Aberrant ^3H labelling of ATP during *in vivo* labelling of Ehrlich mouse ascites tumour cells with $[2\text{-}^3\text{H}]\text{inositol}$ is significant in the study of isomers of InsP_3 and InsP_4

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Neutralized perchloric acid extracts of intra-abdominally proliferating Ehrlich mouse ascites-tumour cells harvested after 24 h exposure to $[2\text{-}^3\text{H}]\text{inositol}$ were analysed by Mono Q HR5/5 anion-exchange h.p.l.c. using an ammonium formate/phosphoric acid gradient or by ambient-pressure small-column anion-exchange chromatography (Bio-Rad AG 1-X8, 200–400 mesh). The results showed that cellular ATP contained aberrant ^3H label in excess of ^3H in the isomers of InsP_3 and InsP_4 . The

putative ATP ^3H radioactivity showed: (i) h.p.l.c. run time as the material causing the largest A_{254} peak traced, (ii) precise spiking with ATP and $[^{14}\text{C}]\text{ATP}$ and (iii) specific absorption to charcoal. Moreover, enzymic conversion of ATP into ADP changed putative ATP ^3H into putative ADP ^3H . In addition, aberrant ^3H labelling of cellular ADP and GTP was detected, although at a lower level.

INTRODUCTION

This report shows that, after whole-animal labelling with $[2\text{-}^3\text{H}]\text{inositol}$, ATP may become labelled and thereby interfere with analysis of *in vivo* labelling of inositol phosphates. ATP is present in cells in millimolar concentrations, whereas inositol phosphates are present at micromolar concentration or even less. From this it is evident that aberrant labelling of ATP may become significant even before the molar specific radioactivity of ATP has risen to 1/1000th of that for, say, InsP_3 or InsP_4 isomers.

In mammalian inositol catabolism, the major, perhaps exclusive, pathway starts by oxidative opening of the inositol ring between carbon atoms 1 and 6, catalysed by inositol oxidase, followed by removal of C-1 in a decarboxylation reaction and then by reactions leading to D-xylulose 5-phosphate. D-Xylulose 5-phosphate can then be transformed via the pentose phosphate interconversions to intermediates of glycolysis, and thence to glucose and other products. In D-xylulose 5-phosphate, C-5 is the former C-2 from inositol with its hydrogen atom retained, as demonstrated using ^3H (Charollais and Posternak, 1965). This short survey, also based on the work of Hanks et al. (1969), indicates that, in cells labelled with $[2\text{-}^3\text{H}]\text{inositol}$, existing metabolic pathways, in particular those leading to ribose, might result in labelling of ATP, with preservation of the bond originally present between ^3H and C-2 of inositol. More widespread labelling has been demonstrated by Fliesler et al. (1985), who reported that both glycerol and fatty acids were labelled in frogs 1 week after injection of $[2\text{-}^3\text{H}]\text{inositol}$ into the circulation. In mammals, at least 90% of the oxidative opening of the inositol ring occurs in the kidney (Weinhold and Anderson, 1967). Therefore aberrant ^3H labelling of ATP during *in vivo* labelling of mice with $[2\text{-}^3\text{H}]\text{inositol}$ perhaps depends on the passage of labelled inositol into the blood and hence to the glomerular filtrate from which it is reabsorbed by tubular kidney cells.

