Aspects of Radiofolate Absorption, Metabolism and Plasma Binding

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SUMMARY

After \(^{14}C\)-methyl folate (\(^{14}C\)-MeTHF) was taken by mouth, progressive incorporation of this isotope into the dialysis-resistant plasma folate fraction occurred. At 6 hours 68.8% of the total plasma radioactive folate was dialysis-resistant. We have previously shown that \(^{14}C\)-folic acid (\(^{14}C\)-PGA) taken by mouth is not similarly bound at 6 hours. Chromatography of plasma on DEAE A50 after \(^{14}C\)-PGA absorption, showed that PGA in plasma (peak 1) was gradually converted to MeTHF (peak 2) and the absence of bound radiofolate 6 hours after \(^{14}C\)-PGA ingestion probably reflects this conversion phase. No radiofolate appeared in red cells up to 11 days after isotope ingestion. Initial divergence between plasma biofolate and radiofolate indicated that "cold" storage folate was being displaced by absorbed radiofolate. Urinary radiofolate resolved into 3 fractions (peaks 2, 3 and 4) on DEAE A50 chromatography. One of these (peak 2) corresponded to MeTHF, but PGA (peak 1) was absent. Plasma showed peaks 1, 2 and 3, but at 3 hours no equivalent of urinary peak 4 was evident. Further studies are indicated to characterise fractions 3 and 4.


There is good evidence that part of the endogenous plasma folate is bound to macromolecules, probably protein. Based on L. casei bio-assay of eluates from gel filtration columns, Markkanen et al. have suggested that in normal serum this binder consists of 3 main fractions: an \(\alpha\)-macroglobulin, a \(\beta\)-globulin (possibly transferrin) and albumin. These binders were fairly constant under normal conditions. Characteristic variations during certain disease states were reported. Whether these saturated serum binders are identical with the unsaturated binders described by Waxman and Schreiber and Da Costa and Rothenberg is unknown. Our own studies failed to confirm significant unsaturated serum binders. It is also uncertain how these binders relate to the unsaturated folate binders present in milk, leucocytes of leukaemia and pregnancy, brush borders of small intestinal mucosa, and hog kidney.

Johns et al. reported that more than 60% of radioactive isotope of folate which appears in plasma after intravenous injection of tritiated folic acid, was protein-bound. It thus seemed possible that the saturated binder(s) in plasma could perhaps be radioactively labelled in vivo, and in this study the characteristics of radiofolate present in plasma/serum and urine after an oral dose of \(^{14}C\)-labelled folic acid, were investigated.

PATIENTS AND METHODS

Radioactive Folate

Two labelled folate preparations, obtained from the Radiochemical Centre, Amersham, UK, were used: folic acid 2-\(^14\)C (potassium salt), specific activity 122 \(\mu\)Ci/mg, radiochemical purity 98% (\(^{14}C\)-PGA); and 5 (methyl-\(^14\)C)-tetrahydrofolic acid (barium salt), specific activity 92 \(\mu\)Ci/mg, radiochemical purity 99% (\(^{14}C\)-MeTHF).

Each batch of radiofolate was assayed for biological activity by means of the L. casei technique to ensure that bio-activity correlated to within 5% with predicted values calculated from specific activity. Radiofolates were dissolved in sodium hydroxide and ethanol and kept in the dark at -20°C for a maximum of 3 weeks before use.

Patient Studies

Blood and urine samples were collected from 7 volunteer patients (4 folate-replete, 3 folate-deficient) after ingestion of radioactive folic acid. Written consent was obtained from these subjects after the purpose and implications of the experiments had been explained to them. Tests were performed immediately prior to discharge from hospital, when maximal convalescence had occurred. The protocol was scrutinised and passed by the Medical Faculty Ethical Research Committee.

A total of 20 \(\mu\)g/kg folic acid, labelled with 50 \(\mu\)Ci \(^{14}C\)-PGA, was ingested after an overnight fast. Heparinised venous blood was taken in Vacutainer tubes at 0 hours, 30 minutes, 1 hour, 2 hours, 3 hours, 6 hours and 24 hours after administration of the radiofolate and the fast was continued up to the 6th hour. Plasma and red cells were separated and stored at -20°C.
fasting urine specimen, further total collections were made for the periods 0 - 3 hours and 3 - 6 hours. The volumes were recorded and aliquots stored at -20°C.

