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INHIBITION BY METHOTREXATE OF THE STABLE INCORPORATION OF 5-FLUOROURACIL INTO MURINE BONE MARROW DNA*

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Abstract—As a consequence of the inhibition of *de novo* purine synthesis by methotrexate (MTX) there is an increase in 5-phosphoribosyl-1-pyrophosphate (PRPP) concentration. In cells where 5-fluorouracil (FUra) is activated via orotate phosphoribosyltransferase (OPRtase), increased PRPP results in greater conversion of FUra to nucleotides. In the murine CD8F1 breast tumor system, MTX markedly enhances the antitumor activity of FUra, increasing both the activation of FUra to FUMP and the incorporation of FUTP into RNA. However, in contrast to reported tumor tissue culture studies, MTX pretreatment *in vivo* prevents the stable incorporation of FUra into CD8F1 bone marrow DNA. Pretreatment with MTX (300 mg/kg) 2.5 hr prior to [^{3}H]FUra (100 mg/kg), with a 2-hr labeling, reduced the level of FUra in DNA from 921 pmol to 66 pmol/mg of DNA. Without MTX pretreatment, 59% of the total incorporation of FUra into nucleic acids was into DNA when FUra was administered. After MTX the percentage of incorporation into DNA was reduced to 9%. Additionally, the ratio of [^{3}H]FUra to ^{32}P in DNA when both were given simultaneously was reduced by greater than 90%, suggesting that MTX must be specifically blocking the incorporation of FUra rather than nonspecifically preventing its incorporation by inhibiting DNA synthesis. In contrast, MTX failed to reduce the formation of DNA containing fluorouracil residues from FdUrd. To test whether MTX prevents the initial incorporation of FUra into DNA, or acts to enhance removal by the DNA glycosylase repair system, DNA was prelabeled *in vivo* with [^{3}H]FUra, and MTX or MTX plus dThd was then administered. The level of FUra in bone marrow DNA was not reduced by subsequent treatment with MTX, or MTX plus dThd, indicating that MTX does not enhance the removal of FUra from DNA. The level of total free fluorodeoxynucleotides formed from FUra was reduced by two-thirds following MTX pretreatment, suggesting that the action of MTX in preventing the stable incorporation of FUra into DNA was to reduce the availability of FdUTP.

It is now well established that FUra‖ can become incorporated into cellular DNA, both in tissue culture and *in vivo* [1–6]. While some effects of FUra incorporation into RNA on RNA processing and function have been documented [7–10], little, if anything, is known of the effects of FUra incorporation into DNA, although Schuetz *et al.* [11] have suggested that in mouse bone marrow cells toxicity is related to levels of FUra in DNA. In the only *in vivo* system reported so far, the CD8F1 murine mammary carcinoma, substantial stable incorporation of FUra into DNA seems to be limited to normal tissues; only trace amounts are detected in tumor DNA [5].

In murine bone marrow, incorporation appears to take place via competition between dTTP and FdUTP since (FUra)DNA formation is inhibited in a dose-related manner by dThd administration [5]. Similar conclusions have been reported by Major *et al.* [12] *in vitro* where high dThd concentrations (10 μM) inhibit (FUra)DNA formation. In contrast, Tanaka *et al.* [13] showed that, in HL-60 cells, a 3-hr preincubation with 0.1 or 1.0 mM dThd stimulates [^{3}H]FdUrd incorporation, whereas co-incubation with dThd and [^{3}H]FdUrd does not alter (FUra)DNA levels.

The latter two groups also have examined the effect of MTX on (FUra)DNA formation. In HL-60 cells following a preincubation with MTX, if the MTX is first removed, [^{3}H]FdUrd incorporation into DNA is stimulated. If, however, the drugs are present together, even 0.1 μM MTX substantially reduces incorporation [13]. In MCF-7 cells, MTX has little effect on the formation of (FUra)DNA from either FUra or FdUrd. However, MTX plus dThd does seem to stimulate excision of FUra from DNA [2].

