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Retained Folates in the Rat

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The retention of radioactivity after doses of ^{14}C- and ^{3}H-labelled folic acid is described. Radioactivity was retained in liver, kidney and gut of rats for some time after administration of the dose. The retained radioactivity could not be displaced by large doses of unlabelled folic acid or unlabelled 5-methyltetrahydrofolate. ^{14}C- and ^{3}H-labelled folates showed similar chromatographic behaviour on ion-exchange chromatography to 5-methyltetrahydrofolate, and on ion-exchange and gel-permeation chromatography to synthetic pteroylhepta-γ-glutamate.

Animal tissues, particularly liver, contain substantial quantities of folates, most of which is *N*-5-methyltetrahydrofolate and its derivatives (Bird *et al.*, 1965). Microbiological assay has shown that much of the folate in liver is not available to *Lactobacillus casei* until the liver folate has been treated with the enzyme conjugase (glutamate carboxypeptidase, EC 3.4.12.10) (Herbert, 1963), which is said to release folate monoglutamates from a high-molecular-weight form of folate, the folate polyglutamates. There is some controversy about the exact chemical nature of the high-molecular-weight folate in liver (Barford *et al.*, 1976) and its biological role (see Hoffbrand, 1976). Several workers have suggested that coenzyme forms of folate may be the folate polyglutamates, and not, as has previously been thought, the folate monoglutamates (Baugh & Krumdieck, 1971; Rowe *et al.*, 1975).

After a dose of [^{14}C]folic acid to rats, several metabolites are identified in the urine (Blair & Dransfield, 1971): 6h after an oral dose of [^{14}C]folic acid, labelled 5-methyltetrahydrofolate is detected in rat urine. Thus folic acid appears to enter the folate monoglutamate pool rapidly. However, Blair & Dransfield (1971) also showed that unlabelled folic acid displaced labelled folate 3.5h, but not 24h, after a dose of labelled folic acid. [^{3}H]Folic acid has been used to study folate metabolism in human subjects (e.g. Landon *et al.*, 1975; Retief *et al.*, 1976) and at the end of the experiment (usually several days after administration of [^{3}H]folic acid) a large dose of unlabelled folic acid has been used to 'flush out' any residual folate that may remain in the subject. An appreciable quantity of the administered radioactivity was unaccounted for at the end of these experiments. Little information is available on the effectiveness of these flushing doses, and on the half-life of the liver folate under normal conditions.

The present paper describes studies on the half-life of labelled folate in various rat tissues, attempts to flush residual labelled folate out of liver 24h after administration of the dose with several monoglutamates, and preliminary investigations into the chemical nature of the stored folate.

Materials and Methods

Animals

Male Wistar rats (150–200g body wt.) received doses of either [2-^{14}C]folic acid or [3′,5′,9-^{3}H]folic acid orally or by intraperitoneal injections. Animals were then either housed in metabolism cages [Jencons Metabowls; Jencons (Scientific) Ltd., Hemel Hempstead, Herts., U.K.] designed for the separate collection of urine and faeces, or in cages designed to prevent coprophagy. At the end of the experiment animals were killed with ether, and organs (liver, kidney, brain, intestine) removed into ice-cold beakers. For flushing-dose experiments, animals received either oral or intraperitoneal doses of unlabelled folates after a dose of labelled folate. Throughout all experiments rats were allowed food and water *ad libitum*.

Measurement of total radioactivity in animal organs

Animal organs were freeze-dried, and then ground to give homogeneous powder. Samples (100mg) of the powder were oxidized in a Beckman biological-material oxidizer, and the products were collected into 15ml of Fisons absorber P (Fisons, Loughborough, Leics., U.K.) a scintillation 'cocktail' designed for the collection of $^{14}CO_2$. Duplicate determinations of each sample were made, together with appropriate controls. Samples were counted for radioactivity in a Nuclear Enterprises liquid-scintillation counter type NE 8310 until constant count rates were obtained. Appropriate corrections were made for quenching and background.

Extraction of folates from livers

After opening the abdominal cavity of the animal, the liver was perfused with ice-cold 0.15M-NaCl via the hepatic portal vein. The liver was then quickly removed into an ice-cold beaker. To prevent breakdown of folate polyglutamates livers were extracted by one of two methods: (1) the livers were cut into 2mm cubes and plunged into a solution of boiling 0.05M-sodium phosphate buffer, pH7.0, containing 2% (w/v) sodium ascorbate and 5mg of dithiothreitol/100ml, and maintained at 100°C for 5min; (2) that of Stokstad & Thenen (1973). After heating, the liver extracts were cooled to room temperature (18°), centrifuged to remove precipitated protein, and stored at −15°C in 0.05M-phosphate buffer, pH7.0, containing 5mg of dithiothreitol/100ml, until required.

