The Use of Zonal Centrifugation to Study Membrane Formation during the Life Cycle of Mammalian Cells

SYNTHESIS OF 'MARKER' ENZYMES AND OTHER COMPONENTS OF CELLULAR ORGANELLES

By A. M. H. WARMSLEY,* BEVERLY PHILLIPS AND C. A. PASTERNAK†
Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

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1. The activity of enzymes characteristic of microsomes (NADPH–cytochrome *c* reductase and uridine diphosphatase) and of inner mitochondrial membranes (cytochrome *c* oxidase and succinate–cytochrome *c* reductase) increases during the cell cycle of P 815Y neoplastic mast cells in concert with total protein. The activity of glutamate dehydrogenase, an enzyme of the mitochondrial matrix, increases in a somewhat different manner. 2. The specific activity of mitochondrial structures involved in energy-coupling measured with a fluorescent probe remains constant during the cell cycle. 3. Mitochondrial and microsomal protein increases during the cycle at the same time as total protein; nuclear protein increases rather more sharply. 4. The rate of incorporation of labelled choline or inositol into nuclear, mitochondrial or microsomal phospholipid during the cell cycle follows the rate of incorporation into total phospholipid. 5. It is concluded that the major components of cellular membranes are synthesized, like total protein or phospholipid, throughout most of the intermitotic period.

Separation of cells according to size by zonal centrifugation has proved a useful tool for studying the synthesis of cellular constituents during the life cycle of mammalian cells (Warmesley & Pasternak, 1970). The formation of membranes is of special interest and initial experiments (Warmesley, Bergeron & Pasternak, 1969; Bergeron, Warmesley & Pasternak, 1969, 1970; Warmesley & Pasternak, 1970) showed that the synthesis of phospholipid, like that of protein and RNA, begins in the G₁ phase, increases throughout the S phase and declines again in the G₂ phase, resulting in a gradual increase of total phospholipid during interphase. Because these results conflict with those reported for L5178Y cells (Bosmann & Winston, 1970) and in order to study the synthesis of membrane in greater detail, we have now measured the synthesis of phospholipid and protein in subcellular fractions of P 815Y neoplastic mast cells. Although changes in the activity of several enzymes during the cell cycle have been followed, such studies (Mitchison, 1969) have not, with few exceptions (Bosmann,

1970*a,b*), included any 'marker' enzymes characteristic of different cellular organelles. The activity of some membrane marker enzymes during the cell cycle has therefore been determined and the results are presented together with data on the binding of a fluorescent probe (Brocklehurst, Freedman, Hancock & Radda, 1970) indicative of functionally active mitochondrial proteins.

EXPERIMENTAL

Chemicals. NADH, NADPH, NADP⁺, ADP, cytochrome *c*, sodium oxoglutarate and sodium pyruvate were obtained from the Boehringer Corporation (London) Ltd., London W.5, U.K. UDP was obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. All other chemicals were obtained as described by Bergeron *et al.* (1970), Pasternak & Bergeron (1970) or Warmesley & Pasternak (1970).

Cell culture. P 815Y neoplastic mast cells, kindly given by Dr Margaret Fox, were cultured and their number and size determined as previously described (Pasternak & Bergeron, 1970; Warmesley & Pasternak, 1970).

Zonal centrifugation. Cells were labelled with radioactive precursor and separated according to their position in the cell cycle by zonal gradient centrifugation exactly as described by Warmesley & Pasternak (1970). Cells were collected and resuspended in 2–10 ml of ice-cold 50 mM-tris-HCl buffer, pH 7.4, containing 0.9% NaCl, referred to

* Present address: Shell International Chemical Co. Ltd., Shell Centre, London S.E.1, U.K.

† Present address: Department of Neurosciences, School of Medicine, University of California, San Diego, La Jolla, Calif. 92037, U.S.A.

subsequently as tris-buffered saline. When necessary, cells from two or more fractions were pooled so that each sample finally contained at least $1.5-2 \times 10^7$ cells.

Preparation of extracts. (a) Sonic oscillation. Cells in 2 ml of tris-buffered saline were disrupted by sonic oscillation at 0°C for 60 s with an M.S.E. Ultrasonic Disintegrator (100 W) fitted with a titanium probe (end diameter 9.5 mm) tuned for maximum output. Most enzyme activities were measured in such extracts.

