The Metabolic Fate of [2-¹⁴C]Folic Acid and a Mixture of [2-¹⁴C]- and [3',5',9-³H]-Folic Acid in the Rat

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(Received 2 December 1977)

The metabolism of [2-¹⁴C]folic acid over 13 days and a mixture of [2-¹⁴C]- and [3',5',9-³H]-folic acid in rats over a 6-day period is described. Both ¹⁴C and ³H are excreted in urine over the 6-day period, but ³H and ¹⁴C are only detectable in faeces for 2 days. A breakdown product of folic acid labelled with ³H only was found in some urine samples, but no metabolite corresponding to the part of the molecule containing ¹⁴C was detected. These experiments show that in the whole animal a substantial portion of orally administered folic acid undergoes scission shortly after administration [Blair *Biochem. J.* (1957) **68**, 385–387] and that the retained folates are a storage form for folate monoglutamates.

The metabolic fate of folic acid in laboratory animals is usually studied using a radioactive tracer. Two such tracers are commonly used, [2-¹⁴C]folic acid and [3',5',9-³H]folic acid. It was thought that the site of the radioactive nuclide within the molecule was not important since the folate molecule was considered to be stable *in vivo* and the only radioactive products that were isolated from biological materials after a dose of labelled folic acid were identifiable as folates. Barford & Blair (1975) reported that after a dose of a mixture of [2-¹⁴C]folic acid and [3',5',9-³H]folic acid the recovery of ³H and ¹⁴C in urine samples was not identical and urinary metabolites labelled with ³H only were detected. Because Barford & Blair (1975) and Beavon & Blair (1975) failed to find a pterin fragment in urine samples they ascribed this result to ³H exchange.

Futterman (1956) reported that some liver preparations cleave folic acid to *p*-aminobenzoyl-L-glutamic acid. Dinnings *et al.* (1957) reported finding diazotizable amines on incubating folic acid or 5-formyltetrahydrofolate with liver slices. They reported that this cleavage of the folate molecule was strongly inhibited by ascorbic acid, and that the urinary excretion of diazotizable amine in rats was

directly proportional to the dose of folic acid or 5-formyltetrahydrofolate.

A similar finding has been reported by Murphy *et al.* (1976) who identified ³H-labelled *p*-acetoamidobenzoyl-L-glutamic acid in the urine of rats 13 days after a dose of [3',5',9-³H]folic acid. They noted two other minor labelled metabolites, but were unable to identify them.

The present paper describes a series of experiments using a mixture of [2-¹⁴C]folic acid and [3',5',9-³H]-folic acid, and [2-¹⁴C]folic acid alone, to study the possible fragmentation of folic acid in rats and its time course.

Materials and Methods

Animals

Male Wistar rats (150–200g body wt.) received doses of either [2-¹⁴C]folic acid or a mixture of [2-¹⁴C]- and [3',5',9-³H]-folic acid orally or by intraperitoneal injections. Animals were then housed in metabolism cages (Jencons Metabowls; Jencons (Scientific), Hemel Hempstead, Herts. U.K.) designed for the separate collection of urine and faeces. To prevent oxidation of folates urine samples were

Folic acid (¹⁴C label, position 2; and ³H label, positions 9, 3' and 5')

collected into 10ml of 0.05M-sodium phosphate buffer, pH7.0, containing 2% (w/v) sodium ascorbate, and flasks were covered with aluminium foil to exclude light. Urine collection flasks were changed 6 and 24h after administration of the dose and thereafter every 24h. Faeces collection flasks were changed every 24h. At the end of the experiment animals were killed and the liver removed into ice-cold beakers. Throughout all experiments animals were allowed food and water *ad libitum*.

Measurement of total radioactivity in livers and faeces

Livers and faeces were freeze-dried and then ground to give an homogeneous powder. Samples (100mg) of the powder were oxidized in a Beckman biological-material oxidizer. The oxidized material was first passed through a cold trap to collect water and $^3\text{H}_2\text{O}$, and then into a trap containing 15ml of Fisons adsorber P (Fisons, Loughborough, Leics., U.K.), a scintillation 'cocktail' designed for the collection of $^{14}\text{CO}_2$. Duplicate determinations of each sample were made together with appropriate controls. Samples were counted for radioactivity in a Nuclear Enterprises scintillation counter type 8310. Appropriate corrections were made for quenching and background.

