Uptake and metabolism of *myo*-inositol by L1210 leukaemia cells

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The initial rate of uptake of [^3H]*myo*-inositol by L1210 murine leukaemia cells is directly proportional to the extracellular concentration and unaffected by several analogues of *myo*-inositol even at millimolar concentrations. Scyllitol, a geometric isomer of *myo*-inositol, partially inhibited the uptake of *myo*-inositol (40 % at 0.1 mM). A portion of the uptake of *myo*-inositol was not inhibited even at 5 mM-scyllitol. At steady-state the intracellular concentration of [^3H]*myo*-inositol is directly proportional to the extracellular concentration. Addition of *myo*-inositol to medium does not enhance the growth of L1210 cells; these cells can maintain an extracellular concentration of 20 μM -*myo*-inositol even when grown in *myo*-inositol-free medium. Synthesis of *myo*-inositol from glucose by L1210 cells was demonstrated by use of [^{13}C]glucose and m.s. L1210 cells maintain *myo*-inositol pools by a combination of synthesis *de novo* and uptake of exogenous *myo*-inositol by either passive diffusion or a low affinity carrier.

INTRODUCTION

myo-Inositol is an essential nutrient for many mammalian cells growing in culture; deprivation leads to degeneration and 'inositol-less' death for some cells [1–4]. Interestingly, some cells in cultures, even if dependent on its presence in the medium for growth, can synthesize *myo*-inositol from glucose, but apparently not at an adequate rate to permit optimal replication rates [5]. Recently, the role of phosphatidylinositol hydrolysis in the action of growth factors and hormones has been the subject of intense investigation. In these studies of phosphatidylinositol turnover, radiolabelled *myo*-inositol is frequently added to the cultures to provide a tracer for measurement of various metabolites. Despite this widespread use, however, many details of the uptake, metabolism and growth-stimulatory effects of *myo*-inositol remain unclear.

Because *myo*-inositol is highly water-soluble, several investigators have examined the transport of this compound in order to determine whether or not it enters cells by diffusion, facilitated transport or an active transport system. Biden & Wollheim [6] studied *myo*-inositol uptake in rat pancreatic islets and observed concentrative, saturable uptake with an EC_{50} of 75 μM . This concentrative transport of *myo*-inositol may be linked to the concentration gradient of Na^+ across the cell membrane, because marked reductions in the rate of uptake were seen in Na^+ -free medium and on addition of ouabain [6]. Similarly, the uptake of *myo*-inositol into rabbit lung slices and membrane vesicles from mammalian kidney is in part mediated by a saturable, Na^+ -linked carrier [7–9]. In contrast, in isolated rat hepatocytes, rat skeletal muscles and a number of cultured cell lines, the uptake of *myo*-inositol was not saturable [10–13], although in some instances there appeared to be both a high-affinity/low-capacity carrier and non-saturable uptake. The characteristics of *myo*-inositol uptake vary markedly among the various cells and tissues examined.

The current study examines the uptake and metabolism of *myo*-inositol by L1210 leukaemia cells. To probe the specificity of the uptake and metabolism of these

compounds we have employed a newly synthesized series of *myo*-inositol analogues. We also have measured the intracellular concentration of *myo*-inositol under a number of conditions by g.c.-m.s. and have demonstrated the *de novo* synthesis of *myo*-inositol from glucose by these cells.

MATERIALS AND METHODS

Scyllitol (*scyllo*-inositol) was purchased from Calbiochem (San Diego, CA, U.S.A.). D-[1,2,3,4,5,6- ^{13}C]-Glucose (2.5% $^{13}\text{C}_4$, 5.5% $^{13}\text{C}_5$, 94% $^{13}\text{C}_6$) was purchased from MSD Isotopes (Rahway, NJ, U.S.A.). Acetonitrile and *N*-trimethylsilylimidazole were from Pierce Chemical Co. (Rockford, IL, U.S.A.). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Amersham Co. supplied [^3H]*myo*-inositol at 14.2 Ci/mmol. The *myo*-inositol analogues were synthesized by C. Jiang and David C. Baker at the University of Alabama [14].

