Folate binders in body fluids

F. P. RETIEF AND YVONNE J. HUSKISSON
From the Haematology Division, Department of Internal Medicine, Stellenbosch University Medical School, Bellville, CP, Republic of South Africa

SYNOPSIS In normal serum, saline dialysis for 48 hours in Visking casing resulted in folate clearance closely comparable to that produced by haemoglobin-coated charcoal adsorption, except in kwashiorkor where charcoal removed a greater proportion of folate. Pre- and post-dialysis values probably represented total and bound folate, respectively. Urinary folate consisted almost exclusively of dialyzable or free folate. Folate in saliva, bile, and erythrocytes consisted of dialyzable and non-dialyzable fractions; gastric juice contained minimal amounts of folate.

In spite of low serum albumin in kwashiorkor the bound folate fraction was increased rather than decreased; in myeloma with hyperglobulinaemia there was no increase in the bound folate fraction. Nephrotic urine did not contain excess folate, but pregnancy urine (third trimester) showed increased total folate.

Serum, chromatographed on Sephadex G-25, produced two folate peaks, only the first being associated with serum proteins. Urine contained only a second folate peak corresponding to the elution peak of pteroyl-monoglutamic acid (PGA). Adsorption studies with charcoal coated with 'molecular sieves' of varying size suggested that the predominant serum folate binder was of molecular weight 70,000-120,000. It is unlikely to be albumin.

Serum is known to be a poor binder of pteroyl-monoglutamic acid (PGA) (Condit and Grob, 1958; Hampers, Streiff, Nathan, Snyder, and Merrill, 1967; Metz, Zalúsky, and Herbert, 1968) but serum binding of natural endogenous folate has been scantily studied (Markkanen, 1968).

We previously reported a non-dialyzable folate fraction present in serum but absent from urine (Retief and Huskisson, 1969). In the present study this non-dialyzable, and possibly protein-bound, folate fraction was further investigated.

Methods

Folate activity was measured microbiologically with Lactobacillus casei by the aseptic addition technique of Herbert (1966). Serum and urine specimens obtained simultaneously from test subjects were stored without preservative at −20°C; no demonstrable folate deterioration occurred during our maximal storage time of three months. The pH of urine samples was adjusted to 7.0 before storage.

Aliquots, each of 1 to 2 ml, of urine, serum, saliva, gastric juice, bile, and haemolyzed normal whole blood (1 part blood: 9 parts distilled water) were dialyzed in Visking casing against 0.9% NaCl in the dark at 4°C for 48 hours (Retief and Huskisson, 1969). Folate levels measured before and after dialysis were referred to as 'total folate' and 'bound folate', respectively.

Aliquots, each of 1 to 2 ml, of serum and urine were agitated for five min. with 50 mg pellets of various coated charcoal preparations (see below). The folate content of the supernatants (after 15 minutes centrifugation at 1,400 g), were then
compared with the initial folate level. Norit A\(^1\) pharmaceutical grade neutral charcoal was coated with haemoglobin (Gottlieb, Lau, Wasserman, and Herbert, 1965), fibrinogen\(^2\), human gamma globulin\(^3\) or Dextraven\(^4\) by mixing it with these agents on a weight by weight basis: 5 parts charcoal to 1 part coating material (Herbert, 1969). Centrifuging 2 ml of a 2.5% aqueous charcoal suspension produced a convenient 50 mg charcoal pellet, capable of adsorbing from serum more than 50 mg free folate.

Aliquots, each of 2 ml, of normal and folate-deficient sera were incubated at 37°C for 30 min with varying amounts of added pteroylmonoglutamic acid (PGA) and N5-methyltetrahydrofolic acid (methyl THF). (Methyl THF and PGA were assayed with L. casei for biological activity and aqueous working solutions (100 ng) of biologically active material per ml were used in the experiment.) After incubation, the sera were adsorbed with 50 mg haemoglobin-coated charcoal (see above), centrifuged, and the supernatant folate activity was measured.

Aliquots, each of 2 ml, of serum, urine, and a PGA solution (100 ng) were chromatographed at room temperature on a Sephadex G-25 column (30 cm \(\times\) 1.5 cm). A phosphate buffer of \(pH\) 6.1 (similar to that used in the L. casei assay) was used as elution medium, and passed through the column by hydrostatic pressure. Approximately 50 2 ml aliquots were collected at a rate of 0.2 ml per minute. Eluates were assayed for folate content and total protein was determined as ultraviolet extinction at 280 \(\mu\)l in a Beckman DU spectrophotometer.

Preservatives such as ascorbic acid or mercaptoethanol were not added to the samples, because Markkanen (1968) detected no significant folate sparing with these substances under very similar experimental conditions.

