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STABILITY OF THE TRITIUM LABEL IN DIFFERENT POSITIONS OF THE PYRIDOXOL MOLECULE DURING ITS METABOLISM IN RATS

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Abstract—The stability of the tritium label of pyridoxol during metabolism has been studied in rats following the injection of pyridoxol labelled by the Wilzbach and an exchange procedure. During the metabolism of pyridoxol to pyridoxic acid a 10 per cent loss of tritium occurred if the pyridoxol was labelled by an exchange procedure and 60 per cent if labelled by the Wilzbach method. The latter was regarded unsuitable for metabolic studies. Results from rat urine experiments show the tritium to be stable in both types of labelled pyridoxol, unless the molecule is structurally altered during its metabolism. Tritium lost during metabolism of pyridoxol was incorporated into body water, some of which was excreted in urine. The presence of possible new metabolites of pyridoxol has been detected in rat urine.

CERTAIN aspects of the metabolism of pyridoxol in human pregnancy would be easier to study if a labelled molecule was used. Tritium label is regarded as more acceptable than Carbon-14, but it is also notorious for its instability under different working conditions; for example, the label may be stable when tested by a variety of organic reactions but "metabolically" unstable when used in *in vivo* experiments. The use of such a compound could lead to erroneous results and conclusions. Therefore, before starting a study in humans, we decided to test two different types of tritiated pyridoxol that were commercially available for their "metabolic" stability so that the results could be evaluated on a quantitative basis. Since we were able to obtain pyridoxol labelled with ^{14}C in a stable position, we used the ratio of tritium to Carbon-14 as an index for providing stability data during the metabolism of double-labelled pyridoxol in rats.

MATERIALS AND METHODS

^{3}H-Pyridoxol was obtained from the Radiochemical Centre, (Amersham, Bucks., England).

(i) Batch 8 (specific activity 64 mc/mM) prepared by the Wilzbach[1] technique; radiochemical purity > 95 per cent.

(ii) Batch 10 (specific activity 2·23 c/mM prepared by an exchange procedure: radiochemical purity > 99 per cent. Twenty per cent of the label is in the C'_4 and C'_5 positions of the pyridine ring.

^{14}C pyridoxol (di-carbinol-$^{14}C_2$; specific activity 11 mc/mM) was obtained from Hoffman la Roche, Basle, Switzerland.

4-pyridoxic acid was prepared by the method of Heyl.[2]

PURITY OF LABELLED COMPOUNDS

The purity of ^{3}H and ^{14}C-POL was investigated by thin-layer chromatography on cellulose using two different solvent systems. The compounds were detected by scintillation autography (Contractor and Shane),[3] radiochromatogram scanning (Packard Inst. Co.), fluorescence, and by Gibb's reagent.

Standard solutions of the labelled compounds were subjected to the same chromatographic procedures as described for urine in this paper.

ANIMALS AND URINE COLLECTION

Male Wistar rats (225–250 g), injected intraperitoneally with 0·3 ml saline solution containing in the case of (a) batch 8: ^{14}C (3·29 μc) — + ^{3}H (27·6 μc) — POL, (b) batch 10: ^{14}C (4·66 μc) — + ^{3}H (56·0 μc) — POL were placed in metabolic cages fitted with urine–faeces separators and 24-hr urines collected. Urines plus washings were filtered, lyophilised and the residues dissolved in sufficient 0·33 N acetic acid to adjust pH to 3·3–3·5. An aliquot of each sample was separated into fractions on a phosphocellulose column as described by Contractor and Shane.[4] Fraction I contained 4-pyridoxic acid (PIC), pyridoxol phosphate (POLP), and pyridoxal phosphate (PALP) and fraction II contained pyridoxal (PAL) and pyridoxol (POL).

Salts and aliphatic compounds were removed from these two fractions by active charcoal adsorption of the B_6 compounds at pH 3, which were eluted with 60% ethanol in 0·1 N ammonia. The eluates were evaporated to dryness and the residues chromatographed on DEAE cellulose (fraction I) and phosphocellulose (fraction II) to separate the individual B_6 compounds.