L1210 cells were purchased from ATCC (Rockville, MD, U.S.A.) and cultured in Fischer's medium (Gibco) with 10 % horse serum in an atmosphere of 5 % CO_2 in air. Cell number was determined with a Coulter counter. The *myo*-inositol-free and glucose-free Fischer's media were prepared by the NIH media unit. Dialysed horse serum was prepared by dialysis in membrane tubing of 12000 to 14000 M_r cut-off against 20 vol. of 0.9 % NaCl at 4 °C for two consecutive 24 h periods with one change of diffusate and continuous stirring. This dialysis removed > 99 % of added [^3H]*myo*-inositol when we evaluated this procedure by adding 10 μCi [^3H]*myo*-inositol to a 100 ml portion of serum prior to dialysis.

Analysis of *myo*-inositol by g.c.-m.s.

Cell extracts were prepared for g.c.-m.s. analysis after adding scyllitol (1.0 nmol, 10 μl of 100 μM) as an internal standard. The sample was then desalted by loading on a column of 0.15 ml of Dowex 50 (50–100 mesh, hydrogen form) over 0.15 ml of Dowex AG1X2 (acetate form, 300–400 mesh) in a pipette and eluted with 1.5 ml of water. The eluted samples were lyophilized prior to g.c.-

m.s. analysis. Studies with radiolabelled nucleotides and amino acids indicated that charged species were $\geq 95\%$ removed by this procedure, with $89 \pm 12\%$ (mean $\pm$ S.D.) recovery of *myo*-inositol. Standards of 1.0 nmol of scyllitol and 20–0.01 nmol of *myo*-inositol were processed in parallel for construction of a calibration curve. *myo*-Inositol and scyllitol were analysed by g.c.-m.s. as their hexa-trimethylsilyl derivatives, which were formed by adding 20 μ l of acetonitrile and 20 μ l of *N*-trimethylsilyl-imidazole to the eluted samples and heating at 80 °C for 30 min. The derivatized sample (1–2 μ l) was injected into a split/splitless injector operated in the split mode with a ratio of 10:1. Separation of the compound was achieved on a 15 m $\times$ 0.2 mm SE 54 capillary column (Supelco Inc., Bellefonte, PA, U.S.A.) held at 200 °C. Helium at 30 cm/s was used as the carrier gas. Under these conditions, the retention times of hexa-trimethylsilyl-scyllitol and hexa-trimethylsilyl-*myo*-inositol were 4 and 5 min, respectively. A Nermag R10-10-C mass spectrometer (Delsi Instruments, Fairfield, NJ, U.S.A.) equipped with a Vector/one workstation (Teknivent Corp., St. Louis, MO, U.S.A.) for data reduction was operated in the electron impact mode. Prominent ion fragments at m/z 305 and m/z 318, observed for both compounds [15], were monitored and the integrated ion current ratios m/z 305 (*myo*-inositol) to m/z 305 (scyllitol), and m/z 318 (*myo*-inositol) to m/z 318 (scyllitol) were used for quantification of *myo*-inositol. Analysis of control L1210 cell extracts revealed no detectable scyllitol.

The electron impact mass spectrum of *myo*-inositol contained an ion fragment at m/z 507 as observed by Sherman [15]. This ion fragment corresponds to the loss of $(\text{CH}_3)_3\text{SiOH}$ and CH_3^+ from the molecular ion observed at m/z 612. Since all six carbon atoms of the *myo*-inositol ring are present in the ion fragment at m/z 507, the ^{13}C isotopic content was determined after monitoring the ion cluster at m/z 507–514. Incorporation of ^{13}C derived from [1,2,3,4,5,6- ^{13}C]glucose into *myo*-inositol was determined by the method of Biemann [16].

