A long-term study of the excretion of folate and pterins in a human subject after ingestion of ^{14}C folic acid, with observations on the effect of diphenylhydantoin administration[1, 2]

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ABSTRACT After the administration of 2-^{14}C folic acid to a human volunteer, urinary and fecal radioactivity, as well as urinary excretion of folate (*Lactobacillus casei* assay) and biopterin-like material (*Crithidia fasciculata* assay) were determined at intervals over a 129 day period of observation. From two 24 hr urine samples erythroneopterin, bioterin, threoneopterin, pterin, isoxanthopterin, and xanthopterin were isolated by chromatographic procedures, quantitated, and their specific activities were determined. The effect on the pattern of elimination of urinary radioactivity and biological activity resulting from the administration of diphenylhydantoin was studied on two occasions. Urinary radioactivity plots suggest the decay of two forms of folates with markedly different biological half lives. One short-lived ($t^1/_2 \cong 31.5$ hr), corresponding to newly absorbed folate, and one long-lived ($t^1/_2 \cong 100$ day) thought to represent the decay of body pools. Diphenylhydantoin does not alter the rate of elimination of the long-lived component but may accelerate losses of newly absorbed folate. The analysis of pterins does not support the hypothesis that diphenylhydantoin increases the breakdown of folates to pterins. *Am. J. Clin. Nutr.* 31: 88–93, 1978.

In recent years there has been considerable interest in folate metabolism and turnover in man both as a means to determine nutritional requirements and to study the effect of interfering drugs.

It is well recognized that the amount of folate in the diet (1, 2) is at least ten times as great as the quantity found in the urine by microbiological assay (3). This might suggest very incomplete intestinal absorption were it not for the fact that 50 to 75% of tracer doses are not recoverable in 72-hr stool collections (4). An alternative explanation would be that folate is converted into metabolites that do not support growth of assay organisms such as *Lactobacillus casei*. Such metabolites could include, for example, various pterins (5) or fragments thereof, as well as *p*-aminobenzoylglutamate derivatives such as the acetamidobenzoylglutamate recently demonstrated in rat urine (6).

It has been known since 1952 that diphenylhydantoin is capable of producing a folate-responsive megaloblastic anemia under certain conditions. Since the report of Mannheimer et al. (7) in that year, there have been many articles indicating that the prolonged use of anticonvulsants leads to a reduction in plasma folate and occasionally to overt deficiency symptoms (8–10). Neither the mechanism of this depletion nor of the anticonvulsant effect of these drugs is known. Worsening of seizure control after administration of folate to folate-depleted epileptics (11) suggests that an alteration of folate metabolism may play a role in the

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pathogenesis of some forms of this disease or be essential to the mechanism of action of anticonvulsant drugs. The hypothesis has been advanced that diphenylhydantoin interferes with folate absorption by inhibiting intestinal conversion of dietary pteroylpolyglutamates to monoglutamyl forms but it was not possible to confirm such an effect in vitro (12). Evidence that diphenylhydantoin impedes the normal intestinal absorption of free folic acid in the rat has been provided by Hepner (13), and human studies indicating inhibition of free folic acid absorption by therapeutic doses of diphenylhydantoin have been reported by Gerson et al. (14). The possibility remains however that diphenylhydantoin enhances the excretion or degradation of folates. In this report we present the results of a study designed to test the hypothesis that diphenylhydantoin administration might increase the elimination of folates or the degradation of folate coenzymes to pterins or other breakdown products leading eventually to a deficiency state. Data on the biological half-life of folate and an assessment of the contributions of urinary and fecal losses have also been obtained.

