Inhibition of Nucleic Acid Synthesis by Folic Acid Antagonists*

HOWARD E. SKIPPER, PH.D., JACK H. MITCHELL, JR., PH.D., AND LEONARD L. BENNETT, JR., PH.D.

(From the Organic and Biochemistry Division, Southern Research Institute, Birmingham, Alabama)

Woolley and Pringle (13) have recently reported the formation of 4-amino-5-carboxamidoimidazole during growth of *Escherichia coli* in the presence of 4-aminopteroylglutamic acid (aminopterin). Some of us have observed that excessive doses of folic acid speed up the leukemic process in mice and that folic acid will reverse the well known anti-leukemic action of aminopterin (11). This reversal of the anti-leukemic action of folic acid antagonists by means of large doses of folic acid had previously been reported by Burchenal (4) and Law (7).

In the face of the above information, the suggestion that formyl folic acid might be involved in introduction of a single carbon unit into purines (5) and the observation that formate is a precursor of the 2-carbon atom of uric acid in pigeons (12), certain postulates were made regarding the rate-controlling function of folic acid in leukemia (11). This unoriginal hypothesis, based largely on Gordon's observations (5), suggested that folic acid might be involved in the last step of biosynthesis of the purine skeleton, that step being the transfer of formate via formyl folic acid to the 2-position of the purine skeleton. Thus, if the requirement for purines (for formation of cellular nucleoprotein) was greater in the more dynamic leukemic cell-forming tissue than in normal cells, then an excess of folic acid might be expected to speed up the leukemic process, and a deficiency of this vitamin (or co-enzyme) caused by a folic acid antagonist might preferentially depress malignant mitosis.

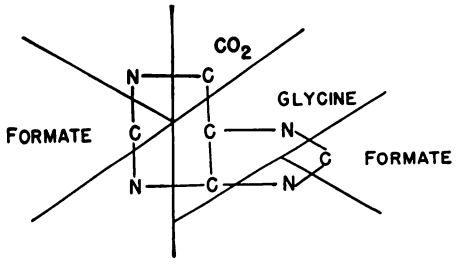


FIG. 1.—Possible precursors of the purine skeleton (2, 10)

The present series of experiments was designed to study the effect of well known folic acid antagonists, aminopterin and A-methopterin (4-amino-N^{10}-methylpteroylglutamic acid), on the incorporation of formate and CO_2 into nucleic acids and nucleic acid purines of normal mice.

EXPERIMENTAL

To ascertain the rate at which formate carbon and CO_2 are incorporated into the nucleic acids and nucleic acid purines (Fig. 1) of normal mice, control experiments have been carried out with carbon 14-labeled sodium formate and sodium bicarbonate. Groups of four mice each were injected with the labeled compounds and immediately placed in a metabolism chamber. After a period of 6 hours, the animals were sacrificed, and the viscera (liver, spleen, testes, kidneys, washed intestines, and pancreas) pooled and mixed with an equal weight of viscera homogenate from non-injected mice. An aliquot of the mixed viscera homogenate (from Waring blendor) was saved for direct oxidation and activity assay. From the remainder, the combined nucleic acids and/or the desoxyribose nucleic acid (DNA) were isolated using the procedure of Plentl and Schoenheimer (8) and Brues, Tracy and Cohen (2), respectively. Aliquots of the nucleic acids obtained were assayed for C^{14} content. The remainder of these samples was used for isolation of the nucleic acid purines as the silver salts. These twice-precipitated silver purines were also measured for radioactivity. All such measurements were carried out by a gas phase procedure which has already been described (10).

* This work was supported by the American Cancer Society, upon recommendation of the Committee on Growth of the National Research Council; the Research Fund of the Southern Research Institute; and Mr. Ben May, Mobile, Alabama. Certain data reported herein were obtained in a program supported by the Atomic Energy Commission, Division of Biology and Medicine. The authors wish to acknowledge the technical assistance of Mrs. Constance Nolan, Miss Linda Simpson, Miss Margaret Ann Newton, Miss Martelia Bell, and Mrs. Janita Chapman.

Received for publication May 6, 1950.

In additional experiments, mice (groups of 4 each) have been injected daily with the maximum tolerated dose of aminopterin or A-methopterin for 6 days; on the seventh day these mice were given an LD_{50} dose of the anti-folic acid compound and were then injected with $HC^{14}OONa$ (1.4 μc.) or $NaHC^{14}O_3$ (11.8 μc.), immediately placed in a metabolism chamber, and at 6 hours the pooled viscera subjected to the isolation procedures mentioned above. The objective of these experiments has been, of course, to observe the effect of folic acid antagonists on the incorporation of formate and CO_2 into the nucleic acid purines. The results obtained are set down in Tables 1 and 2. All values have been multiplied by 2 to correct for addition of inactive viscera homogenate.

