

EFFECTIVE ABSORPTION AND UTILIZATION OF ORAL FORMYLtetrahydrofolate IN MAN

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Abstract Reversal of the effects of antifolates requires effective expansion of the body pool of reduced folate coenzymes. In the past, this has required parenteral use of 5-formyltetrahydrofolate. By use of radiolabeled 5-formyltetrahydrofolate, we showed that its oral administration also expands the body reduced folate pool. After oral administration of 5-formyl-¹⁴C-tetrahydrofolate-³H to fasting subjects, the labels appeared in the serum, peaking at 60 minutes. Chromatographic analysis showed

that the labeled serum folate was principally the naturally occurring 5-methyltetrahydrofolate-³H. Close to 90 per cent of orally administered 5-formyltetrahydrofolate appeared to be absorbed. The most constant labeled urinary folate was found to be 10-formyltetrahydrofolate or 5, 10-methenyltetrahydrofolate. Renal excretion of labeled 5-formyltetrahydrofolate occurred at undetectable serum levels whereas renal excretion of labeled 5-methyltetrahydrofolate was proportional to its serum concentration.

OF the reduced folates, the one that is chemically most stable^{1,2} and is therefore used therapeutically to reverse³⁻⁵ the effects of folate antagonists, such as methotrexate, is 5-formyltetrahydrofolate (leucovorin, citrovorum factor, 5-formylFH₄). However, in vitro, under acid conditions such as exist in the stomach, 5-formylFH₄ isomerizes to the stable 5, 10-methenylFH₄, which, under neutral pH conditions such as exist in the jejunum, isomerizes in turn to the readily oxidized and unstable 10-formylFH₄.¹ Therefore, in the past, 5-formylFH₄ has always been administered parenterally, and, indeed, its oral absorption has only recently been the subject of investigations.⁵⁻⁸

Increasing experience with, and use of, cancer chemotherapeutic agents suggests a place for an effective orally administered reduced folate for reversal of the effects of methotrexate. When 5-formylFH₄ was administered 24 or 36 hours after methotrexate, very high doses of the folate antagonists were safely administered to patients with acute lymphatic leukemia or epidermoid carcinoma of the head and neck, with apparent improvement of the therapeutic index.^{4,9,10} The basis for this differential rescue is not clear but may depend upon the differences in growth characteristics of these malignant cells and the normal bone marrow and gastrointestinal stem cells. Such an agent may also prove desirable for use in conjunction with antifolates in the chemotherapy of bacterial or protozoal infections. For example, studies indicate that 5-formylFH₄ can be administered concomitantly with pyrimethamine (Daraprim) to prevent the toxic but not the therapeutic effects of this antifolate in the

treatment of toxoplasmosis.¹¹ The present study was designed to assess, by radiotracer technics, whether orally administered 5-formylFH₄ can be absorbed in a form that contributes to expand the body pool of reduced folate coenzymes, and so potentially to reverse the effects of antifolates.

MATERIALS AND METHODS

Subjects

Four patients, each candidates for cancer chemotherapy, were selected for the studies. All had normal values for serum creatinine, blood urea nitrogen, serum vitamin B₁₂ and routine liver-function tests. Of the four, only A.S. suffered any symptoms or clinical signs of gastrointestinal disease.

This subject, a 20-year-old man weighing 87 kg, had undergone pelvic radiotherapy for a testicular tumor three months before the study and at the time of the study complained of nausea, and had a hematocrit of 29 per cent and a serum folate level (*Lactobacillus casei* method) of 3.5 ng per milliliter. The remaining patients suffered from local tumors of the head and neck region, and each had a normal hematocrit. J.S., a 57-year-old man weighing 75 kg, had a serum folate of 3.9 ng per milliliter and had received 5 mg of 5-formylFH₄ intramuscularly two days before the study to ensure that he was folate replete. M.S., a 74-year-old woman weighing 53 kg, had a serum folate level of 7.5 ng per milliliter. E.R., a 75-year-old woman weighing 36 kg, had a serum folate of 4.0 ng per milliliter after 3 mg of 5-formylFH₄ intramuscularly two days before the study.

