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THE EFFECT OF REDUCED DERIVATIVES OF FOLIC ACID ON TOXICITY AND ANTILEUKEMIC EFFECT OF METHOTREXATE IN MICE

J. A. R. MEAD, J. M. VENDITTI, A. W. SCHRECKER, A. GOLDIN and J. C. KERESZTESY

Laboratory of Chemical Pharmacology, National Cancer Institute; and Laboratory of Nutrition and Endocrinology, National Institute of Arthritis and Metabolic Diseases, Bethesda, Md. U.S.A.

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Abstract—Mice inoculated with leukemia L1210 were treated with combinations of methotrexate (amethopterin) and one of the following folic acid derivatives: (a) folic acid, (b) dihydrofolic acid, (c) tetrahydrofolic acid, (d) 10-formyltetrahydrofolic acid, (e) 5-formyltetrahydrofolic acid (citrovorum factor), and (f) prefolic A (5-methyltetrahydrofolic acid).

Daily treatment was started 3 days after leukemic inoculation. Treatment with methotrexate (MTX) alone, or in combination with folic acid, resulted in a considerable prolongation of lifetime. All other treatments resulted in leukemic death of the animals at the same time as the untreated controls; i.e. the antileukemic effect of methotrexate was blocked by the metabolite.

Delayed administration of ctirovorum factor and prefolic A, after large doses of MTX, showed that both compounds were effective in reducing toxicity but had little or no effect on the antileukemic activity when given 12 to 24 hr after MTX. At 48 hr after administration of MTX neither compound protected against the toxicity of the drug.

In view of the extreme sensitivity of dihydrofolic reductase to inhibition by MTX *in vitro*, it was of particular interest that dihydrofolic acid was able to bring about extensive reversal of the antileukemic effect and toxicity of this substance. A study of folic and dihydrofolic reductase activity in the livers of mice which had received combinations of MTX and folic acid derivatives showed that there was no difference in the extent of inhibition, regardless of whether the combination was toxic or nontoxic to the animals.

BOTH folic acid and 5-formyltetrahydrofolic acid (citrovorum factor) have been shown to decrease the lethal toxicity and antileukemic effect of folic acid antagonists.[1, 2] The extent of this decrease is dependent not only upon the relative dose levels of metabolite and antagonist employed, but also on the temporal relationship of their administration. [3, 4] An essential difference between the behavior of the two metabolites, with respect to their ability to nullify the effect of a folic acid antagonist in mammalian systems, is that folic acid is active only when given before the drug, whereas citrovorum factor retains its activity on simultaneous or even subsequent administration.

The protection afforded by administration of folic acid is attributable to its conversion to an active form (5,6,7,8-tetrahydrofolic acid) related to citrovorum factor. The enzyme system, folic reductase, which catalyzes this transformation, is strongly inhibited by low doses of folic acid antagonists, and thus the conversion of folic acid to an active form *in vivo* is prevented when the metabolite is administered simultaneously with or after the antagonist.

Jukes[5] has reported that the lethal toxicity of aminopterin in mice can be effectively blocked by concomitantly administered tetrahydrofolic acid, anhydroleucovorin, or citrovorum factor. More recently, Lemlein *et al.*[6] observed that the inhibitory effect of 6,7-diisopropyl-2,4-diaminopteridine on the growth of a folic acid-requiring strain of *Streptococcus faecalis* could be completely reversed by the addition of dihydrofolic acid to the medium. In the present investigation we have included dihydrofolic acid among the folic acid derivatives examined. Also included was prefolic A, a naturally-occurring form of folic acid which was isolated from horse liver by Donaldson and Keresztesy[7] and which is probably identical with 5-methyltetrahydrofolic acid.[8]

The current investigation was undertaken as part of a program to characterize in more detail the metabolite–antimetabolite relationships exhibited by the folic acid antagonists. The biological interaction involving methotrexate and such reduced derivatives of folic acid as dihydrofolic acid, tetrahydrofolic acid, 10-formyltetrahydrofolic acid, and prefolic A is presented in this report, from the point of view of both antileukemic activity and toxicity in mice. This program is considered of particular interest in view of our observations on the antimetabolite and antileukemic properties of the reduced derivatives of folic acid antagonists and their demonstrated relationship to citrovorum factor.[9, 10]

MATERIALS AND METHODS

Hybrid male CDBA mice [(BALB/cAn × DBA/2J)F_1], 10 to 12 weeks old and weighing 24 to 28 g, were used. They were maintained on Purina laboratory chow pellets and water *ad libitum*. In studies with leukemic animals, ten mice were used in each experimental group; in toxicity studies on normal mice there were six mice per group.

