Turnover of Mammalian Phospholipids

STABLE AND UNSTABLE COMPONENTS IN NEOPLASTIC MAST CELLS

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(Received 11 May 1970)

1. Choline- and inositol-labelled phospholipids of exponentially growing or static neoplastic mast cells turn over by degradation and resynthesis of the entire molecule. Turnover follows a biphasic pattern, the unstable rapidly turning-over component accounting for 60–80% of labelled phospholipid. The residual stable component does not turn over any more than does protein or DNA. 2. Subcellular fractions and surface membranes of choline-labelled P815Y cells contain the same proportion of stable and unstable components as do whole cells. The unstable component is largely phosphatidylcholine; the stable component is relatively richer in sphingomyelin. 3. It is concluded that the phospholipids of neoplastic mast cells are of two classes, one of which is susceptible to continual enzymic degradation and resynthesis, and the other of which is metabolically stable.

In an attempt to study the biosynthesis of membranes in cultured neoplastic mast cells (Bergeron, Warmsley & Pasternak, 1969; Warmsley, Bergeron & Pasternak, 1969), we have used radioactively labelled choline (e.g. Nagley & Hallinan, 1968; Plagemann, 1968) as a precursor of phospholipid. It was noted (Pasternak, 1969) that if labelled cells are washed and reincubated in non-radioactive medium, radioactivity is lost from the cells by turnover. Although the turnover of animal phospholipids is well documented (e.g. Dawson, 1957, 1966), it seemed noteworthy that turnover occurs during exponential growth, when little turnover of protein, for example, takes place. Another aspect that emerged (Pasternak, 1969) is that the decay of radioactivity in cells follows a biphasic pattern, indicative of two metabolically distinct components. These might be chemically distinct entities or might reflect compartmental differences within the cell. The present experiments were carried out to investigate further these aspects of phospholipid turnover.

EXPERIMENTAL

Chemicals. [*Me*-¹⁴C]Choline chloride (CFA 138, sp. radioactivity 32–54 mCi/mmol), [*Me*-³H]choline chloride (TRA 179, sp. radioactivity 2 Ci/mmol, or TRK 179, sp. radioactivity 15.4 Ci/mmol), [¹⁴C]thymidine (CFA 219, sp. radioactivity 60 mCi/mmol), [¹⁴C]valine (CFB 21, sp. radioactivity 6.9 mCi/mmol), sodium [¹⁴C]-acetate (CFA 13, sp. radioactivity 50 mCi/mmol) and

[³H]glycerol (TRA 118, sp. radioactivity 100–500 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [1,2-¹⁴C₂]Choline chloride (NEC 527, sp. radioactivity 3.1 Ci/mmol), [³H]myo-inositol (NET 114, sp. radioactivity 3.5 mCi/mmol) and [³³P]phosphoric acid, (NEZ 808, carrier-free) were obtained from New England Nuclear Chemicals G.m.b.H., Dreieichen, Germany. All solutions of radioactively labelled compounds were sterilized by filtration. All other chemicals were obtained from normal commercial sources, the purest grade available being generally used.

Cell culture. P815Y and HC cells (kindly given by Dr Margaret Fox and Dr G. A. Fischer) were cultured as described by Wheldrake & Pasternak (1968). Commercially prepared Fischer's medium (Grand Island Biological Company, Grand Island, N.Y., U.S.A.) was used in later experiments. Horse serum was from Wellcome Research Laboratories, Beckenham, Kent, U.K. (horse serum no. 5), Flow Laboratories Ltd., Irvine, Ayrshire, U.K. (membrane-filtered horse serum), or Tissue Culture Services, Slough, Bucks., U.K. (aseptic horse serum). Cells were grown from an initial density of approx. 1×10^5 /ml for 2–3 generations; radioactive compounds (generally 0.4–4.0 μ Ci/ml) were present as indicated. Incorporated radioactivity was measured as described by Thomas & Pasternak (1969), or by filtering samples through Whatman GF/C papers (2.1 cm diam.), washing with 0.9% NaCl or 5% (w/v) trichloroacetic acid (see below) and transferring the paper to 7 ml of scintillation fluid (see below). Turnover was estimated either by addition of unlabelled compound as indicated or by washing and reincubating cells in fresh medium, or both.