MATERIALS AND PROCEDURES

Dr. Harriet Kiltie, of Lederle Laboratories, kindly supplied unlabeled 5-formylFH₄ (calcium leucovorin). Starting with folic acid-³H (Amersham-Searle) and formaldehyde-¹⁴C (New England Nuclear), respectively, 5-formylFH₄-³H with a specific radioactivity of 250 μ Ci per micromole and 95 per cent radiochemical purity, and 5-formyl-¹⁴C-FH₄ with specific radioactivity of 50 μ Ci per micromole and 97 per cent radiochemical purity were synthesized

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and purified in our laboratory.¹² Amounts of each were mixed with unlabeled 5-formylFH₄ for administration to fasting subjects. Hereafter, this mixture will be referred to as 5-formyl-¹⁴C-tetrahydrofolate-³H.

A.S. and J.S. each received a total oral dose of 10 μg per kilogram of body weight; M.S. received 50 μg per kilogram orally, and E.R. 5 μg per kilogram by rapid intravenous injection. Samples of serum, all urine and, in M.S., all stools, were collected through the study period. Urine and serum samples to be chromatographed contained 2-mercaptoethanol to prevent oxidation of reduced folates.

Measurement of radioactivity of samples was optimized for double-label counting of ³H and ¹⁴C, and the radioactivity due to each label, in disintegration per minute (dpm), was computed. The results were expressed as a percentage of the administered dose of each label.¹²

Samples of urine were desalted by chromatography through Sephadex G-15. The resultant fractions containing radiolabeled materials, together with marker compounds, were analyzed by ion-exchange chromatography by use of DEAE-Sephadex.¹³ Samples of serum were similarly analyzed by ion-exchange chromatography.

RESULTS

Oral Absorption of Radiolabels

In all three subjects, after orally administered 5-formyl-¹⁴C-FH₄-³H, both radiolabels appeared in the serum, peaking at about 60 minutes after administration. The time course of appearance and disappearance of labels from the serum, expressed as the percentage of the administered dose per liter of serum, is shown in Figure 1 for a representative subject (J.S.). It was similar in the other subjects, and maxima reached were 3.5 to 5.0 per cent of the dose per liter of serum, for ³H, and 2.3 to 2.5 per cent for ¹⁴C.

Figure 1 also shows the cumulative urinary clearance of radiolabels from J.S., expressed as a percentage of the administered dose. In the period one to 10 hours, the rates of urinary excretion of radiolabels were quantitatively close to, but less than, the rates of fall in concentration of serum radiolabels per liter of serum. Since the circulating plasma volume is more than double this value, and since the radiolabels probably equilibrate, in this time, through a body-fluid volume many times larger still, the urinary clearance in this period represents only a small portion of the radiolabels that have passed into and disappeared from the circulating fluid volume. Data presented below exclude the possibility of appreciable loss by gastrointestinal excretion. The largest proportion of absorbed radiolabels must therefore have been taken up into tissues.

At 24 hours after administration of labeled 5-formylFH₄, J.S. received a therapeutic methotrexate infusion. In the next 24-hour period, the rate of uri-

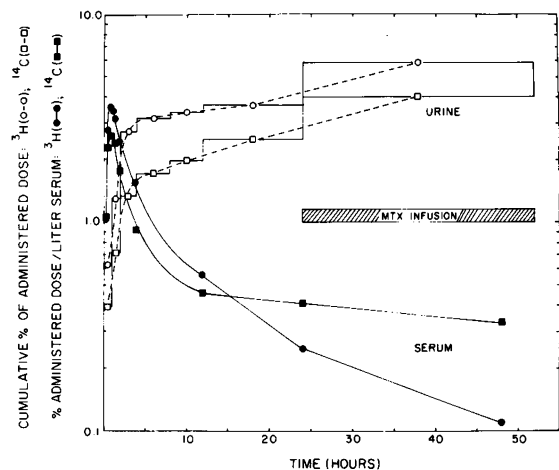


Figure 1. Time Course of Appearance and Disappearance of Serum Radiolabels and Cumulative Urinary Radiolabels after 5-Formyl-¹⁴C-FH₄-³H Given Orally to J.S. at a Dosage of 10 μg per Kilogram.

From 24 to 54 hours J.S. received a therapeutic infusion of methotrexate, at a dosage of 80 mg per square meter of body-surface area over 30 hours. The ordinate is logarithmic.

nary excretion of ³H increased whereas that of ¹⁴C did not, suggesting that, by this time, the two radiolabels were chemically dissociated. Moreover, the rate of urinary excretion was more than 10 times higher than that of serum disappearance for ³H, in the period of 24 to 48 hours; therefore, the increased urinary excretion of ³H that resulted from methotrexate administration was not derived from serum tritium, but at least partly from tissue tritium pools also.

