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The Identification of *p*-Acetamidobenzoate as a Folate Degradation Product in Rat Urine

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Within 48h of administration of radiolabelled 10-formylfolate, folic acid and the polyglutamate derivative 10-formylfolate tetraglutamate to the rat, fragmentation products are found in the urine. The major catabolite was identified as *p*-acetamidobenzoate by chromatography and reverse isotope-dilution analysis.

The folate molecule can undergo fragmentation *in vivo* giving rise to several largely unidentified metabolites in the urine (Murphy *et al.*, 1976; Connor *et al.*, 1977). Blair (1958) and more recently Murphy *et al.* (1978) suggested that a possible metabolic route would be via cleavage of the C-9–N-10 bond in the tissues and subsequent excretion of the resultant fragments and their metabolites. Other workers have shown that the structurally related antifolate drug methotrexate (2,4-diamino-4-deoxy-10-methylfolic acid) undergoes catabolism by loss of the glutamic acid residue, yielding a pteroate analogue, probably arising from metabolism by the gut microflora (Valerino *et al.*, 1972). To clarify the mechanism of folate fragmentation we have attempted to identify the various degradation products, and in the present paper we report the identity of the major folate catabolite found in rat urine within 48h of administration of a radiolabelled tracer.

Materials and Methods

p-Aminobenzoic acid, *p*-aminohippuric acid and *p*-aminobenzoyl-L-glutamic acid were obtained from Sigma (London) Chemical Co. (Kingston upon Thames, Surrey, U.K.); *p*-acetamidobenzoic acid was obtained from the Aldrich Chemical Co. (Wembley, Middlesex, U.K.). *p*-Acetamido-benzoyl-L-glutamate was synthesized from *p*-amino-benzoyl-L-glutamate (Baker *et al.*, 1964), and radio-labelled 10-formylfolate was synthesized from a mixture of [3′,5′,9(n)-^{3}H]folic acid (specific radioactivity 500mCi/mmol) and [2-^{14}C]folic acid (specific radioactivity 58.2mCi/mmol) (The Radiochemical Centre, Amersham, Bucks., U.K.) by the method of Blakley (1959) on a micro scale. 10-Formylfolate tetraglutamate was used as isolated from rat liver (Connor *et al.*, 1977).

Male Wistar rats (200g) were dosed orally with a mixture of ^{14}C- and ^{3}H-labelled folate (folic acid, 10-formylfolate or 10-formylfolate tetraglutamate) in doses ranging from 10 to 100μg per kg body wt. Rats were housed as described by Barford & Blair (1978), and urine was collected into 10ml of 0.05M-sodium phosphate buffer, pH7, containing 2% (w/v) sodium ascorbate. Urine samples were sequentially chromatographed on DE-52 DEAE-cellulose (Whatman, Maidstone, Kent, U.K.) and on Sephadex G-15 (Pharmacia, Uppsala, Sweden) (Barford & Blair, 1978). DEAE-cellulose resolved the radioactivity into several dual-labelled peaks (intact folates), a small amount of tritiated water (probably derived from the C-9 position during cleavage) and a complex mixed peak eluted at 0.4–0.5M-NaCl containing most of the catabolites. The last-mentioned peak was resolved into several components on Sephadex G-15. The major compound (X) was solely ^{3}H-labelled and was eluted well clear of *p*-amino-benzoyl-L-glutamate or *p*-acetamido-benzoyl-L-glutamate, which have been claimed as long-term metabolites (Murphy *et al.*, 1976). Labelled *p*-amino-benzoyl-L-glutamate was not detected in the urine, although small amounts of a minor catabolite, which may be *p*-acetamidobenzoyl-glutamate, were found in the 24–28h urine samples.

The column eluate containing compound X was then freeze-fried, and the residue was taken up in a small volume of methanol and subjected to paper chromatography (Whatman 3MM paper) in (1) propanol/aq. NH_3 (sp.gr. 0.880)/water (200:1:99, by vol.), (2) 1% (v/v) acetic acid in water and (3) butanol/ethanol/aq. NH_3 (sp.gr. 0.880)/water (10:10:1:4, by vol.) by the descending method. A mixture of the column-purified compound X and *p*-acetamido-benzoic acid was hydrolysed in 2M-HCl for 1h at 100°C. The solution was then extracted with 5vol. of diethyl acetate and the extracts were evaporated to dryness. The residue obtained was dissolved in a small volume of methanol and chromatographed on paper in solvents 1 and 2.

p-Acetamidobenzoic acid (50mg) and compound X were dissolved in the minimum amount of boiling

water. After cooling of the solution the crystals that formed were recovered and recrystallized a further five times from boiling water. The specific radioactivity of the crystals was obtained by counting the radioactivity of samples of each recrystallization product and determining the concentration spectrophotometrically ($\lambda_{max.} = 262$nm at pH7.0). It was found to be constant throughout the recrystallizations, with a correlation coefficient between radioactivity and concentration of unity ($r = 1$).

