The reproducibility and use of the tritiated folic acid urinary excretion test as a measure of folate absorption in clinical practice: Effect of methotrexate on absorption of folic acid

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SYNOPSIS Investigations into the standardization and reproducibility of the urinary excretion method for determining the absorption of tritiated folic acid were carried out. By proper timing of tissue-loading doses of folic acid a clear difference between the percentage excretion ranges of normal and coeliac subjects was obtained. In addition conditions were found whereby the procedure could be repeated on an individual up to four times with reproducible results. Methotrexate in pharmacological amounts was found to have no inhibitory effect on the human intestinal absorption of a small oral dose (300 μg) of folic acid as determined by this method. This indicates that reduction of folic acid is not necessary for its absorption in man.

The tritiated folic acid (3H-pteroylglutamic acid, 3H-PGA) excretion test was originally described by Anderson, Belcher, Chanarin, and Mollin (1960) and has been used since by several authors (Klipstein, 1963; Kinnear, Johns, MacIntosh, Burgen, and Cameron, 1963; Halsted, Griggs, and Harris, 1967; and Kremenetchupky, Musso, Hoffbrand, and Rochna-Viola, 1967). The absorption of an oral dose of labelled folic acid is assessed by measuring the percentage which is excreted in the urine. The quantity of the test dose used has varied considerably but it is possible to use 300 μg which is approximately equivalent to the folate content of a normal meal (Butterworth, Santini, and Frommeyer, 1963; Hurdle, 1967; Chanarin, 1958). The test can provide a quantitative measure of the absorption of folic acid under nearly normal conditions, and is simple and convenient for both the patient and the investigator.

The test employs a flushing dose of folic acid to displace the labelled compound from the body. Most authors have, at various intervals before the test, attempted to saturate the body tissues with folic acid so as to reduce the amount of the labelled PGA taken up by the tissues. This precaution is particularly necessary in vitamin B12 or folate-deficient patients (Chanarin, Anderson, and Mollin, 1958; Klipstein, 1963).

Previous studies with normal subjects have shown a mean 24-hour excretion of approximately 45%, but there has been considerable variation in the values, thus limiting the usefulness of the test for comparative studies. In addition, the possibility of carrying out sequential investigations using a patient as his or her own control has never been assessed. In this communication certain modifications of the test procedure are presented which improve its reproducibility and utility.

The intestinal absorption of folic acid in man is of interest for many reasons. While it is probable that in the intact cell all of the vitamin exists in reduced and conjugated forms and no folic acid as such is present, it seems likely that during the preparation of food oxidation gives rise to at least some folic acid (Butterworth et al, 1963). Due to its stability and commercial availability both in radioactive and non-radioactive form, folic acid has tended to be used as a model compound to study folate absorption (Chanarin, 1969). Some controversy has arisen as to whether folic acid is reduced and methylated during transfer through the mucosal cells. It has been suggested by some workers that such reduction takes
place (Baker, Frank, Feingold, Ziffer, Gellene, Levey, and Sobotka, 1965; Cohen, 1965), but more recent studies have shown that while partly reduced folates may be further reduced and methylated (Chanarin and Perry, 1969) folic acid itself is absorbed largely unchanged into the portal blood (Whitehead and Cooper, 1967; Butterworth, Baugh, and Krumdieck, 1969; Leeming, Portman-Graham, and Blair, 1972). Chanarin and Perry (1969) suggest that it may be absorbed partly unchanged since dihydrofolate reductase has a limited ability to reduce it. At odds with these latter observations is the marked inhibition of folic acid absorption elicited by methotrexate in the rat (Burgen and Goldberg, 1962; Hepner, 1969). If methotrexate, which is a potent inhibitor of folic acid reduction, does in fact inhibit absorption, it would suggest that reduction of folic acid is in fact involved in its absorption. We have investigated the effect of large oral doses of the drug on folic acid absorption and conclude that it has no significant effect in the human intestine.

