THE METABOLISM OF TRITIATED FOLIC ACID IN MAN *

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Several microbiological methods are available for the assay of folic acid and related coenzymes in tissues and body fluids (1, 2). Many conjugated forms of folic acid are not detected by such assays unless first converted enzymatically to active forms (3). The presence of folic acid antagonists or of antibiotics can also render difficult the quantitative determination of folic acid and its conjugates by present microbiological techniques. In order, therefore, to facilitate the study of the metabolism of folic acid in normal subjects and in patients with hematological and other disorders, tritiated folic acid has been prepared and some aspects of its metabolism in man studied by non-microbiological assay methods. The present paper presents the results of these studies, preliminary accounts of which have appeared (4, 5).

METHODS AND MATERIALS

Preparation of tritiated folic acid

Finely powdered USP grade folic acid, 500 mg, was exposed to 15 c tritium at 27°C and 0.4 atm pressure for 10 days. The crude tritiated material had an activity of 220 μc per mg; 40-mg batches of this material were dissolved in 0.2 per cent $NaHCO_3$ and chromatographed in the dark at room temperature on a 2×20 cm column packed with DEAE-cellulose (Eastman Kodak 7392) equilibrated by the method of Sober, Gutter, Wyckoff and Peterson (6). Elution was started with 0.01 M sodium phosphate buffer at pH 6.9. At 280 ml, buffer strength was raised to 0.2 M. A flow rate of 2 ml per minute was used. The effluent was passed through an ultraviolet absorptiometer (Uvicord, LKB, Stockholm) and collected in 10-ml portions in an automatic fraction collector (RadiRac, LKB, Stockholm). The absorptiometer lamp was switched off during the passage of the folic acid peak to minimize photochemical decomposition. Major impurities were found by ultraviolet analysis of the fractions to be 2-amino-4-hydroxy-6-methylpteridine, 2-amino-4-hydroxypteridine-6-carboxylic acid, *p*-aminobenzoylglutamic acid and pteroic acid. The folic acid fractions were pooled and rechromatographed. The specific activity of the folic acid was found to be 12.2 ± 0.2 μc per mg. Additional chromatographic purification did not further lower this value. In order to minimize radiation decomposition, a weight of glucose 20 times that of the weight of the pooled folic acid was added and the resulting folic acid-sodium bicarbonate-glucose solution lyophilized and stored in the dark at -5°C. The microbiological activity of this preparation of tritiated folic acid was determined by *Lactobacillus casei* assay by the method of Baker and co-workers (2) and was equal to that of an equivalent weight of USP folic acid.

Distribution of radioactivity within the folic acid molecule. Two mg of tritiated folic acid of specific activity 1.40×10^{10} dpm per mmole was dissolved in 2 ml of 0.2 per cent $NaHCO_3$; 3 ml of 2 N H_3PO_4 and 200 mg of zinc were added and reduction allowed to proceed for 30 minutes; 600 mg of $CaCO_3$ was then added and the mixture left to stand overnight. The clear supernatant was decanted and applied to a column of DEAE-cellulose, 1×7.5 cm. Elution was started with 0.01 M sodium phosphate buffer, pH 6.9, the effluent being passed through an ultraviolet absorptiometer. After the passage of the 2-amino-4-hydroxy-6-methylpteridine peak, buffer strength was increased to 0.2 M. The pteridine and *p*-aminobenzoylglutamic acid fractions were collected. No unsplit folic acid was detectable in the effluent. The concentration of *p*-aminobenzoylglutamic acid was determined by the Bratton-Marshall method (7), and the concentration of 2-amino-4-hydroxy-6-methylpteridine by the measurement of ultraviolet absorption at 252 mμ and 363 mμ, at pH 13. The specific activity of the *p*-aminobenzoylglutamic acid was 1.06×10^{10} dpm per mmole or 75.7 per cent that of the starting material, and the specific activity of the pteridine was 0.31×10^{10} dpm per mmole or 22.1 per cent that of the starting material; 97.8 per cent of the radioactivity present in the folic acid was therefore accounted for after reductive cleavage.

To 0.5 mg of tritiated *p*-aminobenzoylglutamic acid in 9.5 ml of 0.2 M sodium phosphate buffer collected by the foregoing procedure was added about 9.5 mg of nontritiated *p*-aminobenzoylglutamic acid. An aliquot was taken for the determination of specific acivity, which amounted to 5.07×10^8 dpm per mmole. Sufficient concentrated H_2SO_4 was added to bring the solution to 2 N. Hydrolysis was allowed to proceed for 10 hours at 100°C. The reaction mixture was then brought to 50 ml with distilled water and allowed to stand overnight with a large excess of $BaCO_3$.

i) Ten ml of the reaction mixture was brought to pH 3 with 5 N HCl and extracted with 10 ml of ethyl ace-

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tate. The extract was then taken to dryness and the residue redissolved in 3 ml distilled water. The solution was chromatographed on DEAE-cellulose and the *p*-aminobenzoic acid fraction collected. No α-amino nitrogen was detectable by the ninhydrin reaction. The concentration was determined by the Bratton-Marshall method. The specific activity was 1.43×10^8 dpm per mmole or 28.2 per cent that of the starting material.

ii) Ten ml of the reaction mixture was chromatographed on DEAE-cellulose and the *p*-aminobenzoic acid (PABA) and *p*-aminobenzoylglutamic acid fractions collected. The PABA fraction was brought to pH 3 with 5 N HCl and extracted with 10 ml of ethyl acetate. The extract was washed with 5 ml distilled water and the washings discarded. The ethyl acetate extract was then taken to dryness and the residue redissolved in 5 ml distilled water. No α-amino nitrogen was detectable by the ninhydrin reaction. The concentration of *p*-aminobenzoic acid was determined by the Bratton-Marshall method. The specific activity was 1.45×10^8 dpm per mmole or 28.6 per cent that of the starting material.

The distribution of radioactivity in this preparation of tritiated folic acid is therefore 22.1 per cent in the pteridine fraction, probably on carbon 9, 28.4 per cent in the *p*-aminobenzoic acid, and 47.3 per cent in the glutamic acid. These together account for 97.8 per cent of the total radioactivity.