Fractionation of cells and preparation of surface membranes. Cells were harvested, washed with 0.16 M-NaCl and suspended in 1 or 2 ml of 0.16 M-NaCl. Fluorescein mercuric acetate (2.2 mM in 0.02 M-tris-HCl buffer, pH 8.1; 3 vol.) (Warren & Glick, 1968) was added and the

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suspension was left at room temperature for 5 min and then chilled to 3°C. The cell suspension was disrupted in a Silverson homogenizer (for approx. 1 min) and a portion was then fractionated into crude nuclear, mitochondrial and microsomal portions by centrifuging at 900g_{av.} for 10 min, 8000g_{av.} for 30 min (Warren & Glick, 1968) and 100000g_{av.} for 60 min respectively. Each pellet was washed by resuspension in 0.25M-sucrose and recentrifuged at the same speed as before; the pellet was then resuspended in 1 ml of 0.25M-sucrose. The remainder of the disrupted cell suspension was used for the preparation of surface membranes by the method of Warren & Glick (1968). Pooled debris sedimented during the first two centrifugations and surface membranes were suspended in water before assay of radioactivity. The presence of fluorescein mercuric acetate precludes the assay of marker enzymes in the various subcellular fractions; since conventional centrifugation is reliable for the preparation of crude organelles at least, and since no metabolic differences between the various fractions were in fact found (Tables 1, 2 and 4), their purity was not checked further.

Fractionation of phospholipids. Lipids were extracted from cell pellets by the method of Folch, Lees & Sloane-Stanley (1957). The percentage of radioactivity present in the lower (lipid) phase from extracts of cells that had been exposed to radioactive choline was approximately equal to that which was precipitated when cells were treated with 5% (w/v) trichloroacetic acid as noted by Plagemann (1968). Treatment of cells on filters with 5% (w/v) trichloroacetic acid was accordingly sometimes used as a convenient method for measuring incorporated radioactivity directly. T.l.c. of the lower phase was carried out by the method of Skipski, Peterson & Barclay (1964) by using silica gel H obtained from E. Merck A.-G., Darmstadt, Germany. Added markers were used to locate, by exposure to I₂ vapour, the areas corresponding to the following choline- and inositol-containing phospholipids: lysophosphatidylcholine (lysolecithin), sphingomyelin, phosphatidylcholine (lecithin) and phosphatidylinositol. After the yellow colour had been allowed to fade, portions of the dried gel were scraped directly into 7 ml of scintillation fluid and the radioactivity was assayed.

The lower lipid phase was also fractionated by silicic acid chromatography (Bezman-Tarcher, Otway & Robinson, 1965) into a neutral and phospholipid fraction. No radioactivity was present in the neutral fraction after incubation of cells with radioactive choline, and this step was subsequently omitted. Cells labelled with radioactive palmitate or acetate contained 70–80% of the total lipid radioactivity in the neutral fraction (A. M. H. Warmsley, unpublished work).

Radioactivity assay. Radioactive solutions, filters or dried silica gel were added to 7 ml of scintillation fluid consisting of 8 g of 5-(4-phenyl)-2-(4-tert-butylphenyl)-1-oxa-3,4-diazole [CIBA (A.R.L.) Ltd., Duxford, Cambs., U.K.] and 80 g of naphthalene in 1 litre of toluene-methyl-Cellosolve (3:2, v/v), and assayed in a Beckman liquid-scintillation spectrometer (efficiency 90% for ¹⁴C and ³³P; 40% for ³H; radioactivity of quenched samples was corrected by means of standard curves for ¹⁴C and ³H). In some experiments, radioactivity on filters was measured with an end-window probe as described by Thomas & Pasternak (1969).

RESULTS

Nature of incorporated choline and inositol. Of the labelled choline incorporated by P815Y or HC cells during growth for 2–3 generations, 60–80% is present as lipid-soluble material (lower phase of Folch *et al.* 1957). By t.l.c., approx. 84% of the label in phospholipids is found to be in the phosphatidylcholine area, 10% in sphingomyelin and 2% in lysophosphatidylcholine. The same result is obtained with [*Me*-³H]choline and [1,2-¹⁴C₂]choline. Approx. 80% of incorporated inositol, like choline, is lipid-soluble. Of this over 80% is present as phosphatidylinositol, as judged by t.l.c.