Uptake of [^3H]*myo*-inositol

L1210 cells at $(2\text{--}10) \times 10^5$ cells/ml were collected by centrifugation, washed twice with inositol-free Fischer's medium and resuspended in the same medium at 2×10^7 cells/ml. Versilube F50 silicone oil, 0.5 ml (Read Plastics, Rockville, MD, U.S.A.) was placed in a 1.5 ml conical tube and 0.5 ml of inositol-free Fischer's medium layered over the oil. The tubes were centrifuged briefly in an Eppendorf 5414 centrifuge to sharpen the layer interface. The L1210 cells were incubated at 37 °C with [^3H]*myo*-inositol at the indicated concentration. The L1210 cell suspension was sampled at the indicated times by removing 0.5 ml and placing it in the upper layer of the tubes containing oil and centrifuging at 10000 g for 10 s. The cell pellet was isolated by washing the top of the oil layer twice with 1 ml of medium and then aspirating the oil from the tube. The acid-soluble fraction, containing unmetabolized *myo*-inositol, was obtained by adding 1.0 ml of 0.3 M-perchloric acid to the cell pellet, mixing well with a vortex mixer and removing the precipitated material by centrifugation. The acid insoluble material contained the cellular phospholipids and this was dissolved in 1.0 ml of 0.3 M-KOH prior to determination of radioactivity by scintillation

spectrometry. All determinations of radioactivity were performed in ACS scintillation cocktail (Amersham).

Intracellular volume was calculated by pre-equilibrating the cell suspension with a known concentration of $^3\text{H}_2\text{O}$ and corrected for extracellular volume by the use of [^{14}C]inulin as described [23].

Thin layer chromatography

Phospholipids were chromatographed on silica H plates (Analtech Co., Newark, DE, U.S.A.) with a solvent of chloroform/methanol/20% methylamine, 30:18:5 (v/v) [17], and on silica G plates with a solvent of chloroform/methanol/glacial acetic acid/0.9% NaCl, 100:50:16:5 (v/v) [18]. The acid-soluble extracts were chromatographed on cellulose plates with a solvent of acetone/water, 4:1 (v/v) and on silica plates with a solvent of butanol/acetic acid/water, 2:1:1 (v/v) [19]. In each instance the plates were cut into 1 cm strips and the solid phase was scraped into a scintillation vial for measurement of radiolabel by counting in 10 ml of ACS scintillation cocktail. The position of the standards on the plates was determined by use of radiolabelled compounds.

RESULTS AND DISCUSSION

Influx and incorporation of [^3H]*myo*-inositol

The accumulation of free [^3H]*myo*-inositol and incorporation into cellular phospholipid was examined by use of a method which separates cells from the medium by centrifugation through a layer of oil (Fig. 1), thereby avoiding the possible efflux during a washing step. Through the range of concentrations of *myo*-inositol examined the concentration of intracellular *myo*-inositol reached an apparent steady state within 4 h, whereas incorporation of radiolabel into phospholipid continued in a nearly linear fashion throughout the 4 h of incubation. The concentration of intracellular unmetabolized *myo*-inositol obtained at steady-state was proportional to the external concentration. Calculation of intracellular concentration at 4 h of incubation from the data in Fig. 1 and the intracellular volume, measured as described under Materials and methods, indicated that the intracellular concentration of unmetabolized [^3H]-*myo*-inositol derived from the medium was 7000 μM and 14 μM at a medium concentration of 5000 μM and 10 μM respectively. Thus over a concentration range of 1000 in extracellular *myo*-inositol concentration, the intracellular concentration was only slightly higher than the extracellular. This discrepancy may be accounted for by intracellular binding or by errors in measurement. It is clear, however, that *myo*-inositol is not highly concentrated intracellularly by an active transport in these cells. No saturation of initial rates of *myo*-inositol uptake was observed over a concentration range of 0.01–5 mM-external *myo*-inositol (Fig. 2). The rate observed was directly proportional to external concentration over this range. These results are compatible with entry of *myo*-inositol by either passive diffusion or by a facilitated transport of low affinity ($K_m > 5$ mM).