Extraction of folates from livers

Folate was extracted from livers as described previously (Barford *et al.*, 1977).

Column chromatography

(a) *Liver extracts.* Liver extracts were chromatographed on DEAE-cellulose (Whatman DE52) and Sephadex G-15 as described previously (Barford *et al.*, 1977).

(b) *Urine samples.* Urine samples from several animals were pooled and samples (usually 10ml) were chromatographed on DEAE-cellulose and Sephadex G-15 with the same conditions as those used for liver extracts. The position of peaks containing radioactivity on columns was determined by counting 50 μl samples of each fraction in a liquid-scintillation counter with 10ml of Fisons Dioxan D 'cocktail'. Samples were counted in channels previously calibrated for ^{14}C and ^3H . Appropriate corrections were made for quenching, background and ^{14}C spillover into the ^3H channel.

Chemicals and reagents

[2- ^{14}C]Folic acid (specific radioactivity 54.3 Ci/mol) and [3',5',9- ^3H]folic acid (specific radioactivity 500 Ci/mol) were from the Radiochemical Centre, Amersham, Bucks., U.K.

Folates for calibration purposes were prepared as described previously (Barford *et al.*, 1977). *p*-Aminobenzoyl-L-glutamic acid was from Sigma London

Chemical Co., Kingston upon Thames, Surrey, U.K. All other chemicals were of Analar grade or its equivalent.

Results

Experiments with a mixture of [2- ^{14}C]- and [3',5',9- ^3H]-folic acid

(a) *Urinary radioactivity.* Quantitative analysis of urine samples for ^3H and ^{14}C showed that in all urine samples up to day 6 except the 0-6h urine sample a higher percentage of the dose of ^3H than the dose of ^{14}C was recovered (Table 1). In some urine samples as much as 4 times more ^3H than ^{14}C was recovered. Over the 6-day period 22% of the dose of ^{14}C and 43% of the ^3H was recovered.

Urine samples from a single day were pooled and analysed by column chromatography using DEAE-cellulose and Sephadex G-15. Fig. 1 shows the chromatograms at 0-6 and 6-24h urine samples. On both Sephadex G-15 and DEAE-cellulose the 0-6h urine sample was shown to contain three peaks of radioactivity, all of which were labelled with both ^3H and ^{14}C . Peak I co-chromatographed with 4a-hydroxy-5-methyltetrahydrofolate, peak II with 10-formylfolate and peak III with 5-methyltetrahydrofolate. The major metabolite was 5-methyltetrahydrofolate. The 6-24h urine chromatograms were different from the 0-6h chromatograms. On both columns the 6-24h urine sample was shown to contain a large ^3H - and ^{14}C -labelled peak corresponding to 5-methyltetrahydrofolate, and two much smaller ^3H - and ^{14}C -labelled peaks corresponding to 4a-hydroxy-

Table 1. Recovery of ^3H and ^{14}C in the urine, faeces and livers of rats

Animals received intraperitoneal doses of a mixture of [3',5',9- ^3H]folic acid and [2- ^{14}C]folic acid (117.5 $\mu\text{g/kg}$ body wt.). The results are expressed as percentage of the dose. The number of animals is given in parentheses, except that two animals only were used for each determination of liver radioactivity. Abbreviation: nd, not detectable.

Time after dose	Recovery of radioactivity (%)					
	Urine		Faeces		Liver	
	^3H	^{14}C	^3H	^{14}C	^3H	^{14}C
0-6h (12)	5.1	9.8	9.6	11.0	14.0	18.0
6-24h (12)	21.7	5.0				
1-2 days (10)	4.2	2.6	4.0	5.2	16.3	20.1
2-3 days (8)	3.3	1.9	1.1	0.7	23.7	23.1
3-4 days (6)	6.4	1.4	nd	nd	17.9	23.4
4-5 days (4)	1.5	0.9	nd	nd	16.1	24.0
5-6 days (2)	0.7	0.4	nd	nd	15.4	18.1
Total	42.9	22.0	14.7	16.2	—	—

Fig. 1. DEAE-cellulose chromatography of urine samples from rats. Animals received doses of a mixture of [2-¹⁴C]- and [3',5',9-³H]-folic acid (117.5 µg/kg body wt.). ○, ¹⁴C; △, ³H. Elution positions are: 1, 4a-hydroxy-5-methyltetrahydrofolate; 2, 10-formylfolate; 3, 5-methyltetrahydrofolate. Urine samples were for 0-6 h (a) and 6-24 h (b).

