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A Simple Method of Estimating Folic Acid Absorption (A Modified Faecal Excretion Method)[1]

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Folic acid absorption can be measured by observing the blood folic acid levels following an oral load of unlabelled folic acid (1), but this method has several disadvantages. One disadvantage is that it does not give an accurate idea of percentage absorption and, secondly the load of folic acid alters the haematological status of the patients. Furthermore, since the oral load of folic acid amounts to a few mgms, it is unphysiological, because it is greatly in excess of the normal daily intake.

Recently, the method of measuring folic acid absorption by using ^{3}H folic acid has become popular (2). Absorption with this tracer can be estimated in one of two ways. First, after an oral dose, absorption can be measured by subtracting the amount excreted in feces from the dose given. Second, it can be done by flushing out the absorbed tracer in the urine with an injection of unlabeled folic acid, a method similar to the Schilling test for vitamin B_{12}. The first method largely overcomes the problems associated with the use of methods utilising unlabeled folic acid, but the second method is semiquantitative and the injection of folic acid alters the haematological status of the patients.

The faecal excretion method, though technically very satisfactory, has two main difficulties: first, the preparation of fecal samples for tritium counting requires some form of oxidation, either with explosive agents like perchloric acid (3) or by the use of expensive combustion bombs. Second, collection of feces for many days is required to ensure complete recovery of the unabsorbed tracer. The latter process makes the procedure unattractive to the patients and may necessitate admission to ensure satisfactory collection. Furthermore, complete collection is possible only with very cooperative patients.

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These two difficulties have made the fecal excretion methods rather complicated. This paper presents a method which largely overcomes these difficulties and compares it with the results of absorption measured by total fecal collections done simultaneously in control subjects and patients with various intestinal conditions.

PRINCIPLE OF METHOD

The preparation of the fecal samples for tritium counting was accomplished by wet oxidation with acid potassium permanganate and hydrogen peroxide. This method does not require any elaborate apparatus and there is no danger of an explosion.

The necessity for collecting feces for many days was overcome by feeding chromium-51, (as chromic oxide) an unabsorbable substance simultaneously with ^{3}H folic acid. The absorption was calculated from the ratio of ^{51}Cr to ^{3}H folic acid in a single stool collection made in the first 24 hours after the dose. This method has already been used to measure vitamin B_{12} absorption (4).

MATERIALS AND METHODS

RADIOACTIVE COMPOUNDS

Hydrogen-3 folic acid was prepared by exchange labeling of pure cyrstalline folic acid with tritium followed by chromatography, at the Isotope Division of the Atomic Energy Establishment, Trombay, a method similar to that described by Johns *et al* (5). The specific activity was 250 μC/mg.

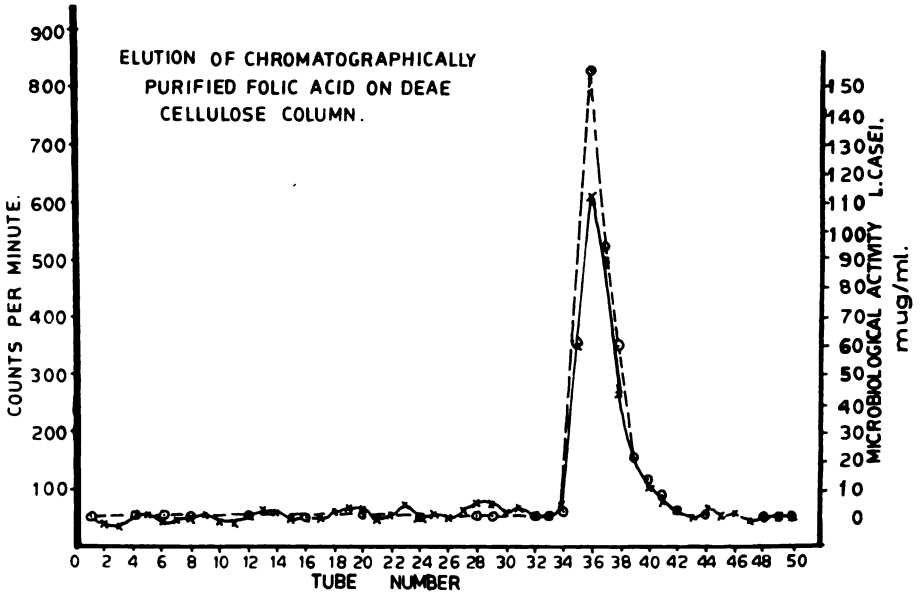

Fig. 1. Elution of chromatographically purified folic acid on a DEAE cellulose column showing the radiochemical purity of the compound fed. Solid line represents counts per minute and dashed line microbiological activity.