EXPERIMENTAL

Materials

All chemicals were of analytical grade. Phytic acid (as InsP_6 sodium salt), Triton X-100, tri-*n*-octylamine and Freon were from Sigma, St. Louis, MO, U.S.A. BSA (fraction V from Sigma) was extensively dialysed against water. Perchloric acid (60%) and disodium EDTA were from Merck, Darmstadt, Germany. AMP, ADP, ATP, GTP and UTP were from Boehringer, Mannheim, Germany. *myo*- $[2\text{-}^3\text{H}]\text{inositol}$, $[2\text{-}^3\text{H}]\text{Ins}1\text{P}$, $[2\text{-}^3\text{H}]\text{Ins}(1,4)\text{P}_2$, $[2\text{-}^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ and $[8\text{-}^{14}\text{C}]\text{ATP}$ were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. $[1\text{-}^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ and $[1\text{-}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ were from NEN, Boston, MA, U.S.A. Hionic-Fluor (LSC-cocktail) was from Packard, Downers Grove, IL, U.S.A. ^3H -labelled *GroPInsP* and *GroPInsP*₂ were obtained from the $[2\text{-}^3\text{H}]\text{inositol}$ -labelled Ehrlich cells, essentially as described by Creba et al. (1983), the formic acid and ammonium formate being removed from the appropriate eluate fractions by lyophilization overnight. Activated acid-washed charcoal was Norit A from Serva, Heidelberg, Germany. Cellular ATP was converted into ADP by using hexokinase (Sigma H 5375). ATP was measured by means of an ATP kit (Sigma).

In vivo labelling of cells with $[2\text{-}^3\text{H}]\text{inositol}$

Six days after intra-abdominal inoculation of Ehrlich ascites-tumour cells into white female mice (Pan:NMRI), the proliferating cells were exposed to 300 or 500 μCi of *myo*- $[2\text{-}^3\text{H}]\text{inositol}$ (code TRK 911) using abdominal wall puncture. After 24 h, the cells were harvested into a buffered mammalian Ringer solution (Hoffman et al., 1986), gently sedimented, washed once and then resuspended in the Ringer solution, enriched with 1% BSA. The cytocrit value was 3–8% of total volume. The cell suspension was then preincubated at 37 °C for 30 min in a water bath with

Abbreviation used: *GroPInsP*₂, glycerophosphoinositol 4,5-bisphosphate.

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gentle mechanical shaking before sampling was started from magnetically stirred portions (4–10 ml) of the cell suspension, still at 37 °C.

Perchloric acid extraction of cell suspensions

Samples (0.5 ml) of the cell suspension were transferred to 1.5 ml Microfuge tubes containing 0.5 ml of ice-cold 1.0 M perchloric acid, 0.2% Triton X-100 and partially hydrolysed InsP_6 (Wreggett et al., 1987) corresponding to about 20 μg of total P (an amount that represents nmol quantities of any of the six groups of inositol phosphate isomers produced by hydrolysis) and immediately vortex-mixed. Three additional mixes were performed over a 20 min period, and the ice-cooled tubes were then spun for 1 min at 20000 g at 4 °C to obtain the perchloric acid extract, 800 μl of which was mixed in a 2 ml Microfuge tube with 100 μl of 36 mM disodium EDTA. The mixture was then neutralized with 600 μl of tri-*n*-octylamine/Freon (1:1, v/v) as described by Sharpes and McCarl (1982). The upper phase obtained had a pH value of 5.8–6.0 and an estimated ionic strength of about 0.08 (due to cells, Ringer solution and 4 mM EDTA). A 700 μl portion of this upper phase (the neutralized extract) was stored in Microfuge tubes at –20 °C for subsequent separation of inositol phosphates. Each 700 μl sample represents from 10 to 25 μl cell volume, and 10 to 25 pmol of isomer of cellular origin present at 1 μM in the cells.

Chromatography

H.p.l.c. separation

Pharmacia Mono Q HR 5/5 f.p.l.c. anion-exchange column, two LKB 2150 pumps and a 2152 controller were used. Elution was with 10 mM ammonium formate as buffer A, and 1.8 M ammonium formate adjusted to pH 3.7 with phosphoric acid (Irvine et al., 1985) as buffer B. The gradient break points, given as run time (min) over percentage of buffer B, were: 0/0, 5/0, 8/9, 23/16, 25/22, 38/22, 50/40, 70/100, 90/100. During elution the absorbance at 254 nm (A_{254}) was continuously recorded. The fractions collected measured 0.375 ml.