(b) Homogenization and subcellular fractionation. Cells resuspended in 1 ml of a solution containing 10 mM-tris-HCl buffer, pH 7.4, 10 mM-KCl, 2 mM-MgCl₂ and 0.1 mM-ZnSO₄, subsequently referred to as homogenizing medium, and kept at 0°C for 10 min, were disrupted by hand with a glass-ball homogenizer (clearance 0.2 mm). The suspension was centrifuged for 5 min at 350 *g*_{av} in a BTL Bench Centrifuge to give a nuclear pellet. The supernatant was centrifuged for 15 min at 10000 *g*_{av} in a Beckman model L preparative ultracentrifuge to give a pellet which was resuspended in 0.5–1 ml of homogenizing medium and recentrifuged for 15 min at 10000 *g*_{av} to give a crude mitochondrial fraction. This contained more than 96% of the total cytochrome oxidase activity. The combined supernatants from the last two centrifugations were centrifuged for 60 min at 100000 *g*_{av} to give a crude microsomal pellet and a clear supernatant fraction which contained more than 95% of the total lactate dehydrogenase activity. Morphological confirmation of the identity and purity of the subcellular fractions was obtained by electron microscopy.

Assay of enzyme activity. Spectrophotometric assays were carried out in a final volume of 1 ml (1 cm light-path) with a Beckman D.B.6 spectrophotometer fitted with a constant-temperature device, coupled to a Sargent S.R. recorder. The molar extinction coefficient for NADH and NADPH was taken as $6.2 \times 10^6 \text{ l mol}^{-1} \text{ cm}^{-1}$ (340 nm) and for cytochrome *c* as $18.5 \times 10^6 \text{ l mol}^{-1} \text{ cm}^{-1}$ (550 nm). All assays were carried out at two enzyme concentrations such that rate was proportional to concentration. Activities are given as μmol of substrate transformed or product formed/min (U), either per cell or per mg of protein. Lactate dehydrogenase (EC 1.1.1.27) activity was measured at 37°C by the method of Bergmeyer, Bernt & Hess (1963). Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) activity was measured at 30°C by the method of Löhner & Waller (1963). Glutamate dehydrogenase (EC 1.4.1.4) was measured at 37°C by the method of Schmidt (1963) as modified by Williamson, Lund & Krebs (1967). Cytochrome *c* oxidase (EC 1.9.3.1) activity was measured at 37°C by the method of Appelmans, Wattiaux & de Duve (1955). NADPH-cytochrome *c* reductase (EC 1.6.2.a) activity and succinate-cytochrome *c* reductase (EC 1.3.99.1) activity were measured at 37°C by the method of Sottocasa, Kuylensstierna, Ernster & Bergstrand (1967). Uridine diphosphatase (EC 3.6.1.6) activity in extracts prepared by homogenizing cell pellets in 10 mM-tris-HCl buffer, pH 7.4, containing 0.1% Triton X-100 was measured at 37°C by the method of Plaut (1963). Such extracts contained only negligible amounts of 5'-nucleotidase (EC 3.1.3.5), measured by either of the methods described by Murray & Friedrichs (1969).

Fluorescent-probe studies. The method of Brocklehurst *et al.* (1970) was used. The reaction mixture contained 225 μmol of mannitol, 75 μmol of sucrose, extract corre-

sponding to 0.1–0.4 mg of protein and 20 μmol of tris-HCl buffer, pH 7.4, in a final volume of 1 ml. To this was added 32.5 μmol of 1-anilinonaphthalene-8-sulphonate and the resulting fluorescence was measured with a spectrofluorimeter as described by Brocklehurst *et al.* (1970). Excitation was at 390 nm and emission at 490 nm. The increase in fluorescence resulting from the addition of 50 μmol of succinate (reversible by the addition of 2.5 μmol of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) was measured.

Other determinations. DNA was measured by the method of Burton (1956). Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951). Incorporated radioactivity was determined as described by Pasternak & Bergeron (1970).

RESULTS

Activity of marker enzymes. NADPH-cytochrome *c* reductase, a microsomal marker enzyme (Dallner, Siekevitz & Palade, 1966), shows a gradual increase in activity per cell relative to the increase in DNA

Fig. 1. NADPH-cytochrome *c* reductase activity in cells separated by zonal centrifugation. Cells (1×10^9) were separated, disrupted by sonic oscillation and assayed as described in the Experimental section. (a) Activity (U)/cell; (b) specific activity. □, Total DNA; ●, NADPH-cytochrome *c* reductase. The approximate allocation of different parts of the gradient to the G₁, S and G₂ phases was made on the basis of cell number, cell volume, thymidine incorporation (Warmesley & Pasternak, 1970) and radioautographic analysis of the cell cycle (Bergeron *et al.* 1970).