Table 2. Percentage of total ¹⁴C in urine sample associated with folates

Animals received intraperitoneal doses of a mixture of [2-¹⁴C]- and [3',5',9-³H]-folic acid. (117.5 µg/kg body wt.). Each value represents the results of two chromatograms of pooled urine samples.

Time	¹⁴ C in folates (%)		
	5-Methyltetrahydrofolate	10-Formylfolate	4a-Hydroxy-5-methyltetrahydrofolate
0-6h	63	33	2.7
6-24h	42	26	10.4
1-2 days	4.5	0	38
2-3 days	23	5.5	58
3-4 days	60	8.5	0
4-5 days	45	40	10

5-methyltetrahydrofolate and 10-formylfolate. The major peak on these chromatograms was labelled only with ³H and chromatographed in the same place as *p*-aminobenzoyl-L-glutamate on both columns. This peak labelled with ³H alone was not detected

Fig. 2. DEAE-cellulose chromatography of urine samples. Animals received doses of a mixture of [2-¹⁴C]- and [3',5',9-³H]-folic acid (117.5 µg/kg body wt.). ○, ¹⁴C; △, ³H. Urine samples were for 24-48 h (a), 48-72 h (b) and 72-96 h (c).

in the 24-48 h and 2-3 day urine sample (Fig. 2) although there was more ³H than ¹⁴C recovered in these urine samples. In these urine samples the major metabolite was 4a-hydroxy-5-methyltetrahydrofolate. The minor metabolites were unidentified. The major metabolite in the 3-4 day urine sample was 5-methyltetrahydrofolate, there was one other minor metabolite labelled with ³H and ¹⁴C, and two metabolites labelled with ³H only. One of the ³H-labelled metabolites chromatographed in the same place as *p*-aminobenzoyl-L-glutamate, the second ³H-labelled metabolite has not been identified, but is not ³H₂O.

The relative amounts of the three folates, 5-methyltetrahydrofolate, 10-formylfolate and 4a-hydroxy-5-methyltetrahydrofolate, excreted over the time of the experiment vary from day to day. 5-Methyltetrahydrofolate excretion is high in the first sample, falls up to day 2 then rises again (Table 2). 4a-Hydroxy-5-methyltetrahydrofolate concentrations are low in the early urine samples, reach a maximum on day 3 and subsequently fall. These results suggest two pools of folate exist. The first is a short-term pool and its excretory products are 5-methyltetrahydro-

folate and 10-formylfolate. These two metabolites are formed rapidly after administration of the isotope and as the time after dosage increases, there is a fall in the relative excretion of these two products and a rise in excretion of breakdown products, i.e. 4a-hydroxy-5-methyltetrahydrofolate and fragmentation products. The second rise in the relative excretion rate of 5-methyltetrahydrofolate and 10-formylfolate corresponds to breakdown and excretion of the second pool of folate, probably the folate polyglutamates, and a similar pattern occurs.

Faeces

Faeces were freeze-dried and analysed for total radioactivity. Although the recovery of ^{14}C was higher than that of ^3H the differences were not significant.

Liver radioactivity

The pattern of recovery of radioactivity in liver was different for ^3H and ^{14}C . ^{14}C recovery from livers reached a peak after 5 days, whereas ^3H recovery reached a peak after 3 days and thereafter decreased. The large loss of ^3H from livers between day 3 and day 4 (approx. 6% of the dose) is paralleled by an increase in the recovery of ^3H in urine, and the re-appearance of metabolites labelled with ^3H only in urine samples. Liver extracts obtained by hot extraction procedures were chromatographed on Sephadex G-15 and DEAE-cellulose. In all samples there was a single peak of radioactivity, labelled with both ^3H and ^{14}C that chromatographed in the same place as pteroyl-heptaglutamate and tetraglutamate form of 10-formylfolate (Connor *et al.*, 1977). In no liver samples prepared by hot extraction procedures was it possible to detect metabolites labelled with ^3H only, or a metabolite that could be a pterin.