ISOTOPE DETERMINATION

Radioactive assays were carried out using a liquid scintillation spectrometer (Tri-carb, Packard Inst. Co.). 0·1-ml aliquots were added to a scintillator mixture containing methanol (4 ml) and 0·4% PPO and 0·01% dimethyl POPOP (scintillator grade, Packard Inst. Co.) in toluene (10 ml).

The instrument was set to provide maximum efficiency of ^{14}C in channel 1 and tritium in channel 2. Discriminators were set to cut out tritium in channel 1 and to give the maximum ^{3}H:^{14}C ratio in channel 2. Absolute efficiencies of the two isotopes were determined for each channel by an external standard count in channel 3. Approximate values in the mixture described above for ^{14}C and tritium were respectively, 22 and 0·01 per cent in channel 1, and 6 and 5 per cent in channel 2. The standard error in the ^{3}H:^{14}C ratio after conversion to disintegrations/min was less than 5 per cent.

RESULTS

Thin-layer chromatography of the ^{3}H and ^{14}C POL standards did not reveal any impurities. Ion-exchange chromatography of double isotope mixtures of the labelled POL standards revealed impurities in the electronegative "fraction I". Table 1 shows the nature and amount of these impurities in a mixture of ^{3}H (Batch 10) and ^{14}C-POL and compares the isotope ratios of the impurities and purified POL to that of the original unchromatographed standard solution. Similar impurities were found when a mixture of ^{3}H (Batch 8) and ^{14}C-POL was chromatographed.

TABLE 1. ISOTOPE RATIOS OF STANDARD SOLUTION OF ^{3}H (BATCH 10, EXCHANGE PREPARATION)—AND ^{14}C-POL BEFORE AND AFTER CHROMATOGRAPHIC PROCEDURES

Fraction	^{14}C disintegrations/min $\times 10^{-5}$	Recovery (%)	^{3}H disintegrations/min $\times 10^{-5}$	Recovery (%)	$^{3}H/^{14}C$	^{3}H Label (% of original label)
Standard	64·4	100	741	100	11·5	100
Standard:						
Freeze-dried	63·9	99	717	97	11·2	97
Fraction I	0·37	0·6	41·0	5·5	111	965
Fraction II	63·0	98	674	91	10·7	93
DEAE column						
Peak 1	0·21	0·3	20·2	2·7	111	965
Peak 2 (PIC)	0	0	0		—	—
Peak 3	0·04	0·1	14·2	1·9	392	3410
Peak 4	0·04	0·1	2·10	0·3	64	557
Peak 5	0·07	0·1	4·43	0·6	71	617
Phosphocellulose column						
POL	61·6	96	653	88	10·6	92

Recoveries from rat urine and the stability of the tritium label in B_6 metabolites following an injection of ^{3}H (Batch 8) and ^{14}C-POL are shown in Table 2. The elution pattern of the more acidic metabolites (fraction I) are shown in Fig. 1. Corresponding results for a mixture of ^{3}H (Batch 10) and ^{14}C-POL are shown in Table 3. The isotopic ratios determined for POL, PAL and PIC (Tables 2 and 3) were found to be the same after thin-layer chromatography.

TABLE 2. 24-hr RECOVERIES AND ISOTOPIC RATIOS OF B_6-METABOLITES FROM RAT URINE AFTER INJECTION OF ^{3}H (BATCH 8; WILZBACH PREPARATION)—AND ^{14}C-POL (122 μg)