DISCUSSION

It seems apparent from the data presented in Tables 1 and 2 that the multiple injection of normal mice with a folic acid antagonist (aminopterin or A-methopterin) profoundly decreases the rate of synthesis of nucleic acids. The fact that administration of aminopterin did not inhibit CO_2 fixation into the control tissue suggests that the nucleic acid effect may be a somewhat specific inhibition, at least within the period involved. Carbon dioxide is now considered a member of the pool of glycogen precursors and is known to be rapidly incorporated into amino acids and fats. Thus, over-all tissue CO_2 fixation serves as a sensitive rate index for a number of metabolic processes. In Experiment No. 13, where a single injection of aminopterin (an LD_{50} dose) was administered to mice immediately before injection of $NaHC^{14}O_3$, no significant decrease in nucleic acid or nucleic acid purine synthesis was observed. This might be considered preliminary evidence that multiple injections of the folic acid antagonists are necessary to produce a folic acid deficiency which, in turn, is responsible for the failure in purine synthesis. The folic acid antagonists in question reduced the specific activity of the viscera of formate-injected mice by only about 25 per cent, while incorporation of formate carbon into nucleic acid purines was reduced almost 15-fold. The decrease in incorporation of CO_2 into nucleic acid purines following treatment with folic acid antagonists is interpreted to indicate that CO_2 is incorporated into a purine precursor which, because of the purine inhibition accompanying treatment with these compounds, is never completed and thus never enters into purine anabolism.

TABLE 1

THE EFFECT OF AMINOPTERIN ON INCORPORATION OF FORMATE INTO NUCLEIC ACIDS AND NUCLEIC ACID PURINES (1.4 μC. INJECTED INTO ALL MICE)

Experiment no.	Treatment	Specific activities (μc/mol carbon): Viscera homogenate	Combined nucleic acids	Combined nucleic acid purines
1	Control	8.48	57.6	138.4
2	Control	4.42	71.8	176.8
3	Control	5.14		
4	Control	5.16		
Average		5.8	64.7	157.6
5	Aminopterin*	4.32	6.20	10.82
6	A-methopterin†	4.4	4.7	9.17

* Mice injected at the level of 0.23 mg/kg on 1, 2, 3, 4, 5, and 7 days and at the level of 8.33 mg/kg on the eighth day (immediately before injection of active formate).

† Mice injected at the level of 3 mg/kg on 1, 2, 3, 4, 5, and 7 days and at the level of 90 mg/kg on the eighth day (immediately before injection of active formate).

TABLE 2

THE EFFECT OF AMINOPTERIN ON INCORPORATION OF C^{14} FROM $NaHC^{14}O_3$ INTO NUCLEIC ACIDS AND NUCLEIC ACID PURINES (11.8 μC. INJECTED INTO ALL ANIMALS)

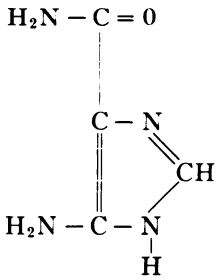
Experiment no.	Treatment	Specific activities (μc/mol carbon): Viscera homogenate	Combined nucleic acids	DNA	Combined nucleic acid purines	DNA purines
7	Control	0.68				1.41 (0.48)
8	Control	0.77				1.76 (0.44)
9	Control	0.90	1.65 (0.54)	1.95 (0.46)		2.10 (0.44)
10	Control	0.71	2.32 (0.31)	2.22 (0.32)		
11	Control	0.98	2.50 (0.35)		3.27 (0.3)	
12	Control	0.99		2.63 (0.38)		
Average		0.87				
13	Aminopterin*	0.88	1.71 (0.52)	1.236 (0.71)		2.20 (0.40)
14	Aminopterin†	1.32	1.05 (1.26)		0.54 (2.45)	

* A single injection of 8.33 mg/kg immediately before administration of $NaHC^{14}O_3$.

† Injection at the level of 0.23 mg/kg on 1, 2, 3, 4, 5, and 7 days and at the level of 8.33 on the eighth day (immediately before administration of $NaHC^{14}O_3$).

NOTE: The values in parentheses are the ratios of the specific activities of original viscera homogenate to the various fractions isolated therefrom.

The results obtained by Woolley and Pringle showing the build-up of the following compound by *E. coli* when inhibited by aminopterin:



4-amino-5-carboxamidoimidazole

along with the present observation that aminopterin or A-methopterin-treated mice are greatly inhibited with respect to nucleic acid purine synthesis (formate utilization) strongly suggests that folic acid, possibly acting as formyl folic acid or folinic acid is involved in completing the purine skeleton.