In this paper, we present evidence that *in vivo* MTX pretreatment specifically inhibited the stable incorporation of FUra into murine bone marrow DNA, probably by reducing the availability of

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‖ Abbreviations: FUra, 5-fluorouracil; OPRtase, orotate phosphoribosyltransferase; MTX, methotrexate; FdUrd, 5-fluorodeoxyuridine; dR, deoxyribose; PRPP, 5-phosphoribosyl-1-pyrophosphate; FdUTP, 5-fluorodeoxyuridine triphosphate; FdUMP, 5-fluorodeoxyuridine monophosphate; TS, thymidylate synthetase; (FUra)DNA, DNA containing fluorouracil residues; TCA, trichloroacetic acid; PCA, perchloric acid; FUMP, fluorouridine monophosphate; and FUTP, fluorouridine triphosphate.

FdUTP. In contrast, MTX had no effect on the incorporation of [^{3}H]FdUrd into DNA and, when administered after FUra, it did not enhance the removal of FUra from the DNA.

MATERIALS AND METHODS

Source of biochemicals and radiolabeled compounds. FUra was obtained from Hoffmann-LaRoche (Nutley, NJ); FdUrd, methotrexate, tetrahydrofolate, FdUMP and dThd were from the Sigma Chemical Co. (St Louis, MO); Cs_2SO_4 was from Sigma and the J. T. Baker Co. through Thomas Scientific (Philadelphia, PA); [6-^{3}H]FUra and [^{3}H]FdUMP were from Moravek Biochemicals (Brea, CA); and ^{32}P was from New England Nuclear (Boston, MA). All drugs were made up in 0.85% NaCl solution (saline) and were injected i.p. Thymidylate synthetase was obtained from the Biopure Corp. (Boston, MA).

Animals and preparation of bone marrow nucleic acids for cesium sulfate density gradients. The experiments detailed in this paper utilized BALB/c × DBA/8 F1 mice (hereafter called CD8F1), both normal and mice bearing first-generation transplants of the CD8F1 spontaneous murine mammary carcinoma [14, 15]. They were allowed food and water *ad lib*. Radioactive precursors were injected i.p. in saline. After the labeling period, the animals were killed by cervical dislocation. Bone marrow was collected by flushing the marrow cavity of the femur with ice-cold saline. The bone marrow pellet was suspended in 0.01 M Tris–HCl, pH 7.6; 0.15 M NaCl; 0.02 M EDTA buffer, treated with sodium dodecyl sulfate, and sonicated; nucleic acids were purified by two extractions with phenol, one extraction with chloroform: isoamyl alcohol, and ethanol precipitation. The precipitated nucleic acids were dissolved in 0.5 ml of 50% formamide, heated at 85° for 20 min, quick-chilled, and then mixed with 4.5 ml of water and 5.5 ml of a saturated Cs_2SO_4 solution containing 5 mM EDTA. The gradients were centrifuged in a Beckman Ti 50 rotor for 60–66 hr at 44,000 rpm and 20°. Gradients were fractioned from the bottom, and the DNA peak was located by refractive index and A_{254}. The amount of DNA in the peak was estimated from the A_{254}. Acid-precipitable radioactivity in each fraction was collected on glass fiber filters. The filters were dried and radioactivity was determined by liquid scintillation counting.

Preparation of bone marrow and plasma for HPLC analysis. Animals were anesthetized with either sodium pentobarbital or ether. Bone marrow was collected by flushing the marrow cavity of the femur into a homogenizer. The marrow was pelleted by a short centrifugation. Marrows from four animals were pooled into a single sample and homogenized in 1.2 N perchloric acid. After centrifugation to remove acid-insoluble material, the supernatant fraction was heated (100°, 15 min) to convert pyrimidine nucleotides to the monophosphate form. The extracts were then neutralized by extraction with a 1:2 mixture of tri-*n*-octyl amine in freon. Recoveries were normalized to the amount of protein in the acid-insoluble pellet as determined by the procedure of Lowry *et al.* [16].

Plasma was deproteinized with trichloracetic acid and, again, the extract was neutralized by extraction with tri-*n*-octyl amine in freon.

High pressure liquid chromatography (HPLC). HPLC analyses utilized a Dupont model 850 HPLC system equipped with a Waters-Millipore WISP automatic sampler and Z-module and Spectra-Physics model 4100 calculating integrator. Plasma dThd was measured using a Waters C_8 column and a buffer of 50 mM KH_2PO_4, pH 3.0. Bone marrow extracts were analyzed for TMP and dUMP using a Waters C_8 column and a buffer of 10 mM KH_2PO_4 in 5 mM tetrabutylammonium hydrogen sulfate, pH 2.7.

