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# Distribution of $^3$H within purine nucleotides of Ehrlich mouse ascites tumour cells after intraabdominal injection of *myo*-[2-$^3$H]inositol

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## SUMMARY

In Ehrlich mouse ascites tumour cells, exposed intra-abdominally to [2-$^3$H]inositol, ATP and GTP presented enough aberrant $^3$H-label to cause potential interference in the chromatographic analysis of inositol phosphates involved in signal transduction. After acid extraction and charcoal adsorption/desorption the nucleotides were dephosphorylated, enriched with [U-$^{14}$C]adenosine, and exposed to purine-nucleoside specific enzymes. Reverse phase HPLC and radioactivity measurement demonstrated that for adenosine about 82% of total stable $^3$H label was in ribose and thus about 18% in adenine. For guanosine about 89% was in ribose and 11% in guanine. This aberrant $^3$H labelling could be avoided using [1-$^3$H]inositol.

*Key words: Aberrant $^3$H. Nucleotides. [2-$^3$H]Inositol. Cell signalling. Ehrlich cell.*

## INTRODUCTION

Labelling of cells with [2-$^3$H]inositol *in vivo* or *in vitro* is often used in studies of inositol-$P_3$ and inositol-$P_4$ isomers that are assigned key roles in intracellular signalling [1,2]. Following intra-abdominal injection of [2-$^3$H]inositol to host mice carrying an intra-abdominally multiplying culture of Ehrlich mouse ascites tumour cells it was previously found that the nucleotides in acid extracts of the tumour cells were stably labelled with aberrant $^3$H; in particular ATP and GTP presented analytically significant contaminants to the above-mentioned isomers present as [2-$^3$H]labelled compounds [3]. Based on purine nucleoside-specific enzymatic cleavages we here report that such aberrant stable $^3$H-label in ATP and GTP is located primarily in ribose but found also in the respective base components, adenine and guanine.

The basis for this aberrant $^3$H labelling is the opening of the inositol ring-structure through the action of *myo*-inositol oxygenase. The D-glucuronate formed is further metabolized such that the C-1 from inositol is removed in an early decarboxylation. The remaining five-membered carbon chain is passed to the metabolism of pentoses with carbon atoms 2 through 6 renamed as 5 through 1, meaning that the $^3$H at C-2 in inositol is found in *e.g.* ribose, as $^3$H at C-5. It is an important feature, in

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mammals at least, that the opening of the *myo*-inositol ring-structure is 98-100% dependent on intact kidney function [4-9].

## RESULTS AND DISCUSSION

In Fig. 1, showing reverse phase HPLC data for cell Expt. I, the upper panel (Control) shows $^{3}$H-peaks at known run times for adenosine and guanosine, the former in exact co-elution with marker [U-$^{14}$C]adenosine. Guanosine is further identified (middle panel) through its removal (143 DPM) when exposed to purine-nucleoside phosphorylase (NP). As guanine carried only $^{3}$H at trace level, most of the 143 DPM in guanosine would be found in ribose as reflected in a significant increment to the early eluting $^{3}$H-peak. In Expts. II and III guanosine came out at 156 and 292 DPM, guanine at 18 and 35 DPM, and ribose thus at 138 and 257 DPM,respectively, and, also here, with reasonably consistent increments to the early peak. Overall-values would be that 89-90% of $^{3}$H in guanosine was in ribose and 11-12% in guanine.

As to adenosine, the lower panel is for material incubated with adenosine deaminase (ADA) and next with NP: At the expense of both guanosine and adenosine an increment of 906 DPM is given to the early eluting $^{3}$H-peak. About 122 of the 906 DPM would represent $^{3}$H-ribose from guanosine and 784 DPM accordingly the $^{3}$H-ribose from adenosine. The total of 906 DPM co-eluted with the marker $^{14}$C-ribose-1P derived from [U-$^{14}$C]adenosine. The marker $^{14}$C-hypoxanthine from [U-$^{14}$C]adenosine is accompanied by $^{3}$H ascribable mainly to hypoxanthine, with a few DPM to guanine which co-elutes. $^{3}$H and $^{14}$C peaks with run time corresponding to inosine (deaminated adenosine) indicated that the NP reaction had not gone to completion, such that only 88% of adenosine contributed to the labelled hypoxanthine and ribose-1P. These results are all as predicted for guanosine and adenosine, since both guanosine and inosine, but not adenosine, are substrates for purine-nucleoside specific phosphorylase (NP).