Turnover in whole cells. P815Y cells that had been grown in the presence of [*Me*-¹⁴C]choline, [¹⁴C]valine or [¹⁴C]thymidine were centrifuged, washed and resuspended in fresh non-radioactive medium and reincubated. Growth was identical in each flask. Fig. 1 shows that approx. 60% of incorporated [*Me*-¹⁴C]choline is lost within 30 h,

Fig. 1. Turnover of phospholipid, protein and DNA in P815Y cells. Cells that had been grown ($1.9 \times 10^5/\text{ml}$ to $4-6 \times 10^5/\text{ml}$) in [*Me*-¹⁴C]choline (0.2 $\mu\text{Ci/ml}$), [¹⁴C]valine (0.6 $\mu\text{Ci/ml}$) or [¹⁴C]thymidine (0.2 $\mu\text{Ci/ml}$) for 5 days were washed, resuspended ($1.4 \times 10^5/\text{ml}$) in non-radioactive medium and samples removed for assay of cell number and radioactivity by the method of Thomas & Pasternak (1969). (a) Radioactivity: $\circ$, ¹⁴C-labelled phospholipid; $\square$, ¹⁴C-labelled protein; $\triangle$, ¹⁴C-labelled DNA. (b) Cell number: $\bullet$, [¹⁴C]choline-labelled cells; $\blacksquare$, [¹⁴C]valine-labelled cells; $\blacktriangle$, [¹⁴C]thymidine-labelled cells.

Fig. 2. Turnover of choline- and inositol-labelled phospholipids. Cell suspension (50 ml, 2×10^5 cells/ml) was incubated with $[Me-^{14}\text{C}]$ choline ($0.4 \mu\text{Ci/ml}$) and $[^3\text{H}]$ inositol ($1 \mu\text{Ci/ml}$). Portions (5 ml) were removed at the times indicated by the arrows and turnover was estimated by the addition of unlabelled choline (30 mM) and inositol (6 mM). Samples (0.1 ml) were removed for the assay of cell number and radioactivity by filtration as described in the Experimental section. (a) ▲, Uptake; △, turnover of $[^{14}\text{C}]$ choline; (b) ■, uptake; □, turnover of $[^3\text{H}]$ inositol; (c) ●, cell number.

whereas incorporated $[^{14}\text{C}]$ valine and $[^{14}\text{C}]$ thymidine are decreased by approx. 7% (cf. Eagle, Piez, Fleischman & Oyama, 1959) and 5% respectively during the same interval. Thereafter incorporated $[Me-^{14}\text{C}]$ choline is as stable as incorporated $[^{14}\text{C}]$ valine or $[^{14}\text{C}]$ thymidine. Loss of label from HC cells follows a similar biphasic pattern (60% of incorporated $[Me-^3\text{H}]$ choline is lost in 35 h during growth from 8×10^4 cells/ml to 1.7×10^5 /ml; less than 5% is lost in the following 15 h). Turnover of $[^3\text{H}]$ inositol also follows a biphasic pattern, approx. 60–80% being unstable (Fig. 2).

Independence of turnover on cell growth. It might at first be thought that turnover of incorporated $[Me-^{14}\text{C}]$ choline (Fig. 1) is due to the fact that the cells were in lag phase during the first 30 h. Several experiments show that this is not the case. Fig. 2 shows the results of an experiment in which turnover of $[Me-^{14}\text{C}]$ choline- and $[^3\text{H}]$ inositol-labelled phospholipids was measured during the exponential and stationary phases, and were found to be similar (approx. 60% of incorporated choline and approx. 80% of incorporated inositol was lost in 40 h) at all stages. Moreover, $[Me-^{14}\text{C}]$ choline or $[^3\text{H}]$ inositol incorporated during a 30 min pulse by cells at various stages of growth also shows the biphasic pattern of turnover. Omission of serum from the medium prevents growth of P815Y cells, but has no effect on turnover of incorporated choline or inositol; nor has inhibition of growth by fluoro-deoxyuridine or by excess of thymidine (Bergeron, 1969). Chilling the cells, on the other hand, prevents turnover, which resumes when cells are warmed to 37°C ; turnover is also decreased in non-viable cells (permeable to eosin) (A. M. H. Warmsley, unpublished work). The same degree of turnover is detected whether incorporation is stopped by addition of unlabelled compound or by reincubation of cells in fresh medium.

Turnover in cell organelles. To test the possibility that the biphasic decay of labelled phospholipid reflects differential turnover within the various cell organelles, the following experiment was carried out. A batch of cells (A) was grown in the presence of $[Me-^3\text{H}]$ choline ('phase 1'), washed, regrown in non-radioactive medium ('phase 2'), resuspended in medium containing $[Me-^{14}\text{C}]$ choline and again allowed to grow ('phase 3'). The experiment was repeated with another batch of cells (B), which was treated in the same way except that it was exposed to $[Me-^{14}\text{C}]$ choline during phase 1 and to $[Me-^3\text{H}]$ choline during phase 3. At the end of phase 3, the cells of each batch were harvested, suspended in fluorescein mercuric acetate and homogenized. Half of each suspension was then fractionated into crude nuclear, mitochondrial, microsomal and supernatant portions. The results with batch A and