Column chromatography

(*a*) *Sephadex G-15 chromatography*. Liver extracts were applied to a column (2cm² × 60cm) of Sephadex G-15 in 0.05M-phosphate buffer, pH7.0, containing 5mg of dithiothreitol/100ml and columns were eluted with the same buffer; 60 5ml fractions were collected from each column.

(*b*) *DEAE-cellulose chromatography*. (i) Liver extracts were applied to DEAE-cellulose (Whatman DE-52) columns (2cm² × 50cm) that had been previously equilibrated with 0.05M-sodium phosphate buffer, pH7.0, containing 5mg of dithiothreitol/100ml. Columns were eluted with linear gradients of 0–1.2M-NaCl in 0.05M-phosphate buffer, pH7.0, containing 5mg of dithiothreitol/100ml. The column effluent was collected in 5ml fractions, and the conductivity of every tenth fraction was determined with a Mullard conductivity cell.

(ii) Liver extracts were applied to DEAE-cellulose (Whatman DE-52) columns (0.65cm² × 24cm) that had been previously equilibrated with 0.01M-sodium phosphate buffer, pH6.0, containing 2% (w/v) sodium ascorbate. The columns were eluted with an exponential gradient of 0.01–0.5M-phosphate buffer, pH6.0, containing 2% (w/v) sodium ascorbate: 100 2.5ml fractions were collected. The conductivity of every tenth fraction was determined.

All columns were calibrated with authentic unlabelled folate monoglutamates which were detected by using an LKB Uvicord II instrument and a chart recorder (LKB Instruments, Croydon, Surrey, U.K.). Some columns were calibrated with [5-^{14}C]methyltetrahydrofolate, [2-^{14}C]folic acid, [3′,5′,9-^{3}H]folic acid and [3′,5′,9-^{3}H]pteroylhepta-γ-glutamate.

Identical gradients on different DEAE-cellulose columns were maintained by using an LKB UltraGrad gradient maker attached to a peristaltic pump. The position of the radioactive peaks on columns was determined by counting the radioactivity of 50μl portions of each fraction in a liquid-scintillation counter, by using 10ml of either a 'cocktail' of toluene (500ml), Fisons emulsifier E (250ml) and 2,5-diphenyloxazole (PPO) (2.5g) for ^{14}C counting, or Fisons Dioxan D for ^{3}H counting. Samples were counted for radioactivity to a constant count rate, and suitable corrections for background and quenching were made.

Unless otherwise stated, recovery of radioactivity from columns was complete.

Chemicals and reagents

[2-^{14}C]Folic acid (specific radioactivity 54.3μCi/μmol), [5-^{14}C]methyltetrahydrofolate (specific radioactivity 57.7μCi/μmol) and [3′,5′,9-^{3}H]folic acid (specific radioactivity 500μCi/μmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

5-Methyltetrahydrofolate, prepared by the method of Blair & Saunders (1970), was supplied by Dr. K. Ratanasthien (Department of Clinical Chemistry, University of Chiang Mai, Chiang Mai, Thailand). 4a-Hydroxy-5-methyltetrahydrofolate and 5-methyl-5,6-dihydrofolate, prepared by the method of Gapski *et al.* (1971), were supplied by Dr. K. Ratanasthien. 10-Formyltetrahydrofolate was prepared from 5-formyltetrahydrofolate by the method of Beavon & Blair (1972). 5-Methyl-5,8-dihydrofolate was prepared by acidification of a solution of 5-methyl-5,6-dihydrofolate (Gapski *et al.*, 1971). 10-Formylfolate was prepared by the method of Blakley (1959).

All other chemicals were of AnalaR grade or its equivalent.

Synthetic [^{3}H]pteroylheptaglutamate was a gift from Dr. O. K. Ahmad and Professor I. Rosenberg, University of Chicago.

Results

Half-life of the retained radioactivity from a dose of [^{14}C]folic acid in rat tissues

Male rats received oral folic acid at doses of 102, 51.3, 25.7 and 12.9μg/kg body wt. Eight animals were used for each dose, and two animals were killed at intervals of 1, 5, 9 and 13 days. Total radioactivity was measured in the liver and kidneys of each animal and in the gut of some animals. The results are shown in Table 1. At 24h after administration of a dose of [^{14}C]folic acid, substantial amounts of radioactivity are retained in the liver, kidney and gut of rats. Both kidneys and gut showed a continual loss of radioactivity with time, until 13 days after administration of the dose, only trace amounts of radioactivity (less than 1% of the dose) remained in these tissues.