In order to test purity, a sample of the ^{3}H folic acid used was subjected to chromatography on DEAE cellulose, using a modification of the method described by Johns *et al* (5). The elutes were subjected to microbiological assay for folic acid using *Lactobacillus Casei* (6) and also counted for ^{3}H activity.

To ascertain the stability of ^{3}H label in the intestine, 10μC of ^{3}H folic acid was fed to two rats. After one hour, the rats were sacrificed and the small intestine was removed and divided into two equal halves. The intestinal contents in each half was washed out with five ml phosphate buffer 0.01M, pH 6.8 containing 100 mg% ascorbic acid. An aliquot of the contents were counted as such and then dried, reconstituted with buffer and again counted. This procedure would indicate if there was any free $^{3}H_2O$ produced during digestion. The contents were then applied to a DEAE column and chromatographed (5). The elutes were subjected to microbiological assay for folic acid using *Lactobacillus Casei* and were also counted for ^{3}H radioactivity.

Eighty μC of ^{3}H folic acid was mixed with 10-15 μC of ^{51}Cr in a waxed paper cup and fed to fasting patients. The cup was washed twice and the washings were also fed.

The first 24 hours' fecal sample was collected and a further five daily 24-hour collections were made after the dose.

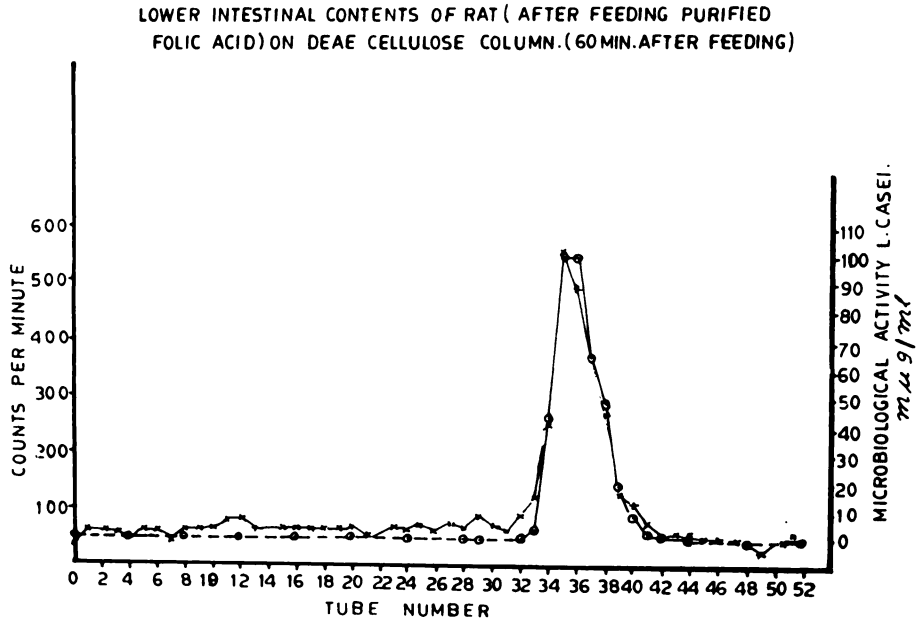

Fig. 2. Chromatographic pattern of the lower intestinal contents of a rat after feeding purified folic acid. The figure shows that the radioactivity was firmly bound to the folic acid activity during intestinal digestion. Solid line represents counts per minute and dashed line microbiological activity.

The fecal samples were thoroughly and separately homogenised, weighed and aliquots were taken for counting of hydrogen-3. The ^{51}Cr excretion in the first 24-hour collection was also counted.

The first 24-hour fecal collection was counted between two sodium iodide crystals in Nuclear Chicago Model GA-5 multiprobe unit with model 1620 c. Ratemeter and Model 1810 Radiation Analyser. The counts were referred to a standard made from the dose fed and expressed as percentage.