Table 1 gives molar specific activities for adenosine calculated for Expts. I, II and III. An average of about 18% of adenosine $^{3}$H was in adenine (analyzed as hypoxanthine), and thus by subtraction 82% per cent in the ribose moiety of the molecule (column *a*). In column *b* the three values for ribose are based on the $^{3}$H-increments to the early eluting peak caused by ribose from adenosine: In Expt.I, 784 DPM corresponding to 15.6 DPM/nanomole. In Expts. II and III, the increments were 786 and 1704 DPM, respectively, corresponding to 13.5 and 18.8 DPM/nanomole. The overall average becomes 16.0 DPM/nanomole indicating that 81% of $^{3}$H in adenosine was found in ribose. The approaches *a* and *b* thus showed reasonable consistency.

Based on the present results it seems justifiable to conclude that Ehrlich cells, labelled intra-abdominally for 24 h with [2-$^{3}$H]inositol, do contain stably bound $^{3}$H in both the ribose and purine base moieties of ATP and GTP. It may be noted in this context that the average value for DPM/(nanomole adenosine) in Table 1 is 19.7, *i.e.* not far from the 16.2 DPM/nmole which can be derived from the discussion of Fig. 3 in [3].

The catabolism of inositol (*Introduction*) may result in the incorporation of $^{3}$H-labelled C-5 of ribose-5-phosphate into nucleotides either by *de novo* synthesis or by salvage pathway reactions. In the purine ring stably bound $^{3}$H would be covalently

 

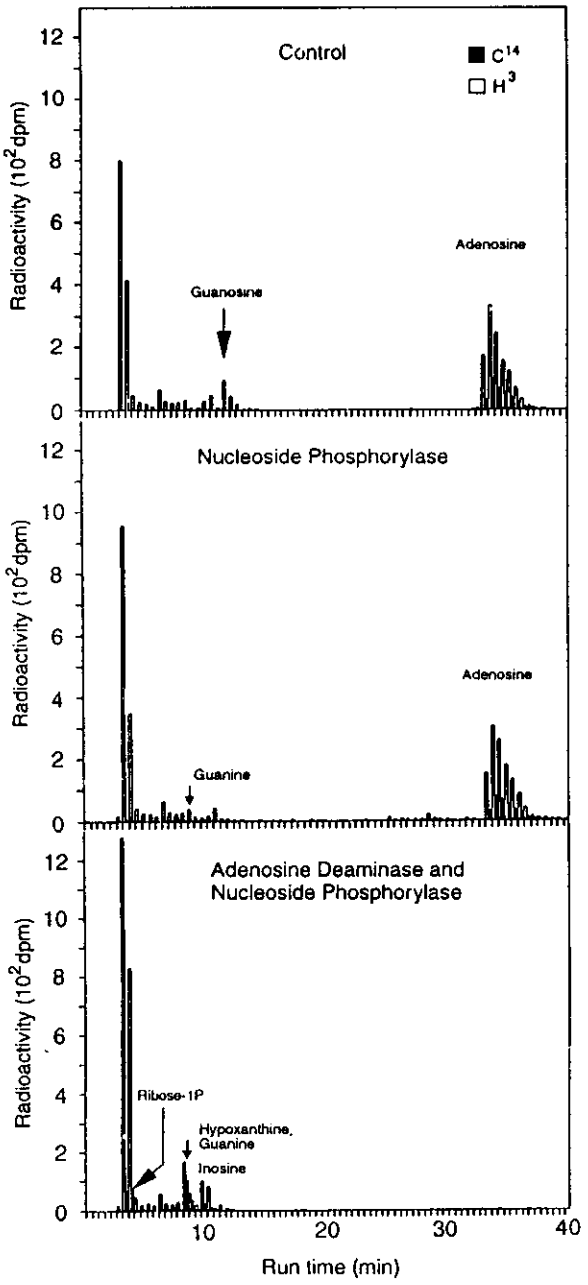


Figure 1 Cells labelled in mouse abdominal cavity with [2-$^3$H]inositol were saved and extracted at 0.5 M TCA. Nucleotides were removed from the extract with charcoal, eluted, and dephosphorylated. The nucleosides formed were enriched with [U-$^{14}$C]adenosine and two of three sub-volumes treated enzymatically as indicated in the panels, before reverse phase HPLC.