Fraction	^{14}C disintegrations/min $\times 10^{-5}$	Recovery (%) injected dose	Recovery (%) urine content	^{3}H disintegrations/min $\times 10^{-5}$	Recovery (%) injected dose	Recovery (%) urine content	$^{3}H/^{14}C$	^{3}H label retained (%)
Injected dose	73·0	100	—	613	100	—	8·40	100
Urine	32·1	44	100	223	36	100	6·94	83
Urine:								
freeze-dried	31·8	44	99	150	24	67	4·72	56
Fraction I	24·3	33	76	105	17	47	4·33	52
Fraction II	4·26	6	13	29·0	5	13	6·81	81
DEAE column								
Peak 1	3·92	5·6	12·2	—	—	—	6·26	75
Peak 2 (PIC)	14·6	21	45·6	—	—	—	3·42	41
Peak 3 (POLP)	2·70	3·9	8·4	—	—	—	8·13	97
Peak 4 (PALP)	2·18	3·2	6·8	—	—	—	5·15	61
Peak 5	0·96	1·4	3·0	—	—	—	1·69	20
Phosphocellulose column								
PAL	2·50	3·6	7·8	—	—	—	6·82	81
POL	1·67	2·4	5·2	—	—	—	8·70	104

The ordinate of Fig. 1 is adjusted to give a ^{3}H:^{14}C ratio of unity for the original POL solution so that any loss in the tritium label of the metabolites can be easily recognised.

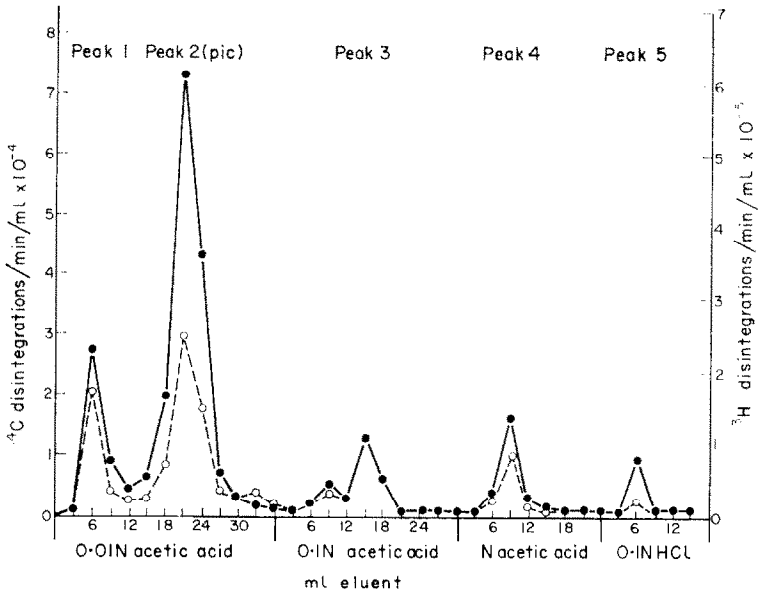

FIG. 1. Elution pattern from DEAE cellulose column of B_6-metabolites in desalted "fraction 1" of rat urine after injection of ^{3}H (Batch 8) and ^{14}C-POL. ●——● ^{14}C; ○— — —○ ^{3}H (Batch 8).

TABLE 3. 24-hr RECOVERIES AND ISOTOPIC RATIOS OF B_6-METABOLITES FROM RAT URINE AFTER INJECTION OF ^{3}H (BATCH 10; EXCHANGE PREPARATION)—AND ^{14}C-POL (75 μg)

Fraction	^{14}C disintegrations/min × 10^{-5}	Recovery (%) injected dose	Recovery (%) injected content	^{3}H disintegrations/min × 10^{-5}	Recovery (%) injected dose	Recovery (%) injected content	^{3}H/^{14}C	^{3}H label retained (%)
Injected dose	103·4	100	—	1243	100	—	12·0	100
Urine	29·6	28·6	100	402	32·3	100	13·6	113
Urine: freeze-dried	30·3	29·3	102	389	31·3	96·8	12·8	107
Fraction I	24·4	23·6	82·4	313	25·2	77·9	12·8	107
Fraction II	4·78	4·6	16·1	54·7	4·4	13·6	11·4	95
DEAE column								
Peak 1	2·60	2·5	8·8	—	—	—	14·5	121
Peak 2 (PIC)	15·9	15·4	53·7	—	—	—	10·7	89
Peak 3 (POLP)	2·07	2·0	7·0	—	—	—	12·0	100
Peak 4 (PALP)	2·19	2·1	7·4	—	—	—	14·5	121
Peak 5	1·66	1·6	5·6	—	—	—	9·33	78
Phosphocellulose column								
POL	1·79	1·7	6·0	20·6	—	—	11·5	96

