Phospholipid Synthesis and Degradation during the Life-Cycle of P815Y Mast Cells Synchronized with Excess of Thymidine

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1. P815Y cells synchronized with excess of thymidine incorporate choline, proline and uridine throughout the cell cycle; the rate increases two- to four-fold during the S phase, when thymidine incorporation increases more than 15-fold. 2. Choline incorporated at any stage of the cell cycle turns over in a biphasic manner; stable and unstable components are each labelled maximally during the S phase. Total phospholipid also doubles predominantly during the S phase. 3. It is concluded that, despite turnover, choline incorporation is a useful measure of net phospholipid formation during the cell cycle.

Phospholipids are a characteristic constituent of cellular membranes. Since little phospholipid appears to exist free within cells (e.g. Getz *et al.* 1961; Kuff, Hymer, Shelton & Roberts, 1966), the timing of its synthesis during the cell cycle may throw light on the stage at which membranes are made. Preliminary studies (Bergeron, Warmesley & Pasternak, 1969) have shown that the incorporation of choline into the phospholipids of synchronously dividing P815Y cells is continuous throughout the cell cycle, the rate increasing during the S phase when DNA synthesis takes place. However, the phospholipids of P815Y cells turn over during exponential growth (Pasternak & Bergeron, 1970) and it therefore becomes important to separate synthesis from degradation. This has now been achieved and is described together with details of some of the results briefly reported earlier (Bergeron *et al.* 1969).

EXPERIMENTAL

Chemicals. L-[G-³H]Proline (TRA 82, sp. radioactivity 550 mCi/mmol) and [2-¹⁴C]uridine (CFA 315, sp. radioactivity 58 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Other labelled compounds were obtained as described by Pasternak & Bergeron (1970).

Cells. P815Y neoplastic mast cells kindly given by Dr G. A. Fischer and Dr Margaret Fox were cultured as described by Pasternak & Bergeron (1970). Radioautographic (Warmesley & Pasternak, 1970) analysis of the cell cycle by using a mitotic inhibitor (Puck & Steffen, 1963; Puck, Sanders & Petersen, 1964) gave the following

result: M phase, 0.9 h; G₁ phase, 4.5 h; S phase, 6.5 h; G₂ phase, 3 h; mean generation time, 15 h.

Synchrony. An exponentially growing culture (2×10^5 – 3×10^5 cells/ml) was exposed to 2 mM-thymidine (Xeros, 1962) for 15 h during which time cell number increased by about 10%. Cells were washed free of inhibitor and resuspended (1.5×10^5 – 2.0×10^5 cells/ml) in fresh medium. Growth recommenced after a short lag and when cell number had increased by approximately half (total 'recovery time' 7 h) the culture was again exposed to 2 mM-thymidine for 15 h. After the cells had been washed and reincubated (at approx. 2×10^5 cells/ml) in fresh medium ($t = 0$), synchronous growth commenced after a lag of approx. 5 h (Bergeron *et al.* 1969). Although cells increase in size during thymidine treatment (Petersen, Tobey & Anderson, 1969), perhaps as a result of unbalanced growth (Bergeron, 1969; Mueller, 1969), the timing of biochemical events during subsequent synchrony is the same as that in cells synchronized by a purely physical method (Bergeron *et al.* 1969; Warmesley & Pasternak, 1970).

Incorporation of radioactivity. Samples of synchronously growing cells (1.5×10^5 – 5.0×10^5 cells/ml) were removed at intervals and incubated with radioactively labelled compound (generally 2 μ Ci of ³H-labelled compound/ml and 1 μ Ci of ¹⁴C-labelled compound/ml) for 30 min. Separate experiments showed that uptake of radioactive isotope is linear for at least this time. Incorporated radioactivity was determined by filtration, washing with 5% (w/v) trichloroacetic acid and assaying of filters as described by Pasternak & Bergeron (1970). Turnover was measured by concentrating the cell suspension tenfold to prevent further growth and adding unlabelled choline (30 mM).

Other methods. Phospholipid phosphorus in a chloroform-methanol (2:1, v/v) extract of trichloroacetic acid-precipitated cells was determined by the method of Bartlett (1959).

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Fig. 1. DNA and phospholipid synthesis in synchronized P815Y cells. Samples (10ml) were incubated with $2\mu\text{Ci}$ of [$M\epsilon$ - ^3H]choline and $1\mu\text{Ci}$ of [^{14}C]thymidine for 30 min and incorporated radioactivity [(a) and (b)] and cell number (d) were determined. Turnover of phospholipid in samples concentrated tenfold was followed; the amount of stable component (Table 1) is plotted in (c). $\blacktriangle$, Cell number; $\blacksquare$, ^{14}C -labelled DNA; $\bullet$, total ^3H -labelled phospholipid; $\circ$, stable ^3H -labelled phospholipid.