In the studies dealing with the reversal of the antileukemic effect of methotrexate, the mice were inoculated with the lymphoid leukemia L1210 as previously described.[11] Treatment was started before the appearance of a local tumor at the site of inoculation, usually day 3 after the inoculation. In the toxicity experiments, treatment of non-leukemic mice was continued for the time indicated.

Methotrexate and citrovorum factor (calcium leucovorin) were obtained from the Lederle Division, American Cyanamid Co. Folic acid was obtained from the California Corp. for Biochemical Research. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) (enzymically reduced) was purchased from Sigma Chemical Co. or from the California Corp. for Biochemical Research. Dihydrofolic acid, tetrahydrofolic acid, and anhydroleucovorin were prepared and stored in the dark under high vacuum prior to use.[12–14]

Solutions of various drugs were injected subcutaneously in the scapular region (0·01 ml/g body weight). Citrovorum factor was administered as an aqueous solution. The barium salts of prefolic A, isolated from horse liver and the synthetic compound,[8] were converted, prior to injection to the sodium salt by dropwise addition of 5% sodium sulfate solution to an aqueous solution of the metabolite. The precipitate of barium sulfate was removed by centrifugation and the solution then diluted as required with a solution of 0·6 g ascorbic acid/100 ml, previously neutralized to pH 6 with potassium hydroxide. All other compounds were dissolved in a vehicle of 2% sodium bicarbonate containing the neutralized ascorbic acid. The pH of this vehicle was 8·5. Solutions of folic acid and MTX were prepared and stored in the refrigerator.

Fresh solutions of dihydrofolic acid and tetrahydrofolic acid were prepared immediately before use. Anhydroleucovorin was allowed to stand in the ascorbate vehicle at room temperature for 15 min before injection. This was done in order to convert completely anhydroleucovorin to 10-formyltetrahydrofolic acid (as measured by the disappearance of the ultraviolet absorption peak at 355 mμ).[14]

For the enzyme studies, liver acetone powders were prepared[15] at −40° to −20° and stored in a freezer. Extracts were prepared from the powders with 15 to 20 volumes of the appropriate buffer at 3° and centrifuged at 35,000 × *g* prior to the reductase assays. Alternatively, the fresh livers were homogenized[16] with 4 volumes of 0·25 M sucrose containing 0·01 M mercaptoethanol and 0·001 M ethylenediamine tetraacetate (EDTA) at 3°, and the supernatant fractions were obtained by centrifugation at 100,000 × *g* for 45 min. Folic reductase activity was determined according to the method of Zakrzewski[17] by incubating 0·2 to 0·4 ml of acetone powder extract with 0·12 μmole of folate and 0·75 μmole of NADPH in 0·1 M sodium citrate buffer, pH 5·2 (total volume, 1·5 ml), at 37° under argon in a Dubnoff metabolic incubator for 20 min. The amount of diazotizable amine derived from tetrahydrofolate was measured colorimetrically,[17] and a blank obtained in the absence of NADPH was subtracted from the reading.* Enzyme activity was essentially linear with respect to concentration when the absorbance differences were in the range of 0·05 to 0·15. Dihydrofolic reductase activity was determined, according to a slight modification[15] of the method of Osborn and Huennekens,[18] by measuring the decrease in absorbance at 340 mμ at 1-min intervals for 10 to 20 min at 28°. The reaction was carried out in cuvets containing 0·25 to 0·5 ml of acetone powder extract or supernatant fraction, 0·1 μmole of dihydrofolate, and 0·24 μmole of NADPH in 0·05 M potassium phosphate buffer (pH 7·4) with 0·01 M mercaptoethanol–0·001 M EDTA (total volume, 3 ml). A blank obtained in the absence of dihydrofolate was subtracted from the experimental reading. Dihydrofolic reductase activity was also determined in 0·1 M citrate buffer, pH 5·2, containing 0·01 M mercaptoethanol. Acetone powders were extracted directly with the buffer, while supernatant fractions were adjusted to the appropriate pH and recentrifuged. To avoid precipitation of residual protein during the assay at pH 5·2, it was necessary to preincubate the enzyme solutions at 35° for 3 min and to centrifuge them again. The amount of NADPH was increased to 0·48 μmole. In either buffer, enzyme activity for the controls was linear with respect to concentration up to absorbance changes of 0·05 per min. The amounts of enzyme used in the assays were such as to yield absorbance changes of 0·015 to 0·040 per min for the controls and 0·002 to 0·008 per min when methotrexate had been administered. Protein was determined by the method of Lowry *et al.*[20] Specific activities (mμmole/hr/mg protein) are based, respectively, on the amount of diazotizable amine formed[17] or on the decrease in absorbance at 340 mμ caused by the oxidation of NADPH and reduction of dihydrofolate.[15]

