Observations on Inhibition of Nucleic Acid Synthesis Resulting from Administration of Nitrogen Mustard, Urethan, Colchicine, 2,6-Diaminopurine, 8-Azaguanine, Potassium Arsenite, and Cortisone*

HOWARD E. SKIPPER, JACK H. MITCHELL, JR., LEONARD L. BENNETT, JR., MARGARET ANN NEWTON, LINDA SIMPSON, AND MARY EIDSON

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

It has long puzzled those interested in the chemotherapy of cancer why such diverse agents as are mentioned in the title of this paper should provide temporary palliative effects in certain types of neoplastic disease, while hundreds of other compounds, some closely related structurally, should be without effect. Most of the known anti-cancer agents which have been studied extensively have been shown to be carcinogenic and under certain experimental conditions cause nuclear damage, inhibition of mitosis, chromosome breaks, and the production of mutations (1). This knowledge would suggest that one underlying similarity in the heterogeneous list of anti-cancer agents might have to do, directly or indirectly, with chromosome metabolism.

It is well established that the chromosome is composed largely of desoxyribonucleoprotein (10).

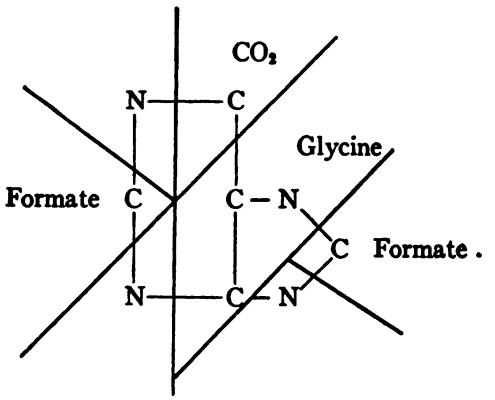
The fact that two well-known purine antagonists in bacteria, 2,6-diaminopurine and 8-azaguanine, will in certain instances preferentially inhibit neoplastic cell growth (3, 9) is strong evidence that the mechanism of these compounds at least has to do with nucleic acid metabolism. Our recent observations that 8-azaguanine, as such, is apparently fixed in mouse nucleic acids (11) and that folic acid antagonists inhibit purine synthesis (16) are further evidence implicating the nucleoprotein system in temporarily effective cancer chemotherapy. It has also been reported that x-radiation significantly depresses nucleic acid synthesis (7, 15).

* 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. The authors wish to acknowledge the assistance of Miss Martelia Bell, Mrs. Juanita B. Chapman, and Mrs. Constance W. Nolan.

Received for publication October 21, 1950.

In view of the above-mentioned facts, it was considered of interest to study the effect of a series of known anti-cancer agents on the *in vivo* synthesis of nucleic acids and fractions thereof.

The formation of the carbon chain of uric acid, therefore, presumably also of the purines of nucleic acid, has been studied with the aid of carbon 13 (2, 17). It was concluded that the sources of the component atoms of the purine skeleton were as follows:



Totter *et al.* (18) have recently shown that, in the rat, formate carbon is indeed a precursor of desoxyribonucleic acid guanine, adenine, and thymine (methyl group) and ribonucleic acid guanine and adenine.

It is theoretically possible, with the use of a labeled precursor of the nucleic acid purines, to determine the rate of synthesis of these genetically important compounds. Also, once a base line for the incorporation of the labeled precursor is established, one should be able to determine the effect of various agents on the synthesis of nucleic acid purines in the intact animal. Attempts to determine the effect of nitrogen mustard (methyl bis-

[2-chloroethyl]amine), urethan, colchicine, 2,6-diaminopurine, 8-azaguanine, potassium arsenite, and cortisone on nucleic acid synthesis have been made and are reported herein.