Assay for FdUMP. Measurement of FdUMP utilized the competitive binding assay developed by Moran *et al.* [17]. Animals were killed 30 min after receiving FUra or FdUrd. Perchloric acid extracts were prepared from the pooled bone marrow from four animals. After centrifugation, the acid supernatant fraction was heated (15 min, 100°) to convert any FdUDP and FdUTP to FdUMP. A 10x solution of 5,10-methylenetetrahydrofolate (5,10-CH_2-H_4PteGlu) was prepared by dissolving a 25-mg ampule of tetrahydrofolate in 9.5 ml of buffer A (2 ml 1 M KH_2PO_4, pH 7.2, 17 ml H_2O, 2 ml bovine serum albumin, 10 mg/ml, and 40 μl mercaptoethanol) containing 0.5 ml of 1 M sodium ascorbate, pH 6.5, and 25 μl of 37% formaldehyde. This was diluted 1:10 with Buffer A just prior to use. Cofactor, [^{3}H]FdUMP, 20 Ci/mmol, 0.9 pmol per reaction, and known amounts of FdUMP for the standard curve or aliquots of the acid extracts from bone marrow were mixed prior to the addition of purified thymidylate synthetase. The reaction was started by the addition of enzyme. After a 2-hr incubation at 30°, unreacted [^{3}H]FdUMP was removed by the addition of dextran-treated charcoal followed by centrifugation. The amount of [^{3}H]FdUMP–cofactor–enzyme ternary complex remaining in the supernatant fraction was determined by liquid scintillation counting.

Statistical analysis. Statistical significance was determined using Student's *t*-test. A P value of 0.05 or less indicated a significant difference between groups.

RESULTS

In the course of studies on the use of MTX to raise PRPP levels (thereby increasing the conversion of FUra to FUMP), we observed that pretreatment with MTX substantially eliminated the stable incorporation of FUra to bone marrow DNA. (Very little FUra is found in CD8F1 tumor DNA with or without MTX [5].) In the experiment detailed in Table 1, the bone marrow of animals receiving only [^{3}H]FUra (100 mg/kg) incorporated 921 pmol/mg of DNA of [^{3}H]FUra into acid-precipable material with a buoyant density in cesium sulfate of DNA. This represented 59% of the total incorporation into nucleic acids. Pretreatment with MTX (300 mg/kg) 2.5 hr prior to [^{3}H]FUra resulted in a greater than 90% reduction in incorporation of FUra into DNA (group 2). Following MTX pretreatment, only 9% of the total FUra incorporation was into DNA.

That the effect of MTX was to specifically reduce

Table 1. Comparison of the effect of methotrexate administered before or after 5-fluorouracil on the level of 5-fluorouracil in murine bone marrow DNA

Treatment	pmol FUra in DNA/ mg DNA	[^{32}P]DNA (cpm/mg DNA × 10^{-4})	$\frac{[^3H]FUra\ DNA}{[^{32}P]DNA} \times 10^{2*}$
1. [^{3}H]FUra	921	4.2	4.4
2. MTX→ [^{3}H]FUra	66	3.2	0.4
3. [^{3}H]FUra	954	5.7	3.4
4. [^{3}H]FUra→ MTX	1048	6.8	3.1

Normal male CD8F1 mice, four per group, each received 100 mg/kg of [^{3}H]FUra plus ^{32}P. Group 2 received MTX, (300 mg/kg) 2.5 hr prior to [^{3}H]FUra/^{32}P. For groups 1 and 2, the labeling period was 2 hr. Group 4 received MTX, again at 300 mg/kg, 2 hr after [^{3}H]FUra/^{32}P. For groups 3 and 4, the labeling period was 6.5 hr. Bone marrow from each group was pooled into a single sample per group, and the incorporation of label into RNA and DNA was determined by cesium sulfate gradients.