Livers retain a much higher percentage of the dose, between 15.7 and 26% at 24h after administration of the dose, and between 20.5 and 29% of the dose is found in liver 5 days after administration of the dose. Thereafter the amount of radioactivity in the liver falls with increasing time, until at 13 days after ad-

Table 1. *Retention of radioactivity in rat organs*

[^{14}C]Folic acid was given orally, and total radioactivity, expressed as a percentage of the dose, was assayed. For details see the text.

Organ	Dose (μg/kg body wt.)	Time after administration of dose (days) ...	Radioactivity (%) 1	5	9	13
Liver	102.0		15.7	25.5	23.6	15.7
	51.3		19.7	20.5	20.3	14.4
	25.7		25.0	25.7	19.8	16.7
	12.9		26.0	29.0	20.0	17.0
Kidney	102.0		2.6	2.4	1.1	0.4
	51.3		3.1	2.0	1.4	0.6
	25.7		4.2	2.4	1.3	0.4
	12.9		3.9	2.5	1.1	0.8
Gut	51.3		6.4	2.1	1.2	0.4
	25.7		7.3	2.4	1.3	1.0
	12.9		7.4	2.2	0.7	0.7

Table 2. *Retention of an oral dose of [^{14}C]folic acid in rat organs*

(A) 48h after administration of the dose; (B) 48h after administration of [^{14}C]folic acid and 24h after a flushing dose of unlabelled folic acid (285.7mg/kg body wt.); (C) 48h after administration of [^{14}C]folic acid and 24h after a flushing dose of unlabelled 5-methyltetrahydrofolate (571mg/kg body wt.). Results are expressed as μg of folic acid retained/g dry wt. of tissue.

Dose (μg/kg) body wt.)	Liver A	B	C	Kidney A	B	C	Gut A	B	C
102.0	1.3	1.3	—	0.8	0.5	—	—	—	—
51.3	2.2	1.9	—	0.3	0.3	—	0.25	0.36	—
25.7	0.24	0.25	—	0.19	0.17	—	0.07	0.05	—
12.9	0.2	0.18	0.18	0.17	0.18	0.08	0.04	0.04	0.02

ministration of the dose, about 16% of the administered radioactivity is found in the liver.

The amount of radioactivity found in liver, kidneys and gut (expressed as μg of folic acid/g dry wt. of tissue) 24h after administration of the dose shows a linear relationship with dose, which indicates that the capacity of all the tissues for [^{14}C]folic acid is not saturated even at a dose of 102μg/kg body wt. In addition, it appears that the kidneys and livers have similar binding capacities/g dry wt.

In a single experiment that was continued for 70 days radioactivity was detectable in the livers of animals at this time. Radioactivity can still be detected in gut 28 days after dosage, and in kidneys 35 days after dosage. At a dose of 12.9μg/kg body wt., half-life values for loss of radioactivity from rat tissues were 8 days for livers and kidneys and 4 days for gut. A first-order rate plot of the amount of radioactivity retained in liver between 5 and 70 days gives a straight line.

Displacement of radioactivity from rat tissues by large doses of unlabelled folates

Male rats received [^{14}C]folic acid orally at doses of 51.3, 25.7 and 12.9μg/kg body wt., and 24h after dosage the animals were given intraperitoneal injections of either unlabelled folic acid (285.7mg/kg body wt.) or unlabelled 5-methyltetrahydrofolate (571mg/kg body wt.). Then 48h after administration of [^{14}C]folic acid, animals were killed and livers, kidneys and gut were removed and the total amount of radioactivity in each tissue was measured. Table 2 shows the retention of radioactivity in rat tissues after a large flushing dose of unlabelled folic acid or unlabelled 5-methyltetrahydrofolate. There is no evidence that either of the unlabelled folates flushed retained radioactivity from rat organs.