Table 1. *Distribution of radioactivity in the organelles and surface membranes of cells grown in the presence of [*Me*-³H]choline and [*Me*-¹⁴C]choline*

A batch (50 ml) of cells (A) was grown (1.7×10^5 /ml to 5×10^5 /ml) in the presence of [*Me*-³H]choline (0.4 μ Ci/ml) for 50 h (phase 1), washed, resuspended in non-radioactive medium (100 ml) and regrown (1.7×10^5 /ml to 8×10^5 /ml) for 32 h (phase 2); cells were then resuspended in medium (250 ml) containing [*Me*-¹⁴C]choline (0.024 μ Ci/ml) and regrown for 35 h (phase 3). An identical batch of cells (B) was treated in the same way except that it was exposed to [*Me*-¹⁴C]choline (0.12 μ Ci/ml) in phase 1 and to [*Me*-³H]choline (0.08 μ Ci/ml) in phase 3. At the end of phase 3 cells were disrupted, divided into equal portions and fractionated into organelles or surface membranes as described in the Experimental section.

	Total radioactivity (d.p.m.)				¹⁴ C/ ³ H ratio Batch A	³ H/ ¹⁴ C ratio Batch B
	Batch A		Batch B			
	³ H	¹⁴ C	³ H	¹⁴ C		
Fractionation into organelles						
Homogenate	600 000	2 700 000	7 200 000	345 000	4.5	21
Nuclear fraction	55 000	190 000	430 000	20 000	3.5	21
Mitochondrial fraction	215 000	970 000	3 030 000	130 000	4.5	29
Microsomal fraction	150 000	340 000	900 000	41 000	2.3	22
Supernatant	325 000	860 000	2 150 000	110 000	2.6	20
Fractionation the surface membranes						
Homogenate	440 000	2 500 000	8 750 000	344 000	5.7	25
Surface membranes	7 700	28 000	68 000	3 800	3.6	18
Remainder	390 000	2 100 000	6 000 000	290 000	5.4	21

batch B are in good agreement (Table 1), almost half (40–50%) of the radioactivity being in the mitochondrial fraction, as found by Plagemann (1968) with cultured hepatoma cells. Radioactivity in all fractions was lipid-soluble except in the supernatant fraction, in which it was mainly water-soluble. The other half of the suspension was used to isolate a surface-membrane fraction. Table 1 shows that in each batch, all fractions have approximately the same ¹⁴C/³H ratio. The values of 2.3 and 2.6 for microsomal and supernatant fractions of batch A are probably not significantly different from the mean, and reflect technical difficulties of assaying low amounts of ³H in the presence of high concentrations of ¹⁴C. This difficulty also accounts for the apparently anomalous distribution of ³H among the different cell fractions of batch A; in any case, their values are not offset by a higher ratio in any other cell fraction. The fact that the ratio in the supernatant fraction (water-soluble) is similar to those of the other (which are lipid soluble) fractions is surprising. It may indicate the retention of certain water-soluble degradation products that do not equilibrate readily with the free choline pool (Pasternak & Friedrichs, 1970). The whole experiment was twice repeated and essentially similar results were obtained. Although the fractionation procedure is somewhat crude it is unlikely that a concentration of stable or unstable phospholipid in any particular cell organelle would have escaped detection. One may therefore conclude that turnover of choline-

labelled phospholipid occurs to the same extent at the major sites within P815Y cells.

The situation with respect to inositol-labelled phospholipids was examined in a similar manner. In this case, cells were grown in the presence of [³H]inositol and [*Me*-¹⁴C]choline. Cells were centrifuged, washed and suspended in a non-radioactive medium and a portion (*t* = 0 h) was removed, centrifuged and frozen. The remainder was reincubated and allowed to grow for 47 h before being harvested and frozen. During the first 30 h of this period, approx. 70% of the ³H and 60% of the ¹⁴C incorporated by the cells was released; subsequent analysis of cell fractions similarly indicated a somewhat greater loss of inositol relative to choline (Table 2). Both samples were then thawed, homogenized and fractionated as described in the previous experiment. Cell breakage was less effective in the 47 h sample than in the 0 h sample (and neither batch was disrupted as efficiently as in the experiment described in Table 1). Nevertheless, the ³H/¹⁴C ratio was approximately the same in all fractions (Table 2). This implies that inositol-containing phospholipids are metabolically similar (cf. Gurr, Prottey & Hawthorne, 1965) to those containing choline, each fraction containing stable and unstable phospholipids in the same proportion.