EXPERIMENTAL

Isolation procedure.—Carbon 14-labeled sodium formate (1.4 μc.) was injected intraperitoneally into groups of four adult CFW strain mice. After 6 hours these mice were sacrificed, and the viscera (liver, spleen, washed intestines, kidneys, and testes) were mixed with an equal weight of viscera from noninjected mice in order to provide sufficient starting material for isolation of fractions of particular interest. This mixture of tissues was then homogenized in the cold, with a jacketed Waring Blendor. Aliquots of the homogenate were assayed for radioactivity. The viscera homogenate was dehydrated with several portions of absolute ethanol and ethyl ether. The dehydrated tissue was extracted with 10 per cent NaCl and the crude sodium salts of the nucleic acids precipitated with alcohol and redissolved in water; the nucleic acids were precipitated with HCl and alcohol, washed with water, and dried with alcohol and ether (18). To obtain desoxyribonucleic acid (DNA), a portion of the combined nucleic acids was twice hydrolyzed for about 18 hours at room temperature in 2 N sodium hydroxide and reprecipitated in acid alcohol (13). Another portion of the combined nucleic acids was hydrolyzed in 0.5 N HCl for 1 hour and the purines precipitated in acid solution with silver nitrate. These purines were redissolved in 0.5 N HCl and reprecipitated with silver in acid solution.

Examination of isolation procedures.—In order to assure ourselves of the adequacy of the isolation technics for these exploratory experiments, several studies were carried out. A large group of leukemic mice was injected with 1.4 μc. each of sodium formate, and after 6 hours the viscera nucleic acids and nucleic acid purines were isolated and carried through repeated purifications. The results of this investigation are presented in Table 1. Representative samples of the crude twice-precipitated combined nucleic acids used herein were analyzed by the orcinol and diphenylamine color reactions and shown to be about 90 per cent nucleic acids (40 per cent RNA and 50+ per cent DNA). The twice-purified DNA was found to contain 90–95 per cent DNA and no more than 5 per cent RNA by these somewhat inadequate colorimetric analyses.

Reichard has reported that, on acid precipitation of silver purines, precipitation of part of the pyrimidines ("in the form of thymic acid") takes place (12). We have carried out phosphorus determinations on purines (extracted with acid from silver purines) after 1, 2, 4, and 6 precipitations with silver nitrate in acid solution. The results of these analyses are given in Table 2. An HCl extract from the twice-precipitated purines has also been acid-hydrolyzed in a sealed tube for 2 hours at 180° C. (in order to free pyrimidines, if any) and chromatographed on paper with a solvent system

TABLE 1

THE EFFECT OF REPEATED PURIFICATION ON THE SPECIFIC ACTIVITY OF NUCLEIC ACIDS AND SILVER PURINES FROM FORMATE-INJECTED MICE

No. of purifications	Specific activity (μc/mole C) Combined nucleic acids*	DNA†	Ag purines from combined NA‡
1	61.0	67.6	184.2
2	46.6	51.0	
3	56.4	50.4§	
4		55.0	204.0
6		51.1	200.0

* Repeated purification through sodium salt.

† Repeated treatment with 2 N NaOH (for 18 hours) and precipitation with acid.

‡ Repeated extraction with 0.5 N HCl and precipitation with silver nitrate.

§ This sample is an aliquot of the twice-purified DNA, which was carried through the lanthanum salt, as has been suggested by Hammarsten (6).

NOTE: These isolations were carried out on the viscera from 50 leukemic Akm mice (6 days after inoculation of Ak4 leukemia).

TABLE 2

PHOSPHORUS CONTENT OF SILVER PURINES AFTER REPEATED PRECIPITATION IN ACID SOLUTION

No. of precipitations	Per cent phosphorus
1	0.47
2	0.17
4	0.14
6	0.15

containing 4 parts butanol, 1 part diethylene glycol, and 1 part water in an ammonia atmosphere. This chromatogram showed a barely discernible trace of thymine but no evidence of other pyrimidines.

Injections with anti-cancer agents.—In the experimental groups, mice were treated with a single dose (usually the LD_{50}) of a given anti-cancer agent, or six doses (maximum tolerated level) on successive days followed by an LD_{50} dose. The last injection of the anti-cancer agent was followed immediately by an injection of $HC^{14}OONa$. These experiments were all of 6 hours' duration, and isolation of nucleic acids and purines was carried out in the same manner as has been described above.