A two-gram aliquot of the first 24-hour collection which had been counted for ^{51}Cr was taken in a double-necked flask and mixed with 5 cc. of concentrated sulphuric acid. A reflux condenser was fitted to the central neck and the contents were stirred continuously with a magnetic stirrer. From the side neck, two grams of potassium permanganate were gradually added and a brisk reaction was seen in the flask. The stirring was continued until the reaction subsided. Then 0.9 — 1 ml of hydrogen peroxide, 20 volumes per cent was gradually introduced until

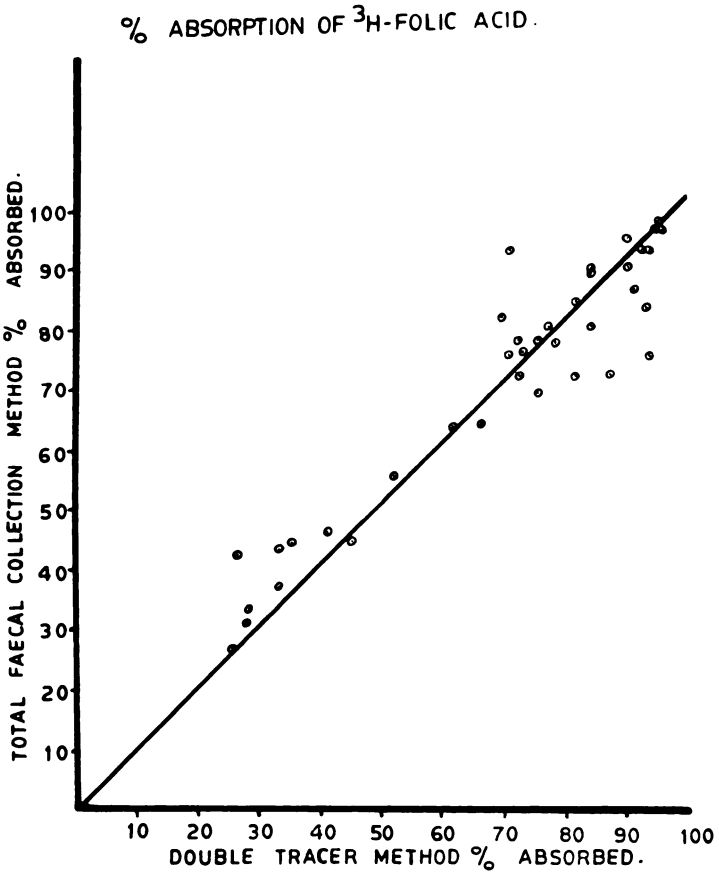

Fig. 3. The results of absorption measured by the double tracer method compared to the absorption measured by the total faecal collection.

the solution in the flask became colorless. Fifteen to twenty milliliters of 40% sodium hydroxide were added to the acid solution until a permanent black precipitate had just formed. The pH at that point was about eight. The whole solution was transferred to a measuring cylinder and volume was noted. An aliquot was centrifuged and 0.5 ml of the clear supernatant was counted in 20 ml of Dioxane scintillation fluid containing four per cent thixotropic gel. The counts were referred to an internal standard made up of an aliquot of the dose fed. The same procedure was adopted with subsequent 24-hour collections. The calculation of tritium excreted is illustrated in the example given below.

Weight of the 24-hour collection	.. 100 gms	
Weight of aliquot taken	.. 2 gms	
Volume of the oxidised faecal mixture	.. 30 ml	
Net counts in 0.5 ml	.. 1000 cpm	
Net counts in internal standard	.. 35×10^3 cpm	
Dilution of dose used to prepare internal standards.	.. 1/2000	
Counts of tritium excreted in 24 hour collection.	.. $\frac{1000 \times 30 \times 2 \times 100}{2}$	$= 3 \times 10^6$
Counts fed to patients.	.. $35 \times 10^3 \times 2000$	$= 70 \times 10^6$
% excretion of tritium.	.. $\frac{3 \times 10^6 \times 10^2}{70 \times 10^6}$	$= \frac{30}{7} = 4.3\%$