The identities of the radiolabelled compounds in the aqueous and lipid phases of L1210 extracts prepared as shown in Fig. 1 were examined by chromatography in several thin-layer systems as described under Materials and methods. The radiolabel in the lipid phase was

Fig. 1. Uptake of exogenous $[^3\text{H}]\text{myo-inositol}$ by L1210 cells

The accumulation of radiolabel into acid-soluble pools ($\blacktriangle$) and phospholipids ($\bullet$) was determined as described under Materials and methods. The results are the average of duplicate determinations which agreed within 10%. The values in nmol are calculated based on the specific activity of the added *myo*-inositol. The results here are representative of those observed in three independent experiments. The concentration of *myo*-inositol in the medium was: a, 5 mM; b, 1 mM; c, 0.1 mM; d, 0.01 mM.

Fig. 2. Initial rate of uptake of exogenous *myo*-inositol by L1210 cells

The total amount of radiolabel from *myo*-inositol accumulated both unmetabolized and as phospholipid in the first 20 min of incubation as in Fig. 1 is plotted versus the external concentration. The values are the average of two determinations within 10% agreement and are representative of three independent experiments.

> 92% phosphatidylinositol, and radioactivity in the aqueous phase was > 85% *myo*-inositol, with < 5% of the radiolabel found in inositol phosphates as determined by h.p.l.c. [20].

Efflux of $[^3\text{H}]\text{myo-inositol}$

L1210 cells prelabelled with $[^3\text{H}]\text{myo-inositol}$ released > 80% of the water-soluble radioactivity to the medium within 3 h (Fig. 3). The radiolabelled phospholipid increased only slightly over this period. This experiment shows that the uptake of *myo*-inositol is rapidly reversible

in L1210 cells. In contrast, C-6 glioma cells released < 10% of their internal *myo*-inositol in a 60 min chase [8].

Specificity of $[^3\text{H}]\text{myo-inositol}$ uptake

The effect of three inositol analogues on the uptake of *myo*-inositol and its incorporation into phospholipid was measured by incubation of L1210 cells with the analogues at 1 or 5 mM (Table 1). These analogues represent only very minor structural modifications of the parent compound. None of the analogues decreased the uptake of *myo*-inositol into the water-soluble pools, but each reduced incorporation into phospholipid. In two additional experiments, three other analogues, 5-bromo-5-deoxy-*myo*-inositol, 5-chloro-5-deoxy-*neo*-inositol and 4-deoxy-4-fluoro-*epi*-inositol produced < 10% inhibition of $[^3\text{H}]\text{myo-inositol}$ uptake into the acid-soluble pool when examined as described for Table 1 (data not shown). These results support the conclusion that either passive diffusion or a low-affinity carrier is responsible for uptake in L1210 cells, as these analogues are very similar in structure to *myo*-inositol and would probably compete for binding to a high-affinity transport site. The reduction in incorporation of $[^3\text{H}]\text{myo-inositol}$ into phospholipid produced by the *myo*-inositol analogues of Table 1 probably reflects a competition for phosphatidylinositol synthase [21].

scyllo-Inositol was an inhibitor of the uptake of *myo*-inositol by L1210 cells at concentrations as low as 0.01 mM (Fig. 4). The concentration dependence of this effect suggested that a fraction (about 60%) of the uptake was by a process inhibited by *scyllo*-inositol with an IC_{50} of < 0.05 mM, whereas the remaining uptake was

Fig. 3. Efflux of [^3H]myo-inositol from L1210 cells

L1210 cells at 5×10^6 cells/ml were incubated for 2 h with 1 mM-[^3H]myo-inositol ($5 \mu\text{Ci/ml}$), immediately placed on ice and concentrated to 1×10^7 cells/ml in fresh cold medium. The L1210 cell suspension was incubated at 37°C and sampled at the indicated times. The amount of radiolabel released into the medium (○), present in acid-soluble pools (△) and in phospholipids (●) was determined as described under Materials and methods. The value in nmol is calculated on the basis of the specific activity of the myo-inositol added for the labelling period.

not greatly reduced. Thus, 0.1 mM-scyllitol reduced uptake of [^3H]myo-inositol into the soluble pool by 40 %, and a 50-fold increase in scyllitol only increased the inhibition to 55 % of control.