Experimental design

Purified 2-^{14}C folic acid was administered orally in four equal doses of 10 μCi given every 12 hr to a fully informed human volunteer. A total of 40 μCi (0.725 μmoles, 320 μg of folic acid) was given. The subject, a highly intelligent and cooperative 36-year-old woman, was diagnosed as having Hodgkin's disease at age 25 and received intermittent chemotherapy for 8 years thereafter. She had been in a state of complete remission for 3 years at the time of this study and remains symptom free. She was hematologically normal, weighed 75 kg, and was receiving no medication other than estrogen replacement (Evex 1.25 mg/day, Syntex Laboratories, Inc. brand of esterified estrogens, expressed as estrone sulfate) to compensate an ovarian insufficiency secondary to antitumor therapy. Studies in the hospital were conducted under metabolic ward conditions. Complete daily collection of urine (under toluene) and stools were made for 9 consecutive days. A 300 mg dose of diphenylhydantoin was administered orally (100 mg three times daily) on the sixth day after the first dose of radioactive folic acid, at which time the amount of radioactivity in the urine had begun to level off. She was discharged 4 days later. At home she collected 24-hr specimens of urine and feces for several periods of observation. The last collection period was ended 129 days after ingestion of the isotope. A second period of diphenylhydantoin administration (300 mg/day) was started on day 20 and continued for 4 days. The urine samples were assayed for radioactivity, and bioassayed with *L. casei* and *Crithidia fasciculata*. Total radioactivity was determined in the fecal samples. If the administration of diphenylhydantoin were to cause increased loss of folate in the urine, an elevation of both radioactivity and *L. casei* active material would be anticipated; if, on the other hand, the drug should cause increased breakdown of folate to pterins or other degradation products bearing the 2-C^{14} label, the urinary radioactivity would increase with no concomitant elevation of *L. casei* activity. The isolation and characterization of urinary pterins was carried out to search for possible conversion of radioactive folate into this class of compounds.

Materials and methods

2-^{14}C folic acid (specific activity 55.3 mCi/mmole) was purchased from Amersham Corporation. It was purified by DEAE cellulose chromatography as previously described (15). Ecteola-Sephadex and phospho-Sephadex were prepared from Sephadex G-25 (fine) by the method of Peterson and Sober (16) for the corresponding celluloses. The pH 6 or pH 7 Ecteola-Sephadex's were prepared as described before (5).

Microbiological assays

Urinary folates were determined by bioassay with *L. casei* as described by Baker and Frank (17). Urinary levels of Crithidia active substances (as biopterin equivalents) were determined by *C. fasciculata* by a modification of the procedure described by Dewey and Kidder (18). The medium was prepared to give a 5-fold increase in the concentration of all the ingredients and Kanamycin, 500 μg/ml was added. The concentrated medium was stored at −10 C and, for use, it was appropriately diluted and sterilized by use of a 0.45 μm Millipore filter. The filtered-sterilized medium can be stored at 5 C for several weeks. Urinary radioactivity was determined by counting 0.5 ml of untreated urine using Aquasol as counting fluid. All counts were obtained with the automatic quench correction mode

in a carbon[14] channel that excluded counts due to chemiluminescence. The efficiency of counting was 51%. Fecal radioactivity was obtained after homogenizing the weighed samples in 1.0 liter of 0.05 N NaOH and oxidizing an aliquot with an excess of potassium permanganate as previously described (4). Packard Tricarb (model 3375) and Beckman (LS-250) liquid scintillation spectrometers were used to measure radioactivity.

Analysis of pterins in urine

The pterins in urine were purified by the column chromatography procedures previously reported (5, 19). These compounds were identified by thin layer chromatography (MN-Polygram Cell 300 sheets, Brinkman, Inc.) employing five solvent systems (5). The R_f values of biopterin, xanthopterin, pterin, erythroneopterin, threoneopterin, and isoxanthopterin were the same as those reported earlier (5).

Spectrophotometric quantitations of pterins were performed whenever possible using the appropriate extinction coefficients (20-23). A fluorescent method was applied when the amount of pterin was small (5).

Results

Table 1 summarizes the results of the radioactivity measurements in urine and feces as well as the urinary levels of folates and biopterin-like compounds.

It may be seen that maximal urinary excretion of ^{14}C occurs on the second day of labeled folate ingestion, and declines rapidly for several days thereafter. A very efficient absorption is indicated by the small residual radioactivity, amounting to 3.16 μCi of the 40 μCi dose (7.8% of the administered dose), recovered in the feces collected during the first 6 days of the experiment. Urinary losses during the same period amounted to approximately 1.44 μCi, or 3.9% of the absorbed material. Figure 1A shows the semilogarithmic plot of urinary radioactivity (cpm/24 hr) versus days after administration of the labeled folic acid. The graph suggests the decay of two forms of folates with markedly different biological half-lives. By averaging the results obtained during the five periods of urine collection indicated in Figure 1B a half-life of approximately 100 days is obtained for the long-lived component. Sub-