RESULTS

Figures 1 through 3 show the results of three separate experiments in which reduced

* This blank, in a number of control experiments, was identical with a blank determined by adding the folate after acidification.[17]
At pH 7·4 identical rates were observed when chemically or enzymically reduced NADPH was used in the assay. At pH 5·2, however, enzymically reduced NADPH gave about twice the rate obtained with the chemically reduced compound. Increasing the amount of the latter did not lead to an increased rate. With the enzymically reduced NADPH, the reaction rate in citrate buffer, pH 5·2, was 3 to 4 times that observed in phosphate buffer, pH 7·4, in agreement with previous findings.[19]

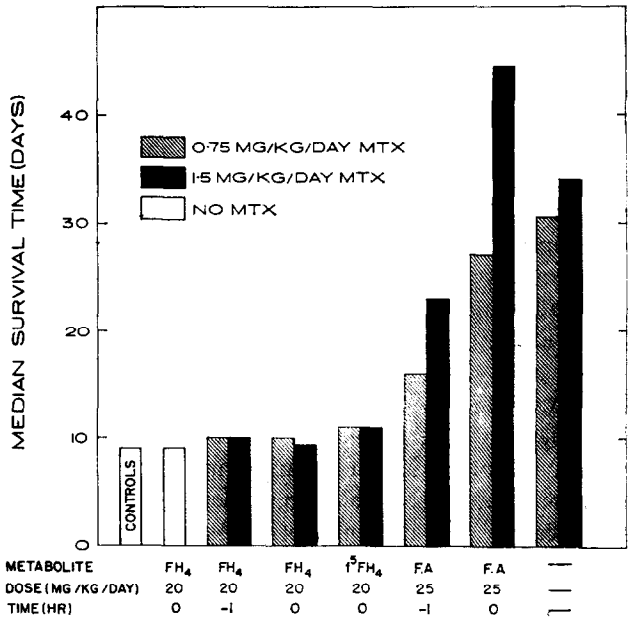
MEDIAN SURVIVAL TIME (DAYS)
0·75 MG/KG/DAY MTX
1·5 MG/KG/DAY MTX
NO MTX
CONTROLS
METABOLITE
DOSE (MG/KG/DAY)
TIME (HR)
FH4 FH4 FH4 f5FH4 F.A F.A —
20 20 20 20 25 25 —
0 -1 0 0 -1 0 —

FIG. 1

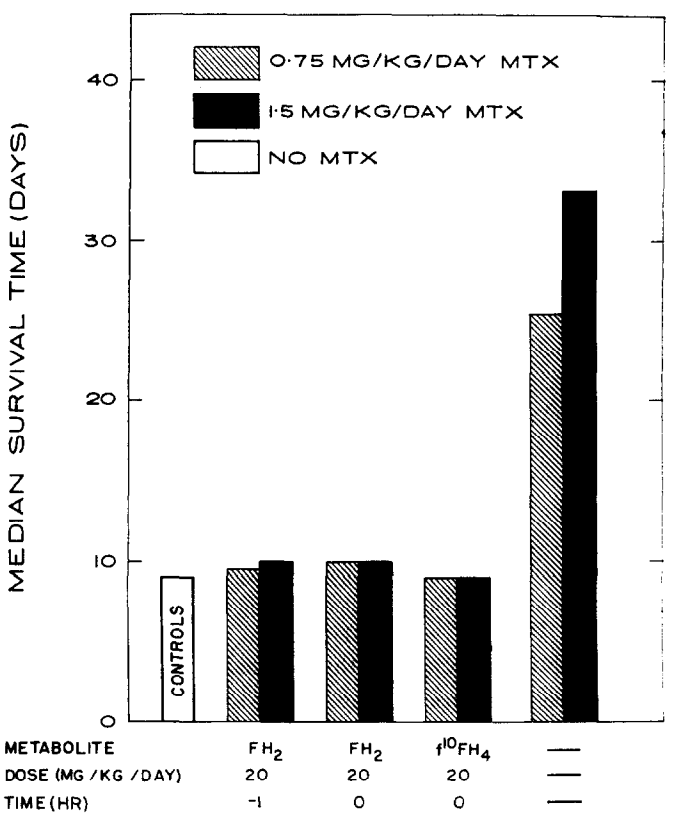
MEDIAN SURVIVAL TIME (DAYS)
0·75 MG/KG/DAY MTX
1·5 MG/KG/DAY MTX
NO MTX
CONTROLS
METABOLITE
DOSE (MG/KG/DAY)
TIME (HR)
FH2 FH2 f10FH4 —
20 20 20 —
-1 0 0 —