Materials and Methods

Preparation of Tritiated Folic Acid ($^{3}H$-PGA)

Folic acid (Cyanamid of Great Britain, Gosport, Hants) uniformly labelled by tritium gas exposure (Johns, Sperti, and Burgen, 1961) was purified by DEAE cellulose anion exchange chromatography. Chemical and radiochemical purity exceeded 98% and the specific activity based on extinction at 282 nm pH 7.0 ($\epsilon = 27.6 \times 10^{3}$) was 12 $\mu$Ci/mg. Doses contained 300 $\mu$g $^{3}H$-PGA in 100 ml water with a total activity of 3.75 $\mu$Ci. Dose solutions were found to be unstable when stored at 5°C in the dark for periods exceeding two weeks. They were therefore regularly subjected to microbiological assay with *Lactobacillus casei* and examined spectrophotometrically. Commercially available $3^{5}'9$ ($^{3}H$-) PGA, obtained from the Radiochemical Centre, Amersham, Bucks (specific activity 62 mCi/mg), was also used with similar results.

The non-radioactive folic acid for the flushing doses (Folvite) was supplied by Lederle, England. Both the radioactive and non-radioactive folic acid used in the oral dose were purified by DEAE cellulose chromatography before use. Methotrexate (4-amino-10-methylpteroylglutamate) was a gift of the Lederle Division, American Cyanamid Co, Pearl River, NY.

Test Procedure

Serum taken from all subjects before the test was stored at $-20^\circ$C in 1% ascorbate and subjected to microbiological assay. Tissue folate saturation was obtained by administering a 15 mg intramuscular injection of folic acid (Folvite, Lederle) 24 hours before the initial test. This is termed the 'loading dose'. In reproducibility studies the 'flushing dose' of the previous test served as the loading dose for the subsequent test. The interval between the tests was varied from 24 to 144 hours. Subjects were either fasted overnight or allowed to have an early light breakfast. Their bladders were emptied just before the test. The $^{3}H$-PGA dose was taken orally in 100 ml of water and followed by 200 ml of water. A further 15 mg of folic acid was then given intramuscularly in order to flush all the absorbed $^{3}H$-PGA into the urine, the so-called 'flushing dose'. Urine was then collected for 24 hours, the volume recorded, and aliquots were stored at $-20^\circ$C. Intake of fluids was encouraged during this period and a normal diet was resumed two hours after dose administration.

Assay of Urine Radioactivity

Urine samples (1-0 ml) were pipetted in triplicate into glass scintillation counting vials containing 10 ml toluene Triton X-100 scintillant fluid (Turner, 1969). The scintillation fluid was prepared by adding 700 ml Triton X-100 to 1 400 ml toluene containing 0.14 g POPOP and 5-6 g PPO. Activity was determined in a Tricarb liquid scintillation spectrometer (Packard model 3380) at 5°C and the counting error never exceeded 2.5%. The vials were recounted after the addition of 60 $\mu$l (approximately 5000 dpm) 1, 2, 3-trichlorobenzene ($^{3}H$) n hexadecane (The Radiochemical Centre, Amersham, Bucks, UK) which served as an internal standard. Overall efficiencies of counting in the urine by this method varied from 16 to 30%.

Microbiological Assay of Serum, Bile, and Test Doses

Blood was collected in 5 ml disposable plastic syringes and allowed to clot for two to four hours. The serum was removed and centrifuged at 3000 $g$ for 10 min to compact any cells not removed in the clot. It was then transferred to a plastic tube containing 0.05 ml 50% (w/v) potassium ascorbate pH 6.1 and mixed thoroughly. Bile was collected from patients with T-tube drainage following cholecystectomy into plastic tubes as above. In addition 3 ml samples of test dose were stored in plastic tubes as above. All samples were frozen at $-20^\circ$C until analysis.

All specimens were assayed with a chloramphenicol-resistant strain of *L. casei* (NCIB. 10463) following a modification of the method described by Millbank, Davis, Rawlins, and Waters (1970). Dehydrated folic acid (PGA) assay medium BBL was used throughout at half the recommended strength.
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The drug was administered in some instances 60 minutes before the test dose of oral folic acid or in solution simultaneously with it.

Results

\[ ^3\text{H-} \text{PGA} \text{ EXCRETION \textbf{IN NORMAL AND COELIAC SUBJECTS} } \]

Twenty-six control subjects had a mean excretion of 45.4 (SD ± 7.3)\% with a range of 30 to 57\%. Eight patients with coeliac disease had a mean excretion of 13.4 (SD ± 5.6)\% with a range of 7 to 21\%.