TABLE 1

Days from start	Urine		*L. casei* activity	*C. fasciculata* activity, biopterin equivalents	Feces
	Volume	cpm (total)			cpm (total)
	ml		*μg/24 hr*		
0	1340	0	7.4	630	
1[a]	940	216,200	4.4	620	
2[a]	895	365,160	9.0	322	
3	1450	324,800	6.6	435	565,000
4	1500	246,600	7.4	480	1,210,000
5	1150	230,000	6.4	675	1,120,000
6[b]	1980	249,480	9.9	620	688,000
7	925	223,850	6.2	481	
8	1290	216,720	8.4	722	
16	970	184,300	7.7	621	
20[b]	1540	184,800	8.8	755	
21[b]	1260	168,840	6.3	605	
22[b]	1080	145,150	6.4	476	
23[b]	920	168,540	7.4	619	
24	1300	161,720	8.1	536	
25	1240	157,232	11.5	462	224,000
26	1160	169,128	10.5	777	132,000
27	1200	180,720	11.9	754	
54	1690	144,320	10.3	670	
55	1410	148,890	11.2	536	200,600
56	1260	135,070	7.3	642	81,900
74	2290	135,540	11.7		
75	1800	109,440	8.8		
76	1450	95,120	7.8		84,000
77	790	104,280	9.2		70,000
127	1360	92,480			57,900
128	825	77,550			52,200
129	1250	72,500			57,120

[a] 2-^{14}C folic acid administered. [b] Diphenylhydantoin administration.

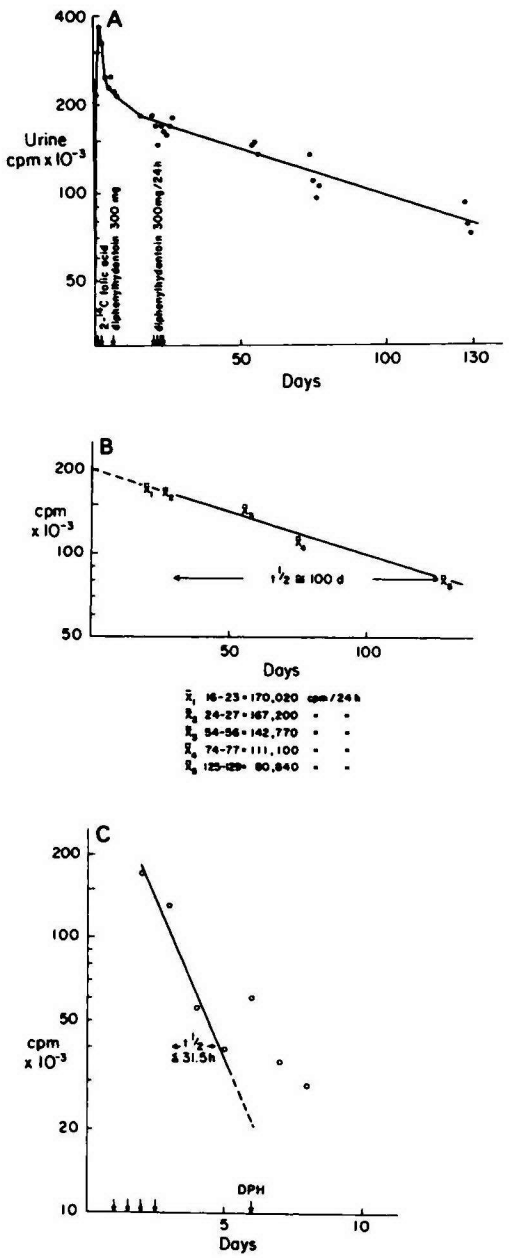

FIG. 1A. *Semilogarithmic plot* of urinary radioactivity. B, *Semilogarithmic plot* of average ($\bar{x}$) urinary radioactivities obtained during five periods of collection. Half-life of long-lived component. C, Half-life of short lived component. See text for explanation.

tracting the counts corresponding to the long-lived component (obtained by extrapolation to zero time) from the actual counts obtained in specimens collected during the first 6-days of the experiment, it is possible to estimate the rate of decay of the short-lived component. This *plot* is shown in Figure 1C and indicates a half-life of about 31.5 hr.

Effect of diphenylhydantoin

Following the administration of diphenylhydantoin on day 6 of the experiment, the *L. casei* activity in the urine increased and the urinary radioactivity also increased failing to continue its downward course (as predicted by the *dotted line* in Fig. 1C).

The second period of diphenylhydantoin administration of 4 days duration (rather than only 24 hr) was started on the 20th day of the experiment in the hope of showing a more pronounced increase in folate and/or radioactivity losses. Contrary to expectations no discernible change in the pattern of elimination was observed.