All glassware was autoclaved to ensure maximal sterility and eluates were stored at \(-20^\circ\text{C}\) until analysis could be performed.

### Results

In Fig. 1 the effect of 48 hours' saline dialysis on serum folate is compared with folate clearance by haemoglobin-coated charcoal adsorption in certain clinical conditions.

In kwashiorkor less folate appeared to be lost by dialysis than by charcoal adsorption, but in the other diseases residual folate was closely comparable in the two methods.

Total and bound folate in serum and urine, measured before and after 48 hours' saline dialysis in a variety of conditions, are recorded in Table I. In most conditions mean serum-bound folate fractions were comparable but kwashiorkor

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**Fig. 1** A comparison between the serum folate clearing effect of 48 hours' dialysis and adsorption with haemoglobin-coated charcoal. O = original serum folate; Dial = post-dialysis folate; Ch = folate after charcoal adsorption PV = erythraemia PA = pernicious anaemia

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**Table I** Total and bound folate in body fluids

\(^1\)10% aqueous haemolysate.
Table II  Effect of excess PGA and methyl THF on folate binding by serum

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial Serum folate (g/100 ml)</th>
<th>PGA Added to Serum</th>
<th>After Hb-charcoal Adsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Native Serum after Hb-charcoal Adsorption</td>
<td>Initial</td>
</tr>
<tr>
<td>1</td>
<td>18.5</td>
<td>7.3</td>
<td>30.0</td>
</tr>
<tr>
<td>2</td>
<td>8.3</td>
<td>1.4</td>
<td>12.7</td>
</tr>
<tr>
<td>3</td>
<td>7.6</td>
<td>1.4</td>
<td>30.0</td>
</tr>
<tr>
<td>4</td>
<td>6.6</td>
<td>0.8</td>
<td>9.8</td>
</tr>
<tr>
<td>5</td>
<td>2.8</td>
<td>0.6</td>
<td>10.2</td>
</tr>
<tr>
<td>6</td>
<td>0.9</td>
<td>0.7</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>1.4 ± 0.4 (SE)</td>
<td>1.8 ± 0.6 (SE)</td>
<td></td>
</tr>
</tbody>
</table>

Table III  Folate clearing effect of uncoated charcoal and a variety of coated charcoals

<table>
<thead>
<tr>
<th>Adsorption Media</th>
<th>Serum A</th>
<th>Plasma A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>12.9</td>
<td>11.3</td>
</tr>
<tr>
<td>Uncoated charcoal</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Charcoal + fibrinogen (MW 400,000)</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Charcoal + gamma globulin (MW 155,000)</td>
<td>0.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Charcoal + Dextraven (MW 120,000)</td>
<td>2.9</td>
<td>2.8</td>
</tr>
<tr>
<td>Charcoal + haemoglobin (MW 70,000)</td>
<td>5.1</td>
<td>4.0</td>
</tr>
</tbody>
</table>

showed a moderately increased value. The mean serum protein and serum albumin values in kwashiorkor were 4.8 (± 1.08) g/100 ml and 1.67 (± 0.27) g/100 ml, which are significantly lower than our mean values for normal adults, 7.05 g/100 ml and 4.15 g/100 ml, respectively. The myeloma patients had mean total serum proteins of 8.7 (± 0.9) g/100 ml and mean gamma globulins of 3.46 (± 1.13) g/100 ml compared with our normal mean serum gamma globulin of 1.22 g/100 ml for adult patients. Bound folate thus appeared to be unrelated to serum albumin and gamma globulin.

Mean urinary folate was increased in the third trimester of pregnancy, compared with normal; the same group of pregnant patients had a mean serum level slightly less than normal. However, these were random urine values, and no attempt was made to calculate total daily urinary folate excretion. The pregnant patients were not on folic acid supplements. In spite of prominent proteinuria the glomerulonephritis group, which included a nephrotic patient, did not show significantly increased total or bound urinary folate fractions. Non-dialyzable folate was present in urine in very small amounts only, and could merely have represented residual folate contamination of the dialysis bags. Three saliva specimens examined had relatively high folate contents; most of the folate was dialyzable. Gastric juice contained very little folate; the values were not appreciably affected by previous neutralization of specimens to pH 7. The folate present in whole blood, and in a single bile specimen contained non-dialyzable fractions, comparable with that of serum. Whole blood folate consists predominantly of red cell folate (Hoffbrand, Newcombe, and Mollin, 1966) so that the above findings are primarily representative of red cell folate.