Twenty-four hours prior to use, the lyophilized folic acid was redissolved in distilled water and rechromatographed. The effluent was precipitated at pH 3 with 5 N HCl, separated by centrifugation, and redissolved in sufficient 0.9 per cent NaCl containing 0.2 per cent $NaHCO_3$ to give a concentration of 200 μg per ml. One per cent benzyl alcohol was added and the solution passed by means of a Swinny syringe adapter (Millipore Filter Corp., Bedford, Mass.) through a Millipore HA filter into a sterile vial. An aliquot was taken for determination of the specific activity and the solution kept in the dark at 4°C until immediately before use. Solutions for oral administration were prepared in the same way except that sterilization was omitted.

Tritiated folic acid prepared by exchange with tritiated water. Seven-tenths mg of tritiated folic acid prepared by an exchange reaction with tritiated water (TRA. 34, batch 5, Radiochemical Centre, Amersham, England) was purified by chromatography on DEAE-cellulose. The major impurities were *p*-aminobenzoylglutamate and two unidentified pteridines. The specific activity after purification was 174 μc per mg. Solutions were prepared for administration as described above.

A similar preparation was used by Anderson, Belcher, Chanarin and Mollin (8) in studies of the handling of orally administered tritium-labeled folic acid in patients with megaloblastic anemias; these workers found that radioactivity was distributed almost equally between the *p*-aminobenzoylglutamate and the pteridine portions of the molecule.

Tritiated folic acid prepared by exchange with tritiated acetic acid. Twenty mg of folic acid USP was shaken with 15 mg of pre-reduced platinum catalyst and acetic acid containing 25 c tritium at 100°C for 12 hours. After removing catalyst and acid, the labile tritium was removed by dissolving the residue in 0.1 per cent $NaHCO_3$ and precipitating the folic acid at pH 3 with dilute HCl. The activity of the crude tritiated material was 62 μc per mg. Purification was carried out by chromatography on DEAE-cellulose, the major impurities being *p*-aminobenzoylglutamate and an unidentified pteridine. The specific activity was 52 μc per mg.

In the experiments described below, the preparations of tritiated folic acid used were those labeled by exchange with tritium gas and by exchange with tritiated water. No differences in *in vivo* behavior between the batches of labeled folic acid prepared by the two different methods were noted. Folic acid labeled by the third method, exchange with tritiated acetic acid, was not used in this study, but preliminary experiments suggest that it, too, is handled by the body in the same way.

Procedures

1. Assay of plasma tritiated folic acid. i) Isolation method. To 5 ml of heparinized plasma containing an unknown amount of tritiated folic acid was added 0.5 ml of a 0.2 per cent solution of nontritiated folic acid in 0.2 per cent $NaHCO_3$; 5.3 ml of acetone (reagent grade, Fisher) was added, the solution mixed and centrifuged at 3°C and 2,500 rpm for 10 minutes, and the supernatant decanted into a 50 ml polyethylene test tube; 25 ml of acetone was added, the solution mixed and centrifuged in the cold and the supernatant discarded. The precipitate was redisolved in 3 ml distilled water and poured into a 15 ml centrifuge tube. The polyethylene tube was washed once with 3 ml of 0.2 M phosphate buffer at pH 6.9 and the washings added to the centrifuge tube. The folic acid was precipitated at pH 3 by the addition of 5 N HCl and separated by centrifuging at 3°C. The supernatant was discarded. The precipitate was redissolved in 1 ml of 0.2 per cent $NaHCO_3$; 150 mg of zinc and 1 ml of 2 N H_3PO_4 were added and reductive cleavage allowed to proceed for 2 hours; 200 mg $CaCO_3$ was added and the solution allowed to stand overnight. The solution was then centrifuged and the supernatant decanted into a 15 ml centrifuge tube. The precipitate was washed once with 2 ml of water and the washings added to the supernatant. The supernatant was kept at 80°C for 20 minutes in the oven, recentrifuged and decanted. One-tenth ml of this solution was taken for measurement of diazotizable amine by the Bratton-Marshall method (7). The total folic acid could be calculated from the value obtained. Three ml was added to counting vials and taken almost to dryness in the oven at 80°C and then to complete dryness in a vacuum desiccator; 1 ml of a 10 per cent solution of Hyamine in toluene was added and the vials allowed to stand for several hours until solution was complete; 5 ml of scintillator was then added and the tritium concentration determined in the liquid scintillation counter. Efficiencies averaged 14.2 per cent.

In some cases, all nonfolic acid radioactivity was removed by redissolving the folic acid precipitated at pH 3 during the isolation procedure in 0.2 per cent $NaHCO_3$

TABLE I

Recovery of tritium-labeled folic acid added to plasma and urine

	H^3-folic acid added	Carrier folic acid added	Carrier folic acid after isolation	H^3-folic acid after isolation	Original H^3-folic acid	Recovery
	dpm	*μg*		*dpm*		%
Isolation method, plasma	6,000	1,000	438	2,695	6,145	102.4
	6,000	1,000	414	2,536	6,137	102.3
	6,000	1,000	438	2,640	6,019	100.3
	1,900	1,000	453	842	1,860	97.9
	1,900	1,000	426	784	1,842	96.9
	1,900	1,000	399	764	1,918	100.9
Direct chromatography, urine	5,000	364	120	1,606	4,870	97.3
	5,000	364	100	1,373	4,998	100.0
	5,000	364	107	1,563	5,320	106.4
	22,000	364	96	6,012	22,785	103.6
	22,000	364	84	5,093	22,053	100.2
	22,000	364	92	5,711	22,616	102.8
Isolation method, urine	33,000	1,143	360	10,317	32,808	99.4
	33,000	1,143	354	10,152	32,791	99.4
	33,000	1,143	402	11,260	32,050	97.2
	8,400	1,143	102	738	8,273	98.5
	8,400	1,143	132	994	8,608	102.5
	8,400	1,143	393	2,868	8,346	99.6

and purifying it by column chromatography on DEAE-cellulose. The collected folic acid fraction was then reprecipitated at pH 3 and the usual procedure followed. Nonfolic acid radioactivity ranged from 11 to 23 per cent of the total radioactivity measured by this method.

ii) Assay of total plasma radioactivity. Eight-tenths ml of heparinized plasma containing tritated folic acid was added to 15 ml of Thixcin-Hyamine emulsion and counted at − 5°C by the method of Shapira and Perkins (9). Standards of known amounts of tritiated folic acid in blank plasma were run; efficiencies averaged 5.4 per cent.