**Table 1 Aberrant $^3H$ in adenosine: Distribution after enzymatic breakage of the N-glycosidic bond**
$^3H$ radioactivities in adenosine from Ehrlich cell experiments I, II and III. The amounts of adenosine analyzed per panel were 57, 66 and 103 nmoles in Expts. I, II and III, respectively. Since some inosine escaped NP action, only about 88% of the adenosine delivered the $^3H$ of hypoxanthine and ribose.

| Cell expt. | Specific $^3H$ radioactivities (DPM/nanomole) | | | |
|---|---|---|---|---|
| | Adenosine | Hypoxanthine | Ribose-1-phosphate | |
| | | | *a**) | *b***) |
| I | 20.7 | 3.5 | 17.2 | 15.6 |
| II | 19.9 | 3.7 | 16.2 | 13.5 |
| III | 18.5 | 3.5 | 15.0 | 18.8 |
| Average of I, II and III | 19,7 | 3,6 | 16.1 | 16.0 |
| Percentage distribution | 100% | 18% | 82% | - |

a*) Obtained by subtraction of values for hypoxanthine (deaminated adenine) from values for adenosine (column 2).
b**) Obtained from the increment of $^3H$ radioactivity at run time interval for $^{14}C$-ribose-1P simultaneously formed.

attached to C-2 and C-8 in adenine and to C-8 in guanine. The C-$^3H$ group would probably only be incorporated into the purine ring in connection with *de novo* synthesis and would originate from the beta-position of serine with tetrahydrofolic acid derivatives as metabolic intermediates. The C-3, C-4 and C-5 of ribose-5-phosphate may be metabolized to serine in such a way that the C-5 of ribose becomes the beta-carbon of serine.

Additional Remarks: *(1)* Rat kidney slices were able to catabolize [2-$^{14}C$]inositol over glucuronate to $^{14}CO_2$ [4]. Therefore, problems with aberrant $^3H$ of the type described here might perhaps appear for *in vitro* cultures of those kidney cells which do express the *myo*-inositol oxygenase.

*(2)* In the metabolism of inositol, the C-1 is removed in an early decarboxylation (see *Introduction*). This means that any significant aberrant $^3H$-labelling derived from metabolism of inositol could be reduced (avoided?) if it were possible to work with an [1-$^3H$]inositol as an alternative to the [2-$^3H$]inositol commonly used.

## CONCLUSION

Labelling *in vivo* of proliferating cells with [2-$^3H$]inositol results in significant aberrant $^3H$-labelling of both purine base and ribose moiety of adenine and guanine nucleotides. This aberrant $^3H$ is presumably derived from kidney metabolism of the [2-$^3H$]inositol, and it is large enough to cause significant interference in the chromatographic analysis of co-extracted labelled inositol-$P_3$ and inositol-$P_4$ isomers, since these show cellular concentrations that are orders of magnitude lower than the concentrations of ATP and GTP.

## EXPERIMENTAL

### Materials

All chemicals were of analytical grade. NP (purine-nucleoside:ortho-phosphate ribosyltransferase, *abbrev.*: Nucleoside Phosphorylase), ADA (adenosine-amino-hydrolase, *abbrev.*: Adenosine Deaminase) and alkaline phosphatase were purchased from Boehringer (Mannheim, Germany). [U-$^{14}$C]adenosine and [2-$^3$H]*myo*-inositol (with PT6-271 stabilizer, inositol concn. 56 $\mu$M and 1 mCi/ml) were from Amersham, U.K. Norit A charcoal (Serva, Heidelberg, Germany) was refluxed with 3 N HCl as in [3,10].