TABLE 3

THE EFFECTS OF CERTAIN KNOWN ANTI-CANCER AGENTS ON INCORPORATION OF C^{14} (FROM FORMATE) INTO VISCERA, NUCLEIC ACIDS, AND NUCLEIC ACID PURINES

Exp. No.	Treatment	Dosage (mg/kg)	Specific Activity*			Ratio† of specific activities of viscera to purines ($\times 10^2$)
			Viscera homogenate	Combined nucleic acids	Combined nucleic acid purines	
1	Controls			57.6	138.4	
2	Controls		4.4	71.8	176.8	
3	Controls		4.4	42.4	187.2	
4	Controls		5.2			
5	Controls		4.4			
		(Average)	4.6	57.3	167.5	2.7
6	Nitrogen mustard	4.0	6.6	57.2		
7	Nitrogen mustard	0.75(6×)‡				
		4.0 (1×)	6.8	39.8	135.2	5.0
8	Urethan	1,800.0	7.4	45.4	148.2	5.0
9	Urethan	450.0 (6×)				
		1,800.0 (1×)	8.4	19.8	132.8	6.3
10	Urethan+nitrogen mustard	225+0.5 (6×)				
		900+2.0 (1×)	5.6	23.3	74.8	7.6
11	Benzene	250.0 (6×)				
		1,150.0 (1×)	5.0	38.3	161.0	3.1
12	$KAsO_2$	4.5 (6×)				
		9.0 (1×)	4.2	22.5	79.8	5.3
13	Colchicine	2.4	6.6	52.4	172.4	3.8
14	Colchicine	0.63(6×)				
		2.4 (1×)	5.5	39.3	163.5	3.4
15	2,6-Diaminopurine	100.0 (7×)	5.8	53.2	98.6	5.8
16	2,6-Diaminopurine	100.0 (6×)				
		248.0 (1×)	7.0	18.5	65.6	10.7
17	8-Azaguanine	31.3 (6×)				
		250.0 (1×)	3.7	27.2	99.2	5.0
18	Cortisone	44.0	4.4	36.7	88.0	5.0
19	Cortisone	44.0	6.3	49.0	133.5	4.7
20	Cortisone	44.0 (3×)	5.3	31.9	98.0	5.4

* Specific activities in μc/mole of carbon.

† Ratio of the specific activities of the viscera homogenate to the combined nucleic acid purines isolated therefrom.

‡ Number of injections indicated in parentheses under dosage.

TABLE 4

THE EFFECT OF CERTAIN KNOWN ANTI-CANCER AGENTS ON OVER-ALL INCORPORATION OF C^{14} FROM $NaHC^{14}O_3$ INTO VISCERA

Exp. No.	Treatment	Dosage (mg/kg)	Specific activity of viscera homogenate (μc/mole of carbon)
21	Controls		0.68
22	Controls		0.77
23	Controls		0.90
24	Controls		0.71
25	Controls		0.98
26	Controls		0.99
		(Av.)	0.84
27	Urethan	1,800.0	1.07
28	Urethan	1,800.0	2.04
29	Urethan	1,800.0	2.07
30	Nitrogen mustard	3.0	1.08
31	Nitrogen mustard	4.0	1.18
32	2,6-Diaminopurine	100.0 (7×)	1.15
33	Benzene	1,150.0	1.22
34	Potassium arsenite	18.0	0.62
35	Colchicine	2.4	1.38

NOTE: All treatment was on the basis of a single intraperitoneal injection except 2,6-diaminopurine, which was given at the level of 100 mg/kg daily for a total of seven injections.

TABLE 5

INCORPORATION OF C^{14} FROM BICARBONATE IN NUCLEIC ACIDS AND NUCLEIC ACID PURINES OF CONTROL MICE AT 6 HOURS

Exp. No.	Specific activity (μc/mole C)			
	Combined nucleic acids	DNA	Combined nucleic acid purines	DNA purines
21				1.41
22				1.76
23	1.65	1.95		2.10
24	2.32	2.22		
25	2.50		3.27	
26		2.63		
(Av.)	2.16	2.26		1.76

NOTE: All these experiments have been carried out on pooled viscera from four mice injected with 1.4 μc. of sodium formate each.

TABLE 6

TURNOVER OF C^{14} FROM FORMATE IN NUCLEIC ACIDS AND NUCLEIC ACID PURINES FROM MICE

	Specific activity (μc/mole C)		
	1 hour	6 hours	24 hours
Viscera homogenate	3.9	4.6	3.8
Combined nucleic acids	36.7	57.3	31.7
Combined NA purines	77.2	167.5	75.8

NOTE: The 1-hour and 24-hour experiments represent pooled tissues from four mice each, while the 6-hour experiment is average data involving 12–16 animals. The dosage of formate has been uniformly 1.4 μc. per mouse.