When PGA and methyl THF were added to normal and folate-deficient sera and the sera subsequently adsorbed with haemoglobin-coated charcoal there was no significant binding of added folates in excess of the endogenous bound folate (Table II).

When serum and heparinized plasma from a normal person were adsorbed with charcoal preparations coated with agents of varying molecular weight, folate was maximally cleared when the coating agent had a molecular weight of 155,000 or greater (Table III). A coat of molecular weight 70,000 (haemoglobin) resulted in folate clearing similar to that produced by dialysis in Visking casing (Fig. 1). We did not experiment with charcoal coats of molecular weight less than 70,000. Dextraven-coated charcoal had a folate clearing efficiency midway between that of haemoglobin and gamma globulin, suggesting that the main folate binder in serum (and plasma) may have a molecular weight between 70,000 and 120,000.

Chromatography of serum on a Sephadex G-25 column demonstrated two folate peaks, the first eluting with the serum proteins and the second appearing later (Fig. 2). After haemoglobin-coated charcoal adsorption the second peak was largely abolished while the first remained unchanged. The first peak thus probably represents bound folate and the second peak free folate.

When normal urine was passed through the column only a second folate peak was recorded, corresponding to the single elution peak of PGA (Fig. 3). Nephrotic urine gave a similar result, in spite of an associated protein peak.

Total folate recovery from the columns often
exceeded the folate applied as serum or urine. The reason for this is as yet obscure. However, in a specific experiment the ratio between bound and total folate in the serum sample applied usually compared well with the ratio between protein-bound (first peak) folate and total folate eluted. The serum applied in Fig. 2 had a bound folate fraction of 1.7 ng/ml, with total folate of 10.3 ng/ml (ratio 16.5%). After chromatography the first (bound) folate peak was 4.6 ng and total folate amounted to 30.6 ng (ratio 15.0%).

Electrophoresis and chemical analysis of the serum eluates associated with the second (non-protein) folate peak revealed no demonstrable protein in spite of a minimal ultraviolet light extinction at 280 μ.

Discussion

These studies confirmed our previous findings (Retief and Huskisson, 1969) that serum folate consists of a dialyzable and non-dialyzable fraction. The non-dialyzable fraction is probably protein bound, eluting from the Sephadex G-25 column in association with the serum proteins. Adsorption of serum with haemoglobin-coated charcoal had a folate clearing effect very similar to dialysis in Visking casing, suggesting that the two techniques removed the same (free) folate fraction, and that the bond between folate and binder is a significant one. Charcoal preparations, coated with molecules of varying size, may be used to estimate the molecular size of binder substances (Herbert, 1969). With this procedure the shape of coating molecules must of course also be taken into account. Such an experiment was performed and it suggested that the main serum folate binder may have a molecular weight between 70,000 and 120,000. It is unlikely to be albumin as kwashiorkor (characterized by a markedly decreased serum albumin) showed an increased rather than decreased bound folate fraction. Serum vitamin B₁₂ binding capacity is also increased in kwashiorkor in contrast with most other binding proteins (Grassmann and Retief, 1969). Glomerulonephritic urine, with prominent albuminuria, did not contain increased amounts of total or bound folate. Markkanen (1968) performed serum gel filtration with Sephadex G-200 and recorded two protein-associated folate peaks: a small peak in the 900,000 molecular weight range and a larger fraction in the 70-90,000 molecular weight range. In this article he refers to 'PGA binders' but it is evident from the text that naturally occurring L. casei activity was in fact measured. As we did, Markkanen (1968) found prominent folate elution distal to the protein peak, although prolonged dialysis was in his experience less effective in clearing this peak.

Folate recovery from our Sephadex column...
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was often in excess of 100%. One wondered whether elimination of serum folate antagonists (Cowan, Hoffbrand, and Mollin, 1966) during the chromatography procedure might have been partially responsible for this phenomenon.

We were unable to demonstrate unsaturated folate-binding capacity in normal and folate-deficient sera after the addition of PGA and methyl THF (Table II). The physiological significance of the free and bound folate fractions in serum is unknown, but urinary folate may derive from the free serum folate fraction. However, there was no constant relationship between free serum folate and urinary folate; in pregnancy the mean urinary folate was increased in spite of a decreased mean free serum folate (Table I). Bound folate was not found in urine in significant amounts except in two myeloma urines tested. The trace values recorded in Table I could represent residual contamination of the dialysis bags, and chromatography of urine did not reveal an early folate peak. In a limited study of other clinical conditions there was little significant difference as far as the free:total serum folate ratio was concerned (Table I). We are further investigating the finding in kwashiorkor that membrane dialysis gave higher bound serum folate values than did coated charcoal adsorption.

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References