The results of the uptake studies indicate that in L1210 cells myo-inositol uptake occurs by a combination of two processes. A facilitated process with low affinity for myo-inositol and a greater affinity for scyllitol and a second process, which may be simple diffusion or a facilitated uptake with poor affinity for both scyllitol and myo-inositol, may be involved. Yorek, Dunlap & Ginsberg have observed a similar dual uptake mechanism in four cultured cell lines [12], but did not examine the effect of scyllo-inositol.

Effect of myo-inositol on L1210 cell replication

L1210 cells grow in Fischer's medium with 5 % dialysed horse serum, but without myo-inositol, at nearly the same rate as in medium containing 1.5 mg of myo-inositol/l as in the original formulation. Thus, in three independent experiments the doubling time of control cells in medium with 1.5 mg of myo-inositol/l was 15 ± 1 h, and for cells in medium without myo-inositol,

Table 1. Effect of myo-inositol analogues on uptake and incorporation of [^3H]myo-inositol by L1210 cells

L1210 cells at 2×10^7 cells/ml were incubated at 37°C in Fischer's medium without myo-inositol with [^3H]myo-inositol ($5 \mu\text{Ci/ml}$, $1 \mu\text{M}$) for 2 h with the addition noted. Incubation was in a CO_2/air (1:19) atmosphere. The cells were isolated by centrifugation through oil and intracellular [^3H]myo-inositol and [^3H]phosphatidyl-inositol measured as described under Materials and methods. Each value is the mean of duplicates which agreed within 10 %.

	$10^{-3} \times$ myo-Inositol uptake (c.p.m./ 10^7 cells)	
	Acid-soluble	Lipid
Saline	6.8	39
5-Deoxy-5-fluoro-myoinositol (1 mM)	7.7	26
5-Deoxy-5-fluoro-myoinositol (5 mM)	8.2	14
5-Deoxy-5-fluoro-neo-inositol (1 mM)	8.9	37
5-Deoxy-5-fluoro-neo-inositol (5 mM)	6.3	19
5-Deoxy-myoinositol (1 mM)	6.8	32
5-Deoxy-myoinositol (5 mM)	6.1	17

Fig. 4. Inhibition of [^3H]myo-inositol uptake by L1210 cells by scyllitol

L1210 cells (2×10^7 cells/ml) in inositol-free Fischer's medium, were incubated for 40 min with [^3H]myo-inositol ($1 \mu\text{M}$, $5 \mu\text{Ci/ml}$) and the indicated concentrations of scyllitol. The accumulation of the radiolabel into acid-soluble pools and phospholipids was determined as described under Materials and methods. The percentage inhibition of the radiolabel into acid-soluble pools (○) and phospholipids (●) is plotted versus the concentration of scyllitol. The results are the averages of triplicate determinations which agreed within 10 %, observed in two independent experiments.

16 ± 1 h, mean $\pm$ S.D. In addition, cells were passaged three consecutive times in myo-inositol-free medium containing 5 % dialysed horse serum with an expansion from 10 000 cells/ml to $\sim 300\,000$ cells/ml each passage.

Table 2. Intracellular myo-inositol in L1210 cells

L1210 cells (0.2×10^6 cells/ml) were grown in myo-inositol-free Fischer's medium with 5% dialysed horse serum and the indicated myo-inositol concentration for 24 h, then collected by centrifugation and extracted with 0.7 ml of 5% trichloroacetic acid. myo-Inositol was measured by g.c.-m.s. as described under Materials and methods. Intracellular concentration was calculated from intracellular water volume of $0.61 \text{ ml}/10^9$ cells. Mean $\pm$ S.D., $n = 3-5$ independent cultures, except n is 10 for $0 \mu\text{M}$ medium myo-inositol.