Fig. 2. Cytochrome *c* oxidase and succinate-cytochrome *c* reductase activity in cells separated by zonal centrifugation. Experimental details were as given for Fig. 1. (a) Activity (U)/cell; (b) specific activity. ●, Cytochrome *c* oxidase; ○, succinate-cytochrome *c* reductase. Total DNA is shown as a single continuous line.

Fig. 3. Lactate dehydrogenase and glucose 6-phosphate dehydrogenase activity in cells separated by zonal centrifugation. Experimental details were as given for Fig. 1. (a) Activity (U)/cell; (b) specific activity. ●, Lactate dehydrogenase; ○, glucose 6-phosphate dehydrogenase. Total DNA is shown as a single continuous line.

content (Fig. 1a) following a pattern similar to that of total protein (Warmsley & Pasternak, 1970). This is seen more clearly by the constancy in specific activity (Fig. 1b). However, Bosmann (1970a,b) has reported that the activity of uridine diphosphatase (Novikoff & Goldfischer, 1961) and of several other microsomal enzymes of L 5178Y cells shows a sharp decrease after a peak in the S phase. Assay of uridine diphosphatase in P 815Y cells by the method of Bosmann (1970a) gives a pattern essentially similar to that of NADPH-cytochrome *c* reductase. The reason for this discrepancy may lie not so much in the difference of cell line as in the methodology used. Use of inhibitors of DNA synthesis to achieve synchrony (Bosmann, 1970a) has in our hands also (Bergeron *et al.* 1969, 1970) given sharp peak patterns (for incorporation of macromolecular and other precursors) which are not apparent in cells separated by gradient centrifugation (Warmsley & Pasternak, 1970). What is clear from both these studies is that microsomes are at their most active as regards protein synthesis during the S phase, a conclusion previously reached

with regard to histone (Gallwitz & Mueller, 1969), immunoglobulin (Byars & Kidson, 1970) and other protein (Mueller, 1969) synthesis.

Two markers of the inner mitochondrial membrane, cytochrome *c* oxidase (Schnaitman, Erwin & Greenawalt, 1967) and succinate-cytochrome *c* reductase (Sottocasa *et al.* 1967) shows a variation in activity similar to that of NADPH-cytochrome *c* reductase (Figs. 2a and 2b).

Two soluble enzymes of the cytoplasm, lactate dehydrogenase and glucose 6-phosphate dehydrogenase, follow a pattern (Fig. 3) resembling that described above; this is similar to the changes of these enzymes observed with hepatoma cells by Martin, Tomkins & Granner (1969). In some experiments, fluctuations are superimposed on a steady increase, although these oscillations are not as marked as those found with Chinese-hamster cells (Klevecz & Ruddle, 1968). Experiments in which P 815Y cells of low lactate dehydrogenase activity were mixed with cells of high activity showed that the fluctuations, which occur only with cells in early exponential growth (cf. Hooper, 1967),

are not due to the presence of an inhibitor or activator; a similar conclusion was reached by Klevecz (1969).

Glutamate dehydrogenase, a soluble enzyme of the mitochondrial matrix (Munn, 1969), exhibits a different pattern in that activity increases only after total DNA has begun to double (Fig. 4a). An

Fig. 4. Glutamate dehydrogenase activity in cells separated by zonal centrifugation. Experimental details were as given for Fig. 1. (a) Activity (U)/cell; (b) specific activity. Total DNA is shown as a single continuous line.

initial decrease in specific activity (Fig. 4b) confirms that this enzyme follows a pattern of duplication different to that of total protein.

Fluorescent-probe studies. The results of fluorescence studies, which measure in effect the activity of structures of the inner mitochondrial membrane involved in energy-coupling, are shown in Table 1. Fluorescence intensity increases in parallel with total mitochondrial protein; in other words the 'specific activity' remains constant. There is negligible variation in the percentage of fluorescence that is succinate-dependent at different stages of the cell cycle; the rather low values may be due to insufficient or excessive sonic oscillation. The same result was obtained when whole cells that had been disrupted by sonic oscillation, rather than mitochondrial extracts, were analysed. These results therefore confirm those obtained with enzyme markers.

