stone, Miller & Prescott, 1965) is a determining factor in the synthesis of phospholipid during the cell cycle.

Cell-cycle analysis by zonal centrifugation. A good separation of G₁-phase, S-phase and G₂-phase cells, judged by thymidine incorporation, is achieved by the use of a zonal rotor (Fig. 4a). Mean cell volume increases gradually and there is less spread of smaller and larger cells around the mean (narrower Poisson distribution), especially at the lower region of the gradient, than in conventional centrifugation. Incorporation of precursor (Figs. 4b and 4c) increases two- to three-fold with uridine and proline, two- to four-fold with choline and inositol and more than tenfold with thymidine (Fig. 4). The same pattern of incorporation is observed whether trichloroacetic acid precipitates on filters or hot-trichloroacetic acid extracts (for thymidine) or chloroform-methanol extracts (for choline and inositol) are examined. The increase in mean cell volume and incorporation of thymidine and choline by HC cells follows a pattern similar to that of P815Y cells. As might be expected, the content of phospholipid, protein and RNA per cell doubles more gradually than that of DNA (Fig. 4d).

DISCUSSION

The results presented above clearly show that gradient centrifugation by conventional (Figs. 1 and 2) and zonal (Fig. 4) techniques is a useful tool for

Fig. 4. Analysis of prelabelled cells separated by zonal centrifugation. Cells (1×10^9 in 25 ml) were exposed to 20 μ Ci of [14 C]thymidine and either 60 μ Ci of [3 H]proline, 40 μ Ci of [3 H]uridine, 40 μ Ci of [Me - 3 H]choline, or 40 μ Ci of [3 H]inositol, and fractionated and analysed as described in the Experimental section. The results of four separations, reproducible with respect to cell number, mean cell volume and [14 C]thymidine incorporation, are presented. (a) $\bullet$, Cell number; $\blacksquare$, mean cell volume; $\circ$, [14 C]-labelled DNA. (b) $\bullet$, 3 H-labelled protein; $\square$, 3 H-labelled phospholipid. (c) $\blacksquare$, [3 H]Choline-labelled phospholipid; $\blacktriangle$, [3 H]inositol-labelled phospholipid. (d) $\circ$, Total DNA; $\bullet$, total protein; $\square$, total RNA; $\blacksquare$, total phospholipid. The pattern of 14 C-labelled DNA synthesis shown in (a) is repeated as a continuous line in (b) and (c).

the separation and analysis of mammalian cells at different stages of their life cycle. So far as neoplastic mast cells are concerned, the problem of the lack of correlation between volume and age (Petersen *et al.* 1969) is not a serious one. The advantages of zonal centrifugation over conventional gradient centrifugation are twofold. (1) A better resolution is achieved (Fig. 4 compared with Fig. 1), and (2) about ten times as many cells can conveniently be assayed. The method therefore lends itself well to the measurement of enzymes and other components at different stages of the cell cycle (A. M. H. Warmley & C. A. Pasternak, unpublished work).

The application of centrifugal techniques to the study of phospholipid metabolism has confirmed some of the results obtained with cells synchronized by treatment with excess of thymidine (Bergeron *et al.* 1970). In particular, the fact that incorporation of choline resembles that of uridine and proline, being continuous throughout the cell cycle with a maximum in the S phase, is borne out. The present results also show that incorporation into phospholipid, protein and RNA begins to increase already in the G₁ phase, in cells that are not yet synthesizing DNA. A similar result has been reported by Enger & Tobey (1969) for RNA synthesis in Chinese-hamster cells, whereas Pfeiffer & Tolmach (1968) and Martin, Tomkins & Granner (1969) found little increase in RNA or protein synthesis during the G₁ phase of HeLa or hepatoma cells respectively.

The decreased incorporation of precursor into phospholipid, protein and RNA during the G₂ phase (Fig. 4) is in agreement with results obtained with synchronized cells (Bergeron *et al.* 1970) and, in conjunction with the increase during the G₁ phase, shows that gene-dosage effects cannot account for alterations in rate of synthesis. The fact that the mean cell volume of P815Y cells changes in concert with net phospholipid is compatible with the view that cellular membranes, as exemplified by the plasma membrane, are laid down continuously during the intermitotic period of neoplastic mast cells. A somewhat similar result has been described for the synthesis of membrane constituents in KB cells by Warren (1969).

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