* Ratio of [^{3}H] to [^{32}P] in DNA.

the stable incorporation of FUra into bone marrow DNA rather than to simply stop FUra incorporation by blocking DNA synthesis is demonstrated by the third column in Table 1. The ratio of ^{3}H to ^{32}P in DNA estimates the ratio of [^{3}H]FUra incorporation relative to overall DNA synthesis. If MTX merely prevented FUra incorporation by blocking DNA synthesis, then the ratio of ^{3}H/^{32}P should remain approximately constant. That MTX pretreatment reduced the ^{3}H/^{32}P ratio from 0.044 for [^{3}H]FUra alone (group 1) to 0.004 (group 2) shows that MTX specifically blocks stable FUra incorporation much more than it blocks DNA synthesis. Both the level of FUra in DNA and the ^{3}H/^{32}P ratio were reduced by greater than 90%, while [^{32}P]DNA synthesis was reduced by only 24% during the first 2 hr.

The observed decrease in stable incorporation of FUra into bone marrow DNA associated with MTX

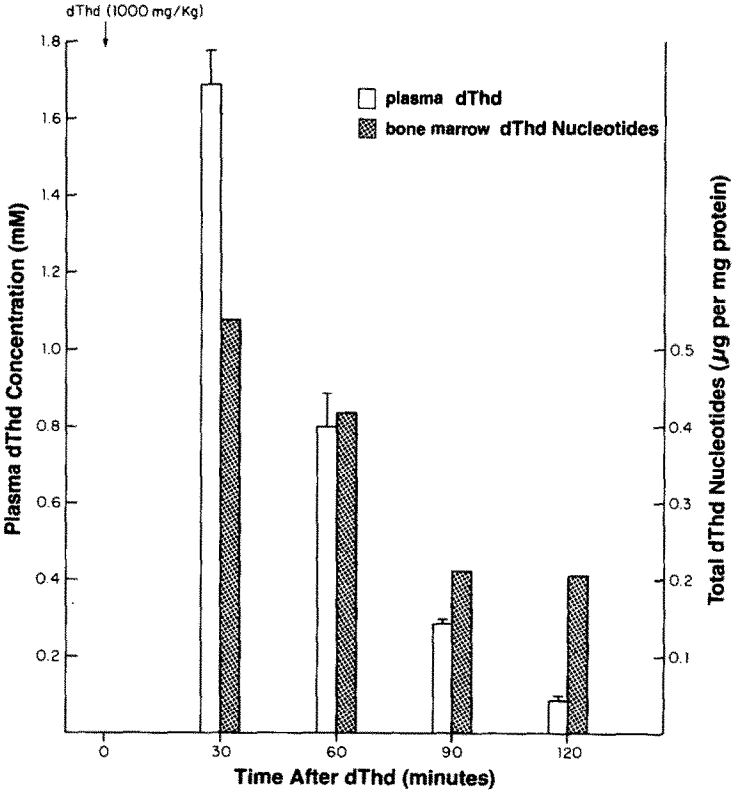

Fig. 1. Effect of high-dose dThd on plasma dThd and bone marrow dThd phosphates. Normal male CD8F1 mice received 1000 mg/kg of DThd, i.p., at zero time. At 30-min intervals, four animals were killed, and blood and bone marrow were collected. Plasma was deproteinized with TCA and dThd levels were quantitated by reverse phase HPLC. PCA extracts were prepared from bone marrow pooled from four animals, heated, and neutralized and TMP was quantitated by ion-pairing HPLC. TMP was normalized to the amount of protein in the acid-insoluble pellet.

Table 2. Failure of methotrexate and thymidine to enhance the specific removal of [^{3}H]5-fluorouracil from CD8F1 bone marrow DNA

	Treatment*	pmol FUra in RNA/ mg DNA	pmol FUra in DNA/ mg DNA	$\frac{[^3H]DNA}{[^{32}P]DNA} \times 10^3$†
1.	[^{3}H]$FUra_{100}$	2769 ± 543	821 ± 250 (22.9%)	9.5 ± 1.4
2.	[^{3}H]$FUra_{100} \rightarrow MTX_{300}$	2602 ± 585	656 ± 80 (20.1%)	9.5 ± 1.9
3.	[^{3}H]$FUra_{100} \rightarrow dThd_{1000} \rightarrow dThd_{1000}$	2629 ± 457	982 ± 316 (27.2%)	10.7 ± 2.2
4.	[^{3}H]$FUra_{100} \rightarrow MTX_{300} + dThd_{1000} \rightarrow dThd_{1000}$	2246 ± 451	564 ± 84 (20.1%)	10.5 ± 2.2

* Normal male CD8F1 mice received 100 mg/kg of 5-fluorouracil containing [6-^{3}H]FUra plus ^{32}P. Two hours later, they received MTX, two doses of dThd, or MTX plus two doses of dThd. Six hours after FUra administration, the animals were killed; bone marrow nucleic acids were purified by phenol extraction and ethanol precipitation and analyzed by cesium sulfate density gradient centrifugation. Bone marrow from three mice was pooled into a single sample. Data are the means ± SD of four separate experiments, three mice per group per experiment. The percentages in parentheses are the percent of the total incorporate present in DNA.