In patient 4 separate small aliquots were taken at 1 hour and 3 hours for subsequent chromatography (see below). Patient 8, a 63-year-old White man with Hodgkin's lymphoma who had normal folate status, was similarly given 20 μg/kg (1 800 μg) ^7^C-MeTHF by mouth and blood samples were collected as above.

**Blood and Urine Samples**

**Bio-activity** was measured, by means of the *L. casei* method.

**Radiofolate** content was determined by liquid scintillation counting on an Intertechnique SL 30 connected to a Multi-8 computer as previously described, and radiofolate was quantitatively calculated from the specific activity of the oral dose of radiofolate given. Red cells were haemolysed and prepared for scintillation counting with the Intertechnique Oxymat model 4104 sample oxidiser.

**Dialysis** was done in Visking tubing against 0.9% NaCl for 24 hours.

**Ion exchange chromatography** was done on DEAE Sephadex A50. Columns of 20 × 0.8 cm were used and 0.5-ml samples were applied and eluted stepwise with 7 × 5 ml buffer NaCl solutions at pH 7.0. Approximately 45 fractions of 1 ml each were collected and total protein was estimated by measuring the extinction at 280 nm. The radioactivity in each fraction was also measured by scintillation counting.

**Haemoglobin-coated charcoal adsorption** (HCCA) was done by using a 50-mg coated charcoal pellet per 0.5-ml test sample, diluted with 1.5 ml 0.9% NaCl.

**RESULTS**

Data of the 7 patients are summarised in Table I. Patient 1 had a serum folate concentration of 3 μg/l, which is at the lower limit of normal for this laboratory, but he was included in the folate-replete group because of a normal blood picture and red cell folate. Patient 3 showed a slightly low red cell folate level (normal greater than 140 μg/l) but no other evidence of folate deficiency.

Maximal folate values were obtained 1 - 3 hours after radiofolate ingestion, when total biofolate exceeded radiofolate.

Urinary folate loss in 3 patients is reflected in Table II. The proportion of radiofolate to total folate excreted during 6 hours, was approximately 50% for patient 1 and nearly 100% for patient 4. Approximately two-thirds of the total folate in the 3-hour urine specimen of patient 6 was radioactive. Radiofolate loss as a percentage of the initial oral dose was 4.3% in patient 4, 1.4% in patient 1 and 2.5% in patient 6.
TABLE II. URINARY FOLATE EXCRETION (µg)

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Folate excretion</th>
<th>Total folate</th>
<th>Radiofolate</th>
<th>Balance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 - 3 hours</td>
<td>3 - 6 hours</td>
<td>Total 0 - 6 hours</td>
</tr>
<tr>
<td>4</td>
<td>Total</td>
<td>51</td>
<td>51</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Per hour</td>
<td>17</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>Total</td>
<td>23</td>
<td>13,3</td>
<td>9,7</td>
</tr>
<tr>
<td></td>
<td>Per hour</td>
<td>7,3</td>
<td>4,4</td>
<td>3,2</td>
</tr>
<tr>
<td>6</td>
<td>Total</td>
<td>45</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Per hour</td>
<td>15</td>
<td>10</td>
<td>5</td>
</tr>
</tbody>
</table>

* Total biofolate with L. casei technique.

Minimal radioactivity (folate 1.0 - 2.4 µg/1 packed cells) was detectable in 1% ascorbate haemolysates of thrice-washed red cells of patient 2, which were collected for 11 days after 14C-PGA ingestion. The patient who was given 14C-MeTHF (patient 8) similarly showed no significant red cell radiofolate incorporation over 6 hours of study.

The results of Visking dialysis and HCCA on plasma samples of patient 8 are represented in Table III. Both techniques showed a progressive increase of apparently bound 14C-MeTHF during the postabsorption phase. The bound fraction was larger with dialysis, but the difference is probably due to the more efficient clearing of non-bound (free) folate by HCCA. The absence of a prominent postabsorption radiofolate peak was an unexpected finding in this patient, although it may have been partly due to the lack of a 1-hour sample.