FIG. 2

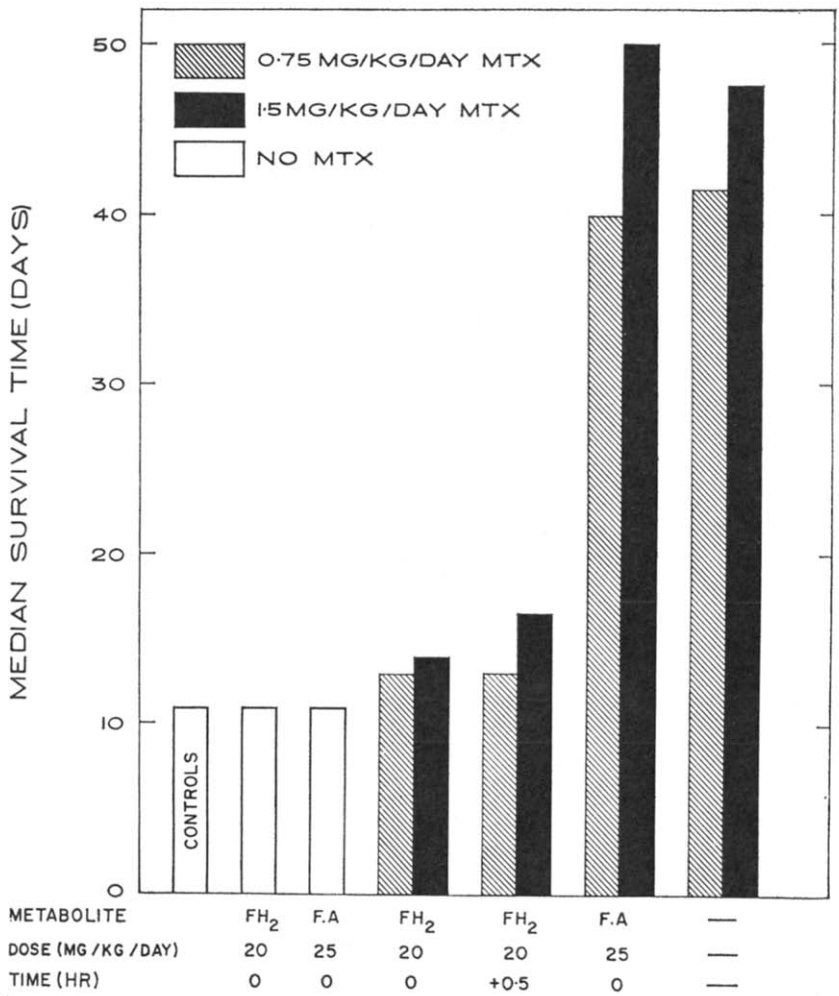

FIG. 3

FIGS. 1–3. Influence of folic acid derivatives on the antileukemic (L1210) effectiveness of methotrexate (amethopterin). Mice inoculated with leukemia L1210 on day 0. Treatment started on day 3. Time refers to time of administration of metabolite relative to that of methotrexate (MTX). F.A. = folic acid, FH_2 = dihydrofolic acid, FH_4 = tetrahydrofolic acid, f^5FH_4 = 5-formyltetrahydrofolic acid (citrovorum factor), $f^{10}FH_4$ = 10-formyltetrahydrofolic acid. Ten mice per group.

derivatives of folic acid were examined with respect to their ability to nullify the antileukemic effect of therapeutic doses of methotrexate. The previously investigated citrovorum factor and folic acid were included for comparison.

It can be seen that tetrahydrofolic acid, 10-formyltetrahydrofolic acid, and citrovorum factor are capable of decreasing the antileukemic effect of MTX when given before or at the same time as the drug. Folic acid, on the other hand, prevented antileukemic activity only when given 1 hr prior to the drug. When folic acid was administered at the same time as MTX, no reduction in antileukemic effect was seen. In fact, when administered together with 1·5 mg MTX/kg, there may have been some increase in antileukemic activity of the drug as measured by the greater median survival time of the mice.