Ambient-pressure anion-exchange chromatography

Small-column separation of inositol phosphates into groups of equal degree of phosphorylation (Berridge et al., 1983) was on 0.75 ml columns of Bio-Rad AG 1-X8 resin (200–400 mesh). In our version the method was extended so as to follow separation also for the InsP_5 isomers from InsP_6 (Christensen and Harbak, 1990), even at flow rates of about 0.5 ml per min.

Measurement of radioactivity in the fractions collected was performed after addition of Hionic-Fluor. To the low-salt eluate fractions (nos. 1–19) from the small ambient-pressure columns was added 0.3 ml of 6 M ammonium formate solution in order to obtain a clear stable fluid phase after mixing with 16.5 ml of the LSC-cocktail. The counting efficiency changed only slightly at the stepwise change in ammonium formate concentration during elution, with correction factors of between 0.95 and 1.05, as evaluated by internal standardization using [^3H]inositol. These factors were taken into account in the conversion of c.p.m. into d.p.m. Where appropriate, a preset $^3\text{H}/^{14}\text{C}$ liquid-scintillation program was used with correction for any spill-over between channels.

Removal of nucleotides by adsorption to charcoal

The procedure for adsorption of nucleotides to charcoal used by

Mayr (1988), and further discussed by Pittet et al. (1989), was adopted with minor modifications. The activated charcoal (Norit A) was, as recommended, boiled in 3 M HCl for 2 h to reduce putative anion-exchange properties, washed to neutrality and dried. Before use, the purified Norit A was resuspended at 5% (w/v) in 0.1 M NaCl/0.05 M sodium acetate, adjusted with 1 M HCl to pH 5. The 700 μl volume of neutralized perchloric acid cell extract was transferred to a disposable glass tube (75 mm $\times$ 10 mm), and, after addition of 56 μl of the 5% (w/v) suspension of Norit A (final concn. 4 mg/ml), the tubes were covered with parafilm and the contents vortex-mixed six times (although three times seems to give identical results) with 3 min intervals at room temperature. The charcoal was finally sedimented at 1400 g for 15 min whereupon 650 μl of the completely clear supernatant was removed for chromatography.

Control experiments with [^{14}C]ATP added to neutralized perchloric acid cell extracts or to solutions designed to mimic

Figure 1 (a) A neutralized perchloric acid extract of Ehrlich cells labelled *in vivo* with [$2\text{-}^3\text{H}$]inositol analysed by h.p.l.c. using a Mono Q HR5/5 anion-exchange column and an ammonium formate/phosphoric acid gradient and (b) duplicate sample of the pooled cell extract depleted of nucleotides by charcoal treatment

The left ordinate gives the ^3H radioactivity (●) in the 0.375 ml fractions collected. Note that InsP_5 d.p.m. values have been reduced 5-fold. A_{254} was continuously traced but is only marked by the symbol (○) indicating peak maxima for the only two deflections recorded (right ordinate). Run time for unresolved isomers of InsP and for AMP was about 11 min, and for InsP_2 and ADP about 16 min. However, the early part of the chromatogram has not been further investigated. Peak identification was helped by spiking with authentic ^3H -labelled inositol phosphate standards, ^{14}C -labelled ATP and authentic ATP and GTP in parallel runs. Two independent experiments were performed with similar results. Both the material causing the A_{254} peaks (ATP and GTP) and the co-eluted ^3H radioactivity are absent after charcoal treatment (b).

neutralized cell extracts (see below) showed that, after the charcoal treatment, ATP was, for our purposes, reduced to insignificant levels (about 1%). Therefore, the charcoal treatment was not extended and is less rigorous than that used by Mayr (1988).