RESULTS AND DISCUSSION

Macromolecular synthesis during the cell cycle. Incorporation of labelled choline, inositol, proline and uridine is maximal during the S phase, when thymidine incorporation takes place (Figs. 1 and 2). Since cells become synchronized at the G_1/S boundary by thymidine treatment, an initial S+ G_2 phase, somewhat shorter (Petersen *et al.* 1969) than that of unsynchronized cells, precedes cell division. In contrast with DNA synthesis, incorporation into phospholipid, protein or RNA [the amount of DNA labelled by uridine was estimated by Pfeiffer & Tolmach (1968) to be less than 10% of the total nucleic acids in cultured HeLa cells] at no time falls to the 'background' value (incubation time 0 min), which indicates that synthesis of these molecules is never completely turned off. This is confirmed by radioautographic analysis of exponentially growing cells exposed to a pulse of labelled compound. Although the actual amount incorporated by any cell varies widely,

Fig. 2. RNA and protein synthesis in synchronized P815Y cells. Samples (1ml) were incubated with $2\mu\text{Ci}$ of [^3H]proline and $1\mu\text{Ci}$ of [^{14}C]uridine for 30 min and incorporated radioactivity (a) and cell number (b) were determined. $\bullet$, Cell number; $\blacktriangle$, ^{14}C -labelled RNA; $\blacksquare$, ^3H -labelled protein.

more than 90% of cells become labelled after exposure to [^3H]inositol, [^3H]choline, [^3H]proline or [^3H]uridine, compared with 40% labelled after exposure to [^3H]thymidine (Bergeron, 1969; Bergeron *et al.* 1969). Moreover, if isotopically labelled compound is present throughout synchronous growth (Bergeron *et al.* 1969), the uptake of [^3H]thymidine shows sharper discontinuities than does that of [^{14}C]choline. The increase in rate of incorporation into phospholipid is three- to four-fold (up to eightfold in some experiments) and two- to three-fold into protein or RNA, compared with more than 15-fold for DNA (Figs. 1 and 2).

If one assumes incorporation of choline, and hence the synthesis of phospholipid (see below), to be a measure of membrane formation (Nagley & Hallinan, 1968), it follows that the synthesis of membrane takes place during most of the intermitotic period, with an increase in rate during the S phase. This result is in general accord with observations (Warren, 1969) on the incorporation of labelled leucine and glucosamine into the surface membrane of KB cells. Greater incorporation of labelled phosphate into the phospholipids of HeLa cells during interphase than in mitosis has been described by Robbins & Scharff (1966). Bosmann & Winston (1970), on the other hand, claim that glycolipid and lipid synthesis in L5178Y cells is restricted to the G_2 and mitotic periods. Increase in the mean cell volume may be an indication of the proliferation of surface membrane. Interpretation of such changes

after thymidine treatment (see the Experimental section) is difficult; on the other hand, separation of cells by gradient centrifugation (Warmley, Bergeron & Pasternak, 1969; Warmley & Pasternak, 1970) does indicate a fairly continuous increase in cell size throughout the intermitotic period.

The fact that the rates of phospholipid, protein and RNA synthesis decrease when DNA synthesis stops (Figs. 1 and 2), shows that a gene-dosage effect of the kind observed by Pfeiffer & Tolmach (1968) for RNA synthesis in HeLa cells and by Martin, Tomkins & Granner (1969) for RNA and protein synthesis in rat hepatoma cells, is not operative in P815Y cells.

A decreased rate of RNA and protein synthesis in the G_2 phase (Figs. 1 and 2) has also been observed with Chinese-hamster cells (Sinclair, 1967). Moreover the decrease of non-nucleolar RNA synthesis during the G_2 phase is sharper in monkey kidney-cell nuclei than in HeLa nuclei (Seed, 1963). It might therefore appear that the neoplastic mast cells under study have more in common with normal than with cancer cells. Although it is true that P815Y cells remain relatively differentiated with respect to biochemical (Schindler, Day & Fischer, 1959) and chromosomal (Green & Day, 1960) characteristics in culture, it is also the case that the

Table 1. *Turnover of [^3H]choline-labelled phospholipid in synchronized P815Y cells*

Samples (10ml) of synchronized cells removed at the times indicated were incubated as described in Fig. 1 and concentrated tenfold, and turnover was determined by sampling (0.1ml) at intervals. A biphasic pattern of turnover (Pasternak & Bergeron, 1970), with all unstable phospholipid lost by approx. 50 h, was observed in all cases. The percentage of label remaining between 50 and 100 h is taken as stable component.

Time of sampling after start of synchrony (h)	Stable ^3H -labelled phospholipid (%)
0	42
2	25
4.5	19
6.5	22
8.5	48
10.5	46
12	34
13.5	17
17	13
21	17
23	12
25	42

Fig. 3. Net phospholipid synthesis in synchronized P815Y cells. Duplicate samples (5ml) were removed at intervals and cell number and phospholipid P were determined. ●, Cell number; ■, phospholipid P.

pattern of net synthesis of phospholipid, protein and RNA relative to that of DNA (Warmesley & Pasternak, 1970) is more akin to the increase in nuclear dry mass in HeLa and mouse L cells than that in monkey kidney cells or embryonic fibroblasts (Seed, 1962, 1963). Limitations of present methodology and differences in culture conditions are more likely to account for such conflicting results than genuine differences between cell types.

Synthesis and degradation of phospholipids during the cell cycle. Incorporated choline turns over in a biphasic manner, indicative of stable and unstable components (Pasternak & Bergeron, 1970), at all stages of the cell cycle. Although the percentage of stable component fluctuates between approx. 15% (when total incorporation is high) and 45% (when total incorporation is low) (Table 1), the timing of its synthesis (Fig. 1c) coincides with that of total labelled phospholipid (Fig. 1b). Moreover, net phospholipid increases at the same time (Fig. 3). Hence one may conclude that incorporation of choline is a useful measure of phospholipid synthesis during the cell cycle.

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