Similar studies have been carried out using $C^{14}O_2$, a less specific precursor of the 6-carbon atom of the purine skeleton. In these experiments $NaHC^{14}O_3$ was injected at a level of 11.8 μc. per mouse, and viscera were assayed for carbon 14 content.

The dosages employed and the comparative specific activities of viscera homogenate, combined nucleic acids, desoxyribonucleic acid (when isolated), and nucleic acid purines at 6 hours after injecting active formate and carbonate are presented in Tables 3, 4, and 5.

We have observed that there is little free radioactive formate or bicarbonate carbon in mouse tissues at 6 hours after injection. In order to obtain some information on the rate of turnover of formate carbon in nucleic acids and nucleic acid purines, experiments were carried out in which activities were determined at 1, 6, and 24 hours after injection (Table 6).

All carbon 14 determinations were carried out by a gas phase procedure, which has been described (14). The specific activities reported have been corrected for the original dilution of active viscera with inactive viscera.

DISCUSSION

From data presented in Tables 1 and 2 and from results mentioned in the experimental section, it appears that the twice-purified DNA and the nucleic acid purines are of a purity adequate for the objectives of these exploratory experiments. Less reliance can be placed on the absolute accuracy of the combined nucleic acid values.

In Tables 3 and 4 it can be seen that, in general, nitrogen mustard, urethan, colchicine, and 2,6-diaminopurine have caused an increase in the over-all incorporation of formate and bicarbonate carbon into the visceral organs at 6 hours. Hevesy has reported a similar increase in tissue incorporation of carbon 14 from carboxyl-labeled acetate following the injection of urethan or the administration of x-radiation (8). Hevesy interpreted this enhanced carbon 14 fixation, observed on administration of urethan, to be due to a decreased catabolism, which, in turn, slowed down the decrease in specific activity of carbon dioxide and acetate with time (dilution with normal catabolic products) and thus promoted incorporation of carbon 14 into tissue constituents. If such an interpretation were adapted to the present data, it would appear that the above-mentioned anti-cancer agents have depressed metabolic processes responsible for production of CO_2 and formate in the animal.

From data summarized in Table 3, it can be seen that in addition to folic acid antagonists (16) and x-radiation (7, 15), which have previously been reported to possess inhibiting action on nucleic acid synthesis, 2,6-diaminopurine, 8-azaguanine, cortisone, potassium arsenite, and the combination of nitrogen mustard plus urethan all markedly reduced incorporation of formate into nucleic acid purines. If one considers the ratios of the specific activities of the visceral organs to the nucleic acid purines from mice treated with nitrogen mustard or urethan alone, a very significant alteration can be seen. After administration of urethan or nitrogen mustard, viscera specific activities at 6 hours after injection of $HC^{14}OONa$ have increased by 40–80 per cent over the control groups, while the nucleic acid specific activities are slightly lower than those of the control animals. Benzene and colchicine have been relatively ineffective as nucleic acid inhibitors. All the present comparisons were necessarily made at levels which take into consideration the relative toxicities of the chemotherapeutic agents.

The inhibition of nucleic acid synthesis observed in certain of the present experiments does not necessarily indicate that the site of action of a given agent is directly on some moiety of the chromosome or nucleoprotein molecule, although this may be the case with the purine antagonists (11). There is the obvious possibility that the inhibition observed might be directed toward the source of energy for nucleic acid anabolism, on some precursor or precursor system, or perhaps more likely on enzyme systems having to do with nucleic acid metabolism. In a single experiment with four mice, we have observed that starvation (3 days) resulted in an apparent increase in over-all tissue fixation of formate in 6 hours and, at the same time, a reduction of the incorporation of carbon 14 from this source in nucleic acids and nucleic acid purines to about half that observed with control mice.

It is of considerable interest that CO_2 fixation in the combined viscera nucleic acids is of the same order as in desoxyribonucleic acid or in the nucleic acid purines (Table 5). This fits in well with the pattern of observations that purine precursors are incorporated into DNA and RNA at rates which are of the same order (4, 18). In contrast, adenine has been shown to be incorporated into RNA at a much higher rate than into DNA (5). In the case of radioactive formate-injected mice, the nucleic acid purine specific activities have usually been 3–4 times that of the crude combined nucleic acids.