Nature of stable and unstable choline-containing phospholipids. Since the biphasic nature of phospholipid turnover in P815Y cells is not explicable in terms of the passage of label from one part of the

Table 2. *Distribution of radioactivity in the organelles of cells grown in the presence of [Me-¹⁴C]choline and [³H]inositol*

Cells were grown (1.4×10^5 /ml to 4.5×10^5 /ml) in the presence of [Me-¹⁴C]choline (0.4 μ Ci/ml) and [³H]-inositol (5 μ Ci/ml) for 48 h and resuspended (1.4×10^5 /ml) in non-radioactive medium containing unlabelled choline and inositol (each 0.1 mM). One-third of the cells was removed ($t = 0$ h) and frozen. The remainder was grown (to 4.6×10^5 /ml) for a further 47 h ($t = 47$ h). Both batches were disrupted and fractionated as described in the Experimental section.

	Total radioactivity (d.p.m.)				¹⁴ C/ ³ H ratio	
	$t = 0$ h		$t = 47$ h		$t = 0$ h	$t = 47$ h
	³ H	¹⁴ C	³ H	¹⁴ C		
Whole homogenate	26 000	57 000	42 000	130 000	2.2	3.1
Nuclear fraction	8 000	18 000	21 000	70 000	2.2	3.3
Mitochondrial fraction	6 800	17 000	2 000	8 000	2.5	4.0
Microsomal fraction	1 000	2 200	4 800	18 000	2.2	3.8
Supernatant	4 000	5 900	7 200	19 000	1.5	2.6

Table 3. *Distribution of radioactivity in phospholipids of P815Y cells exposed to labelled choline*

Cells were grown (22–49 h) in the presence of [Me-³H]choline (0.8–2.8 μ Ci/ml) or [1,2-¹⁴C]choline (0.4 μ Ci/ml), washed and diluted by resuspension in non-radioactive medium containing unlabelled choline (0.1 mM). A sample of cells was removed ('before turnover') and frozen. Cells were regrown for 31–139 h ('after turnover') and the remainder sampled. Both samples were analysed by t.l.c. as described in the Experimental section. The numbers of experiments are given in parentheses.

	Radioactivity (as % of total recovered after t.l.c.)			
	Before turnover		After turnover	
	[Me- ³ H]Choline	[1,2- ¹⁴ C ₂]Choline	[Me- ³ H]Choline	[1,2- ¹⁴ C ₂]Choline
Lysophosphatidylcholine	2 (3)	} 10 (1)	4 (2)	} 31 (1)
Sphingomyelin	10 (3)		30 (2)	
Phosphatidylcholine	84 (3)		60 (2)	

cell to another, the chemical nature of stable and unstable phospholipid was examined. By using the method of Skipski *et al.* (1964) to separate the major classes of phospholipid, it was found that the percentage of label present in the phosphatidylcholine area of the chromatogram decreases during turnover (Table 3). The distribution of label at the end of turnover suggests that most of the unstable phospholipid is phosphatidylcholine, with stable phospholipid being relatively richer in sphingomyelin. The same result was obtained with [1,2-¹⁴C₂]choline (Table 3) and with [Me-³H]choline (Table 5) and with HC cells. The mean distribution at the end of turnover for several experiments with P815Y cells was 60% in the phosphatidylcholine area, 30% in sphingomyelin and 4% in the lysophosphatidylcholine area. Smith (1969) has found that in rat myelin also, phosphatidylcholine turns over faster than sphingomyelin. The phospholipids of the mitochondrial and surface-membrane fractions obtained from the experiment described in Table 1 were separated by t.l.c. The distribution

of radioactivity is the same as that in whole cells, with phosphatidylcholine accounting for the bulk of unstable phospholipid. The ¹⁴C/³H ratio in phosphatidylcholine reflects that of total phospholipid (Table 1). The ratio in the lysophosphatidylcholine and sphingomyelin fraction on the other hand, is significantly lower in every case (Table 4), indicating a relative enrichment of stable phospholipid.

Nature of turnover: synthesis and degradation versus exchange. It is unlikely that random exchange of choline or inositol units accounts for the turnover of unstable phospholipid, in view of the fact that only some 70% of phosphatidylcholine (Figs. 1 and 2; Table 3) and 80% of phosphatidylinositol (Fig. 2) turns over. Nevertheless, the possibility of exchange was examined as follows. P815Y cells were grown in the presence of [Me-³H]choline and [³³P]phosphate, [Me-³H]choline and [¹⁴C]acetate, [Me-¹⁴C]choline and [³H]glycerol or [³H]inositol and [³³P]phosphate. After 48 h samples were removed and phospholipids were extracted and

Table 4. *Radioisotope ratio in phospholipids of P815Y cells and their organelles grown in the presence of [Me-³H]choline and [Me-¹⁴C]choline*

Samples of the whole homogenate, mitochondrial and surface-membrane fractions obtained in the experiment described in Table 1 were analysed by t.l.c. as described in the Experimental section.