2. Assay of tritiated folic acid in the urine. i) Direct chromatography. To 5 ml of urine containing an unknown amount of tritiated folic acid was added 1 ml of a 0.2 per cent solution of nontritiated folic acid in 0.2 per cent $NaHCO_3$; 2 ml of this solution was then applied to a 0.5 × 5 cm column of DEAE-cellulose, followed by 20 ml of 0.01 M sodium phosphate buffer at pH 6.9. The folic acid, visible as a yellow band, was eluted with 0.2 M sodium phosphate buffer, pH 6.9. The folic acid was collected and precipitated at pH 3 with 5 N HCl. The precipitate was centrifuged, redissolved, reduced, chemically assayed and counted by the same method used for the plasma folic acid.

ii) Isolation method. For highly pigmented urines, direct chromatography as described above was not suitable. To these urines, carrier folic acid was added in the same way, and the total folic acid was precipitated at pH 3 with 5 N HCl and separated by centrifugation at 3°C. The precipitate was redissolved in 6 ml of 0.2 per cent $NaHCO_3$; 2 ml of this solution was then chromatographed as described above.

iii) Assay of total urine radioactivity. Five-tenths ml urine was added to a counting vial and taken to a volume of about 0.1 ml in the oven at 80°C; 0.4 ml Hyamine was then added, followed by 1 ml ethanol and 5 ml of scintillator. All samples were run in quadruplicate. As an alternative method, 1 ml of urine was added to a counting vial containing 3 g naphthalene (reagent grade, Matheson, Coleman, and Bell); 15 ml of scintillator was then added, made up as follows: 77 ml dioxane, 23 ml ethanol, 25 mg 1, 4-*bis*-2-(5-phenyloxazolyl)-benzene (POPOP) and 1 g 2,5-diphenyloxazole (DPO). The samples were counted at − 5°C. Efficiencies averaged 7.2 per cent. If the deepfreeze temperature were raised, the addition of ethanol to the scintillator solution could be omitted with a consequent improvement in efficiency. This method was not suitable for concentrated urines containing a heavy sediment because of the adsorption of folic acid on the insoluble residue at the bottom of the counting vial and consequent low counts.

3. Assay of tritiated water in the urine. In a number of experiments water was distilled from the urine samples and assayed for tritium by the method of Werbin, Chaikoff and Imada (10).

4. Counting methods. A Packard TriCarb liquid scintillation counter was used for all tritium assays. Unless otherwise stated, samples were taken to dryness in 20-ml glass counting vials (Wheaton Glass Co., Millville, N. J.) and redissolved in 10 or 20 per cent Hyamine in toluene, prepared by the method of Eisenberg (11); 5 ml of

toluene containing 0.6 per cent DPO and 0.02 per cent POPOP (Pilot Chemicals Inc., Watertown, Mass.) was added and the samples counted with a discriminator window setting of 10 to 100, voltage at tap 6, and a deep-freeze setting of −5°C. Because of variations in the degree of quenching encountered, all samples were recounted after the addition of an internal standard (tritiated toluene, New England Nuclear Corp., Boston, Mass.). Efficiencies ranged from 5 to 14 per cent. Solutions containing nontritiated carrier folic acid were assayed chemically by the method of Bratton and Marshall (7).

5. Recoveries and reproducibility of results. The amount of tritiated folic acid originally present in those samples to which carrier folic acid was added, followed by an isolation procedure, was calculated as follows. If the amount of carrier folic acid added was x mg, the amount recovered (as measured by the Bratton-Marshall method) was y mg, and the tritium activity detected was z dpm, then the tritium activity present in the original samples was xz/y dpm. Reproducibility by this method is shown in Table I.

In those procedures in which radioactivity in urine and plasma was counted directly without isolation, the calculated amount of radioactivity present for the total plasma by the Thixcin gel method was 98.1 ± 2.1 per cent (mean of 10 trial determinations ± SD); for total urine radioactivity by the ethanol method, 99.5 ± 3.3 per cent (mean of 8 trial determinations ± SD); and for total urine radioactivity by the dioxane method, 101.9 ± 3.0 per cent (mean of 8 trial determinations ± SD).

6. The stability of tritiated folic acid in plasma and urine. *i*) To centrifuge tubes containing 5 ml of heparinized plasma, 1.12 μg of tritium-labeled folic acid of specific activity 35,700 dpm per μg was added. The tubes were incubated for varying periods at 37°C, carrier folic acid added and the total folic acid then isolated and counted as described above. In 4 tubes in which carrier folic acid was added before incubation, recoveries were 100.4 ± 6.8 per cent (mean ± SD) but in 2 tubes in which the addition of carrier was delayed for 1 hour, recovery averaged 87.5 per cent, and in 2 tubes in which carrier was added at 3 hours, recovery averaged 88.8 per cent. In the experiments to be described, carrier folic acid was therefore added to the plasma immediately after collection.

ii) To 2-ml aliquots of a blank urine which had been allowed to stand overnight with $CaCO_3$, 1.34 μg of tritium-labeled folic acid of specific activity 35,700 dpm per μg was added (see above). The urines were then incubated for periods of 1, 6, and 24 hours, carrier folic acid was added and the total folic acid isolated by chromatography on DEAE-cellulose as described above. Recoveries did not decrease significantly with time.

iii) To six 2-ml aliquots of blank urine which had been brought to neutral pH with $CaCO_3$, 1.12 μg of tritium-labeled folic acid of specific activity 35,700 dpm per μg was added. Two aliquots were brought to pH 5.25 with 0.1 N HCl, two were brought to pH 8.1 with 0.1 N NaOH and two allowed to remain at neutrality. One urine from each group was then incubated for 3 hours at 37°C and the H^3-folic acid assayed by addition of carrier, followed by isolation on DEAE-cellulose. Recoveries were as shown in Table II.

TABLE II

Stability of tritium-labeled folic acid in urine at alkaline and acid pH

pH	Time of incubation	Recovery	Recovery
	hrs	*dpm*	%
5.25	0	38,720	96.8
5.25	3	32,950	82.4
6.9	0	41,010	102.5
6.9	3	39,890	99.7
8.1	0	39,100	97.8
8.1	3	33,235	83.1

Because of this apparent instability of small amounts of folic acid in urine at acid or alkaline pH, urines in the experiments described below were brought to neutrality and carrier folic acid added immediately after collection.