Of particular interest was the observation that dihydrofolic acid was able to decrease the antileukemic activity of MTX, whether it was given before, at the same time, or 30 min after the drug. In view of these results, the ability of dihydrofolic acid to protect nonleukemic mice from the toxic effects of parenterally administered MTX was examined. Folic acid, dihydrofolic acid, and citrovorum factor were compared in this study. The results, shown in Table 1, indicated that, on simultaneous administration,

TABLE 1. PROTECTION AGAINST THE TOXICITY OF METHOTREXATE BY FOLIC ACID DERIVATIVES*

Daily dose of metabolite (mg/kg)	Mice surviving to day 8 (6 mice per group) Daily dose of methotrexate (mg/kg)							
	80	40	20	10	5	2·5	1·25	0
None				0	2	6	6	6
20 Folic acid				0	2	5	6	6
20 Dihydrofolic acid	2	6	6	6	6	6		6
20 Citrovorum factor	6	6	6	6	6	6		6

* Treatment was initiated on day 0, and each group received a total of 8 injections. Methotrexate and the metabolites were given simultaneously as indicated.

dihydrofolic acid more closely resembled citrovorum factor than folic acid in its pharmacological behavior. Thus, a dose of 10 mg MTX/kg/day, frankly toxic by itself, was rendered essentially nontoxic by the simultaneous administration of 20 mg dihydrofolic acid/kg/day. At doses of MTX in excess of 10 mg/kg/day, simultaneous administration of 20 mg dihydrofolic acid/kg/day did not prevent weight loss in the treated animals, but reduction of toxicity was apparent as judged by the number of animals surviving to day 8 after initiation of treatment. Folic acid was completely ineffective in reducing the toxicity of MTX at any dose level in this experiment, whereas citrovorum factor prevented any weight loss when the dose of MTX was as high as 40 mg/kg/day.

It has recently been shown that, when the preparation of dihydrofolic acid by dithionite reduction[12] was carried out at elevated temperatures, the principal reaction product was tetrahydrofolic acid.[21] For the present work it was important to show that

dithionite reduction of folic acid at room temperature did not produce appreciable quantities of tetrahydrofolic acid. Blakley has shown that dihydrofolic acid prepared by dithionite reduction of folic at room temperature caused no detectable activation of the enzymic synthesis of serine, and this led him to conclude that tetrahydrofolic acid was absent from his preparation.[22] We have carried out additional experiments which confirm this conclusion.

In Table 2 are shown the results of a microbiological assay carried out on a sample of dihydrofolic acid used in the present studies. *Pediococcus cerevisiae*, which requires preformed tetrahydrofolic acid for growth, was used as the test organism, and the

TABLE 2. MICROBIOLOGICAL ASSAY OF TETRAHYDROFOLIC ACID ACTIVITY IN DIHYDROFOLIC ACID

FH_2 added per tube (μg)	FH_4 added per tube (mμg)	FH_4 activity found (mμg)	FH_4 activity in FH_2 (%)
100		0·28	0·00028
250		0·56	0·00022
500		1·12	0·00022
	1	1·03	
50	1	1·42	0·00078
100	1	1·95	0·00092
250	1	3·15	0·00084

FH_2, dihydrofolic acid; FH_4, tetrahydrofolic acid. Standard growth curve obtained with tetrahydrofolic acid according to Bakerman[23], with *P. cerevisiae* as test organism. Assays represent average of two determinations; tetrahydrofolic acid alone the average of four determinations.

bioassay was carried out according to Bakerman.[23] It can be seen that there was less than 0·001% of tetrahydrofolic acid activity associated with the dihydrofolic acid. Excess dihydrofolic acid did not inhibit the growth of the organism when tetrahydrofolate was present.

In a further biological study in mice it was shown that 10 mg methotrexate/kg/day could be rendered nontoxic by the simultaneous administration of 10 mg dihydrofolic acid/kg/day. In the same experiment, simultaenous administration of 0·625 mg citrovorum factor/kg/day, equivalent to 0·3 mg *l*-tetrahydrofolate activity/kg/day, did not protect the animals from the toxic effects of the same dose of MTX. However, when the dose of citrovorum factor was raised to 1·25 mg/kg/day, the animals showed no signs of toxicity. Thus, if the biological activity of dihydrofolic acid was attributable to the presence of some tetrahydrofolic acid, the minimal contamination would have to be greater than 3% *l*-tetrahydrofolate, and this was not borne out by the microbial assay.

Further proof of the lack of contamination of the dihydrofolic acid with tetrahydrofolic acid was obtained from chromatography of dihydrofolic acid on a DEAE-cellulose column. A linear gradient elution system with ammonium bicarbonate buffer, pH 8·3, containing 0·01 M mercaptoethanol, was used. No detectable amounts of tetrahydrofolic acid were found in the eluate. Control experiments showed that it

was possible to achieve complete separation of the two compounds when as little as 1% of tetrahydrofolate was present in a mixture of the two. Similar complete separation had previously been achieved with a different elution system.[24]

The influence of prefolic A, as compared with that of citrovorum factor, on the antileukemic effectiveness of methotrexate is shown in Table 3. Essentially complete reversal of antileukemic effect was observed with both metabolites when administered at the same time as the drug.