Column chromatography of liver extracts

(*a*) *Sephadex G-15 chromatography.* Animals receiving oral doses of [^{14}C]folic acid or [^{3}H]folic acid were killed 48h after administration of the dose. Radioactivity was extracted from livers by either Method 1 or Method 2. Extracts of liver from rats receiving [^{14}C]folic acid were eluted from Sephadex G-15 columns with 0.05M-phosphate buffer, pH7.0, containing dithiothreitol. Most of the radioactivity, both ^{14}C and ^{3}H, was eluted from the columns close to the void volume and before any authentic folate monoglutamates (see Fig. 1). In addition, on some chromatograms a second peak of radioactivity leaving the column in the same region as the folate

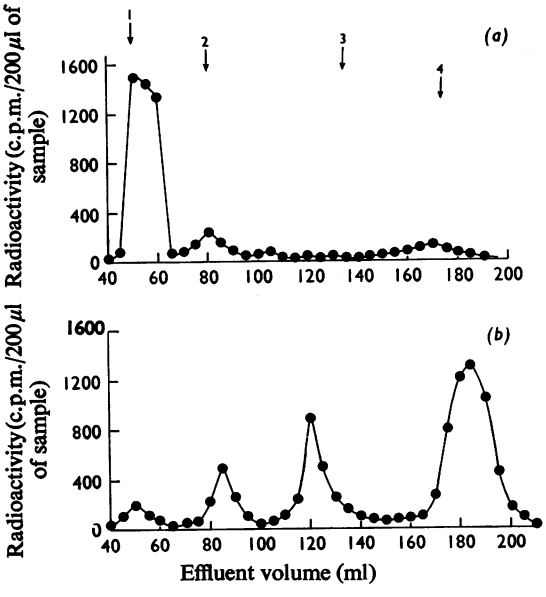

Fig. 1. *Sephadex G-15 chromatography of radioactivity from rat liver*

(*a*) The liver radioactivity was extracted by Method 2. (*b*) The liver was homogenized in 0.05M-phosphate buffer at room temperature. Animals received [^{14}C]folic acid (102μg/kg body wt.). For details, see the Materials and Methods section. Elution positions of authentic folates are: 1, [^{3}H]pteroylheptaglutamate; 2, 4a-hydroxy-5-methyltetrahydrofolate; 3, 10-formyltetrahydrofolate. 4, 5-methyltetrahydrofolate.

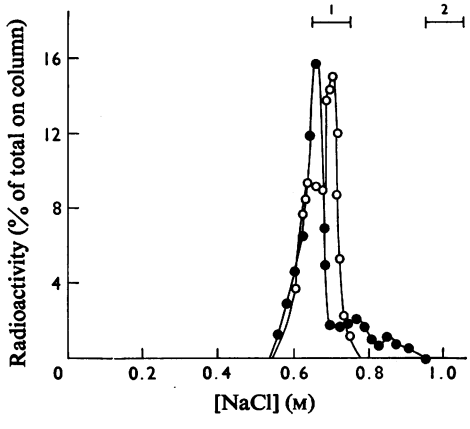

Fig. 2. *DEAE-cellulose chromatography of liver extracts*

Animals received orally either [^{14}C]folic acid (102μg/kg body wt.) or [^{3}H]folic acid (293μg/kg body wt.). The livers were extracted by Method 1. Elution positions of authentic folates are: 1, 5-methyltetrahydrofolate; 2, folic acid. ●, ^{14}C radioactivity; ○, ^{3}H radioactivity.

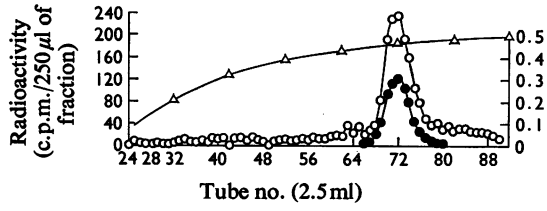

Fig. 3. *DEAE-cellulose chromatography of liver extracts*

Animals received oral doses of [^{3}H]folic acid (300μg/kg body wt). 5-[^{14}C]Methyltetrahydrofolate was added to the liver extract before chromatography on DEAE-cellulose. ○, ^{3}H radioactivity; ●, ^{14}C radioactivity; △, phosphate concentration.

monoglutamate markers was seen (usually less than 10% of total column radioactivity).

On Sephadex G-15 chromatograms of liver extracts prepared without heating, only peaks of radioactivity chromatographing in the folate monoglutamate region could be detected (Fig. 1).

(*b*) *DEAE-cellulose chromatography.* Animals receiving oral doses of [^{14}C]folic acid were killed 48h after administration of the dose. Radioactivity was extracted from livers as described in the Materials and Methods section.