The seemingly most logical assumptions which might be made at the present time are that folic acid or folinic acid attached to an active center (protein, see Allfrey and King [1]) is acting as an enzyme or co-enzyme in formate transfer. Perhaps aminopterin and A-methopterin compete for this center and, when given in excess, prevent the formation of enough of the folic acid complex to allow for purine synthesis. Another possibility is that a direct inhibition of the synthesis of pteroylglutamic acid might be a result of administration of folic acid antagonists.

It has been shown that folic acid given in excess will speed up the leukemic process and a deficiency of this metabolite will preferentially inhibit leukemic cell division (11); therefore, since neoplasms apparently synthesize nucleic acid more rapidly than most host tissue, it would appear that one might explain the mechanism of action of folic acid antagonists in cancer chemotherapy on a purely differential rate basis as suggested by Hitchings *et al.* (6).

It also seems possible that the mechanism discussed above may be involved in the well known hematopoietic response observed in certain types of anemia following folic acid therapy.

SUMMARY

Aminopterin and A-methopterin, well known folic acid antagonists, have been shown to inhibit the incorporation of C^{14} from formate into nucleic acids and nucleic acid purines. Under the conditions of these experiments, incorporation of $C^{14}O_2$ into purines was partially blocked, but no decrease was observed in tissue CO_2 fixation. The relationship of these observations to the mechanism of action of folic acid in purine synthesis and the anti-leukemic mechanism of "folic acid inhibitors" is discussed.

REFERENCES

1. Allfrey, V. G., and King, C. G. An Investigation of the Folic Acid-Protein Complex in Yeast. J. Biol. Chem., **182**:367–84, 1950.
2. Brues, A. M.; Tracy, M. M.; Cohn, W. E. Nucleic Acid of Rat Liver and Hepatoma: Their Metabolic Turnover in Relation to Growth. J. Biol. Chem., **155**: 619–33, 1944.
3. Buchanan, J. M.; Sonne, J. C.; and Delluva, A. M. Biological Precursors of Uric Acid. II. The Role of Lactate, Glycine, and Carbon Dioxide as Precursors of the Carbon Chain and Nitrogen Atom 7 of Uric Acid. J. Biol. Chem., **173**:81–98, 1948.
4. Burchenal, J. H.; Kushida, M. N.; Johnston, S. F.; and Cremer, M. A. Prevention of Chemotherapeutic Effects of 4-Amino-N^{10}-methylpteroylglutamic Acid on Mouse Leukemia by Pteroylglutamic Acid. Proc. Soc. Exper. Biol. & Med., **71**:559–62, 1949.
5. Gordon, M.; Ravel, J. M.; Eakin, R. E.; and Shive, W. Formyl Folic Acid: A Functional Derivative of Folic Acid. J. Am. Chem. Soc., **70**:878–79, 1948.
6. Hitchings, G. H.; Elion, G. B.; Falco, E. A.; Russell, P. B.; Sherwood, M. B.; and Vanderwerff, H. Antagonists of Nucleic Acid Synthesis. I. The Lactobacillus Casei Model. J. Biol. Chem., **183**:1–9, 1950.
7. Law, L. W.; Dunn, T. B.; Boyle, P. J.; and Miller, J. H. Observations on the Effect of a Folic Acid Antagonist on Transplantable Lymphoid Leukemias in Mice. J. Nat. Cancer Inst., **10**:179–92, 1949.
8. Plentl, A. A., and Schoenheimer, R. Studies in the Metabolism of Purines and Pyrimidines by Means of Isotopic Nitrogen. J. Biol. Chem., **153**: 203–17, 1944.
9. Schmidt, G., and Tannhauser, S. J. A Method for the Determination of Desoxyribonucleic Acid, Ribonucleic Acid, and Phosphoproteins in Animal Tissue. J. Biol. Chem., **161**:83–89, 1945.
10. Skipper, H. E; Bryan, C. E.; White, L., Jr.; and Hutchison, O. S. Techniques for *in Vivo* Tracer Studies with C^{14}. J. Biol. Chem., **173**:371–81, 1948.
11. Skipper, H. E.; Chapman, J. B.; and Bell, M. Studies on the Role of Folic Acid in the Leukemic Process. Cancer (in press).
12. Sonne, J. C.; Buchanan, J. M.; and Delluva, A. M. Biological Precursors of Uric Acid. I. The Role of Lactate, Acetate, and Formate in the Synthesis of the Ureide Groups of Uric Acid. J. Biol. Chem., **173**:69–79. 1948.
13. Woolley, D. W., and Pringle, R. B. Formation of 4-Amino-5-carboxamidoimidazole during the Growth of *Escherichia coli* in the Presence of 4-Aminopteroylglutamic Acid. J. Am. Chem. Soc., **72**:634, 1950.