Recovery of ^3H -labelled inositol phosphate standards in supernatants after treatment with charcoal was measured under conditions identical with those in neutralized cell extracts with regard to EDTA, phytic acid hydrolysate and ATP, and at various ionic strengths and pH values. This was achieved by adding the three components to 0.1 M NaCl/0.05 M sodium acetate buffer (pH 5), variously diluted with water to create differences in ionic strength, or with different pH values produced by the addition of 1 M HCl or 1 M NaOH. Supernatants from charcoal-treated and untreated samples were then analysed by ambient-pressure chromatography. The inositol phosphate standards showed negligible adsorption to charcoal at ionic strengths ranging from 0.02 to 0.18 (at pH between 5 and 6), and also at pH values ranging from 4 to 6.9 (at ionic strength 0.08). The measured recoveries are included in Table 1 (column 6). Figure 1 shows that charcoal treatment of neutralized cell extract did not significantly influence the ^3H radioactivity ascribed to InsP_5 . In the case of InsP_6 , data obtained by ambient-pressure chromatography also indicate essentially complete recovery in the supernatant over sedimented charcoal (results not shown).

Conversion of ATP into ADP by hexokinase treatment

To neutralized perchloric acid cell extracts (containing 4 mM EDTA, 0.5 mM Ca^{2+} and 0.5 mM Mg^{2+}) was added 5 mM MgSO_4 , 1 mM EGTA, 2 mM glucose and 11 mM Tris base to a final pH of 7.5. One half of the extracts then received 0.5 unit/ml hexokinase (where one unit phosphorylates 1 μmol of D-glucose/min at pH 7.6 and 25 °C), and the other half served as control. After incubation for 12 min at room temperature, the extracts were stored at -20 °C until they were processed for ambient-pressure chromatography. From the eluted fractions, a 0.8 ml portion was transferred to a quartz cuvette with 1 cm light path for determination of A_{259} . For purine nucleotides a reading of 0.015 would correspond to 1 nmol/ml of eluate analysed. The volumes were 4 and 5 ml for the fractions containing ADP and ATP respectively, and these volumes were taken into account when calculating the amount of ATP converted into ADP during hexokinase treatment. Measurements of ATP concentration confirmed that it had been effectively removed by the hexokinase treatment (residual concentration less than 2%).

RESULTS

An h.p.l.c. profile for neutralized perchloric acid extracts of $[2\text{-}^3\text{H}]$ inositol-labelled cells is shown in Figure 1(a). The chromatogram shows a major peak of ^3H radioactivity co-eluted with the material causing the dominant A_{254} peak, suggesting that ATP had become ^3H -labelled. In separate runs, this peak was precisely spiked when $[8\text{-}^{14}\text{C}]$ ATP or authentic unlabelled ATP had been added to the column load (results not shown). The chromatogram in Figure 1(b) was obtained for a duplicate sample which had been exposed to charcoal. Essentially all the material causing the A_{254} peak and the co-eluted ^3H radioactivity shown in Figure 1(a) is now absent. These findings indicate that the ^3H -labelled material is ATP.

Another candidate for the ^3H radioactivity could be $[^3\text{H}]\text{GroPInsP}_2$, as this compound was found to be eluted slightly

before, but to a great extent overlapping with, unlabelled ATP, when these substances were loaded together for h.p.l.c. (results not shown). However, charcoal was found not to adsorb

Figure 2 Localization of $[8\text{-}^{14}\text{C}]$ ATP relative to inositol phosphate standards during elution from small ambient-pressure anion-exchange columns

The four commercial labelled standards were loaded in trace amounts on to Bio-Rad AG 1-X8 (200–400 mesh) columns, as additions to an NaCl solution with the same ionic strength as in cell samples. The boxes at the top indicate concentrations of ammonium formate/formic acid in the three eluents. Two columns were identically loaded and the average values obtained for each of the two radiolabels are presented as a histogram. The bars indicate half the difference between the duplicate determinations. Note that, under these conditions, ATP is co-eluted with InsP_2 , whereas it is eluted between InsP_2 and InsP_3 isomers during elution from the Mono Q h.p.l.c. column (see Figure 1). $\text{Ins}(1,4,5)\text{P}_3$ was the InsP_3 isomer used.