7. Administration of tritiated folic acid. The folic acid solution was injected into an antecubital vein by means of a pre-set delivery syringe through a 3-way stopcock and followed with 10 ml of normal saline. In most experiments a dosage of 15 μg per kg was used. Blood samples were taken into heparinized syringes from an antecubital vein of the other arm by an indwelling siliconed Cournand needle.

Experimental subjects

The subjects for this series of experiments were 16 healthy adult males, members of the Resident Staff of the Montreal General Hospital, ranging in age from 22 to 29 years and in weight from 59 to 86 kg. All were in a good state of nutrition. No subject was used for more than four experiments nor did any subject receive more than a total of 25 μc of tritium. At least 4 weeks was allowed to elapse between successive experiments on the same subject. The plasma disappearance curve of tritium-labeled folic acid after a standard dose of 15 μg per kg was determined for 10 of these subjects. The limits of variation were small; for instance, 30 minutes after injection the mean plasma concentration was 2.35 ± 0.25 per cent of the dose per L (mean ± SD); i.e., the coefficient of variation was 10.6 per cent.

RESULTS

After the intravenous administration of 1 μg per kg of tritiated folic acid to healthy adults, the concentration in the plasma falls very rapidly so that in a typical experiment, after 3 minutes only 2 per cent of the dose per L remained, and by 30 minutes only 0.1 to 0.2 per cent per L (Figure 1). This amount is equivalent to only about 0.1 μg per L. Thereafter the fall in concentration is slow and

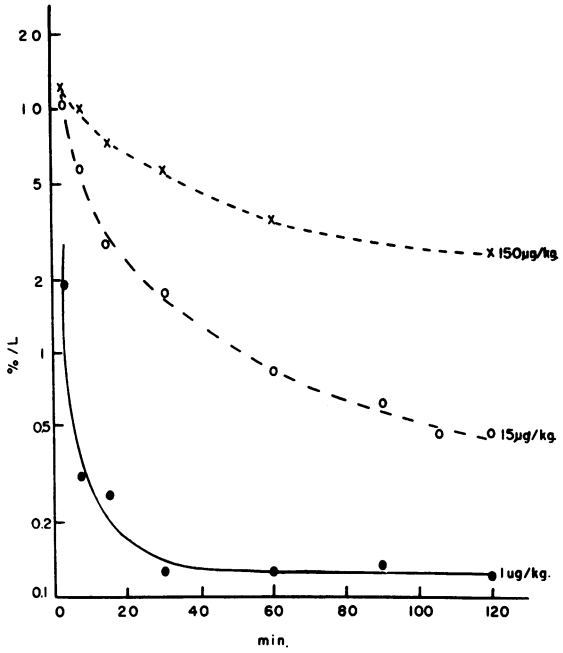

FIG. 1. PLASMA CONCENTRATION OF FOLIC ACID RADIOACTIVITY AFTER THE INTRAVENOUS INJECTION INTO NORMAL SUBJECTS OF 9 TO 14 µC TRITIATED FOLIC ACID DILUTED WITH CARRIER TO GIVE TOTAL DOSES OF 1, 15, AND 150 µG PER KG, RESPECTIVELY. Ordinate: % injected dose/L plasma.

difficult to follow with precision because of the extremely low levels of radioactivity involved (0.01 to 0.03 µc per L). This extremely rapid disappearance of tritiated folic acid from the plasma is not due to urinary excretion, since in the same experiment less than 2 per cent of the injected activity had been excreted in the urine by 2 hours (Figure 2) and the later excretion was even slower (less than 0.5 per cent per hour).

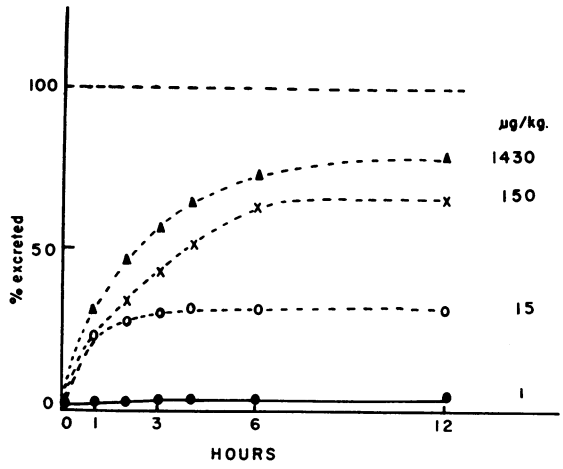

FIG. 2. URINARY EXCRETION OF RADIOACTIVITY AFTER THE INTRAVENOUS INJECTION INTO NORMAL SUBJECTS OF TRITIATED FOLIC ACID DILUTED WITH CARRIER TO GIVE TOTAL DOSES OF 1, 15, 150, AND 1,430 µG PER KG, RESPECTIVELY. Ordinate: cumulative excretion of radioactivity in the urine as a percentage of the injected dose.

When the dose of folic acid injected was increased to 15 µg per kg [1] the fall in plasma concentration was considerably slower, so that in a typical experiment 3.0 per cent of the dose was still present per L of plasma after 15 minutes and 0.5 per cent per L after 2 hours. In two subjects in whom satisfactory measurements were obtained 6 hours after injection, the residual fractions were 0.15 and 0.1 per cent per L, respectively. This level is therefore similar to that found after the lower dose, although it is attained about one-twentieth as fast and in absolute amount is equivalent to 1.3 to 1.6 µg per L of folic acid. With this higher dose of folic acid the urinary excretion of radioactivity is much greater (Figure 2) so that by the end of 2 hours, 20 to 30 per cent of the injected activity has been recovered in the urine; subsequently, the excretion rate falls steeply so that only 5 to 10 per cent more is excreted in the following 24 hours and it thereafter falls to less than 2 per cent per day.

Raising the dose to 150 µg per kg leads to a further slowing of the fall of plasma concentration and to a greater and more sustained urinary excretion. After 12 hours, 60 per cent of the radioactivity had been recovered in the urine (Figure 3). Increasing the dose of folic acid to 1,430 µg per kg had little additional effect on either the rate of disappearance of plasma activity or the urinary excretion.