Area on plate	¹⁴ C/ ³ H ratio (batch A)		
	Whole homogenate	Mitochondrial fraction	Surface membranes
Lysophosphatidylcholine + sphingomyelin	1.1	1.3	1.3
Phosphatidylcholine	2.7	2.7	1.8
Area on plate	³ H/ ¹⁴ C ratio (batch B)		
	Whole homogenate	Mitochondrial fraction	Surface membranes
Lysophosphatidylcholine + sphingomyelin	6.3	7.4	3.6
Phosphatidylcholine	19	22	11

Table 5. *Radioisotope ratios in phospholipids of P815Y cells during turnover*

Four batches of cells were grown ($1.4\text{--}1.8 \times 10^5/\text{ml}$ to $3.6\text{--}6.8 \times 10^5/\text{ml}$) in the presence of [*Me*-³H]choline ($2.8 \mu\text{Ci}/\text{ml}$) and [³³P]phosphate ($2.5 \mu\text{Ci}/\text{ml}$), [*Me*-¹⁴C]choline ($0.4 \mu\text{Ci}/\text{ml}$) and [³H]glycerol ($1.0 \mu\text{Ci}/\text{ml}$), [*Me*-³H]choline ($1.0 \mu\text{Ci}/\text{ml}$) and [¹⁴C]acetate ($0.4 \mu\text{Ci}/\text{ml}$) and [³H]inositol ($1.0 \mu\text{Ci}/\text{ml}$) and [³³P]phosphate ($2.0 \mu\text{Ci}/\text{ml}$) for 48 h. Each batch was resuspended in non-radioactive medium containing unlabelled choline, inositol, acetate and glycerol (each at 0.1 mM), a sample removed ('before turnover') and the remainder regrown ($0.8\text{--}1.4 \times 10^5/\text{ml}$ to $1.6\text{--}7.2 \times 10^5/\text{ml}$) for 31–58 h. Both sets of samples were analysed by t.l.c. as described in the Experimental section. Radioactivity left at the origin is not included under 'remainder'.

Area on plate	Radioactivity (as % of total recovered after t.l.c.)				³ H/ ³³ P or ³ H/ ¹⁴ C ratio	
	Before turnover		After turnover		Before turnover	After turnover
	[<i>Me</i> - ³ H]-Choline	[³³ P]-Phosphate	[<i>Me</i> - ³ H]-Choline	[³³ P]-Phosphate		
Lysophosphatidylcholine	3	<1	4	<1	—	—
Sphingomyelin	15	7	33	12	133	130
Phosphatidylcholine	80	42	58	24	124	117
Remainder	<2	47	<2	57	—	—
	[³ H]-Glycerol	[<i>Me</i> - ¹⁴ C]-Choline	[³ H]-Glycerol	[<i>Me</i> - ¹⁴ C]-Choline		
Lysophosphatidylcholine	2	3	13	13	0.54	0.67
Sphingomyelin	12	20	21	38	0.52	0.38
Phosphatidylcholine	50	74	29	33	0.56	0.50
Remainder	35	<2	22	<1	—	—
	[<i>Me</i> - ³ H]-Choline	[¹⁴ C]-Acetate	[<i>Me</i> - ³ H]-Choline	[¹⁴ C]-Acetate		
Lysophosphatidylcholine	8	7	17	13	9	9
Sphingomyelin	29	22	47	30	11	11
Phosphatidylcholine	60	29	27	25	25	11
Remainder	1	38	1	24	—	—
	[³ H]-Inositol	[³³ P]-Phosphate	[³ H]-Inositol	[³³ P]-Phosphate		
Lysophosphatidylcholine	<2	2	<2	1	—	—
Sphingomyelin	<2	10	<2	16	—	—
Phosphatidylcholine	2	38	<2	25	—	—
Phosphatidylinositol	95	33	97	39	6.1	4.3
Remainder	<2	18	<2	16	—	—