† Ratio of [^{3}H] to [^{32}P] in DNA.

pretreatment could be the result of decreased incorporation of FUra into DNA, or of an enhancement of excision-repair as has been suggested by Herrick *et al.* [2]. To distinguish between these possibilities, we investigated whether MTX could diminish the level of FUra in bone marrow DNA when it was administered 2 hr *after* FUra. Two hours duplicates the labeling period for groups 1 and 2 (Table 1), and allows for the initial incorporation of FUra into DNA. MTX was then administered to the animals in group 4 and labeling continued for an additional 4.5 hr to mimic the 4.5-hr exposure to MTX of group 2. In this second part of the experiment, both FUra alone (group 3) and FUra–MTX (group 4) had a total labeling period of 6.5 hr. By comparing groups 1 and 3, it can be seen that the majority of the initial incorporation of FUra into DNA occurred in the first 2 hr after FUra administration. Once incorporation had taken place, administration of MTX, group 4, did not decrease the level of FUra in DNA, indicating that MTX was unable to induce removal of the FUra already present in bone marrow DNA.

Previous *in vitro* data from Herrick *et al.* [2] suggested that both MTX and dThd were required to effect removal of FUra from DNA in the MCF-7 cells that they examined. Therefore, we evaluated the MTX after FUra sequence, adding two 1000 mg/kg doses of dThd. The first dose was administered 2 hr after FUra, and again 2 hr later. This schedule was chosen to maintain substantially elevated plasma dThd and bone marrow dThd nucleotides during the 4 hr following MTX administration (Fig. 1). The effects of MTX, dThd, and the combination of MTX plus dThd on the levels of FUra remaining in DNA are presented in Table 2. In these experiments, treatment with MTX plus dThd appeared to cause some loss of both ^{3}H and ^{32}P from RNA and DNA, but the differences between groups were not statistically significant ($P > 0.1$ for all groups). The data argue against there being an MTX–dThd enhancement of the uracil–DNA–glycosylase repair activity since the ratio of ^{3}H in DNA to ^{32}P in DNA remained constant.

Tissue culture studies have shown that treatment with MTX can cause a substantial increase of intracellular dUTP pools [18–20]. As was the case with expanded TTP pools [5], greatly expanded dUTP pools would be expected to competitively reduce utilization of FdUTP by DNA polymerase. HPLC chromatograms of heated PCA extracts from control bone marrow contained a very small peak which co-chromatographed with a dUMP marker (Fig. 2). This peak was not increased in chromatograms of extracts from MTX-treated bone marrows, sug-

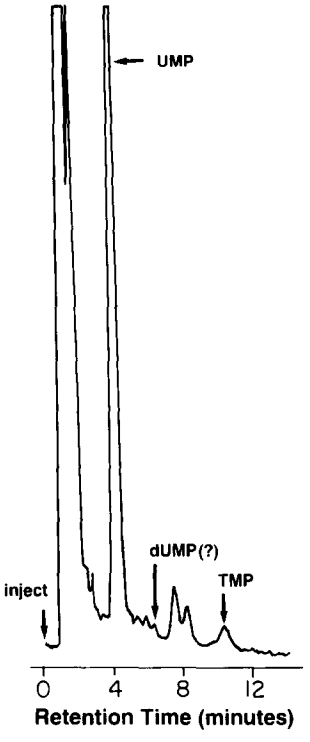

Fig. 2. Ion-pairing HPLC analysis of perchloric acid extracts from CD8F1 bone marrow. The heated PCA extracts from bone marrow of normal male CD8F1 mice were analyzed using as buffer 0.01 M KH_2PO_4 in 5 mM tetrabutylammonium hydrogen sulfate, pH 2.7, and a Waters radial compression C_8 column. The peak marked dUMP co-chromatographed with a dUMP marker. This chromatogram is of control bone marrow.

gesting that there was no HPLC-detectable accumulation of dUTP (measured here as dUMP), nor did MTX have much effect on bone marrow thymidine nucleotides (Table 3).