Citrovorum factor has also been shown to give some therapeutic advantage in situations where toxic levels of aminopterin have been administered and treatment

TABLE 3. INFLUENCE OF PREFOLIC A ON THE ANTILEUKEMIC (L1210) EFFECTIVENESS OF METHOTREXATE*

Prefolic A (mg/kg)	Citrovorum factor (mg/kg)	Methotrexate (mg/kg)	Median survival time (days)
10		60	14
10		30	14
	10	60	15·5
	10	30	13
		60	26·5
		30	22
10			11
	10		11
Untreated controls			11

* The compounds were administered subcutaneously on days 3 and 7 after leukemic inoculation. Prefolic A or citrovorum was given 5 min prior to methotrexate. There were 10 mice in each experimental group.

with the metabolite was withheld for varying periods of time. Citrovorum factor administered at these times reduced the toxicity of aminopterin for the host but did not reduce its antileukemic effect.[25] In view of the current interest in regional chemotherapy, in which systemic toxicity from massive local perfusion of methotrexate is controlled by systemic administration of citrovorum factor, it was of interest to compare the relative effectiveness of prefolic A and citrovorum factor in reducing the toxicity or antileukemic effect, or both, in mice when administration of the metabolite was delayed. Table 4 shows the results obtained in an experiment in which the administration of the two metabolites was delayed for periods up to 48 hr after treatment with MTX. In this experiment mice received a large single dose of MTX on day 3 after leukemic inoculation. Administration of citrovorum factor or synthetic prefolic A was withheld for 12, 24, and 48 hr after MTX. Characteristically, the mice could be separated into two categories: those succumbing to drug toxicity and those succumbing to tumor growth. It can be seen (Table 4) that both prefolic A and citrovorum factor were equally effective in reducing the toxicity of MTX when given 12 or 24 hr after the drug. At the same time there was no appreciable loss of antileukemic effect as judged by the median survival times of the mice dying of leukemia. When either metabolite was given 48 hr after the MTX, there was little protection from toxicity. Mice surviving the toxicity showed increases in life span comparable to those seen when

MTX was given alone. These studies have shown that there is apparently no advantage to be obtained by the use of prefolic A instead of citrovorum factor, under these experimental conditions.

The experiment summarized in Fig. 4 represents an attempt to correlate the inhibition of the enzymic conversion of folic and dihydrofolic acid to tetrahydrofolic

TABLE 4. EFFECT OF DELAYED ADMINISTRATION OF PREFOLIC A AND CITROVORUM FACTOR ON THE RESPONSE OF LEUKEMIC (L1210) MICE TO METHOTREXATE (MTX)

MTX	Dose (mg/kg)			No. of of mice	No. of toxic deaths	Median survival time based on leukemic deaths only (days)	
0 hr	+12 hr	+24 hr	+48 hr				
	Prefolic A						
400				10	8(6–10)*	21·5	(21–22)
200				10	2(7–8)	13	(12–15)
100				10	1(9)	15	(14–16)
400	200			9	0	15	(13–17)
200	200			7	0	13	(11–16)
100	200			7	0	13	(10–13)
400		200		10	0	18	(14–23)
200		200		10	0	14	(11–19)
100		200		10	0	13	(12–22)
400			200	10	6(7–8)	18·5	(16–25)
200			200	10	2(6)	14	(13–16)
100			200	10	0	13	(12–17)
			200	10	0	9	(8– 9)
	Citrovorum factor						
400	200			10	1(9)	15	(11–18)
200	200			10	0	13·5	(12–16)
100	200			10	0	13	(11–17)
400		200		10	0	15	(12–19)
200		200		10	0	13	(12–15)
100		200		10	0	13	(12–15)
400			200	10	4(7–9)	14	(13–18)
200			200	10	2(7–8)	13·5	(13–18)
100			200	10	2(7)	15	(13–19)
			200	10	0	8·5	(8– 9)
Untreated controls				10	0	9	(9–10)

*Figures in parentheses refer to the range (in days) of individual mortalities. Methotrexate was administered on day 3 after leukemic inoculation, citrovorum factor and prefolic A at the times indicated.

acid, with the pharmacological behavior of combinations of methotrexate and folic acid derivatives. In this experiment a toxic daily dose of MTX was administered alone and in combination with the metabolite for 4 days. The mice were killed 24 hr after the last treatment in order to minimize spurious inhibition by free MTX present in the tissues during preparation of the enzyme. Acetone powders were prepared from the pooled livers of three mice in each experimental group. After assay of these preparations for folic and for dihydrofolic reductase activity, the results were expressed as a

percentage of the untreated control values. As can be seen, there was no significant difference in the activity of the enzyme from mice treated with MTX alone or in combination with the folic acid derivatives although, as shown in Table 1, there was a considerable difference in the pharmacological properties of the combinations. In this experiment, the reduction of folic acid was inhibited to a greater extent (95 to 96%) than that of dihydrofolic acid (80%).