Experiments with $[2-^{14}\text{C}]$ folic acid

In a parallel series of experiments animals received

oral doses of $[2-^{14}\text{C}]$ folic acid. Urine and faeces were collected over a 13-day period. At 1, 2, 5, 9 and 13 days after dosage animals were killed and the livers were removed and the total radioactive content was measured. The results (Table 3) were not substantially different from the recovery of ^{14}C in the animals given a mixture of $[2-^{14}\text{C}]$ - and $[3',5',9-^3\text{H}]$ -folic acid, and similar chromatograms were obtained. However, the longer time period used for this experiment demonstrated that a major excretory route for liver ^{14}C could be in the faeces, presumably via the bile. Thus there is an early rapid loss of radioactivity in the faeces, which falls over 2–5 days. Between day 5 and day 9 there is a rise in the faecal excretion of radioactivity, which parallels the fall in liver radioactivity over the same period.

Discussion

The results presented here demonstrate breakdown of the folate molecule to produce a metabolite that is labelled with ^3H only. This metabolite appears within 6h after administration of the labelled folic acid. Both ^3H and ^{14}C are found in livers up to 6 days after dosing the animals and both isotopes are associated with a metabolite that behaves as a folate polyglutamate. ^3H is lost from liver faster than ^{14}C and loss of ^3H from liver is paralleled by a rise in urinary ^3H and the re-appearance of metabolites labelled with ^3H alone in the urine. This result suggests that the ^3H -labelled liver metabolite is giving rise to ^3H -labelled metabolites in urine. Breakdown of liver folates may be by a pathway that involves fragmentation of the folate molecule (Blair, 1957; Murphy *et al.*, 1976). In no urine samples were we able to detect a pterin. The long-term experiment using ^{14}C only suggests that a significant amount of liver ^{14}C is excreted in the faeces. By using a mixture of $[2-^{14}\text{C}]$ - and $[3',5',9-^3\text{H}]$ -folic acid faeces always contain ^3H and ^{14}C . Murphy *et al.* (1976) have suggested that a long-term metabolite formed from $[^3\text{H}]$ folic acid in rats may be *p*-acetamidobenzoyl-L-glutamic acid. We have shown that 6 days after administration of the dose some urinary radioactivity is associated with metabolites that are a mixture of ^{14}C - and ^3H -labelled species and chromatograph as folates, one of which is 5-methyltetrahydrofolate. Our findings of the relative distribution of the labelled metabolites in faeces and urine (Tables 1 and 2) indicate that excretion of ^3H - and ^{14}C -labelled fragmentation products after oral folic acid may be by different routes, the ^3H -labelled metabolite appearing in urine, and metabolites labelled with ^{14}C only being excreted in faeces and as $^{14}\text{CO}_2$ and urea (Connor *et al.*, 1977). 10-Formylfolate is known to yield fragmentation products on metabolism in the rat. Thus Connor *et al.* (1977) showed that after a dose of a mixture of

Table 3. Recovery of radioactivity in urine, faeces and livers of rats

Animals received oral doses of $[2-^{14}\text{C}]$ folic acid ($112\mu\text{g/kg}$ body wt.). The values for liver were determined at the time (in days) indicated in parentheses.

Time (days)	No. of animals	Dose recovered (%)		
		Urine	Faeces	Liver
0–1	10	14.5	49.0	16.3 (1)
1–2	8	2.0	6.0	23.7 (2)
2–5	6	1.8	2.2	25.6 (5)
5–9	4	1.7	10.3	16.0 (9)
9–13	2	1.4	1.5	12.5 (13)

10-formyl-[2-¹⁴C]- and -[3',5',9-³H]-folatetetraglutamate the majority of the urinary radioactivity is scission products and Beavon & Blair (1975) found 10-formylfolate to give non-folate species in the urine. 10-Formylfolate is not a folate coenzyme, but is readily formed from 10-formyltetrahydrofolate by oxidation (Pearson, 1974) and is not readily reduced back to a tetrahydrofolate. 10-Formylfolate formation within an animal could therefore be related to the origin of the excreted breakdown products reported in this paper and by Murphy *et al.*, (1976).

We are grateful to the Cancer Research Campaign for financial support and to the Royal Society for a liquid-scintillation counter.

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