Liver extracts prepared by Method 1 were chromatographed on a DEAE-cellulose column. Radioactivity in all liver extracts eluted from DEAE-cellulose columns with a linear NaCl gradient gave a major peak of radioactivity at a similar position to authentic 5-methyltetrahydrofolate, but before folic acid (see Fig. 2). Chromatography of synthetic pteroylheptaglutamate on DEAE-cellulose columns showed that it was eluted from columns in a similar position to 5-methyltetrahydrofolate, and was not separable from the liver radioactivity.

Chromatograms of radioactive liver extracts from DEAE-cellulose columns eluted with a non-linear sodium phosphate gradient showed a single peak of radioactivity which was not separable from 5-methyltetrahydrofolate. Synthetic pteroylheptaglutamate was eluted from these columns in the same place as the liver radioactivity (Fig. 3).

Discussion

The results of this study demonstrate the considerable accumulation of radioactivity in livers after an oral dose of [^{14}C]folic acid. Up to 29% of a dose of folic acid is found in rat livers 5 days after administration of the dose. At 13 days after dosage there is still a substantial amount of radioactivity retained in the liver. The half-life of the radioactivity retained in liver is 8 days, that in kidney 8 days and that in gut 4 days.

Large doses of unlabelled folic acid or 5-methyltetrahydrofolate had no effect on the amount of radioactivity retained in liver, kidney and gut. A possible explanation for this is that the equilibrium between retained radioactivity in liver, kidney and gut, and circulating folic acid or 5-methyltetrahydrofolate, may be so slow that little or no radioactivity is replaced by large flushing doses of unlabelled folates. This explanation is supported by the long half-lives of the retained radioactivity in liver and kidney, and the slow formation in the liver and kidney of high-molecular-weight folates (Brown *et al.*, 1974). Whatever the reason for non-displacement of radioactivity from rat tissues by high doses of unlabelled folates, the observations in the present paper raise questions as to the validity of administering radioactive folic acid followed by flushing doses of unlabelled folates, supposedly to flush out any radioactivity that may be retained at the end of the experiment, as in agreement with Landon *et al.* (1975).

On Sephadex G-15 chromatography radioactivity extracted from rat livers by hot-extraction methods co-chromatographed with synthetic [^{3}H]pteroylhepta-γ-glutamate. This finding is consistent with the results of other workers on the behaviour of folate polyglutamates on gel-filtration chromatography (Lavoie *et al.*, 1975; Hintze & Farmer, 1975).

On DEAE-cellulose chromatograms, liver radioactivity co-chromatographed with synthetic [^{3}H]-pteroylhepta-γ-glutamate and with 5-methyltetrahydrofolate whether columns were eluted with linear NaCl gradients or exponential sodium phosphate gradients (Stokstad & Thenen, 1973). We were, however, unable to repeat the results of Stokstad & Thenen (1973) on the calibration of the columns with [5-^{14}C]methyltetrahydrofolate. In our experiments [5-^{14}C]methyltetrahydrofolate was eluted from the column at a higher ionic strength than that quoted by these workers. The 5-methyltetrahydrofolate peak matched the major radioactive peak from high-temperature liver extracts when used as an internal or external marker. Some 5-methyltetrahydrofolate samples gave a small second peak, 4a-hydroxy-5-methyltetrahydrofolate, and this compound chromatographs in the same place as the 5-methyltetrahydrofolate standard of Stokstad & Thenen (1973). [5-^{14}C]Methyltetrahydrofolate samples contain small amounts of 4a-hydroxy-5-methyltetrahydrofolate, and the percentage of this compound present increases with increasing time even when 5-methyltetrahydrofolate is stored frozen in the presence of ascorbate.

The chromatographic behaviour on DEAE-cellulose of the radioactive material obtained from liver by hot-extraction procedures differs from that of folate polyglutamates reported by Hoffbrand *et al.* (1976) and Brody *et al.* (1976), and results of both groups differ from that given by Moran *et al.* (1976). In our hands the radioactive material could not be separated from 5-methyltetrahydrofolate on DEAE-cellulose. However, on both Sephadex G-15 and DEAE-cellulose columns the ^{14}C- and ^{3}H-labelled material obtained from hot liver-extraction procedures co-chromatographed with synthetic ^{3}H-labelled pteroylhepta-γ-glutamate. Radioactive material obtained from liver without hot extraction co-chromatographed on Sephadex G-15 with authentic 5-methyltetrahydrofolate. Hot liver extraction is said to prevent folate polyglutamate breakdown (Bird *et al.*, 1965).

The long half-life of the radioactive material in the liver and the failure to displace it by large doses of folic acid or 5-methyltetrahydrofolate show that the liver folates are only in very slow equilibrium with the circulating folate monoglutamates.

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