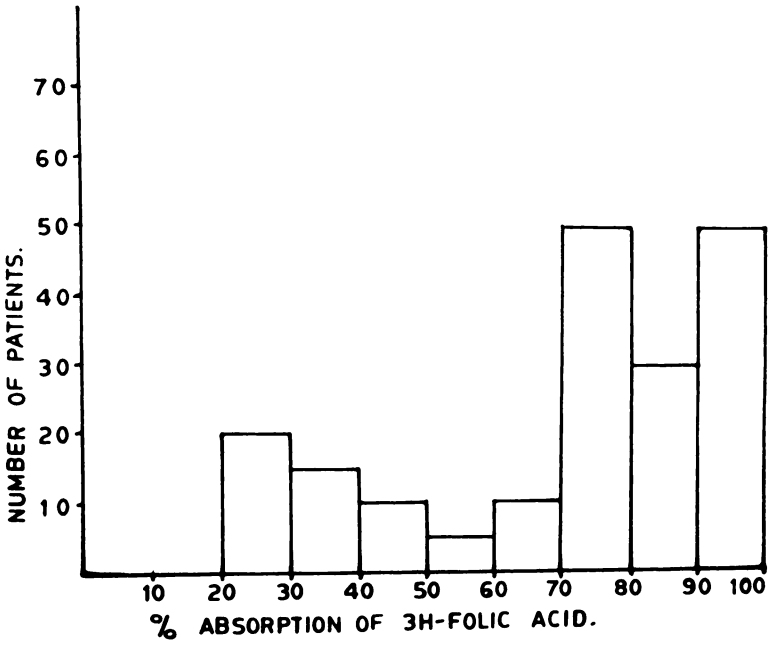

Fig. 4. Distribution of levels of absorption of H^3 folic acid.

Since ^{51}Cr is not absorbed, the percentage of this tracer in a random faecal sample can be used to correct the tritium excretion in the same sample for 100% excretion of ^{51}Cr, the assumption being that ^{51}Cr and ^{3}H have remained intimately mixed in the gastrointestinal tract. For example, if 10% of the orally fed ^{51}Cr was excreted in the 24-hour collection of stool containing 4.3% of ^{3}H folic acid, then the total excretion of unabsorbed tritium would be $\frac{4.3 \times 100}{10} = 43\%$

Hence absorption of ^{3}H folic acid would be $100 - 43 = 57\%$.

Known amounts of ^{3}H folic acid were added to faeces and the recoveries were studied after oxidation by the method described above.

There were 16 control subjects of whom 15 were entirely normal and one was a treated case of iron deficiency anaemia. None of them had gastrointestinal disease.

Three patients had nutritional megaloblastic anaemia, two had low serum L. casei (Folic acid) levels and one a low serum vitamin B_{12} level without evidence of clinical or biochemical malabsorption. There were 19 patients with gastrointestinal disease and 17 of them had tropical sprue with steatorrhoea. The remaining two patients had ulcerative colitis and partial gastrectomy with a low serum L.casei (Folic acid) level, respectively.

RESULTS

PURITY OF ^{3}H FOLIC ACID

The results are given in Fig. 1. It will be seen that the radioactivity comes out as a sharp peak between tubes 34 to 40.

This position corresponded to the elution of a known folic acid standard. Furthermore, the peak of microbiological activity corresponded to the peak of ^{3}H activity confirming the purity of the ^{3}H folic acid used.

TABLE I

Experiment No.	*% recovery of H^3 folic acid added to faeces.*
1	105.2
2	104.2
3	84.6
4	97.6
5	98.0
6	100.0

TABLE II

CONTROL SUBJECTS

No	*Clinical Condition*	*Absorption of ^3H Folic acid*		*% of dose fed excreted in the collection used to calculate absorption in column 3.*
		Double tracer method	*Total collection*	
		% of dose fed		
1	*2*	*3*	*4*	*5*
1.	Iron deficiency (treated)	79	76.9	55
2.	Normal	78	80.3	78
3.	Normal	75.6	69.4	74
4.	Normal	74.2	76.2	4.3
5.	Normal	73.4	78.2	16.5
6.	Normal	73.3	72.1	55.9
7.	Normal	66.8	64.3	40.6
8.	Normal	93.5	92.7	91.5
9.	Normal	72.5	93.3	16.5
10.	Normal	91.5	90.0	69.9
11.	Normal	86.0	79.7	78.0
12.	Normal	83.0	83.8	84.8
13.	Normal	84.7	90.1	37.3
14.	Normal	95.8	96.3	59.0
15.	Normal	96.8	95.6	100
16.	Normal	85.0	89.7	56.4
	PATIENTS WITH NUTRITIONAL MEGALOBLASTIC ANAEMIA			
17.	..	76.4	77.4	21.4
18.	..	52.8	55.6	24.6
19.	..	71.5	75.4	19.5
Mean and standard deviation of absorption of ^3H Folic acid in normal subjects.		79.44 ± 11.1		

TABLE II (cont'd)