fractionated by t.l.c. Cultures were resuspended in non-radioactive medium containing excess of unlabelled compound and reincubated for a further period, at the end of which the remaining culture was treated as before. Cells lost 60% of [*Me*-¹⁴C]-choline, 25–50% of [³³P]phosphate, 60% of [³H]-glycerol, 50% of [¹⁴C]acetate and 70% of [³H]inositol within 30–50 h. Table 5 shows the distribution of radioactivity at the beginning and end of the 'chase' period. The [³H]choline/[³³P]phosphate ratio in phosphatidylcholine (and sphingomyelin) stays constant, despite the fact that approx. 70% of incorporated [³H]choline is lost. Similarly the [¹⁴C]choline/[³H]glycerol ratio remains constant, despite more than 50% turnover. Exchange of choline or phosphorylcholine units is therefore ruled out. The [³H]inositol/[³³P]phosphate ratio falls at most by 30% whereas more than 70% of the incorporated inositol is lost. Hence most of the phosphatidylinositol turns over by a process not involving exchange of inositol units. With [³H]-choline and [¹⁴C]acetate, the ³H/¹⁴C ratio falls to about half the original value in the phosphatidylcholine area, which may reflect a rather more efficient re-utilization of acyl residues, as has been noted with rat liver (Bailey, Taylor & Bartley, 1967; Omura, Siekevitz & Palade, 1967).

DISCUSSION

The loss of labelled choline or inositol from pre-labelled cells appears to be an enzymic process, rather than the removal of labelled compound absorbed on to cells. This is indicated by the fact that for choline (Figs. 1 and 2) the loss takes some 30–40 h to complete (half of the total radioactivity was removed in 25 h; half of the radioactivity of the unstable component alone was removed in 15 h). Moreover, the amount of stable component left is invariably 20–40% of the total, irrespective of the presence of unlabelled compound or of the previous treatment of the cells. Essentially similar results were obtained with inositol. Enzymic exchange of methyl groups in the case of choline is ruled out by the fact that incorporated [*1,2*-¹⁴C₂]choline behaves in the same way as incorporated [*Me*-³H]choline or [*Me*-¹⁴C]choline insofar as turnover and the chemical nature of stable and unstable component (Table 3) is concerned. Enzymic exchange of intact choline or inositol units is ruled out by the experiments (Table 5) discussed above. Hence one must conclude that turnover involves the synthesis and breakdown, perhaps through the action of one or more phospholipases (Dawson, 1966), of the entire phospholipid molecule. The situation is therefore different to that of cultured fibroblasts (Peterson & Rubin, 1969), in which intact phospholipid is released into the growth medium. In the present

case, more than 90% of the radioactivity appearing in the medium from choline- or inositol-labelled cells is water-soluble (upper phase of Folch *et al.* 1957); moreover turnover is detectable whether the medium is changed or not. Release of ¹⁴C- or ³⁵S-labelled material of high molecular weight has been observed with HC cells previously exposed to [¹⁴C]glucosamine or [³⁵S]sulphate (Thomas, 1968).

Turnover of phospholipid during exponential growth of neoplastic mast cells is in contrast with the result of Warren & Glick (1968), who found that the surface membrane of L cells, albeit labelled with [¹⁴C]glucose, [¹⁴C]glucosamine or [¹⁴C]amino acids, does not turn over unless growth is halted. Preliminary results of an experiment with [¹⁴C]-proline-labelled P815Y cells, on the other hand, indicate that during exponential growth membrane protein, like phospholipid, does turn over appreciably. In other words, the present results suggest that membrane turnover, far from indicating cellular inactivity (Warren & Glick, 1968), may be necessary for the very processes of growth and cell division. In exponentially growing bacteria, too, there is a class of phospholipid (phosphatidylglycerol) that turns over, though the major one (phosphatidylethanolamine) is stable (Kanfer & Kennedy, 1963; Ames, 1968).

The exact physiological significance of the turnover described in the present paper is difficult to assess. On the one hand, turnover occurs independently of cell growth (Figs. 1 and 2). In this respect the situation is different from that in cultured lymphocytes, in which stimulation of growth is preceded by an increase in phospholipid turnover (Pasternak & Friedrichs, 1970). Perhaps (Warren, 1969) the turnover rate in neoplastic mast cells is already at a maximum and hence is unresponsive to further metabolic stimulation. Turnover is also continuous throughout the cell cycle, though the amount is greater during the S phase, when synthesis of phospholipid and other molecules is maximal (Bergeron, Warmsley & Pasternak, 1970; Warmsley & Pasternak, 1970). Finally, turnover occurs to an equal extent in all cell organelles (Tables 1 and 2). The latter observation probably reflects rapid equilibration between microsomes and other cell constituents (Wirtz & Zilversmit, 1968, 1969; Beattie, 1969; McMurray & Dawson, 1969). Like these exchange reactions, turnover is decreased at 0°C but is otherwise rather insensitive to environmental variation.

