Decreased *de novo* DNA-Thymine Synthesis and Increased Thymidine-^{3}H Incorporation into DNA by Human Bone Marrow Cultured with Aminopterin.* (32522)

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De novo synthesis of DNA-thymine involves the transfer of an active monocarbon unit into deoxyuridylate to form thymidylate. The transfer of this carbon atom is accomplished by tetrahydrofolic acid (THF), the reduced and metabolically active form of folic acid. Folic acid is reduced *in vivo* by the enzyme dihydrofolic acid reductase(1). The *de novo* synthesis of DNA-thymine has been demonstrated to be inhibited by extremely low concentrations of 4-amino analogues of folic acid such as aminopterin, amethopterin, pyrimethamine and 2,4,7-triamino-6 phenyl-pteridine presumably by inactivation of dihydrofolic acid reductase (2-3). The presence of aminopterin has also been shown to enhance the incorporation of preformed thymidine into DNA in canine bone marrow(4). The present study demonstrates the simultaneous effect of aminopterin on *de novo* DNA-thymine synthesis, and the incorporation of preformed thymidine into DNA in short term normal human bone marrow cultures.

Material and methods. Heparinized bone marrow was obtained by aspiration from the iliac crest of seven normal subjects. Each individual sample was well mixed in order to obtain a homogenous suspension. Two ml aliquots were taken from each sample (cell number $\pm$ s = 2.8%) and suspended in 5 incubation tubes containing media and various additives. Incubation was done in Gey's balanced salt solution‡ at pH 7.6 under nitrogen. Five μCi of L-serine-3-^{14}C§ (20.4 mCi/mmole) and 20 μCi of thymidine-CH_3-^{3}H¶ (2.02 Ci/mmole) were added to each incubation tube. The final concentration of added thymidine (2.5×10^{-6} M) was shown in preliminary experiments with canine bone marrow to be insufficient to influence the rate of *de novo* DNA-thymine synthesis from L-serine-3-^{14}C. Aminopterin‖ was added to the first tube at 10^{-4} M concentration. The second tube served as control and received no additives. B_{12} coenzyme (5′ deoxyadenosyl cobalamine)**(5) was added to the third tube at a final concentration of 1.7 mμg/ml (1.04×10^{-9} M. A combination of deoxyuridine, deoxycytidine, deoxyguanosine and deoxyadenosine‡‡ was added to the fourth tube at a final concentration of 2.5×10^{-6} M each. Uridine‡‡ was added to the fifth tube at a final concentration of 2.5×10^{-6} M. Gey's balanced salt solution was added to each tube to obtain 4 ml final volume. After 3 hours of incubation in a Dubnoff metabolic shaker at 37°C, all tubes were removed and placed in ice. Cells were packed by centrifugation and washed 3 times with ice-cold Gey's balanced salt solution. DNA was isolated using the technique of Kay *et al*(6) adapted for small samples. The isolated DNA was then hydrolyzed in formic acid at 175°C for 35 minutes in a sealed tube free of air(7). One hundred microliter aliquots were taken and spotted on Whatman paper #1 and thymine was separated by paper chromatography in an isopropanol:hydrochloric acid:water solvent system(8). The thymine spot was

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located under UV light and was eluted from the paper with 0.01 N HCL. Aliquots of this eluate were added to a scintillation fluid containing ethanol, toluene, naphthol, dioxane, PPO and POPOP and counted in a 3-channel liquid scintillation counter§§ for ^{14}C and ^{3}H. Total thymine of the eluate was estimated in a Beckman DU spectrophotometer by UV absorption at 264 mμ. The fraction of *de novo* DNA-thymine synthesized from the added L-serine-^{14}C was calculated by dividing the specific activity of DNA-thymine-^{14}C of the incubated cells by the specific activity of the L-serine-^{14}C added to the culture. The fraction of thymidine incorporated into DNA was calculated by dividing the specific activity of the DNA-thymine-^{3}H of the incubated cells by the specific activity of the thymidine-^{3}H added to the culture. Results were expressed as 10^{-4} moles of DNA-thymine synthesized from the added L-serine-3-^{14}C, and 10^{-4} moles of DNA-thymine incorporated from the added thymidine-^{3}H per mole of DNA-thymine.