As shown by the results given in Table 1, as compared to control animals in Table 3 and much

unreported data, we have observed that leukemic mice fix significantly more formate carbon in viscera nucleic acids than do control mice at 6 hours.

Studies are now under way with more refined isolation procedures (ion-exchange chromatography) to determine the effect of known anti-cancer agents and certain hormones on the synthesis of DNA guanine, adenine, and thymine and RNA guanine and adenine.

SUMMARY

Using the technic of measuring the incorporation of formate carbon into nucleic acid purines of control and treated mice, data have been obtained which indicate that 2,6-diaminopurine, 8-azaguanine, cortisone, potassium arsenite, urethan, and nitrogen mustard inhibit nucleic acid synthesis *in vivo*. Benzene and colchicine have been relatively ineffective as inhibitors of nucleic acid synthesis under the conditions of these experiments.

REFERENCES

1. BOYLAND, E. Chemistry of Neoplastic Tissue. Ann. Rev. Biochem., **18**:217–42, 1949.
2. BUCHANAN, J. M.; SONNE, J. B.; 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.
3. BURCHENAL, J. H.; BENDICH, A.; BROWN, G. B.; ELION, G. B.; HITCHINGS, G. H.; RHOADS, C. P.; and STOCK, C. C. Preliminary Studies on the Effect of 2,6-Diaminopurine on Transplanted Mouse Leukemia. Cancer, **2**:119–20, 1949.
4. ELWYN, D., and SPRINSON, D. B. The Extensive Synthesis of the Methyl Group of Thymine in the Adult Rat. J. Am. Chem. Soc., **72**:3317, 1950.
5. FURST, S. S.; ROLL, P. M.; and BROWN, G. B. On the Renewal of the Purines of the Desoxypentose and Pentose Nucleic Acids. J. Biol. Chem., **183**:251–66, 1950.
6. HAMMARSTEN, E. On the Extraction of Nucleotides from Cells. Acta med. Scandinav., Suppl., **196**:634–45, 1947.
7. HEVESY, G. Effect of X-Rays on the Incorporation of Carbon 14 into Animal Tissue. Nature, **164**:269, 1949.
8. ———. Effect of Muscular Exercise and of Urethane Administration on the Incorporation of Carbon 14 into Animal Tissue. *Ibid.*, p. 1007, 1949.
9. KIDDER, G. W.; DEWEY, V. C.; PARKS, R. E., JR.; and WOODSIDE, G. L. Purine Metabolism in *Tetrahymena* and Its Relation to Malignant Cells in Mice. Science, **109**:511–14, 1949.
10. MIRSKY, A. E., and POLLISTER, A. W. Chromosin, a Desoxyribose Nucleoprotein Complex of the Cell Nucleus. J. Gen. Physiol., **30**:117–48, 1946.
11. MITCHELL, J. H.; SKIPPER, H. E.; and BENNETT, L. L., JR. Investigations of the Nucleic Acids of Viscera and Tumor Tissue from Animals Injected with Radioactive 8-Azaguanine. Cancer Research, **10**:647–49, 1950.
12. REICHARD, P. On the Turnover of Purines and Pyrimidines from Polynucleotides in the Rat Determined with N^{15}. Acta chem. Scandinav., **3**:422, 1949.
13. SCHMIDT, G., and TANNHAUSER, S. J. A Method for the Determination of Desoxyribonucleic Acid, Ribonucleic Acid, and Phosphoproteins in Animal Tissues. J. Biol. Chem., **161**:83–89, 1945.
14. 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.
15. SKIPPER, H. E., and Mitchell, J. H., JR. Effect of X-Radiation on the Biosynthesis of Nucleic Acids and Nucleic Acid Purines. Cancer (in press).
16. SKIPPER, H. E.; MITCHELL, J. H., JR.; and BENNETT, L. L., JR. Inhibition of Nucleic Acid Synthesis by Folic Acid Antagonists. Cancer Research, **10**:510–13, 1950.
17. SONNE, J. B.; 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.
18. TOTTER, J. R.; VOLKIN, E.; and CARTER, C. E. Distribution of Isotopically Labeled Formate in the Nucleotides of Ribo- and Desoxyribonucleic Acids. Abstracts, 118th Meeting, Am. Chem. Soc., 1950.