Since neither enhanced uracil–DNA–glycosylase activity nor accumulations of dUTP appeared to be responsible for reducing (FUra)DNA formation, another possibility was that MTX pretreatment might inhibit the formation of fluorodeoxynucleotides from FUra. In these experiments, mice received FUra (100 mg/kg) or MTX (300 mg/kg) 2.5 hr prior to the FUra. Thirty minutes after receiving FUra, mice were killed and perchloric acid extracts were prepared from the bone marrow. Marrow from four mice were pooled into a single sample. The acid extracts were heated to convert all free fluorodeoxynucleotides to FdUMP which was subsequently quantitated by the thymidylate synthetase assay of Moran *et al.* [17]. The data presented in Table 4 are the mean for six samples each of FUra and MTX–FUra bone marrow, a total of twenty-four mice per group. Pretreatment with MTX resulted in a statistically significant two-thirds reduction in total free fluorodeoxynucleotides, $P < 0.02$. As a control, extracts from MTX-treated (no FUra) marrows were found not to interfere with binding of [^{3}H]FdUMP to TMP synthetase in the assay. This fact further supports the conclusion that MTX is not causing an accumulation of dUMP in bone marrow *in vivo*.

Further evidence to suggest that the action of MTX was to limit the availability of FdUTP was obtained by examining the effect of MTX pretreatment on (FUra)DNA formation following FdUrd administration. The results are presented in Table 5, and show that, when FUra was administered as FdUrd, bypassing the ribonucleotide reductase pathway to FdUTP, the total incorporation of [^{3}H]FUra into DNA was not reduced by MTX. The dose of FdUrd (200 mg/kg), was chosen to keep the molar amount roughly equivalent to FUra (100 mg/kg). In these experiments, deoxyribose (dR) was administered just prior to FdUrd to retard phosphorolysis of the deoxyribonucleoside to FUra and deoxyribose. Nonetheless, approximately one-third of the total incorporation was into RNA showing that, *in vivo*, there is substantial cleavage of FdUrd to FUra.

Table 3. Effects of methotrexate and 5-fluorouracil on CD8F1 bone marrow thymidine nucleotides

Treatment		TMP (ng/mg protein)
1.	Control	52 ± 7
2.	MTX, 300 mg/kg	45 ± 6
3.	FUra, 100 mg/kg	43 ± 4
4.	MTX → FUra	43 ± 2

Tumor-bearing male CD8F1 mice received either MTX (300 mg/kg), FUra (100 mg/kg), or MTX 2.5 hr prior to FUra. The animals were killed 2.5 hr after MTX alone or 2 hr after FUra or MTX–FUra. Perchloric acid extracts were prepared from the bone marrow, heated to convert dThd nucleotides to TMP, and the TMP was quantitated by ion-pairing HPLC. Recoveries were normalized to the amount of protein in the acid-insoluble pellet. Data are the average of three experiments, four animals per group per experiment. The bone marrow from the four animals in each group was pooled into a single sample.

Table 4. Effect of methotrexate pretreatment on formation of fluorodeoxynucleotides from fluorouracil *in vivo*

Treatment		FdUMP (pmol/mg protein)
1.	FUra	9.4 ± 1.5
2.	MTX → FUra	3.4 ± 0.6*

Tumor-bearing female CD8F1 mice received either FUra (100 mg/kg) or MTX (300 mg/kg) 2.5 hr prior to FUra. Thirty minutes after FUra, the animals were killed and perchloric acid extracts were prepared from bone marrow. Marrow from four mice was pooled into a single sample. The extracts were heated to convert all fluorodeoxynucleotides to FdUMP which was quantitated by the [^{3}H]FdUMP–TMP synthetase competitive binding assay of Moran *et al.* [17]. Data are the means ± SE for six samples per group, a total of twenty-four mice per treatment group.
* Significantly different from FUra, $P < 0.02$.

Examination of FdUMP levels in FdUrd-treatment bone marrow (last column, Table 5) showed that there was some reduction in free FdUMP in the MTX-treated bone marrows, but that the level of free FdUMP in marrow following MTX–dR–FdUrd was still more than twice that achieved following FUra alone (Table 4).