The results of *C. fasciculata* assays which are presented in Table 1 indicate that there was no discernible effect on the pattern of elimination of Crithidia active pterins following administration of the drug. The urines from days 23 (the last of a 4-day period on DPH administration on 300 mg/day) and of day 25 were analyzed quantitatively for certain pterins. In Table 2 are shown the amounts and specific radioactivities found. Neither seems to be affected by diphenylhydantoin. Pterin and isoxanthopterin were radioactive whereas erythroneopterin, biopterin, and threoneopterin contained little or no radioactivity.

Discussion

The high efficiency of absorption and retention of the radioactive folic acid administered (92.2% of the label absorbed and only 3.9% of the absorbed radioactivity voided during the first 6 days) is in keeping with previously reported observations indicating that small amounts of folic acid of the order of 1 μg/kg of body weight, even when administered intravenously, are almost quantitatively retained in the body (24).

To the best of our knowledge, the biological half-life of folate has not been precisely determined in man because the size of the total body folate pool has not been estab-

TABLE 2
The amount and specific activity of certain pterins excreted in 24-Hr urine on day 23 and 25 of experimental period

Pterins	Day 23 urine		Day 25 urine	
	μg/24 hr	dpm/μmole	μg/24 hr	dpm/μmole
Erythroneopterin	1277[a]	<50	1443[a]	<50
Biopterin	484[a]	<50	439[a]	<50
Threoneopterin	52[a]	<50	56[a]	<50
Pterin	66[b]	3100	56[b]	3300
Isoxanthopterin	16[b]	2100	27[b]	1800
Xanthopterin	+[c]		8.5[b]	

[a] These values were determined by use of the extinction coefficient for each pterin (19). [b] These values were determined by a fluorescence assay described in a previous paper (5). [c] Compound was identified but not quantitated (+).

lished and because the degree of its depletion needed to produce clinical manifestations is unknown. From our data, the biological half-life of folic acid in this subject was approximately 100 days. It is of interest that in the elegant study of Herbert (25), intermediate megaloblasts were observed in the bone marrow on the 99th day of restricted folate intake, and frank megaloblastosis was observed on the 134th day. Thus, it would seem that clear manifestations of deficiency may apear at a time when only about one-half of the body stores have been exhausted.

A second aspect of this study to be emphasized is the recognition of a short-lived pool of newly absorbed folate that is clearly not in equilibrium with other folate forms.

In humans, newly absorbed folate may, according to Eichner and Hillman (26), represent as much as 50% of serum folate and constitute a labile pool which is rapidly depleted on a folate-deficient diet or following hemodialysis. The possibility that diphenylhydantoin may interfere with tissue uptake of newly absorbed folate is suggested by the increased biological activity and radioactivity found in the urine in day six. Further investigations are required to confirm or refute this possibility. Conventional doses of diphenylhydantoin (100 mg three times daily) given on days 20, 21, 22, 23 of the present study failed to produce any noticeable perturbation of the folate excretion pattern. There is no discernible effect on the pattern of elimination of *C. fasciculata* active pteridines following administration of diphenylhydantoin. Pterin and isoxanthopterin were radioactive whereas erythroneopterin, biopterin, and threoneopterin contained little or no radioactivity. The results are consistent with the observations of Fukushima and Shiota (27) concerning pterin biosynthesis in growing cultures of Chinese hamster ovary cells. Based on other studies, erythroneopterin, threoneopterin, and biopterin would not be expected to incorporate label from folic acid since these pterins are synthesized de novo from guanosine 5′triphophate (28–31). On the other hand, pterin and isoxanthopterin, which are formed as breakdown products of folic acid (and from other pterin derivatives), would be expected to be radioactive. It is interesting to compare the levels of Crithidia active substances and the levels of biopterin and erythroneopterin reported here with those reported earlier (5). The present results give an average excretion of biopterin equivalents of 0.7 mg/24 hr by Crithidia assay, with the urines of days 23 and 25 containing respectively 2.6 and 3.3 times more erythroneopterin than biopterin. In the earlier report (5), the Crithidia activity/24 hr assayed 1.5 mg of biopterin equivalents with the daily outputs of biopterin exceeding those of erythroneopterin (1.0 and 0.4 mg/24 hr, respectively). The reason for the inverted ratio of biopterin/erythroneopterin found in this study is not known but could conceivably be attributed to the estrogen supplements that the patient was receiving. The possibility that the estrogenic therapy may in some way influence our results on the half-life of folates, the bimodal curve of elimination of the tracer dose, and the effects of diphenylhydantoin administration is thought to be rather remote.

Our results do not support the hypothesis

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