Figure 2 shows that the serum appearance and disappearance data for M.S., after the fivefold higher oral dose of labeled 5-formylFH₄, was similar to that for J.S., whereas the rates of urinary clearance were approximately doubled. In this patient, radiolabels were measured in the stools as long as five days after the administration of labeled 5-formylFH₄. After five days the total cumulative collection of radiolabels in the stools was only 8 per cent for tritium and 5 per cent for ¹⁴C, suggesting that the radiolabels had almost completely absorbed.

For comparison, 5-formyl-¹⁴C-FH₄-³H was administered to E.R. by rapid intravenous injection of only 5 μg per kilogram of body weight. At the same periods after the first hour, the concentrations of radiolabels remaining in the serum of E.R. (Fig. 3) were comparable to those of J.S. and A.S., and were half those of M.S., as might be expected only if more than 50 per cent of orally administered radiolabels were absorbed. The initial urinary clearance of intravenously administered radiolabels (Fig. 3) was higher than that after oral administration, owing to the higher serum concentrations in the first hour after intravenous administration.

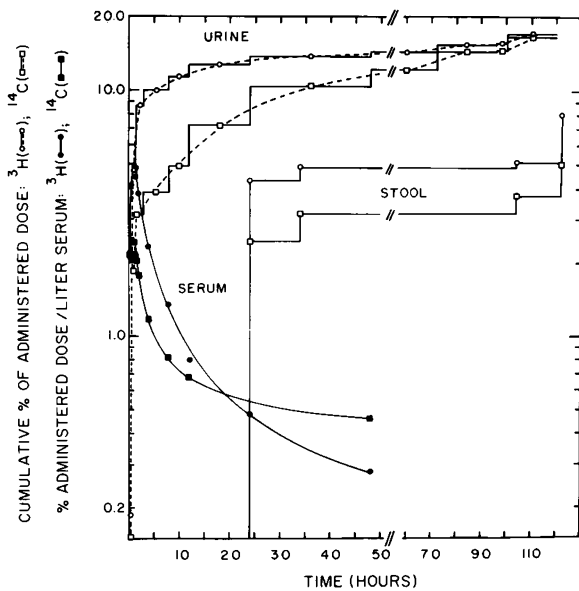


Figure 2. Time Course of Appearance and Disappearance of Serum Radiolabels and Cumulative Urinary and Stool Radiolabels after 5-Formyl- ^{14}C - FH_4 - ^3H Given Orally to M.S. at a Dosage of $50 \mu\text{g}$ per Kilogram.

At 99 and 101 hours M.S. received 6 mg of unlabeled 5-formyl FH_4 intramuscularly.

Identification of Serum Radioactivity

Serum collected 90 minutes after intravenous administration of 5-formyl- ^{14}C - FH_4 - ^3H was chromato-

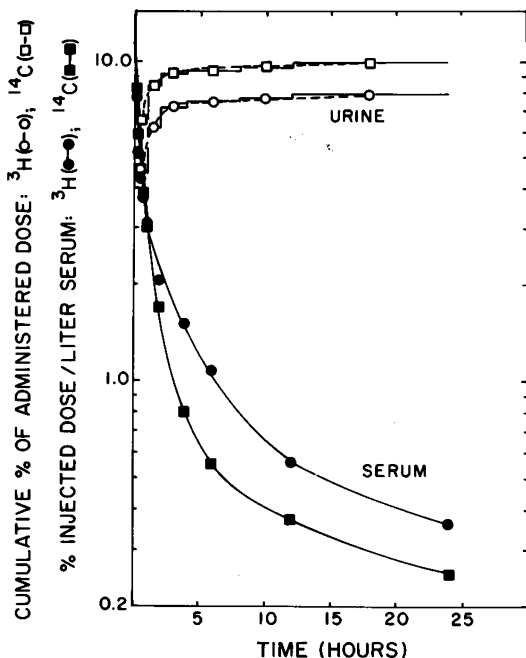


Figure 3. Time Course of Disappearance of Serum Radiolabels and Cumulative Urinary Radiolabels after 5-Formyl- ^{14}C - FH_4 - ^3H Given Rapidly by the Intravenous Route to E.R. at a Dosage of $5 \mu\text{g}$ per Kilogram.