CONTROL SUBJECTS

No	*Clinical Condition*	*Absorption of ^3H Folic acid*		*% of dose fed excreted in the collection used to calculate absorption in column 3.*
		Double tracer method	*Total collection*	
		% of dose fed		
1	*2*	*3*	*4*	*5*
	PATIENTS WITH GASTROINTESTINAL DISEASES			
20.	Ulc. Colitis	63.9	62.8	3.9
21.	Partial gast.	46	44.7	59.5
22.	Sprue	36	44.6	21.9
23.	Sprue	33.5	43.4	21.6
24.	Sprue	94.5	74.8	71.0
25.	Sprue	42	46.6	56
26.	Sprue	26	27	83.0
27.	Sprue	27.8	31.1	17
28.	Sprue	33.6	37.4	27.9
29.	Sprue	26.6	42.5	72.4
30.	Sprue	93.7	83.3	79.4
31.	Sprue	91.9	85.9	69.0
32.	Sprue	87.8	71.9	63.0
33.	Sprue	96.4	96.9	93.0
34.	Sprue	82.3	71.8	77.2
35.	Sprue	90.8	95.0	49.7
36.	Sprue	93.8	92.8	9.8
37.	Sprue	28.5	33.5	23.3
38.	Sprue	70.5	81.7	62
Mean and standard deviation of absorption of ^3H Folic acid in patients with sprue.		62.1 ± 30.3		

STABILITY ^3H LABEL IN THE SMALL INTESTINE

There was no difference in the counts of an aliquot of the intestinal contents in the wet and dried state, indicating that no ^3H_2O was produced during digestion. The chromatographic pattern of intestinal contents was identical in upper and lower small intestine and a representative pattern is seen in Fig. 2. From the figure, it will be observed that the pattern of elution of folic acid from DEAE in the intestinal contents was similar to the pattern of the dose fed and showed no obvious degradation products of folic acid. Furthermore, both microbiological activity and radioactivity coincided, showing that the label was firmly bound to the folic acid.

RECOVERY OF ADDED ^3H FOLIC ACID

The results are given in Table I. Between 84 and 105% of the added ^3H folic acid was recovered by the oxidation method described.

COMPARISON BETWEEN ABSORPTION ESTIMATED BY TOTAL SIX DAYS COLLECTION AND THE ^{51}CR METHOD

The results are given in Table II and Fig. 3. It can be seen that a good correlation exists between the results obtained by the methods described and the results of absorption from total fecal collection in both controls and patients with gastrointestinal disease. Taken as a whole, the coefficient of correlation was $r = 0.9$; however, considering individual results, only in three subjects. (9, 24 and 29) out of 38, the two results were obviously different. Furthemore, good agreement was obtained even when the amount of ^{51}Cr in the collection taken to calculate total absorption was as low as 4% (Table II, column 5) indicating that a small aliquot of stool could be used to measure total absorption.

DISTRIBUTION OF THE LEVELS OF ABSORPTION OF ^3H FOLIC ACID

The results are given in Fig. 4. It will be seen that the absorption of folic acid falls in a bimodal pattern of distribution with all controls absorbing more than 60% of the dose and about half the patients with sprue absorbing less than 50% of the dose. One patient with nutritional megaloblastic anaemia absorbed 52% of the dose; this value for absorption lies between the controls and patient with obvious malabsorption. This patient had no steatorrhoea and the anaemia responded to small oral doses of folic acid.

DISCUSSION

The results show that acid $KMnO_4$ and H_2O_2 is a suitable way of preparing samples of feces for tritium counting. It compares favorably with the other oxidation methods and has the advantage of being both safe and inexpensive. Furthermore, by the use of ^{51}Cr, it is possible to estimate absorption from a single random faecal sample, which makes the procedure feasible in non-cooperative patients and in busy clinical units without adequate facilities for metabolic

studies. Since folic acid absorption is rapidly becoming an important tool in the study of malabsorption syndrome and in megaloblastic anaemias, the technique mentioned above would be useful in making the study feasible in places with relatively meagre facilities. The one problem which remains is the necessity of using an expensive liquid scintillation counter for estimating tritium. Here again, where such facilities are not available, the oxidized fecal samples could be easily transported to a central laboratory for counting, a process which would be of importance in underdeveloped countries.

Using this method, the normal absorption appears to be 60% of the dose and above. This value for normal absorption with ^{3}H folic acid is very similar to that observed in controls by Anderson *et al* (7). Malabsorption appears to be present when less than 50% of the dose is absorbed. Absorption of between 50 and 60% may occur in patients with nutritional megaloblastic anaemia. A slightly impaired absorption of folic acid in nutritional megaloblastic anaemia has been observed by Anderson *et al* (2).

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