Table 1 Effect of nucleotide removal by charcoal treatment on apparent content of ^3H -labelled inositol phosphates extracted from Ehrlich cells labelled *in vivo* with $[2\text{-}^3\text{H}]$ inositol

Four samples of pooled cell extract were analysed independently (two as controls and two after charcoal treatment). Analysis was by ambient-pressure anion-exchange chromatography. For the cell extract, note the reduction in ^3H radioactivity in solution after adsorption of nucleotides to charcoal. This reduction applies in particular to fractions 25–29 in which both InsP_2 and ATP are eluted, and to a smaller extent also to fractions 20–24 in which InsP_3 and ADP are eluted. The ^3H -labelled inositol phosphate standards (column 6) were added to solutions designed to mimic the composition of the neutralized perchloric acid cell extract (see the Experimental section on removal of nucleotides by adsorption to charcoal). For InsP_3 , the $\text{Ins}(1,4,5)\text{P}_3$ isomer was used. Recovery values (means $\pm$ S.E.M.) for InsP_1 , GroPInsP and GroPInsP_2 were $98.0 \pm 0.4\%$ ($n = 4$), $96.3 \pm 2.8\%$ ($n = 4$) and $97.0 \pm 4.3\%$ ($n = 4$) respectively. Values in parentheses are numbers of determinations.

Eluate fraction no.	InsP _n group eluted	Cell extract			Recovery of InsP _n standards in solution after charcoal (%)
		Controls (d.p.m.)	In solution after charcoal		
			(d.p.m.)	(%)	
15–19	InsP ₂	812,838	789,762	97.91	96.5 ± 2.1 (4)
20–24	InsP ₃	210,208	190,170	90.82	99.3 ± 0.6 (8)
25–29	InsP ₄	387,345	142,142	37.41	99.6 ± 1.3 (8)
30–45	InsP ₅₋₆	4946,5115	5085,4974	103.97	

Figure 3 Effect of conversion of ATP into ADP by hexokinase treatment of neutralized perchloric acid extracts of Ehrlich cells labelled *in vivo* with [2-³H]inositol

Ehrlich cells were labelled for 24 h by intra-abdominal injection of [2-³H]inositol. Neutralized cell extracts (○) and extracts treated with hexokinase after addition of glucose (●) were passed through small ambient-pressure anion-exchange columns. Eluate fractions were analysed for A_{259} (a) and ³H radioactivity (b). The volumes were 3, 4 and 5 ml for the fractions containing AMP, ADP and ATP respectively. The arrows indicate the peak absorbance of authentic AMP, ADP, ATP, UTP and GTP as observed in independent runs. A parallel experiment gave similar results. The data show conversion of ATP into ADP, and a concomitant transfer of ³H radioactivity from eluate fractions containing ATP (and InsP_4) to those containing ADP (and InsP_3).

GroPI InsP_2 , as shown by ambient-pressure chromatography (see the legend to Table 1), and h.p.l.c. (results not shown).

Figure 1(a) shows an additional small ³H and A_{259} peak which is also eliminated by charcoal treatment. This peak is co-eluted with authentic GTP (not illustrated) and is taken to represent ³H-labelled GTP. With respect to both ³H and A_{254} , GTP showed values about one-eighth of those found for the putative ³H-labelled cellular ATP. Comparing Figures 1(a) and 1(b), it may

be noted that cellular InsP_3 isomers, InsP_4 , and InsP_5 are all essentially unaffected by the charcoal treatment.

Figure 2 shows an elution sequence obtained by ambient-pressure anion-exchange chromatography. Under these conditions ATP is co-eluted with InsP_4 . This position relative to the inositol phosphates is quite different from the position between InsP_2 and InsP_3 isomers obtained from ATP by h.p.l.c. using Mono Q columns (see Figure 1). Such coelution of ³H-labelled ATP and InsP_4 may result in an overestimate of the cell content of InsP_4 during an analysis of cell extract.