**The Effect of the Interval Between the Loading Dose and the Test Procedure**

Reproducibility of the procedure with a three- to six-day interval between the loading dose and the test

Ten subjects had a series of three to four tests performed. They had a loading dose 24 hours before the initial test. Subsequently the flushing dose of each test served as the loading dose for the next test. A mean excretion value of 47.9 (SD ± 5.5)\% with a range of 38 to 55\% was achieved for the initial test. The test was repeated three to six days later and the mean excretion fell to 29.2 (SD ± 16.5)\%, range 9 to 67\%. When repeated on the third or fourth occasion at seven to 14 days or 15 to 21 days the values were 20.5 (SD ± 13.3)\%, range 5 to 40\%, and 20.0 (SD ± 7.25)\%, range 11 to 28\%, respectively. The intraindividual variation between each test was large and the means were significantly different at \( p < 0.0025 \) level. These results are shown in figure 1.

![Image](http://jcp.bmj.com/) Fig 1 Effect of varying the time between giving the 'loading dose' of folic acid and administration of the oral \(^3\)H-PGA on the percentage excretion in the urine.
Reproducibility of the procedure when performed on consecutive days
Sixteen subjects were preloaded 24 hours before the initial test, the flushing dose of the initial dose serving as the loading dose for the subsequent test. The initial test gave an excretion value of 43·8 (SD ± 7·9)% range 30 to 57%. When the test was performed on the second, third, and fourth consecutive days the values were 41·2 (SD ± 8·5)%, range 26 to 53%, 41·1 (SD ± 8·7)%, range 23 to 56%, and 43·9 (SD ± 12·2)%, range 17 to 58%, respectively. There was no significant difference between the means (fig 2).

Timing of the urinary excretion of 3H-PGA
Ninety per cent of the 3H-PGA excreted in the 24-hour urine sample appears within the first eight hours. When only the flushing dose is administered on the second and third day after the initial test, the amount of 3H-PGA flushed out was less than 4% of the administered dose on the second day and less than 1% on the third day.

Biliary Folate Excretion
The biliary folate levels were determined on the patients with T tube drainage after cholecystectomy. Fasting levels were obtained before the loading and flushing doses of 15 mg folic acid intramuscularly on three consecutive days. The mean fasting level before the administration of the initial flushing dose was 30·0 ng/ml rising to 40·0 ng/ml before the final flushing dose. Biliary levels taken after the administration of 15 mg folic acid im (ie, flushing dose) showed a rise to 85 ng/ml, 290 ng/ml, and 450 ng/ml at 30, 60, and 120 min respectively.

Effect of Methotrexate
The 24-hour urinary excretions of eight subjects

<table>
<thead>
<tr>
<th>Subject</th>
<th>Previous Treatment with Methotrexate</th>
<th>Administration of Methotrexate at Time of Test</th>
<th>Percentage of Oral Dose Excreted in the Urine in 24 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. McN.</td>
<td>90 mg preceding year</td>
<td>5 mg 1 hr before</td>
<td>61</td>
</tr>
<tr>
<td>B. McG.</td>
<td>70 mg preceding year</td>
<td>5 mg one hour before</td>
<td>64</td>
</tr>
<tr>
<td>P. R.</td>
<td>185 mg preceding year</td>
<td>5 mg with dose</td>
<td>60</td>
</tr>
<tr>
<td>M. T.</td>
<td>50 mg preceding 9 months</td>
<td>5 mg with dose</td>
<td>47</td>
</tr>
<tr>
<td>D. F.</td>
<td>None</td>
<td>5 mg with dose</td>
<td>45</td>
</tr>
<tr>
<td>W. K.</td>
<td>None</td>
<td>5 mg with dose</td>
<td>58</td>
</tr>
<tr>
<td>E. H.</td>
<td>None</td>
<td>5 mg with dose</td>
<td>54</td>
</tr>
<tr>
<td>K. B.</td>
<td>None</td>
<td>5 mg with dose</td>
<td>55</td>
</tr>
<tr>
<td>D. F.</td>
<td>None</td>
<td>5 mg with dose</td>
<td>50</td>
</tr>
</tbody>
</table>

Mean (9 observations)
SD (df = 8)

Table I Twenty-four-hour urinary excretion of a 300 μg dose of tritiated folic acid after treatment with methotrexate

Fig 2 Reproducibility of the percentage urinary excretion of 3H-PGA when the interval between the 'loading dose' of folic acid and the oral test is limited to 24 hours.
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(N = 9) after the administration of a physiological test dose of 300 μg of tritiated folic acid are shown in Table I. Some of the subjects had received large doses of methotrexate previously as well as at the time of the test while others had no history of previous methotrexate therapy. In most instances the urinary excretion was considerably higher than the mean value of 45.4 (SD ± 7.3)% (N = 26) found in normal individuals. The mean excretion observed was 54.9 (SD ± 6.5)% with a range of 45 to 64%. This is significantly higher than the normal mean (t = 12.71; P < 0.001).