The radioactivity in samples was determined with a multi-channel liquid-scintillation counter type NE 8310 (Nuclear Enterprises, Edinburgh, Scotland, U.K.), with windows set for the simultaneous determination of ^3H and ^{14}C with allowance made for quenching and overlap by the external-standard ratio method. Aqueous samples (1ml) were dissolved in 10ml of a scintillation 'cocktail' composed of toluene (1 litre) and Fisons Emulsifier Mix no. 1 (500ml) (Fisons, Loughborough, Leics., U.K.) in which was dissolved 2,5-diphenyloxazole (5g) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.1g). Paper chromatograms were cut up and the pieces suspended in the same 'cocktail' but prepared without the emulsifier.

Discussion and Results

In all chromatography systems used compound X co-chromatographed with *p*-acetamidobenzoic acid, and the acid-hydrolysis product of compound X co-chromatographed with *p*-aminobenzoic acid, the hydrolysis product of *p*-acetamidobenzoic acid (see Table 1). The identity of compound X as *p*-acetamidobenzoic acid was confirmed by reverse isotope-dilution analysis, since the two materials co-crystallized through six recrystallizations. The percentage recovery at various times of *p*-acetamidobenzoate from the urine of rats dosed with the various folates is summarized in Table 2. *p*-Acetamidobenzoyl-L-glutamate added to urine chromatographed as a single peak in all systems and the amide bond remained intact; *p*-acetamidobenzoate is therefore not an artifact produced by chemical degradation of *p*-acetamidobenzoyl-L-glutamate. In no urine sample was radioactivity found associated with *p*-aminohippurate markers. Since *p*-acetamidobenzoate is produced from 10-formylfolate, folic acid and 10-formylfolate tetraglutamate and in each case it appeared principally in the 6–48h urine (Table 2), prior metabolism to intermediate metabolites was required. Whether

Table 1. *Summary of the chromatographic behaviour of the metabolite and standard compounds on Whatman 3MM paper (solvents 1, 2 and 3) and elution position from a Sephadex G-15 column*

Sample	R_F on paper chromatography: Solvent 1	Solvent 2	Solvent 3	Sephadex G-15 elution position ($K_{av.}$*)
p-Acetamidobenzoate	0.52	0.56	0.35	1.73
Metabolite	0.52	0.56	0.35	1.73
p-Aminobenzoate	0.39	0.65	0.19	1.67
Hydrolysis product of the metabolite	0.39	0.65	—	—
p-Acetamidobenzoyl-L-glutamate	0.28	0.85	0.09	0.66
p-Aminobenzoyl-L-glutamate	0.21	0.85	0.05	0.66
p-Aminohippurate	0.39	0.83	0.24	1.51

* $K_{av.} = (V_e - V_0)/(V_t - V_0)$.

Table 2. *Variations in the percentage of urinary ^3H with time and the total urinary production over 48h of p-acetamidobenzoate after the oral administration of 10-formylfolate, folic acid or 10-formylfolate tetraglutamate*

Each value was determined by analysis of the pooled urine from six rats. N.D., Not detected.

Folate	Dose (μg/kg) (as folate)	% of ^3H in urine sample as *p*-acetamidobenzoate: 0–6h	6–24h	24–48h	Total % of urinary ^3H as *p*-acetamidobenzoate	Total urinary ^3H as % of dose
10-Formylfolate	10	7.3	19.9	23.4	16.1	25.7
	50	4.3	14.9	7.8	8.5	31.9
	100	N.D.	12.5	9.4	3.9	40.9
Folic acid	100	N.D.	19.0	18.6	9.2	37.4
10-Formylfolate tetraglutamate	100	1.2	52.8	—	8.9	49.9

these intermediates are derived from the folate monoglutamate or the folate polyglutamate pools or both is unknown, since considerable metabolism to both forms would have occurred before 6h (Hillman *et al.*, 1977). The absence of labelled *p*-aminohippurate from the urine suggests that the immediate precursor of *p*-acetamidobenzoate may not be *p*-aminobenzoate, since this would be at least partially excreted as the glycine conjugate, *p*-aminohippurate (Tabor *et al.*, 1951).

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