graphed to identify and quantitate the folate forms with which the two radiolabels were then associated. Figure 4 represents the elution pattern of radiolabels and markers. At this time point, only about 40 per cent of the radiolabels cochromatographed with the 5-formyl FH_4 marker. Almost 60 per cent of ^3H was now identified as 5-methyl FH_4 , the normal circulating serum folate, and almost 40 per cent of the ^{14}C label was labilized to nonabsorbing materials, perhaps amino acids. Only 6 per cent of ^3H cochromatographed with *p*-aminobenzoylglutamate (*p*ABG), which would be expected to retain the largest proportion of ^3H were the reduced folates, labeled in the 9, 3' and 5' positions, degraded. Of the radiolabels chromatographed as 5-formyl FH_4 , the ratio of ^3H to ^{14}C was identical with that of the administered compound.

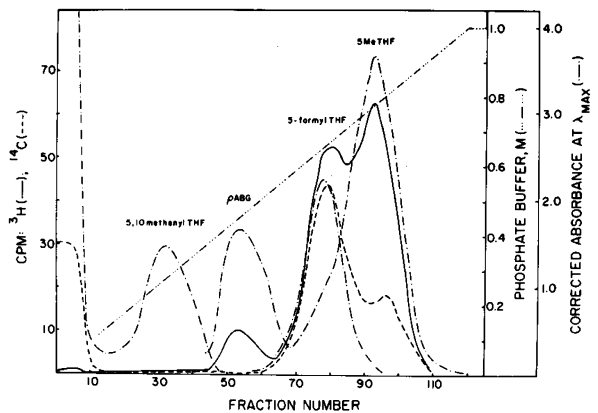


Figure 4. Elution Profile of Radiolabels and Marker Compounds when Serum, Collected 90 Minutes after Intravenous Administration of 5-Formyl- ^{14}C - FH_4 - ^3H , Was Applied to a 0.9×30 Column of DEAE-Sephadex and Eluted by a Concentration Gradient of Potassium Phosphate Buffer, pH-6.0.

The positions of marker compounds were monitored by ultraviolet absorbance spectrums between $240 \mu\mu$ and $360 \mu\mu$ at pH 6 and at $345 \mu\mu$ after incubation for one hour at 37°C at pH 1. Under the latter conditions only the positions of markers 5-formyl FH_4 and 5, 10-methenyl FH_4 were shown, and the absorbances due to overlapping markers could be corrected. The elution position of 10 formyl FH_4 is identical with that of 5, 10-methenyl FH_4 .

After chromatography of serum collected 75 minutes after oral administration of 5-formyl- ^{14}C - FH_4 - ^3H to J.S., a different radiolabel elution pattern (Fig. 5) was obtained. No substantial amounts of either radiolabel cochromatographed with the 5-formyl FH_4 marker. Almost 90 per cent of the ^3H , and 20 per cent of the ^{14}C chromatographed as 5-methyl FH_4 , 8 to 9 per cent of each as 10-formyl FH_4 or 5, 10-methenyl FH_4 (which elute together), and 70 per cent of the ^{14}C was not absorbed to the column. Almost identical elution profiles were obtained when the serum specimens of A.S. and M.S. were chromatographed at similar time points.

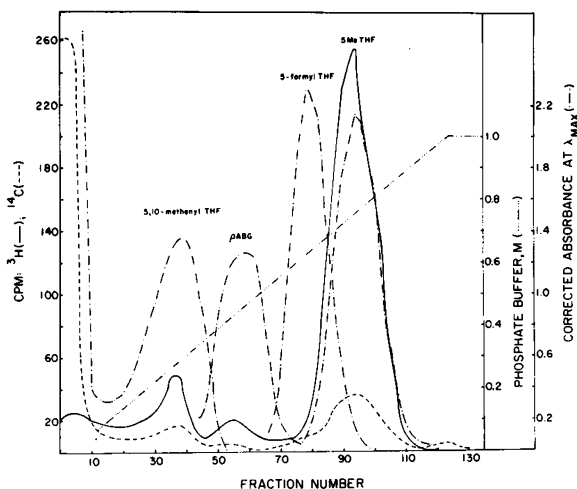


Figure 5. Elution Profile of Radiolabels and Marker Compounds when Serum, Collected 75 Minutes after Oral Administration of 5-Formyl- ^{14}C - FH_4 - ^3H to J.S., Was Chromatographed through DEAE-Sephadex.