Discussion

Folic acid absorption has been measured by giving a small oral dose (300 μg) of tritiated folic acid (³H-PGA) and flushing the absorbed folic acid into the urine. The results recorded in this study demonstrate a reduction in the variance obtained in normal subjects when compared with those obtained by other authors (fig 3). The greater variance demonstrated elsewhere has tended to lessen the value of this test as a measure of upper small intestinal function as no clear demarcation could be shown between normal and coeliac subjects. However, by using the modifications suggested in this communication a definite separation has been obtained between these two groups.

Certain points should be emphasized concerning the changes in the technique advocated. The precision of the test procedure can be enhanced in the following ways. The tritiated folic acid must be of high purity and stock solutions should be monitored routinely by spectrophotometry and bioassay to detect degradation during storage. The use of toluene Triton X-100 scintillant for determining the tritium activity in the urine has given a higher efficiency and reproducibility than the scintillants used in previous studies (Anderson et al, 1960; Kinnear et al, 1963; Halsted et al, 1967). Adherence to these points has enabled the oral dose of tritiated folic acid to be reduced from the 10 to 20 μCi level used by other authors to a 3 to 4 μCi dose, thus enhancing the safety of the procedure and reducing its cost.

Perhaps the most surprising feature of this study has been the importance of the timing of the tissue loading doses of folic acid when assessing the reproducibility of the test in the same individual. If the loading dose is given four or more days before the test the percentage of the dose excreted falls when compared with the level obtained when the loading dose is given 24 hours before the test (figs 1 and 2), which would suggest that the degree of tissue saturation decreases rapidly. This is supported by the findings of Kinnear et al (1963) who demonstrated in 11 control subjects that comparable results could be obtained when the tritiated folic acid absorption test was repeated at a week's interval if a daily parental dose of 30 mg of PGA was given during the week.

Other studies of this procedure have concentrated mainly on the size and timing of the parenteral flushing dose of PGA. Anderson et al (1960) demon-

![Fig 3 Summary of the results obtained by previous authors and the conditions they employed in comparison with the present study.](http://jcp.bmj.com/)

**Fig 3** Summary of the results obtained by previous authors and the conditions they employed in comparison with the present study.
serum folate ship folate absorption is finding does Sobotka, secretion. hours dilution the the day studies have been demonstrated by faecal excretion studies that approximately 80% of a single small dose of tritiated folic acid is absorbed from the human intestine (Anderson et al, 1960; Jeejeebhoy, Ramanath, Mehan, Patthere, Parekh, Nadkarni, and Ganatra, 1967). Of this absorbed folic acid it would appear that by the simultaneous administration of a large flushing dose of unlabelled folic acid, and in addition previously saturating the tissue stores with the vitamin, only some 50% of the oral dose can be found in the first 24-hour urine collection (Anderson et al, 1960). It would appear that even with saturation and flushing some 20% of the tritium label gets taken up by the tissues and retained for a period greater than 24 hours. This is further supported by the intravenous studies of Johns and his coworkers (Johns et al, 1961) who found that even at very high concentrations some of the intravenously administered folic acid was retained longer than 24 hours.

We have found that the administration of methotrexate significantly raises the amount of radioactive folic acid excreted from a small oral dose over what is found in normal circumstances. In the normal group a mean of 45.4 (SD ± 7.3)% of a 300-mg dose of tritium-labelled folic acid was excreted in the first 24-hour urine collection; however, when methotrexate was administered with the labelled folic acid the mean value was elevated to 54.9 (SD ± 6.5)% (table I). In no instance did any 24-hour urinary excretion fall significantly below the mean excretion value of 45.4% found for normals.