DISCUSSION

Tritium-labelled pyridoxol has been used to study vitamin B_6 metabolism in animals[5–11] but no attempt seems to have been made to determine the stability of the label in the metabolically labile positions of the pyridine ring, and the possible incorporation of this label into compounds not on the pathway of pyridoxol metabolism. The results obtained from such experiments could be subject to error.

It has been pointed out by Argoudelis and Kummerow[12] that commercially available ^{3}H-POL labelled by the Wilzbach technique may contain appreciable amounts

of labelled 4 or 5-deoxy pyridoxol. The preparations used in this study were obtained from a different source and though also labelled by the Wilzbach procedure (Batch 8), were shown by thin-layer and column chromatography to be free of these impurities. However, other impurities which could be removed easily by column chromatography were present (fraction I, Table 1). These unidentified impurities account for about 5 per cent of the tritium label and 0·6 per cent of the ^{14}C label.

It was assumed throughout this experiment that the ^{14}C label of POL was metabolically stable so that a fall in the ^{3}H:^{14}C ratio would indicate loss of tritium from the molecule.

After lyophilisation, the urinary tritium content of rats fell by 30 per cent (Wilzbach preparation, Table 2) and about 5 per cent (exchange preparation, Table 3) which was probably due to tritium being incorporated into total body water during metabolism of POL. For example, in the conversion of POL to PAL, one possible tritium atom is removed from the C'_4 position of the pyridine ring whereas oxidation to PIC would result in the loss of all the tritium in this position. The urines of rats injected with the Wilzbach preparation had 80 per cent of the label intact in PAL and only about 40 per cent in PIC (Table 2). On the other hand, conversion of ^{3}H-POL (obtained by an exchange procedure) to PIC resulted in a loss of only 10 per cent of the original label (Table 3), which explains the small amount of "non-B_6" tritium in those urines compared to those of rats receiving the Wilzbach preparation. POL extracted from rat urines (Tables 2 and 3) showed no loss of tritium label for either ^{3}H-POL preparation showing that the label of POL was stable and that any loss of tritium from its metabolites was due to metabolic structural changes and not due to tritium exchange with body fluids. The ratio of ^{3}H:^{14}C of the column fractions representing B_6 metabolites remained the same on further purification of the individual metabolites by thin-layer chromatography. This indicated that none of the tritium lost was incorporated into non-B_6 compounds.

During metabolic studies, the excretion of "non-B_6 tritium" would depend on the urinary output and the degree of labelling in the labile positions of the pyridine ring. The ideal position for the tritium label would be in the metabolically inert methyl group of the C_2 position and the C_6 position of the pyridine ring. The tritiated POL produced by an exchange procedure (Batch 10) with apparently only 10 per cent of the label in the labile C'_4 position would be suitable for most purposes, though for accurate evaluation a suitable correction would have to be applied.

The Wilzbach preparation with about 60 per cent of the label in the labile C'_4 position is unsuitable for metabolic studies.

The fractions with ratios of ^{3}H:^{14}C slightly greater than that of the injected POL (Table 3) may be explained by impurities in the ^{3}H-POL solution (Table 1). This accounts for the excess tritium and therefore tritiated POL has to be purified before use. The presence of other fractions (Fig. 1, Tables 2 and 3: peaks 1 and 5) could be due to breakdown of B_6 metabolites in urine but we think it more probable that they represent hitherto undetected metabolites of POL. These are under further investigation.

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