The residual activity of the enzyme preparations in the above experiment could possibly be due to breakdown of the enzyme-methotrexate complex during preparation of the acetone powder. For this reason an additional experiment was carried out. Again, 10 mg MTX/kg was administered to mice during 4 consecutive days, and both acetone powders and sucrose homogenates were prepared from the livers 24 hr after

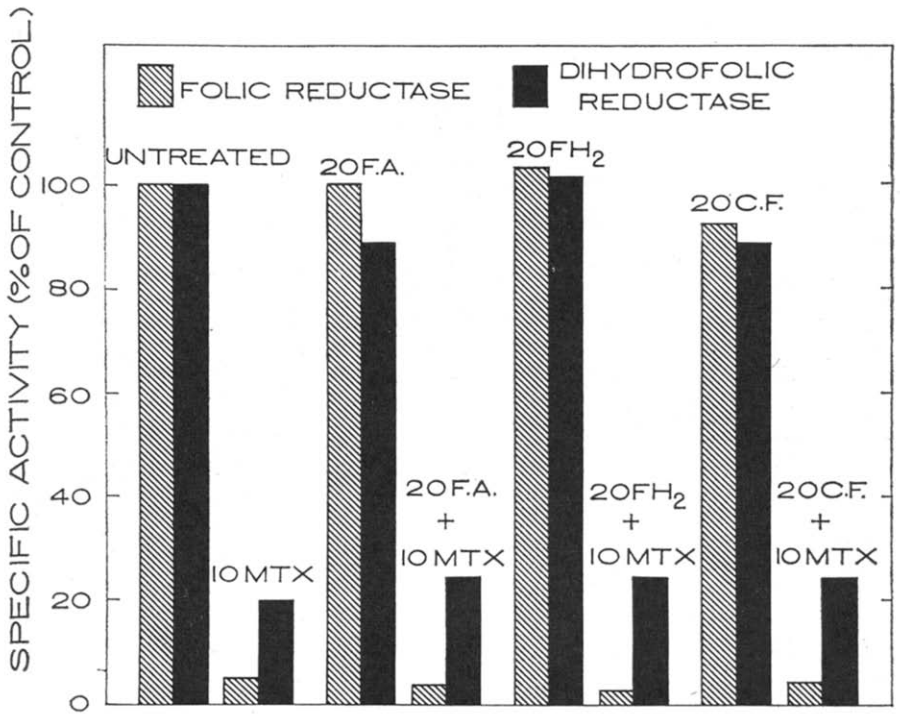

FIG. 4. Folic and dihydrofolic reductase activity of mouse liver acetone powders. The figures above the bars refer to the daily dose of MTX or metabolite, or both, in mg/kg for 4 consecutive days (C.F. = citrovorum factor; other abbreviations as in Fig. 1–3). Mice killed 24 hr after last treatment. Specific activities (mμmoles/hr/mg protein) of untreated controls: folic reductase, 12 (pH 5·2); dihydrofolic reductase, 25–30 (pH 7·4). For details of assays, see Materials and Methods. Three mice per group.

the last injection Reduction of folic acid by extracts of the acetone powders in citrate buffer, pH 5·2, was inhibited 94 to 96% in the treated mice, with a specific activity of 12 mμmoles/hr/mg protein for the untreated controls. The data pertaining to the reduction of dihydrofolic acid are summarized in Table 5. Specific activity of the untreated controls was much higher than in the previous experiment (65 vs. 25 to 30

mμmoles/hr/mg protein), probably because of improvements in the procedure.* The acetone powder extracts and the supernatant fractions from the sucrose homogenates from the livers of methotrexate-treated animals exhibited, at pH 7·4, essentially identical activities based on liver weight. This suggests that, during the preparation of the acetone powders, the enzyme was not denatured, and methotrexate bound to the enzyme was not removed.