On the other hand, the process is specific in that it appears to be restricted to only a fixed proportion of certain classes of phospholipid such as phosphatidylcholine and phosphatidylinositol (Tables 3, 4 and 5). In other words, P815Y and HC cells contain two types of phospholipid, one of which is somehow susceptible to enzymic degradation and

resynthesis and the other one not. Such a situation is probably not unique to neoplastic cells or to cells grown in culture, since metabolic heterogeneity of one sort or another has frequently been observed in tissues of normal animals (see Pasternak & Friedrichs, 1970). Moreover, the particular lability of phosphatidylcholine compared with sphingomyelin (Tables 3, 4 and 5) is clearly seen in a variety of rat tissues labelled with choline *in vivo* (Pasternak & Friedrichs, 1970).

One may conclude that phospholipid turnover is an integral function of cultured neoplastic mast cells and that it is a prerequisite for, rather than an actual part of, the processes of growth and division.

We are indebted to Dr D. S. Robinson for helpful discussion, to Miss Pamela Allan and Mrs Marilyn Jeeves for expert technical assistance and to the Rhodes Trustees and Worcester College, Oxford (J.J.M.B.) for scholarships.

REFERENCES

- Ames, G. F. (1968). *J. Bact.* **95**, 833.
- Bailey, E., Taylor, C. B. & Bartley, W. (1967). *Biochem. J.* **104**, 1026.
- Beattie, D. S. (1969). *J. Membrane Biol.* **1**, 383.
- Bergeron, J. J. M. (1969). D.Phil. Thesis: Oxford University.
- Bergeron, J. J. M., Warmley, A. M. H. & Pasternak, C. A. (1969). *FEBS Lett.* **4**, 161.
- Bergeron, J. J. M., Warmley, A. M. H. & Pasternak, C. A. (1970). *Biochem. J.* **119**, 489.
- Bezman-Tarcher, A., Otway, S. & Robinson, D. S. (1965). *Proc. R. Soc. B*, **162**, 411.
- Dawson, R. M. C. (1957). *Biol. Rev.* **32**, 188.
- Dawson, R. M. C. (1966). In *Essays in Biochemistry*, vol. 2, p. 69. Ed. by Campbell, P. N. & Greville, G. D. London: Academic Press.
- Eagle, H., Piez, K. A., Fleischman, R. & Oyama, Y. I. (1959). *J. biol. Chem.* **234**, 592.
- Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.
- Gurr, M. I., Prottey, C. & Hawthorne, J. N. (1965). *Biochim. biophys. Acta*, **106**, 357.
- Kanfer, J. & Kennedy, E. P. (1963). *J. biol. Chem.* **238**, 2919.
- McMurray, W. C. & Dawson, R. M. C. (1969). *Biochem. J.* **112**, 91.
- Nagley, P. & Hallinan, T. (1968). *Biochim. biophys. Acta*, **163**, 218.
- Omura, T., Siekevitz, P. & Palade, G. E. (1967). *J. biol. Chem.* **242**, 2389.
- Pasternak, C. A. (1969). *Abstr. FEBS 6th Meet., Madrid*, p. 209.
- Pasternak, C. A. & Friedrichs, B. (1970). *Biochem. J.* **119**, 481.
- Peterson, J. A. & Rubin, H. (1969). *Expl Cell Res.* **58**, 365.
- Plagemann, P. G. W. (1968). *Archs Biochem. Biophys.* **128**, 70.
- Skipski, V. P., Peterson, R. F. & Barclay, M. (1964). *Biochem. J.* **90**, 374.
- Smith, M. E. (1969). *Biochim. biophys. Acta*, **164**, 285.
- Thomas, D. B. (1968). *Biochem. J.* **109**, 79.
- Thomas, D. B. & Pasternak, C. A. (1969). *Biochem. J.* **111**, 407.
- Warmley, A. M. H., Bergeron, J. J. M. & Pasternak, C. A. (1969). *Biochem. J.* **114**, 64P.
- Warmley, A. M. H. & Pasternak, C. A. (1970). *Biochem. J.* **119**, 493.
- Warren, L. (1969). *Curr. Topics devl Biol.* **4**, 197.
- Warren, L. & Glick, M. C. (1968). *J. Cell Biol.* **37**, 729.
- Wheldrake, J. F. & Pasternak, C. A. (1968). *Biochem. J.* **106**, 437.
- Wirtz, K. W. A. & Zilversmit, D. B. (1968). *J. biol. Chem.* **243**, 3596.
- Wirtz, K. W. A. & Zilversmit, D. B. (1969). *Biochim. biophys. Acta*, **193**, 105.