Other details are as for Figure 4.

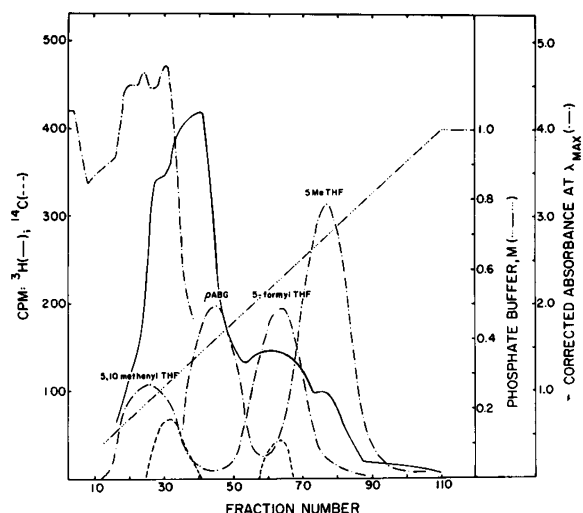


Figure 7. Elution Profile of Radiolabels and Marker Compounds for Chromatography of Urine Collected between Two and Four Hours after Oral Administration of 5-Formyl- ^{14}C - FH_4 - ^3H to J.S.

Other details are as for Figure 6.

Identification of Urinary Radioactivity

When the urine cleared by J.S. in the first zero to one hour after oral administration of 5-formyl- ^{14}C - FH_4 - ^3H was chromatographed, the total ^3H was distributed principally among four compounds (Fig. 6). Less than 20 per cent of ^3H was eluted in the position of 5-methyl FH_4 , whereas 40 per cent was identified as 5-formyl FH_4 , and about 40 per cent eluted as a complex peak including both pABG and 10-formyl FH_4 or 5, 10-methenyl FH_4 .¹² The small amount of ^{14}C absorbed to the column was distributed between the latter folates and 5-formyl FH_4 . Urines collected from J.S. at later periods, two to

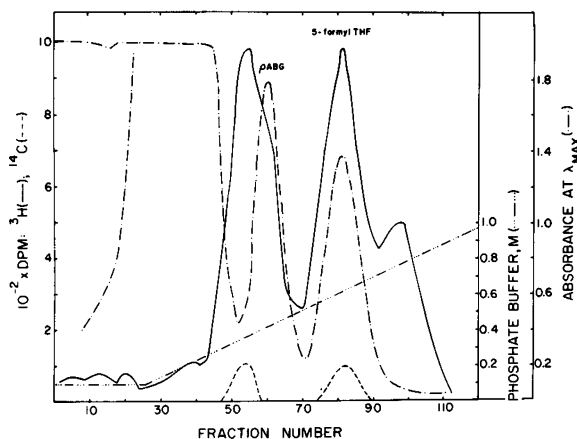


Figure 6. Elution Profile of Radiolabel and Marker Compounds when Urine, Collected between Zero and One Hours after Oral Administration of 5-Formyl- ^{14}C - FH_4 - ^3H to J.S. and Desalted by Molecular Sieving through Sephadex G-25, Was Chromatographed through DEAE-Sephadex.

Other details are as for Figure 4.

four hours (Fig. 7) and four to eight hours, progressively contained much less ^3H identifiable as 5-formyl FH_4 and 5-methyl FH_4 , whereas the excretion of ^3H identifiable as 10-formyl FH_4 or 5, 10-methenyl FH_4 was maintained. Urines collected in corresponding periods from A.S. and M.S. contained radiolabels similarly distributed. In all the urine samples, the proportion of total tritiated urinary folates identifiable as 5-methyl FH_4 varied directly with the serum concentration of 5-methyl FH_4 - ^3H , between the limits observed, which were 6 per cent at 7.5 and 70 per cent at 120 ng per milliliter.

At 99 hours and again at 101 hours after the oral administration of 5-formyl- ^{14}C - FH_4 - ^3H , M.S. received 6 mg of unlabeled 5-formyl FH_4 intramuscularly. The urine cleared in the next 20 hours contained no radiolabeled 5-formyl FH_4 . Rather, 30 per cent of ^3H chromatographed as 5-methyl FH_4 , 70 per cent of ^3H , and 60 per cent of ^{14}C as 10-formyl FH_4 or 5, 10-methenyl FH_4 , and 40 per cent of ^{14}C was not absorbed and presumably converted to nonfolate compounds.