Treatment	myo-Inositol (μM)		myo-Inositol (nmol/ 10^9 cells)
	Medium	Intra- cellular	
None	0.0	20	12 ± 3.6
None	8.5	67	41 ± 15.0
None	50.0	390	236 ± 20.0
LiCl, 10 mM/24 h	0.0	13	7.8 ± 0.6

Thus, this represents a 27000-fold expansion of cell population in the absence of exogenous myo-inositol.

Although a very low concentration of myo-inositol contributed from the dialysed serum or released by hydrolysis of phosphatidylinositol present in serum cannot be excluded, it is clear that the inositol requirements of L1210 cells must be low and that supplementation of medium with myo-inositol is not required for optimal growth. In this regard L1210 cells resemble murine 3T3 or Chinese hamster ovary (CHO) cells [1,3]. The ability of L1210 cells to grow without added myo-inositol prompted measurement by g.c.-m.s. of intracellular myo-inositol concentrations and *de novo* synthesis of myo-inositol.

Measurement of intracellular myo-inositol

The content of myo-inositol in L1210 cells grown in myo-inositol-free medium was $12 \text{ nmol}/10^9$ cells (Table 2), equivalent to an intracellular concentration of $20 \mu\text{M}$ free myo-inositol. Supplementation of medium with myo-inositol resulted in higher intracellular concentrations, whereas incubation of cells in myo-inositol-free medium with LiCl (10 mM), an inhibitor of myo-inositol formation [22] resulted in lower intracellular concentrations of myo-inositol.

The ability of the L1210 cells to grow and maintain myo-inositol pools in myo-inositol-free medium suggested that these cells were capable of synthesis of myo-inositol from glucose. To examine this point, L1210 cells were grown in myo-inositol-free and glucose-free Fischer's medium supplemented with 1 g of $[1,2,3,4,5,6-^{13}\text{C}]\text{glucose}/\text{l}$ for 24 or 48 h. Cell extracts were prepared as described for Table 2 and the isotopic content of glucose-derived ^{13}C in myo-inositol from these incubations was determined by g.c.-m.s. Measurements of the ion fragment cluster at m/z 507 to m/z 513 revealed that 54–73% of the myo-inositol present after 24 h of growth in $[1,2,3,4,5,6-^{13}\text{C}]\text{glucose}$ was synthesized *de novo* (i.e., was derived from the labelled glucose) and by 48 h 90–100% of the myo-inositol present was derived from the labelled glucose. Thus, L1210 cells are able to

maintain pools of myo-inositol adequate for rapid replication by synthesis from glucose. This technique should permit analysis of the relative contribution of *de novo* synthesis from glucose and utilization of myo-inositol from the medium to the maintenance of cellular myo-inositol pools.

These results have several implications for the use of $[^3\text{H}]\text{myo-inositol}$ in the study of phosphoinositide metabolism. Firstly, a steady-state intracellular concentration of $[^3\text{H}]\text{myo-inositol}$ is not obtained for several hours of incubation (Fig. 1) and thus, rates of uptake may influence rates of incorporation into phospholipids in a short pulse experiment. Secondly, the intracellular concentration of myo-inositol can be significantly altered by culture conditions (Table 1). Finally, some cells in culture can maintain myo-inositol concentrations by *de novo* synthesis from glucose. Therefore the specific activity of intracellular $[^3\text{H}]\text{myo-inositol}$ available for phospholipid synthesis may be dependent on factors that alter *de novo* synthesis (possibly glucose concentration) as well as on the specific activity and concentration of myo-inositol added to the growth medium. The ready reversibility of myo-inositol uptake also suggests that cells may contribute myo-inositol synthesized *de novo* to the growth medium, although this was not shown directly.

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