DEAE A50 chromatography of plasma (patient 4) showed a progressive change in the radiofolate elution pattern after absorption of 14C-PGA. At 30 minutes, the bulk of the radioactivity eluted distally to the main protein zone (peak 1 — corresponding to the elution position of 14C-PGA in the saline control). At 3 hours radioactivity had moved proximally under the albumin peak (peak 2 — corresponding to the elution position of 14C-MeTHF in the saline control). More proximal peaks of uncertain origin were also evident, which included at 30 minutes and 2 hours an early fraction in eluates 15 - 20 (peak 3).

After absorption of 14C-MeTHF (patient 8) plasma at 1 and 6 hours showed a 14C-MeTHF peak (peak 2), no PGA (peak 1) but a proximal fraction possibly corresponding to the above peak 3 (Fig. 2).

Saline controls (Fig. 1) also revealed unidentified minor proximal fractions, in addition to PGA and MeTHF, as position 14C-MeTHF in the saline control). More proximal peaks of uncertain origin were also evident, which included at 30 minutes and 2 hours an early fraction in eluates 15 - 20 (peak 3).

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Fig. 1. Elution (Sephadex DEAE A50) of protein and radiofolate from plasma taken 30 minutes, 2 hours and 3 hours after 14C-PGA ingestion, compared with 14C-PGA and 14C-MeTHF standards. Radiofolate fractions referred to in the text: peak 1 (PGA) eluates 45 - 55; peak 2 (MeTHF) eluates 35 - 45; peak 3 elutes 15 - 22.

Fig. 2. Elution (Sephadex DEAE A50) of plasma samples taken 1 and 6 hours after ingestion of 14C-MeTHF, showing absence of radioactivity in the position of PGA and major peaks in the position of MeTHF (as compared with Fig. 1).
Chromatography of urine specimens collected from patient 4 at 1 hour and 3 hours (Table II) revealed peaks of radioactivity corresponding to MeTHF (peak 2) but not to PGA (peak 1) (Fig. 3), although plasma samples taken at 30 minutes and at 1 hour showed both "C-PGA and "C-MeTHF (Fig. 1). Urine also showed earlier peaks of radioactivity. One of these resembled plasma peak 3 (eluates 18-25), and the other was more proximal (eluates 7-15; peak 4).

**DISCUSSION**

We were able to demonstrate by means of Visking tubing dialysis and charcoal absorption (HCCA) that significant macromolecular binding of radioactive MeTHF (but not PGA) occurred within 6 hours of ingestion. This explains our previous inability to label the plasma folate binder by means of oral "C-PGA. If one postulates that only MeTHF, and not PGA, becomes attached to the plasma binder, this finding probably reflects the time necessary for synthetic PGA to be converted to physiological MeTHF.

A more prolonged study\(^6\) has indeed revealed incorporation of label from radioactive PGA into the bound fraction at 24 hours after administration. After absorption of "C-PGA, chromatography showed that radiofolate in plasma becomes progressively more 'protein-associated' (Fig. 1) owing to conversion of PGA to MeTHF, a substance which elutes with albumin in our system (cf. Figs 1 and 2). In Markkanen et al.'s publications on various plasma folate binders\(^1\),\(^7\) it is not clear how the authors differentiated between protein-associated and protein-bound endogenous folate. The various folate fractions reported may indicate non-bound but chromato graphically dissimilar folate entities — perhaps even di- and triglutamates. Our own unpublished data suggest a single albumin-associated binder.\(^8\)