In some experiments the total plasma radioactivity was also measured. When the plasma radioactive folic acid exceeded 100 µg per L, the radioactivity due to compounds other than folic acid was less than 5 per cent of the total. No determinations of nonfolic acid radioactivity were carried out at lower levels.

[1] Many of our studies have been carried out with a dose of 15 µg per kg in order to allow comparison with the extensive studies of Chanarin and co-workers (12–14) who used the same dose but a microbiological assay method. The agreement between the results obtained by the two methods is good. This dose is also convenient in that the plasma radioactivities are higher and thus easier to handle than the lower levels found with doses of about 1 µg per kg.

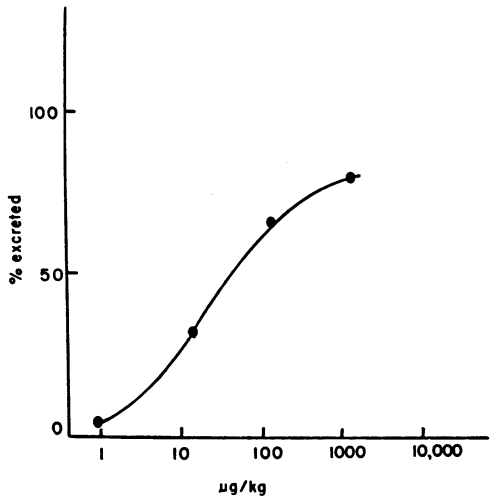

FIG. 3. THE RELATIONSHIP BETWEEN THE AMOUNT OF RADIOACTIVITY EXCRETED IN THE URINE UP TO 12 HOURS AFTER INTRAVENOUS INJECTION OF TRITIATED FOLIC ACID WHEN THE CARRIER MASS WAS VARIED FROM 1 TO 1,430 µG PER KG; 12 HOURS WAS CHOSEN AS THE ENDPOINT, SINCE AT THIS TIME THE RATE OF EXCRETION OF RADIOACTIVITY HAD FALLEN TO A LOW LEVEL.

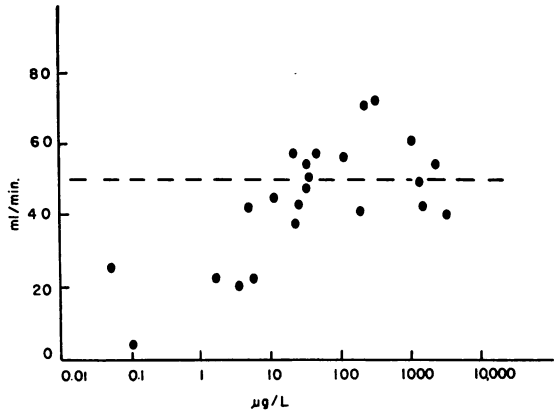

FIG. 4. URINARY CLEARANCE OF FOLIC ACID IN NORMAL SUBJECTS IN RELATION TO PLASMA CONCENTRATION. The dashed line is the clearance expected if filtration of the plasma folic acid that is not protein-bound occurred without tubular reabsorption or secretion. It agrees well with the mean clearance found when the plasma concentration was in excess of 10 µg per L. Ordinate: urinary clearance of radioactive folic acid. Abscissa: plasma radioactive folic acid.

A variable proportion of the radioactivity excreted in the urine was unchanged folic acid; this depended on the dose given. With 150 µg per kg, 75 per cent was folic acid; with 15 µg per kg, 40 to 50 per cent; and with 1.5 µg per kg, only 1 to 30 per cent. The identity of the other radioactive compounds present is dealt with below.

Renal handling of folic acid. During the course of several of the studies described in this report it was possible to collect urine during periods when the plasma radioactive folic acid concentration had been maintained for 1 hour or longer. The range of plasma concentrations studied was 0.05 to 3,000 µg per L.

At plasma concentrations above 10 µg per L the clearance is independent of the plasma level and averages 51 ± 3.1 ml per minute (Figure 4). At lower plasma concentrations the clearance falls. The finding of a fixed upper limit of clearance lower than the glomerular filtration rate (130 to 140 ml per min) made us suspect that a considerable fraction of the plasma folic acid was nonfiltrable because of binding to plasma proteins. This possibility was examined by ultrafiltration of plasma containing radioactive folic acid. The plasma was equilibrated at 37°C with CO_2 at a partial pressure of 40 mm Hg. The results illustrated in Figure 5 show that binding is practically uniform over the whole concentration range of 5 to 3,000 µg per L with an average of 64 per cent bound. Similar results were obtained by equilibrium dialysis.

Making allowance from these figures for the amount of folic acid that is bound and unavailable for filtration, we arrive at a clearance of 140 ml per minute for free folic acid. It appears, therefore, that the renal handling of folic acid for plasma levels above 10 µg per L can be accounted for simply by glomerular filtration of unbound folic

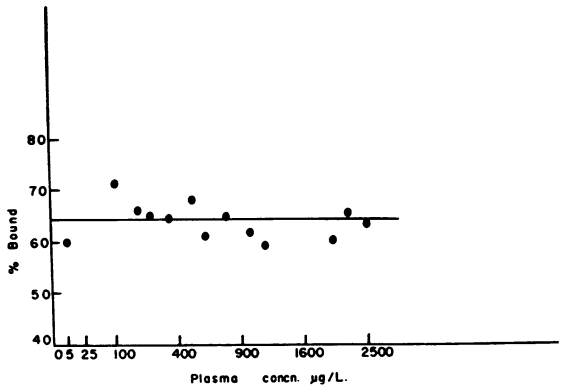

FIG. 5. PERCENTAGE OF RADIOACTIVE FOLIC ACID BOUND TO PLASMA PROTEIN AS DETERMINED BY ULTRAFILTRATION AT 37°C AND 40 MM HG pCO_2.