Protein content of cell organelles. The increase of mitochondrial and microsomal protein during the cell cycle is shown in Fig. 5. Nuclear protein in this case increases sharply during the S phase in a pattern resembling that of the total DNA (Figs. 1-4). This may imply that P 815Y cells resemble non-cancerous cells in so far as Seed (1962, 1966a,b,c,d) has found that the synthesis of nuclear protein is temporally linked to that of DNA in normal but not in tumour cells. However, the crucial parameter is probably not malignancy but the chromosomal pattern, which in the case of P 815Y cells (Green & Day, 1960) is that of the normal mouse host. The fact that nuclear protein behaves differently from total protein resolves the previous discrepancy (Bergeron *et al.* 1970) in this regard. The observation that the synthesis of histones, which account for much of nuclear protein, occurs predominantly during the S phase (Spalding, Kajiwara & Mueller, 1966;

Table 1. *Enhancement of 1-anilinonaphthalene-8-sulphonate fluorescence by mitochondrial proteins*

Mitochondria prepared from cells from different regions of the gradient were suspended in a solution containing 225 mM-mannitol, 75 mM-sucrose and 20 mM-tris-HCl buffer, pH 7.4, exposed to sonic oscillation for 1 min and analysed as described in the Experimental section.

Distance from rotor centre (cm)	Mitochondrial protein added (mg)	Fluorescence intensity (arbitrary units)	Enhancement on adding succinate (% increase)
5.7	0.19	0.52	15
6.0	0.20	0.49	18
6.3	0.20	0.53	21
6.7	0.19	0.53	17
7.0	0.20	0.51	18
7.3	0.16	0.52	17
7.7	0.20	0.50	16
8.2	0.36	0.75	16
8.9	0.28	0.75	16
9.7	0.29	0.81	11
11.1	0.22	0.55	16

Fig. 5. Protein content of organelles after cell separation by zonal centrifugation. Experimental details were as given for Fig. 1. ▲, Nuclear protein; ●, mitochondrial protein; ■, microsomal protein.

Robbins & Borun, 1967; Gurley & Hardin, 1968; Mueller, 1969) is in accord with the present results.

Phospholipid synthesis in cell organelles. P 815Y cells were exposed to a 30 min pulse of labelled choline or inositol (Warmesley & Pasternak, 1970) and radioactivity incorporated into phospholipid, which is an index of phospholipid synthesis (Bergeron *et al.* 1970; Warmesley & Pasternak, 1970), was measured in subcellular fractions obtained after zonal centrifugation of labelled cells. The pattern of variation during the cell cycle in cellular organelles was essentially the same as in whole cells (Warmesley & Pasternak, 1970); that is, it is continuous throughout interphase, being low in the G_1 phase, increasing predominantly during the S phase and decreasing again in the G_2 phase. The same result was also obtained by Gerner, Glick & Warren (1970). Since microsomes are probably the main site of phospholipid synthesis it would seem that transfer to other organelles (Wirtz & Zilversmit, 1968, 1969; Beattie, 1969; McMurray & Dawson, 1969) is equally effective at all stages of the cell cycle.

DISCUSSION

Changes in the activity of enzymes during the cell cycle may reflect several underlying causes (Mitchison, 1969). Where 'peak' patterns are obtained, for example, the decrease may be due to either turnover or inactivation of the relevant enzyme. In the present case, in which the activity of each of the enzymes studied is doubled, it is unlikely that such effects are a contributing factor, and one may tentatively assume that the change in activity represents the net synthesis of new enzyme molecules. This justifies the use of these marker

enzymes as a tool for investigating the biosynthesis of the organelles of which they are a part. Moreover, where the doubling in activity is fairly sharp, the genetic locus of that enzyme relative to others on the chromosome becomes amenable to experimental attack. Of the enzymes studied here, only glutamate dehydrogenase (Fig. 4) falls into this category. The synthesis of the other enzymes (Figs. 1–3) does not appear to be controlled by a mechanism involving gene dosage (see Warmesley & Pasternak, 1970).

Taking together the results obtained with marker enzymes characteristic of microsomes and mitochondria (Figs. 1 and 2), with fluorescence studies of mitochondrial structures involved in energy-coupling (Table 1) and the protein (Fig. 5) and phospholipid content of microsomes and mitochondria, one may conclude that several, and indeed the major, components of intracellular membranes are synthesized throughout most of the intermitotic period of P 815Y cells. The rate of synthesis probably reaches a maximum during the S phase. This result, which confirms our preliminary speculations (Bergeron *et al.* 1970; Warmesley & Pasternak, 1970), is in general agreement with data on the biosynthesis of mitochondria in rat liver and kidney (Droz & Bergeron, 1965), in *Neurospora crassa* (Luck, 1963) and in *Astasia longa* (Kahn & Blum, 1967). It establishes the separation of cells by zonal centrifugation as a promising technique for a more detailed study of the biosynthesis of cellular constituents during the life cycle of mammalian cells.

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