Plasma folate normally consists of a dialysable, 'free' fraction and a dialysis-resistant 'bound' fraction.\(^9\) The latter is bound to a virtually saturated binder, as it takes up no additional folate from the 'free' fraction in vivo, and because in vitro experiments revealed no significant unsaturated binding capacity for PGA or MeTHF,\(^1\) Markkanen and Peltola\(^1\) suggested that some 40% of serum folate is protein-bound, and Retief and Huskisson\(^1\) found that 1-5 μg folate per litre normal serum was dialysis-resistant, irrespective of initial folate values. In folate-deficient serum virtually all the folate appeared bound, and no significant deviations from normal could be detected in myeloma, pregnancy, hepatitis or glomerulonephritis.\(^1\) In kwashiorkor the bound fraction was possibly increased.\(^1\) Markkanen et al.'s\(^1\),\(^7\),\(^8\) extensively investigated the saturated serum binder carrying endogenous folate and suggested that it consists of various fractions — predominantly β-globulin (probably transferrin) and α₂-macroglobulin, but also albumin. They found that β-globulin fraction was increased in pregnancy, in pernicious anaemia, and during diphyllehydantoin therapy, whereas the latter treatment (as well as pregnancy) decreased α₂-macroglobulin binding. Retief and Huskisson\(^1\)
were unable to demonstrate significant increase of nondialysable (bound) serum folate in pregnancy. Additional specific abnormalities were also suggested on the basis of limited studies of various pathological states. 16 Other workers have also favoured transferrin as a folate binder but Jacob and Herbert 20 could not confirm this. After ingestion of "C-PGA minimal red cell-associated radiofolate was detected, but in view of the fact that normal red cell folate is in excess of 140 µg/l, 1-2.4 µg/l cannot be considered significant.

Dialysis experiments have shown a bound endogenous folate fraction in saliva, red cells, bile 21 and urine. 22 Gastric juice, 23 cerebrospinal and synovial fluids 24 contain no bound folate. Tisman and Herbert 25 found that serum inhibits radiofolate uptake by human bone marrow cells, and Waxman and Schreiber 26 noted that the unsaturated binder from folate-deficient serum withheld radiolotope from HeLa cells. This might be interpreted as evidence that the unsaturated plasma folate binder, which binds oxidised folates in preference to reduced folates, 27,28 is inert as a physiological folate carrier in the body and may even be an in vitro artefact.

Whitehead and Cooper 29 and Melikian et al. 30 showed that a physiological dose of oral PGA is absorbed unchanged and then gradually converted in the liver to reduced folates, MeTHF in particular. Our results confirmed this finding (Figs 1 and 2). Smaller radioactive peaks which eluted proximal to PGA and MeTHF in our chromatography system are at present unidentified. They may represent other physiological folate moieties (even di- or triglutamates) originating from the test dose of radiofolate. The finding that total biofolate exceeded radiofolate in postabsorption plasma samples (Table I) confirms previous suggestions that absorbed folate displaces stored folate from the liver. 22,23 Folate-deficient patients showed very low postabsorption plasma folate levels, but even here biofolate exceeded radiofolate. Butterworth et al. 31 could not demonstrate displacement of stored folate after oral administration of "C-polyglutamates.

It has long been known that urine contains a reduced folate which is not present in plasma, and Albrecht and Broquist 32 and Silverman et al. 33 showed that this component, supporting the growth of P. cerevisiae, is predominantly 10-formylfolate. McLean and Chanarin 34 injected "H-PGA intravenously and found 3 folate peaks in urine on DEAE A50 chromatography: a proximal peak, possibly 10-formylfolate, a second peak which appeared to be MeTHF, and a PGA fraction. With the same exchange resin we found urinary radiofolate in the position of MeTHF (peak 2, Fig. 3) as well as proximal fractions possibly corresponding inter alia to the P. cerevisiae fraction. This aspect is being further investigated. We found no PGA peak, but our patients took "C-PGA by mouth while McLean and Chanarin 34 gave the isotope intravenously, thereby producing higher blood levels and probable urinary 'spill-over'. It is interesting to note that PGA never appeared in the urine (Fig. 1) while the plasma of the same patient did initially contain PGA (Fig. 1). Radiofolate peak 3 in urine seemed to correspond to similarly placed peaks in plasma (Figs 1 and 2) but these preliminary results have to be confirmed. Either urinary peak 3 or peak 4 could represent 10-formylfolate. Renal handling of folate is still largely unexplained and the possible role of powerful local binders such as described by Kamen and Caston 35 remains speculative.

This study was supported by an ad hoc grant from the South African Medical Research Council.

REFERENCES