TABLE 5. DIHYDROFOLIC REDUCTASE ACTIVITY OF MOUSE LIVER

Drug	Method*	pH	Protein (mg/assay)	Specific activity†	Tissue activity‡	Percentage of control	
						Specific	Tissue‡
None	Acetone powder	7·4	5·0	64	6·4		
None		7·4	5·0	67	7·0		
MTX		7·4	13·3	3·9	0·41	6	6
MTX		7·4	13·5	5·8	0·62	9	9
None	Supernatant fraction	7·4	3·9– 7·0	94	6·8		
None		7·4	3·9– 7·0	87	6·4		
MTX		7·4	6·1	8·8	0·59	10	9
MTX		7·4	6·9	7·3	0·52	8	8
None	Acetone powder	5·2	2·3	219	23·4		
None		5·2	2·2	241	20·7		
MTX		5·2	3·1	30	2·5	13	11
MTX		5·2	3·6	21	2·0	9	9
None	Supernatant fraction	5·2	1·5– 2·6	383	27·7		
None		5·2	1·6– 2·6	378	27·8		
MTX		5·2	2·3	52	3·1	14	11
MTX		5·2	2·6	50	3·7	13	13

* The treated mice received 10 mg MTX/kg subcutaneously daily for 4 days and were killed 24 hr after the last injection. Duplicate groups had 2 mice each. The same acetone powder or supernatant fraction was used for the assays at both pH 7·4 and 5·2 (see Materials and Methods for details).

† Dihydrofolate reduced, mμmole/hr/mg protein.

‡ Dihydrofolate reduced, μmole/hr/g liver.

DISCUSSION

The current study has demonstrated that dihydrofolate, tetrahydrofolate, 10-formyltetrahydrofolate, and prefolic A can nullify the antileukemic effect of methotrexate as does citrovorum factor. In addition, prefolic A can prevent the manifestation of toxicity in leukemic mice when it is administered up to 24 hr after single large doses of MTX. In these circumstances there was little, if any, effect on the antileukemic action of MTX. There was, however, no apparent difference in the behavior of prefolic A in this situation from equivalent amounts of citrovorum factor.

Folic acid, on the other hand, was ineffective in decreasing the antileukemic effect of methotrexate when given at the same time as the drug, and only partially effective when given 1 hr beforehand on a daily treatment schedule.[3] The effectiveness of pretreatment with folic acid in the reduction of the toxicity of a single dose of MTX has

* Dihydrofolic acid had previously been stored in the refrigerator as a suspension in 0·001 M hydrochloric acid. More recently, according to a suggestion by Professor Morris Friedkin, the freshly prepared material was suspended in 0·001 M hydrochloric acid–0·01 M mercaptoethanol and divided immediately into aliquots, which were kept frozen until used in the assay.

been attributed to its conversion to a protective quantity of citrovorum factor activity in the tissues before the drug is administered.[3] The recent work of Condit has shown that this is, in fact, the case.[26, 27] It was demonstrated that administration of folic acid does increase the level of citrovorum factor activity in normal mouse liver for a few hours. Furthermore, this response to exogenous folic acid could be abolished for a period of 3 to 4 days by the administration of as little as 0·05 mg MTX/kg. Thus, the extreme sensitivity of the folic reductase system to MTX, *in vivo*, parallels that observed *in vitro*.[16, 19]

Zakrzewski and Nichol have shown that, with an enzyme preparation from chicken liver the amount of methotrexate required to inhibit completely the reduction of folate, also inhibited completely the reduction of dihydrofolate.[19] The decrease of MTX toxicity and antileukemic effect by dihydrofolate suggests that dihydrofolic reductase activity may not be inhibited completely *in vivo* by MTX. The differential reduction of folic and dihydrofolic acid in the presence of MTX *in vivo* has been confirmed by Condit and Mead.[28] When 0·1 mg MTX/kg was administered to normal mice, the increase in liver citrovorum factor activity in response to exogenous folic acid was completely inhibited, whereas the response to dihydrofolic acid was unaffected.

The enzyme studies reported in this paper show that the pharmacological effects seen when combinations of methotrexate and folic acid derivatives are given to mice cannot be attributed to the reversal of the inhibitory effect of MTX on folic or dihydrofolic reductase. The same degree of inhibition was seen with both toxic and nontoxic combinations. A possible explanation for the failure of folic acid to afford protection against the effects of MTX lies in its much lower rate of reduction. Zakrzewski and Nichol have shown that the chicken liver enzyme preparation can reduce dihydrofolate 27 times as fast as folate at pH 5·2.[19] It is likely, therefore, that the residual activities observed in our experiments could explain the observed differences in biological behavior between folic and dihydrofolic acid. The degree of inhibition of dihydrofolic reductase from the livers of methotrexate-treated mice is nevertheless quite extensive. The question remains as to whether the residual activity is, in fact, sufficient to convert enough dihydrofolate to tetrahydrofolate to account for the biological activity of dihydrofolic acid. It would also be of interest to determine whether dihydrofolic acid can be converted to tetrahydrofolate, *in vivo*, by a methotrexate-insensitive pathway.

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