DISCUSSION

A priori, it is difficult to predict the effect of MTX on the incorporation of FUra into DNA. MTX might be expected to enhance FUra incorporation into DNA by lowering dTTP levels. In contrast, greatly expanded dUTP levels resulting from MTX inhibition of thymidylate synthetase might effectively out-compete FdUTP utilization by DNA polymerase. Neither effect seemed to play much of a role in murine bone marrow. At 2.5 hr after administration of MTX, the time at which FUra was administered in these experiments, the concentration of dThd nucleotides in bone marrow was only slightly reduced (Table 3). Such a result agrees with our previously published observations that CD8F1 bone marrow contains appreciable levels of dThd kinase [21, 22]. Although FUra rapidly inhibits CD8F1 bone marrow TMP synthetase *in vivo* (>90% inhibition of incorporation of label from [^{3}H]dUrd into DNA), DNA synthesis as measured by ^{32}P incorporation continues at near control rates for several hours, gradually declining as the dThd kinase level falls, suggesting that circulating levels of dThd can maintain dTTP pools when there is an active dThd kinase [21, 22]. Note that FUra had little effect on bone marrow dThd nucleotides at the 2-hr time point (Table 3).

MTX might reduce the level of FUra in bone marrow DNA by three mechanisms. First, increased dUTP could compete with FdUTP for utilization by DNA polymerase. Goulian *et al.* [18] have demonstrated that *in vitro* the MTX block on thymidylate synthetase can result in a 1000-fold expansion of the dUTP pool. However, increased dUTP levels out-

Table 5. Effect of methotrexate on 5-fluorodeoxyuridine incorporation into CD8F1 bone marrow RNA and DNA and formation of FdUMP

	Treatment	pmol FUra incorporated/mg DNA		FdUMP (pmol/mg protein)
		RNA	DNA	
1.	dR → FdUrd	659	1240 (65%)	37.4
2.	MTX → dR → FdUrd	870	1358 (61%)	21.3

Tumor-bearing female CD8F1 mice (three per group) received deoxyribose (dR, 1000 mg/kg) 15 min prior to FdUrd (200 mg/kg). Group 2 received MTX (300 mg/kg) 2.5 hr prior to dR, 2.5 hr prior to FdUrd. The FdUrd used for the incorporation studies also contained [^{3}H]FdUrd (final specific activity, 6 mCi/mmol). Labeling was for 2 hr, and incorporation of ^{3}H into RNA and DNA was measured by cesium sulfate gradients. Marrow from three mice was pooled into a single sample. Data are the average of two experiments, three mice per group, per experiment. The percentages in parentheses represent the percent of total incorporation present in DNA. FdUMP was measured in acid extracts of FdUrd- and MTX–FdUrd-treated bone marrow by the competitive binding thymidylate synthetase assay.

competing FdUTP utilization does not seem to play a role in these *in vivo* studies since there was no detectable increase in dUMP pools (Fig. 2), and MTX did not reduce (FUra)DNA formation from FdUrd (Table 5). Further, extracts of MTX-treated (no FUra) bone marrow did not interfere with the binding of [^{3}H]FdUMP in the TMP synthetase assay. Second, excision-repair of FdUMP residues in DNA might be enhanced. This, too, seems unlikely in view of the fact that MTX administered 2 hr after FUra had no effect on (FUra)DNA levels (Table 1). A third possible mechanism that is suggested by the data is that MTX in some way reduces the availability of FdUTP for utilization by DNA polymerase either by interfering with the formation of FdUTP from FUMP or by increasing the amount of FdUMP bound to thymidylate synthetase (TS), thereby reducing free FdUMP. Elford *et al.* reported that MTX produces a 4-fold elevation in TS levels of HeLa cells [23], and Herrmann *et al.* showed that MTX increases the amount of [^{3}H]FUra bound in TS complex by 2-fold in Sarcoma 180 [24]. Such effects would result in lowered availability of free FdUMP for FdUTP formation. Indeed, the level of free FdUMP fell by one-third following MTX–FdUrd (Table 5), although it still remained more than twice that obtained from FUra in the absence of MTX (Table 4, 21.3 vs 9.4 pmol FdUMP/mg protein). Studies designed to determine whether MTX interferes with FdUMP formation through the ribonucleotide reductase pathway or acts to reduce FdUMP by causing more nucleotide to be bound in TS complexes are underway.

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