Table 1 shows a case in which at most 40 % of the cellular ³H eluted in the appropriate fractions for InsP_4 could be taken as 'true' labelling of InsP_4 , as 60 % of it was removed by charcoal treatment, which leaves InsP_4 in solution to 100 % (see Table 1, column 6). Still with reference to Table 1, an analogous treatment of values related to cellular InsP_3 (known to be co-eluted with ADP from the ambient-pressure columns) showed that at most 80–90 % of the total ³H eluted in the appropriate fractions was 'true' labelling of InsP_3 . With reference to Figure 2, it should be added that labelled ATP may contribute slightly to the 10–20 % overestimate caused by labelled nucleotides eluted with InsP_3 . The overestimate for InsP_4 was 224 d.p.m. and that for InsP_3 29 d.p.m. (see Table 1). The 29/224 ratio is consistent with the cellular ADP content being much lower than the ATP content, as expected and as directly observed from the continuous A_{254} trace during h.p.l.c. (not illustrated) and also from the u.v.-absorbance data presented in Figure 3(a), allowing for the different volumes of the eluate fractions (see the legend to Figure 3). For eluates containing InsP_2 and InsP_{5-6} , the radioactivities measured without resorting to charcoal treatment of the cell extract could apparently be taken as 'true' values (see Table 1, column 5).

Finally, we treated duplicate samples from a pool of neutralized perchloric acid extracts of [2-³H]inositol-labelled cells with hexokinase and glucose in order to convert ATP into ADP. The purpose was to look for the putative displacement of ³H radioactivity from eluates containing ATP to those containing ADP. Analysis was by ambient-pressure chromatography. The results are presented in Figure 3 and show extensive dephosphorylation of ATP to ADP, concomitant with a transfer of ³H radioactivity to fractions containing ADP (and InsP_3) from those containing ATP (and InsP_4), leaving only 30 % of ³H radioactivity in the latter fractions as a more reliable estimate of InsP_4 . This estimate agrees reasonably well with the value of 40 % reported in Table 1 for cells from another experiment. Taking the volume of the fractions into account (4 and 5 ml for ADP and ATP respectively), the estimated values are 87 nmol of ATP (1504 d.p.m.) is that converted into 97 nmol of ADP (1555 d.p.m.). In a parallel experiment, the values estimated are 98 nmol of ATP (1489 d.p.m.) converted into 89 nmol of ADP (1449 d.p.m.). The estimated amount of ATP converted into ADP agrees well with the expected content of ATP in a 20–25 μl volume of proliferating Ehrlich cells, ascribed a 4–5 mM concentration of ATP (Grune et al., 1992), and is consistent with the essentially complete conversion of ATP during hexokinase treatment (see the Experimental section).

DISCUSSION

We report that, after 24 h of *in vivo* labelling of proliferating Ehrlich ascites tumour cells with [2-³H]inositol, the ³H label from inositol can become incorporated into ATP in amounts high enough to interfere significantly with the analysis of cellular inositol phosphates. This was illustrated for the Ehrlich cell when, in an extract of quiescent cells, 1500 d.p.m. of ³H label was

found in ATP, 150 d.p.m. in $\text{Ins}(1,3,4)\text{P}_3$, 500 d.p.m. in $\text{Ins}(1,4,5)\text{P}_3$ and about 200 d.p.m. in InsP_4 (S. Christensen, H. Harbak and L. O. Simonsen, unpublished work).