Results. Table I gives the fraction of DNA-thymine synthesized from L-serine-3-^{14}C added to the bone marrow cultures from 7 normal subjects. It is seen that aminopterin exerts a profound inhibitory effect on such *de novo* synthesis of DNA-thymine. On the other hand, in the presence of aminopterin there is a simultaneous significant ($P<0.005$, Table II) and consistent ($r = 0.945$) increase in the incorporation into DNA of the thymidine-^{3}H added to the culture. At the concentrations used, no effect was observed of added B_{12} coenzyme, deoxyribosides or uridine on such DNA-thymine synthesis, or

TABLE I. Effect of Aminopterin on the Synthesis of DNA-Thymine-^{14}C from L-Serine-3-^{14}C.

Subjects	Aminopterin (10^{-4} M)	Control	B_{12} coenz (1.04×10^{-9} M)	d-ribosides (2.5×10^{-6} M)	Uridine (2.5×10^{-6} M)
JH	1.0	6.1	5.6	5.2	—
JS	.0	9.2	7.0	7.4	—
BT	1.5	5.7	7.2	6.9	—
RH	.8	5.7	5.3	4.8	4.7
DL	1.0	4.4	2.8	3.9	2.5
MK	3.1	7.1	7.5	8.3	—
HA	.0	4.4	4.6	4.6	5.1
Mean ± S.E.	1.1 ± .4	6.1 ± .6	5.7 ± .6	5.8 ± .6	4.1 ± .8

Table I summarizes results of the synthesis of DNA-thymine-^{14}C from L-serine-3-^{14}C added to the cultures of normal human bone marrow. Each column represents values obtained from short-term tissue culture containing the additive heading each column. The values presented are expressed as the fraction $\times 10^{-4}$ of DNA-thymine which was synthesized during the time of incubation utilizing the L-serine-3-^{14}C added to the culture.

TABLE II. Effect of Aminopterin on Incorporation of Thymidine-^{3}H into DNA.

Subjects	Aminopterin (10^{-4} M)	Control	B_{12} coenz (1.04×10^{-9} M)	d-ribosides (2.5×10^{-6} M)	Uridine (2.5×10^{-6} M)
JH	27.2	18.4	18.6	22.5	—
JS	35.5	26.8	15.7	23.7	—
BT	14.9	8.8	11.1	9.6	—
RH	28.4	19.9	16.4	18.3	14.4
DL	27.0	18.7	14.4	17.2	18.6
MK	55.4	36.3	38.7	39.7	—
HA	16.5	18.5	17.8	21.5	18.2
Mean ± S.E.	29.3 ± 5.1	21.0 ± 3.2	19.0 ± 3.4	21.8 ± 3.4	17.1 ± 1.3

Table II summarizes results of thymidine-^{3}H incorporation into DNA in cultured normal human bone marrow. Each column represents values obtained from short-term tissue culture containing the additive heading each column. The values presented are expressed as the fraction $\times 10^{-4}$ of DNA thymidine which was incorporated during the time of incubation, from thymidine added to the culture.

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the incorporation of thymidine-^{3}H into DNA. The total DNA-thymine isolated from each of the cultures derived from a given subject was found to be comparable. The fraction of thymidine added to the culture which was incorporated into DNA was calculated and found to be less than 0.05.

Discussion. Since 70% of "1 C" fragments is normally derived from the number 3 carbon atom of L-serine(9), one may estimate *de novo* synthesis of thymidylate from ^{14}C labeling of DNA thymine following incubation with L-serine-3-^{14}C. Thus, diminution of ^{14}C concentration in DNA-thymine in the experiments described utilizing aminopterin may be interpreted as additional evidence for suppression of *de novo* DNA-thymine synthesis in the presence of aminopterin.

The increased incorporation of thymidine-^{3}H into DNA in human bone marrow that was induced by aminopterin is consistent with similar findings demonstrated in the canine bone marrow studies by Rubini(4). Comparable concentrations of aminopterin and thymidine were used in both of these studies.