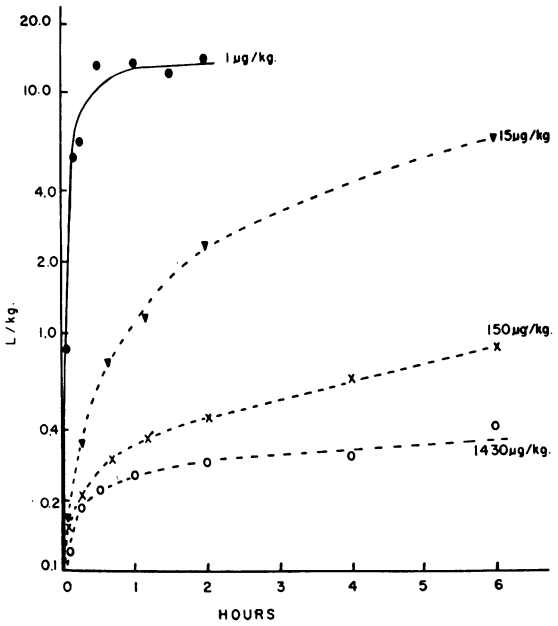

FIG. 6. VOLUME OF DISTRIBUTION OF RADIOACTIVE FOLIC ACID RETAINED IN THE BODY AFTER INTRAVENOUS INJECTION OF 9 TO 14 µC OF TRITIATED FOLIC ACID DILUTED WITH CARRIER TO 1, 15, 150, AND 1,430 µG PER KG, RESPECTIVELY. The extracellular water was taken to be 0.16 L per kg and the total body water 0.56 L per kg. When the carrier dose was 15 µg per kg or less, the radioactivity was rapidly distributed into a space larger than the total body water.

acid without appreciable modification by the tubules. At lower plasma concentrations, however, the clearance is lower than would be predicted from these considerations and probably involves a low rate of tubular reabsorption—not exceeding 0.03 µg per minute. The possibility must be considered that this represents metabolic usage of folic acid by the renal tubules or urinary excretory passages rather than a renal conservation process.

Disposition of retained activity. Calculation of the volume of distribution of the retained radioactivity shows that with doses of 1 to 15 µg per kg it rapidly exceeds the volume of the total body water (ca 0.56 L per kg; Figure 6). Indeed, if the amount of folic acid retained in the plasma and extracellular fluid is calculated (after due allowance for plasma protein binding) and compared with the total activity retained, with a dose of 1 µg per kg by 2 hours it is only 1/150 of the total; with 15 µg per kg at the same time it is 1/27; while for 150 µg per kg it is 2/9.

However, while the distribution of folic acid gives evidence of the rapidity with which radioactivity is taken up into the intracellular space, it does not necessarily show that folic acid itself is concentrated within the cells, since it is possible that it might be trapped within the cells by conversion to a nondiffusible metabolite. A further alternative might be rapid metabolism of folic acid to biologically inactive products. Any extensive metabolism might be expected to yield tritium to the body-water pool. This last possibility seemed improbable because the technique of whole-plasma counting by the emulsion method should be able to detect such products but did not show adequate activity to account for any such metabolic conversion. However, to settle the matter, urine samples were distilled and the radioactivity in the water measured by the method of Werbin and colleagues (10); 24 hours after the administration of 15 µg per kg (10 µc) of folic acid, no radioactivity was detectable in the urinary water (i.e., < 0.01 µc per L), whereas complete metabolism of the 6 to 7 µc of retained activity should have given about 0.15 µc per L of tritium in the body water. We were therefore led to the conclusion that the uptake of folic acid did not involve extensive degradation.

On the other hand, if folic acid itself were being concentrated within the cells, the lower intracellular: extracellular ratio found when large amounts of folic acid are administered suggests that it might be possible to displace activity from the cells by subsequent administration of a large carrier dose of nonradioactive folic acid. This proved to be the case. Figure 7 shows an experiment in which 1.55 µg per kg of tritiated folic acid was given; by 6 hours the plasma radioactive folic acid had fallen to 0.13 per cent of the dose per L and a total of 6.5 per cent of the injected activity had been excreted in the urine. At this time 450 µg per kg of nonradioactive folic acid was injected intravenously; this was followed by a rapid rise in plasma radioactivity to 3 per cent of the dose per L and a greatly increased excretion of radioactivity in the urine. In the next 12 hours a further 27 per cent of the injected radioactivity was excreted, of which 86 per cent was identified as folic acid. In comparable experiments with a dose of 15 µg per kg of radioactive folic acid, results were similar except that as much as 90 per cent of the radioactivity was recovered in 24 hours. In this case also most of the urinary radioactivity

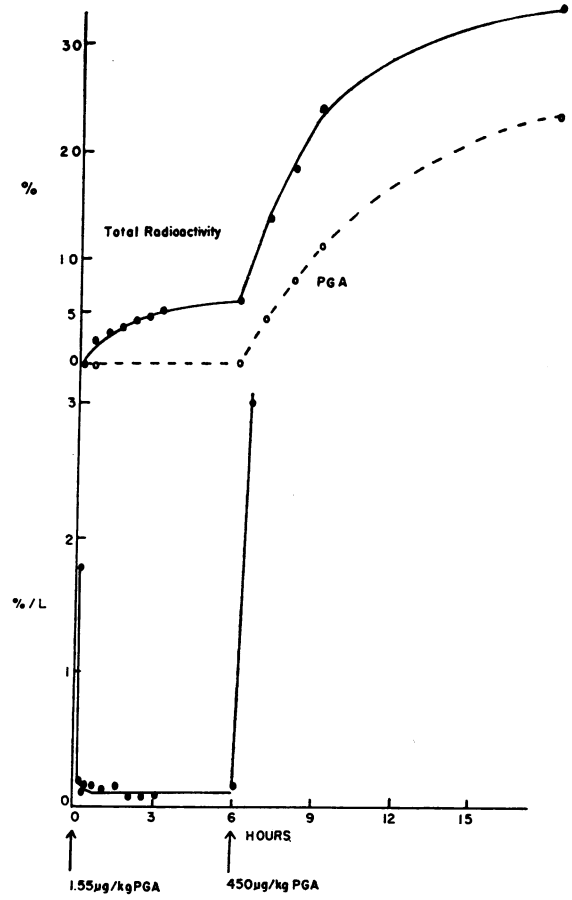

FIG. 7. NORMAL SUBJECT GIVEN 25 µC OF TRITIATED FOLIC ACID INTRAVENOUSLY (TOTAL FOLIC ACID 1.55 µG PER KG). Plasma radioactive folic acid, and urinary radioactive folic acid and total radioactivity were followed. Six hours after the first injection 450 µg per kg of nonradioactive folic acid was injected i.v. This was followed by a prompt rise in plasma radioactive folic acid and in the rate of urinary excretion. Lower curve: plasma folic acid radioactivity as percentage of dose injected. Upper curves: solid line, total radioactivity excreted; dashed line, radioactive folic acid excreted.

was identified as folic acid. At this dose-level, experiments were done in which intervals of up to 3 days elapsed between administration of the tritiated folic acid and the "flushing" dose. The percentage of the retained activity that could be flushed out fell to 68.5 per cent after 24 hours and to 47.2 per cent after 3 days. But even in the latter case 65 per cent of the activity was free folic acid. It seems clear from these experiments that folic acid is initially removed from the circulation and accumulated in the cells as such but is then relatively slowly converted into a nondisplaceable form at a rate that is dependent on the dose of folic acid administered.