### Cellular nucleotides

Intra-abdominal cultures of Ehrlich mouse ascites tumour cells (ELT strain) were labelled with [2-$^3$H]*myo*-inositol by intra-abdominal injection of 0.4, 0.9 and 0.5 mCi for expt. I, II, III, respectively; the mice were alert throughout 24 hours before harvesting and washes of cells as in [3,11,12]. Samples of the cell suspension (250-300 $\mu$l cell volume) were extracted at 0.5 M TCA (Expt.I) or PCA (Expts. II and III) during frequent shaking, for about 20 min. The insoluble material was sedimented, at 1400 *g* x 10 min, and 9 ml of a clear supernate saved for subsequent repeated shaking with 200-250 mg of charcoal. By sedimentation at 1100 *g* x 10 min the nucleotides were recovered as adsorbed material. Carrier material was not added. After seven 10-ml washes with distilled water the charcoal was resuspended in 1-2 ml of distilled water and positioned as an upper layer on a short 1-ml column of water-saturated Whatman cellulose powder. The nucleotides were then eluted with water/ethanol/25% ammonia (3:5:2 by vol.). The effluent was collected in eight volumes of about 1000 $\mu$l, from which 20 $\mu$l sub-volumes were removed to establish an elution pattern for the $^3$H-labelled material. The main volumes were dried during vacuum-centrifugation and the remaining material dissolved and made into one volume, using for this four 200 $\mu$l volumes of a 5 mM $MgCl_2$, 200 mM $KH_2PO_4$ buffer (pH 7.5). Alkaline phosphatase was added, at 10 $\mu$g/ml, and the pH raised to 9.5. The resulting dephosphorylation of nucleotides to nucleosides was followed at room temperature over 16 hours by $A_{254}$ tracing during SAX HPLC runs, and then terminated by a rise in temperature to 65 °C, held for 10 min.

### Ribose, adenine and guanine (detection strategy)

The nucleosides were supplemented with 1-2 nCi of [U-$^{14}$C]adenosine as marker compound during reverse phase HPLC chromatography such as illustrated by Fig.1 for Expt. I. The column loads were of 100 $\mu$l (200 $\mu$l in Expt. III). One load (top panel) was analyzed before, and one load (middle panel) after treatment with nucleoside phosphorylase (NP), 18 $\mu$g/ml for 15 min at room temp., phosphorolyzing guanosine to yield ribose-1P (ribose-1 phosphate) and guanine. A third load (lower panel) was analyzed after treatment with adenosine deaminase (ADA), at 18 $\mu$g/ml for 15 min at room temperature, and a subsequent treatment with NP (as above). Each enzymatic treatment was terminated at 65 °C. In addition to guanine and ribose-1P from guanosine the treatment with NP resulted in hypoxanthine plus ribose-1P from inosine (deaminated adenosine). [U-$^{14}$C]ribose-1P was collected within the fourth run time minute. It held, as did [U-$^{14}$C]hypoxanthine, 5 of the 10 uniformly labelled C atoms in [U-$^{14}$C]adenosine molecules.

**Chromatography and radioactivity registration**

Dephosphorylation of nucleotides was followed by Partisil-10 SAX HPLC. A 2 mm d x 200 mm (Whatman, Clifton, NJ, U.S.A.) column was eluted for 3 min with 0.005 M sodium phosphate (pH 3.5) followed by a gradient for 14 min to 0.400 M sodium phosphate, 0.800 M NaCl (pH 3.5); an isocratic period followed for 19 min, before equilibration with starting buffer.

Reverse phase HPLC was used with a RP-Select B, LiChrosorb (5 $\mu$m), 4 mm d x 250 mm column, run isocratically using 0.15 M potassium phosphate (pH 5.0) mixed with methanol (1%), acetonitrile (1%) and tetrahydrofuran (0.2% by vol.). $A_{254}$ was recorded for all runs, and the peaks automatically integrated based on relevant calibrations with authentic compounds. Flow rate was 0.7 ml/min. Eighty consecutive 0.35-ml eluate portions were collected and mixed with 3 ml of Ultima-Gold (Packard) as scintillant before evaluation in a PACKARD, TRICARB-2500 TR analyzer with use of a preset $^{3}H/^{14}C$ program, tested for accuracy by means of mixtures of [2-$^{3}$H]inositol and $^{14}$C-adenosine at appropriately low DPM levels. The above experimental techniques were based on references [13,14].

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