The simultaneous findings of increased incorporation of thymidine-^{3}H into DNA while *de novo* synthesis of DNA-thymine is diminished (Tables I and II), due to aminopterin inhibition of THF formation, could be explained by any of the following possibilities: 1. *A diminution of either intracellular or extracellular thymidine pool size resulting in increased specific activity of thymidine-^{3}H involved in DNA synthesis.* This possibility appears improbable since the size of the intracellular thymidine pool in normal bone marrow cells at any instant is normally quite small(10) in relation to the total thymidine added to the culture so that its diminution subsequent to decreased formation of thymidine would not be expected to significantly alter the specific activity of the thymidine-^{3}H pool. While a diminution in the pool size of the extracellular thymidine could result from diminished catabolism of thymidine, such a process does not appear to be a function of aminopterin, since *in vitro* studies with canine bone marrow have shown that aminopterin in this concentration does not inhibit thymidine-^{3}H catabolism(4). Moreover, the total quantity of thymidine incorporated into DNA from the thymidine-^{3}H pool represented a small fraction (0.05) of this pool. 2. *An actual increase of DNA synthesis.* Such an increase cannot be definitely established nor ruled out on the basis of present data. It has been shown that moderate increases in deoxyriboside concentration result in increased DNA polymerase activity(11). Since the addition of a balanced combination of deoxyribosides in the present study did not increase the incorporation of thymidine into DNA, the enhancement of thymidine incorporation into DNA due to aminopterin is probably not the consequence of increased DNA polymerase activity. 3. *An increase in preformed thymidine conversion to thymidylate secondary to decreased* de novo *synthesis of thymidylate* via *deoxyuridylate.* Such a process could be induced by a de-repression of enzymes responsible for the incorporation of preformed thymidine into DNA. These enzymes, such as thymidine kinase, may be under direct control of the products of *de novo* synthesis, such as deoxythymidine-, mono-, di-, and tri-phosphate. The presence of such a process also cannot be evaluated from data presented in this paper. It has been noted that there is an increase of enzymes involved in pyrimidine synthesis in bone marrow from patients with vitamin B_{12} deficient megaloblastic anemia(12). Thus, if there were an associated increase in enzymes required for phosphorylation and incorporation of nucleic acids into DNA when THF activity is impaired, one might anticipate an increase in the utilization of preformed thymidine into DNA.

The lack of effect of addition of deoxyribosides, uridine or excess B_{12} coenzyme on either the *de novo* synthesis of DNA-thymine or the incorporation of thymidine into DNA indicates that such syntheses are independent of increases in pool sizes of these materials under these experimental conditions. Prior studies with marrow cells have shown a decreased incorporation of thymidine into DNA when pyrimidine deoxyribosides were added(4,13), but the concentrations of these substances used in most studies were much greater than those used in the present study.

Summary. When incubated with normal human bone marrow cells in the presence of

L-serine-3-^{14}C and thymidine-^{3}H, aminopterin decreases *de novo* synthesis of DNA-thymine-^{14}C to 16%, while incorporation of thymidine-^{3}H into DNA is increased to 140% of control values. These results are interpreted as evidence for increased utilization of preformed thymidine in DNA synthesis in the presence of aminopterin. The possible mechanisms for this increased incorporation of preformed thymidine into DNA in the presence of aminopterin are discussed.

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Developmental Aspects of Glutathione Levels in Dystrophic Mice.* (32523)

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Studies of nutritionally induced and hereditary muscular dystrophy indicate changes in many enzyme and coenzyme levels(1). Greatly elevated glutathione reductase activities have been reported in dystrophic (dy/dy) mice(2,3). High glutathione (GSH) levels have been reported in the skeletal muscle of vitamin E-deficient rabbits(4,5), and in chickens with hereditary muscular dystrophy(6,7). Contrary results have been obtained for GSH levels in vitamin E-deficient rabbits by other workers(8).

In studies of chick skeletal muscle investigators have reported GSH concentrations which varied with age and sex, not only between normal and dystrophic animals but also among the various sub-lines of the dystrophic forms. In vitamin E-deficient rabbits (8) dystrophic individuals exhibit depressed levels for "sulfhydryl groups," which gradually rise to the "normal" value upon continued exposure to the deficient diet. In a study of muscle dialysates from mice of the 129/J strain, sex unspecified(9) it has been shown that SH concentration is higher in normal controls than in dystrophics, while SS concentration is higher in the dystrophic animals. Of special interest is a study of catheptic and other lysosomal enzymes in dystrophic rabbits, chickens and mice, in which the elevation of such enzyme activity in affected animals is always lowest in mice(10,11).

Apparently neither normal nor dystrophic GSH values for mouse muscle have appeared in the literature. The objective, in this preliminary study, was to ascertain whether a developmental pattern for muscle GSH values exists in both normal mice and those with progressive muscular dystrophy from fetal through adult stages.

Methods. Leg and thigh muscles were removed from 17-112-day-old female mice of

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