The analytical interference caused by aberrant ^3H labelling of ATP depends on the chromatographic procedure chosen. Thus, with ambient-pressure small-column anion-exchange chromatography, as used in the present study, ATP is co-eluted with InsP_4 (Figure 2). However, with h.p.l.c. analysis on a Partisil SAX-10 column eluted with an ammonium formate/phosphoric acid gradient, ATP may be eluted very close to $\text{Ins}(1,3,4)\text{P}_3$ (Irvine et al., 1985). With the use of a Mono Q HR5/5 column and an ammonium formate/phosphoric acid gradient, ATP is apparently eluted completely separated from any inositol phosphate (see Figure 1), but would interfere with the analysis of any GroPInsP_2 present. Moreover the small nucleotide peak immediately following $\text{Ins}(1,4,5)\text{P}_3$, and ascribed to [^3H]GTP, could interfere with $\text{Ins}(1,4,5)\text{P}_3$. ADP, which on ambient-pressure chromatography is coeluted with InsP_3 , also carried aberrant ^3H label, but at a much lower level than ATP (see Table 1). AMP is presumably present at a much lower concentration than ATP, particularly in Ehrlich cells in the proliferating phase (Grune et al. 1992). The fairly large absorbance peak recorded in Figure 3(a) for 3 ml fractions known to contain AMP is presumably a composite, with contributions from several minor components absorbing at 259 nm (De Korte et al. 1985). Analytical interference from labelled AMP with unresolved isomers of InsP (Figure 3b) is presumably at the 2–3 % level, but other labelled material (not only nucleotides) may add to the interference. For non-physiological conditions, this quantitative reasoning may be misleading because of possibly considerable deviations from the suggested AMP/ADP/ATP ratios.

In the quiescent cell, interference from aberrant ^3H -labelling will lead to an overestimate of an inositol phosphate coeluted with a labelled nucleotide. Further, such coelution will reduce the observed fractional increase after agonist stimulation of inositol phosphate metabolism. It may be added that aberrant ^3H radioactivity in ATP (and GTP) seems to remain constant during agonist-stimulated inositol phosphate metabolism (S. Christensen, H. Harbak and L. O. Simonsen, unpublished work).

The use of charcoal as described in the present paper is an easy, effective and specific solution to the problem of aberrant ^3H -labelling of nucleotides. The use of phenyl boronate-agarose columns for removal of nucleotides (Ishii et al., 1986) may conceivably be an alternative. The conversion of ATP into ADP by treatment with hexokinase and glucose may help to reveal the analytical problem.

In the intact mammal, the kidneys play a dominant role in inositol catabolism, which starts by oxidative opening of the inositol ring. After whole-animal labelling with [$2\text{-}^3\text{H}$]inositol,

one might therefore expect Ehrlich cells, proliferating in the abdominal cavity, to be exposed to labelled carbon chains in excess of what they may themselves produce (see also the Introduction). The above proposal would imply that most cells grown in culture during even prolonged labelling with [$2\text{-}^3\text{H}$]inositol would suffer less interference of the analysis of inositol phosphates from labelled nucleotides than reported here for the Ehrlich cell after *in vivo* labelling. One might, however, speculate that some kidney cell cultures might show a very pronounced interference.

The position of the ^3H label in the ATP molecule is not known, but C-5 in the ribose moiety is a likely possibility (see the Introduction). If the ^3H label were attached to C-1 of inositol instead of C-2, it might be expected to dissipate in the organism on the oxidative opening of the inositol ring between C-1 and C-6 and the associated removal of C-1 in a decarboxylation reaction. Therefore the use of inositol labelled at C-1 might cause fewer problems for the analysis of cellular inositol phosphates.

Thanks are due to I. Somer (August Krogh Institute), K. Kandborg and B. P. Beck (Institute of Physiology, University of Aarhus) for skilled technical assistance, to Dr. H. Nielsen (Institute of Medical Biochemistry, University of Aarhus) for instructive discussions, to Dr. J. Jensen and Dr. J. P. Andersen (both of the Institute of Physiology, University of Aarhus) for purified [$8\text{-}^{14}\text{C}$]ATP and for help with h.p.l.c. respectively. Financial support was obtained from the Research Foundation of the Aarhus University and from the Danish Natural Sciences Research Council (grant no. 11-9000-3). Ing. T. Nørby is thanked for expert computational work.

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