Disposal of p-aminobenzoylglutamate. In view of the rapid intracellular uptake of folic acid we thought it of interest to compare the behavior of tritium-labeled *p*-aminobenzoylglutamate, which differs from folic acid only in the absence of the pteridine structure. This substance, when given intravenously in a dosage of 7.62 µg per kg (equivalent on a molar basis to a folic acid dose of 15 µg per kg) was found (Figure 8) to distribute itself into a maximal volume of 220 ml per kg, corresponding approximately to the plasma and interstitial water spaces. Urinary excretion was rapid, the clearance being 360 ml per minute, suggesting active tubular excretion. A total of 94 per cent of the dose was recovered from the urine in 24 hours. It appears, therefore, that the pteridine moiety of the molecule is essential for active uptake into the cells.

Distribution of urinary radioactivity. Folic acid isolation procedures suggested that part of the radioactivity present in the urine after an in-

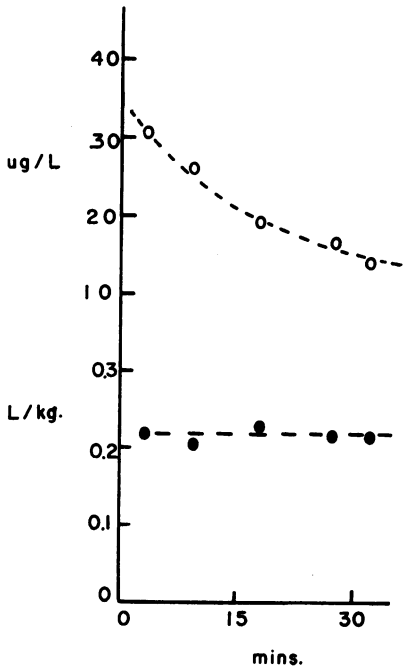

FIG. 8. INTRAVENOUS INJECTION OF 7.6 µG PER KG OF TRITIATED *p*-AMINOBENZOYLGLUTAMATE INTO A NORMAL SUBJECT. O---O, Plasma radioactivity; ●——●, volume of distribution of retained activity.

TABLE III

Distribution of urinary radioactivity before and after a flushing dose of unlabeled folic acid

	0.5–1 hour after initial dose*		0–1 hour after flushing dose		2–3 hours after flushing dose	
	Activity in urine	Excreted	Activity in urine	Excreted	Activity in urine	Excreted
	%	*dpm/hr*	%	*dpm/hr*	%	*dpm/hr*
Pteridine zone	22.2	14.6×10^4	1.9	7.6×10^4	2.2	5.8×10^4
p-Aminobenzoyl-glutamate zone	45.1	29.6×10^4	6.0	24.1×10^4	5.4	14.0×10^4
Folinic zone	26.7	17.5×10^4	1.3	5.2×10^4	1.4	3.6×10^4
Folic zone	3.5	2.3×10^4	90.1	361×10^4	89.6	232×10^4
Pteroyltri-glutamate zone	0.0	0.0×10^4	0.0	0.0×10^4	0.0	0.0×10^4
"NaOH" eluate	2.5	1.6×10^4	0.7	2.8×10^4	1.4	3.6×10^4
Total		65.8×10^4		401×10^4		259×10^4

* Folic acid containing 4.81×10^7 dpm.

travenous dose of tritium-labeled folic acid represented unchanged folic acid. This is in agreement with the finding of Anderson and colleagues (8) that much of the radioactive material excreted in the urine after an *oral* dose of tritiated folic acid is folic acid or its analogs.

In an attempt to identify some of the other radioactive compounds present, a dose of 1.5 μg per kg (22 μc) of tritium-labeled folic acid was administered intravenously to a normal adult male subject and a flushing dose of 30 mg of nonradioactive folic acid given at 6 hours; urines were collected both before and after the flushing dose and chromatographed after the addition of carriers:

One mg each of *p*-aminobenzoylglutamic acid, folinic acid, folic acid, and pteroyltriglutamic acid was dissolved in 1 ml of 0.2 per cent $NaHCO_3$. This solution was added to 10 ml of urine. The urine was diluted to a volume of 120 ml with distilled water and applied to a 1×7 cm column of DEAE-cellulose prepared and equilibrated as described in Methods. A flow rate of 0.4 ml per minute was used and the effluent collected after being passed through a recording ultraviolet absorptiometer. After application of the diluted urine, the column was developed with phosphate buffers of 0.01, 0.03, 0.06, 0.2, and 0.4 M, at pH 6.9. At the end of the run the column was cleared with 0.5 N NaOH solution. The location of the carriers in the collected fractions was determined from the absorptiometer record, identification being aided by determination of diazotizable amine in the various fractions before and after reduction by the Bratton-Marshall method. The distribution of radioactivity found is shown in Table III. All of the radioactivity applied to the columns was recovered in the six fractions listed.

It will be noted that in the second half-hour after injection, the rate of excretion of tritiated folic acid was very low, but there was a considerable excretion of *p*-aminobenzoylglutamate and pteridines. The latter were roughly in the proportions to be expected from the biological splitting of folic acid. Since no precautions were taken in these experiments to maintain a reducing milieu in the urine, at least some of these latter fractions were probably formed in the urine by the breakdown of reduced folates. No pteroyltriglutamate was detected in any of the samples. In the samples obtained after the flushing dose of folic acid there was a great increase in the rate of folic acid excretion but only such gradual decline in the excretion of the metabolites as might have been expected in the absence of flushing.