Chanarin and Bennett (1962) observed an increased urinary Streptococcus faecalis activity after daily parenteral folic acid in one subject who had previously been on methotrexate therapy. These authors considered that the increased excretion of folic acid was related to defective tissue cell uptake. Another possibility would be that methotrexate blocks tissue cell utilization of folic acid rather than transport into the cell per se. Preliminary observations in this laboratory (Brown, Houlihan, Davidson, and Scott, 1973) would suggest that the latter hypothesis is the more probable explanation. Whatever the explanation of the increased urinary folate excretion following methotrexate therapy at high concentrations, the results demonstrated above show that it did not significantly reduce the intestinal absorption of a small oral dose of folic acid in man. This would support the view that while the dihydro derivative may be reduced during its passage through the mucosal cells folic acid remains unchanged.
Summary of Test Procedure

The procedure which is advocated in this communication for the measurement of folic acid absorption is summarized as follows:

A loading dose of 15 mg PGA is given intramuscularly 24 hours before the test. The patient fasts overnight and is given a 100 ml test dose of 300 μg labelled with 3 to 4 μCi 3H-PGA which is washed down with 200 ml of water. A further intramuscular dose of 15 mg PGA is given 30 minutes after the test dose. The urine is collected for 24 hours and repeat tests can be performed on consecutive days since tissue saturation is maintained by the parenteral PGA injections.

Dr D. S. Freedman was in receipt of a grant from the Medical Research Council of Ireland and Dr J. P. Brown is supported by a grant from the Biomedical Research Trust. Both grants are acknowledged with gratitude. We are especially grateful to Dr D. M. Mitchell for introducing us to his psoriatic patients whom he was treating with methotrexate.

References


Chanarin, I., and Perry, J. (1969). Evidence for reduction and methyl-
Letters to the Editor

A Simple Aid to the Administration of Blood Product Concentrates

We have found that nylon catheter mounts are useful for the efficient withdrawal from plastic transfusion bags of blood products such as cryoprecipitate and platelet concentrate.

The mounts (ref 700/180/Luer) are made by Portex Ltd of Hythe, Kent, and can be attached directly to a syringe. Their conical shape makes them a snug fit in the port of a blood bag. They are firm enough to pierce the membrane in the port but the end is not sharp and will not damage the wall of the bag. Their length (5 cm) is sufficient for the tip to lie just within the margin of the bag.

With one of these mounts, the contents of several bags can quickly be aspirated into a single large syringe. The mounts can be autoclaved for re-utilization.

I am grateful to Mr H. Byram, SRN, Superintendent of CSSD, Royal Lancaster Infirmary, who recommended these mounts.

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Type III Hyperlipoproteinaemia and Sinking Prebeta Lipoprotein

In our study of the kindred of a patient with type III hyperlipoproteinaemia (J. clin. Path., 1973, 26, 163), we postulated that a son of the proband exhibited a stage in the development of the type III disorder. His electrophoretic strip revealed chylomicrons and an increased prebeta band without lipoprotein of D<1.006 with beta mobility. On re-examination one year later, this patient has since developed a definite type III hyperlipoproteinaemia in that beta lipoprotein of D<1.006 has now been detected in his plasma; this confirms our previous hypothesis.

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Neonatal Meningitis Caused by Citrobacter koseri

The outbreak described by Gross, Rowe, and Easton (1973) is of interest because we have recently encountered a similar outbreak with four cases in our own premature baby unit (Gwynn and George, 1973) and know of one further case in another baby unit in this city (Bridgewater, 1972, personal communication). Citrobacter koseri may therefore be a more frequent cause of neonatal meningitis than has been recognized in the past and its potential pathogenicity in baby units seems clear.

Studies in our own unit suggested that intestinal carriage was important. The organism was recovered from the bowel of several unaffected infants and also from a member of staff at one stage. The outbreak was controlled, without need for closing the unit, by regular screening of all babies and by isolating carriers. The unit has remained free of the organism in the 10 months since the outbreak. Sensitive methods for detecting the organism were required and we find overnight culture of a saline suspension of stool in selenite F with subculture to MacConkey agar containing 10 µg/ml ampicillin most useful in this respect.

A more detailed account of our methods and of the incidence of this organism in different situations is being prepared for publication.

R. H. GEORGE
Department of Virology, The Medical School, Birmingham

References
Bridgewater, F. A. J. Personal communication.

Correction
In the paper by Freedman et al (J. clin. Path., 1973, 26, 261-267) the illustrations shown for figs 1 and 2 have been transposed.