After intravenous administration of 5-formyl- ^{14}C - FH_4 - ^3H to E.R., urine collected in the period of zero to one hour contained ^3H and ^{14}C , both of which were distributed almost entirely as 5-formyl FH_4 and were present in the same ratio as in the administered material. Urine collected in the period of two to four hours from E.R. differed only in that 30 per cent of the radiolabels chromatographed as 10-formyl FH_4 or 5, 10 methenyl FH_4 .

DISCUSSION

These data clearly show that orally administered 5-formyl FH_4 was well absorbed so as to expand the

serum pool of reduced folate coenzymes. Under fasting conditions, the quantitative absorption of orally administered 5-formylFH₄ was close to 90 per cent of the dose since, even at the higher dosage level of 50 µg per kilogram, only 8 per cent of the administered ³H and 5 per cent of the ¹⁴C was excreted in the stools within five days. The high quantitative absorption of 5-formylFH₄ was confirmed by comparison of the serum radiolabel concentrations achieved by oral administration with those achieved by intravenous administration. At the maximum observed concentrations of serum ³H, the serum folates were elevated by folate-³H to the extent of 27 ng per milliliter at the 5-µg-per-kilogram dosage, and 130 ng per milliliter at 50 µg per kilogram.

During transfer from the gastrointestinal tract to the systemic circulation 5-formyl-¹⁴C-FH₄-³H was substantially metabolized. The formyl-¹⁴C was largely removed from the circulating serum folates, and 90 per cent of the serum folate-³H could be identified as 5-methylFH₄-³H. Since 5-methylFH₄ is the naturally abundant serum folate,¹⁴ and is readily transported into and utilized by tissues,¹⁵ it might be expected that much of the folate administered orally as 5-formylFH₄ would have entered and expanded the tissue pools of reduced folate coenzymes. Comparison of the serum radiolabel disappearance rates with the urinary radiolabel excretion rates suggested that, in fact, the largest proportion of absorbed folate was taken up by tissues.

These data do not indicate whether the metabolism of oral 5-formylFH₄ to serum 5-methylFH₄ occurred in the intestine or the liver or both. However, 90 minutes after rapid intravenous administration of 5-formyl-¹⁴C-FH₄-³H, about 40 per cent of the serum ³H was still in the form of the administered compound. Since metabolism of administered 5-formylFH₄ into serum 5-methylFH₄ is more rapid after oral than after intravenous administration, it seems probable that extensive conversion of 5-formylFH₄ to 5-methylFH₄ does occur in the intestine. That this is true has recently been demonstrated unambiguously by portal venipuncture.⁷

The results of chromatography of urine samples after administration of 5-formyl-¹⁴C-FH₄-³H suggest that there is a fairly constant urinary clearance of folates in the forms of 10-formylFH₄ or 5, 10-methenylFH₄. The fraction of total radiolabeled urinary folates found as 5-methylFH₄ appeared proportional to the serum concentration of radiolabeled 5-methylFH₄. In contradistinction, large amounts of radiolabeled 5-formylFH₄ were found in the urine in the early period (zero to two hours) even when, as after oral administration of 5-formylFH₄, no label was clearly measurable in the serum. In similar studies in our center^{16,17} in which radiolabeled 5-methylFH₄ was administered, we never observed labeled 5-formylFH₄ in the urine. It appears, therefore, that the kidney may have both regulatory and metabolic roles in the excretion of folates: serum 5-methylFH₄

may be conserved by the kidney in preference to 5-formylFH₄, and the most constant urinary folates, 10-formylFH₄ and 5, 10-methenylFH₄, differ from the serum folate.

These data considerably extend preliminary reports⁵⁻⁸ that orally administered 5-formylFH₄ is able to raise the level of serum folates assayed by *L. casei*. Our studies justify the use of oral administration of 5-formylFH₄ to reverse the toxic and therapeutic effects of methotrexate and other antifolates. Preliminary studies in mice¹⁸ as well as man indicate that oral leucovorin may be useful in regimens for the chemotherapy of cancer and infectious diseases.^{10,19}

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