DISCUSSION

The avidity with which folic acid is removed from the plasma by the tissues is quite remarkable. In the experiments in which 1 μg per kg was injected, 90 to 95 per cent was removed in 3 minutes and 60 per cent in one circulation time. This suggests that a high affinity for folic acid must be a property of most tissues. This high affinity leads to a high relative concentration of folic acid inside cells. A similar rate of disappearance for folic acid has been reported by Chanarin, Mollin and Anderson (12) using a microbiologic assay method, and for folinic acid by Spray and Witts (15). On the other hand, Methotrexate (4-amino-

10-methylpteroylglutamic acid) (16) and *p*-aminobenzoylglutamic acid are not so treated and indeed appear to be restricted largely to the extracellular space. Two explanations can be offered for the tissue accumulation of folic acid: specific binding within the cells or a selective uptake process mediated by transport across the cell membrane or both. That the selective uptake mechanism is certainly operational is shown by the differences in behavior between folic acid and the other two compounds. The failure of Methotrexate and *p*-aminobenzoylglutamate to be accumulated in the tissues results from a failure to penetrate into the intracellular space. This defect is most readily explained if there is a specific membrane transport process for folic acid which is unable to transport the other two substances. The process of uptake is thus highly specific and is dependent not only on the presence of the pteridine ring but of specific substituents upon it. It would in any case be difficult to explain the rapid uptake of a molecule as bulky, polar, and lipoid-insoluble as folic acid without invoking a specific transport process. The accumulation of folic acid within the cells appears, therefore, to be due to a "pump and leak" mechanism similar to that described for amino acids and for cations (17). In this kind of process the substance is pumped into the cell but tends to diffuse back, and the steady state cellular concentration depends on the balance of these two processes. The very high intracellular concentration of folic acid observed points to an accumulative process with a high affinity and capacity. Whether a binding of folic acid to specific sites within the cell also occurs we are unable to say from the present data.

It has been estimated that the daily intake of folic acid in the diet is 100 to 200 μg (18); this amount could be transported to the storage sites without the blood level's ever exceeding 0.5 μg per L. Our results are entirely consistent with the view of Herbert (19) that there is little or no free folic acid in the fasting plasma and that the *L. casei* factor of plasma is some other form, perhaps tetrahydropteroyltriglutamate. However, the present data do not provide evidence of any precise level of free folic acid in plasma.

The gradual decrease with time in the amount of folic acid that can be flushed out of the cells can be explained either by a gradual redistribution of the folic acid into tissues that hold folic acid more tenaciously or, more likely, the conversion of free folic acid into tissue storage forms such as the "prefolic A" of Donaldson and Keresztesy (20).

The relatively small dependence of the urinary clearance of folic acid on the plasma concentration points up the unimportant role of the kidney in conservation of this vitamin. Renal conservation appears to be unnecesary in view of the high storage capacity of the tissues and consequent prerenal deviation of ingested folic acid. Our interpretation of the renal clearance data here differs radically from that given by Condit and Grob (21). These authors found a similar clearance but interpreted it as due to free glomerular clearance followed by proportionate tubular reabsorption. This assumption was based on their finding of very little binding of folic acid to plasma. We, on the other hand, find an average of 64 per cent of the plasma folic acid bound, irrespective of concentration. It is of interest in this connection to note that Freeman (16), using similar methods, found an average of 46 per cent of Methotrexate to be protein-bound. Condit and Grob, in their calculation of the clearance of endogenous folic acid, also do not consider the possibility that much of the plasma folic acid activity measured microbiologically may not be due to folic acid itself. Determinations with tritiated folic acid are free from this difficulty and indicate clearances of about 20 to 40 ml per minute with concentrations of 7 to 24 μg per L. This is the level of normal plasma folic acid activity found by Herbert and colleagues (22) by microbiological assay. Condit and Grob find a urinary clearance of less than 1 ml per minute for endogenous folic acid activity. The discrepancy in the clearance values found by the microbiological and radiochemical methods seems to be another indication that little of the endogenous plasma folic activity is actually folic acid.

The other compounds excreted in the urine are of some interest. Considerable amounts of labeled folinic acid appear to be excreted in the urine, but the concentration falls with time and does not seem to be increased by a flushing dose of folic acid. This probably indicates that folinic acid formation parallels the amount of free folic acid within the cells which is decreasing with time after the injection, and thus the rate of production

of folinic acid also decreases with time. After a flushing dose it might be expected that the excretion of *labeled* folinic acid would fall. This appears to occur. Similar changes occur in the pteridine and *p*-aminobenzoylglutamate fractions, which are probably derived from tetrahydropteroylglutamate. The lack of any activity in the pteroyltriglutamate fraction in the urine is perhaps not surprising since, if a pteroylpolyglutamate were flushed out of cells, it would probably be rapidly hydrolyzed by serum folic acid conjugase (23). The inability to demonstrate pteroyltriglutamate does not mean that *reduced* triglutamates are not excreted since, even if they were stable under these conditions, which is unlikely, the buffer concentrations needed for their elution from the column may be quite different from that required for pteroyltriglutamate (24).

It must be pointed out in this connection that identification of folic acid derivatives by the chromatographic procedures we have used are by no means rigorous. Materials are identified by their position in an elution system corresponding to known markers, but the possibility of coincident elution of other labeled compounds with similar chromatographic properties has not been excluded for substances other than folic acid itself. Chromatography in several systems to a constant specific activity would be required but has not yet been attempted.

SUMMARY

1. Tritium-labeled folic acid was administered intravenously in man at dosage levels of 1, 15, 150, and 1,430 μg per kg. Blood levels were followed for several hours. The fraction taken up by the tissues was greater at the lower dosage levels.

2. Tritium-labeled folic acid could be displaced from the cells by large flushing doses of unlabeled folic acid at any time up to 3 days after the initial dose. The amount that could be displaced decreased with time.

3. At low dosage levels most of the urinary radioactivity consisted of metabolic products of folic acid. At high dosage levels or after a flushing dose of unlabeled folic acid, as much as 90 per cent of the urinary radioactivity was found to be due to unchanged folic acid.

4. Tritium-labeled folic acid in plasma was 64 per cent protein-bound, the renal clearance at plasma levels above 5 μg per L averaging 51 ml per minute. At very low plasma levels the renal clearance fell.

5. The uptake of folic acid appeared to be a specific process dependent both on the pteridine structure and on substituents on the pteridine ring.

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