Pyrimidine Studies

III. Effect of Several Compounds with Antitumor Activity on Utilization of Precursors for Synthesis of Nucleic Acid Pyrimidines*

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SUMMARY

Addition of amethopterin to an incubation medium containing slices of the human tumor transplants H.S. #1 and H.Ep. #3 decreased selectively the utilization of orotic acid-C^{14} for the synthesis of DNA thymine.

A substantially larger depression of the specific activity of DNA thymine was observed upon addition of both amethopterin and 5-bromouridine to the incubation medium than upon addition of amethopterin or 5-bromouridine. Seven hours following *in vivo* administration of amethopterin, the utilization of ureidosuccinic acid-C^{14} for the synthesis of DNA thymine was decreased to a greater extent than for the other nucleic acid pyrimidines in tumor (H.S. #1), liver, and intestine. Substantial suppression of the utilization of orotic acid-C^{14} for DNA thymine synthesis in H.S. #1 tumor slices was observed when the incubation mixture contained 5-fluorouridine and 5-fluorodeoxyuridine. Under these conditions, the utilization of thymidine-C^{14} for DNA thymine synthesis was enhanced.

Seven hours following administration of 5-fluorouracil (50 and 150 mg/kg) and 5-fluoroorotic acid (67 mg/kg) to rats bearing H.S. #1 tumors, the utilization of orotic acid-C^{14} for DNA thymine was depressed by a greater factor in liver DNA than in tumor or intestine.

Other compounds studied *in vivo* in a search for selective effects on pyrimidine metabolism included azaserine, urethan, and N-methylformamide.

Several compounds of interest in experimental chemotherapy have been tested in a search for selective effects on the utilization of pyrimidine precursors for the synthesis of nucleic acid pyrimidines. Previous studies with the human tumor transplants H.S. #1 and H.Ep #3 demonstrated selective effects of this nature in the case of 5-diazo-6-oxo-norleucine, 5-bromodeoxyuridine, and 5-fluorouracil (8–10), and the results suggested possible mechanisms of growth inhibition by these compounds. In this study, *in vivo* experiments on rats bearing these tumor transplants as well as *in vitro* incubations of tumor slices were carried out with ureidosuccinic acid-C^{14} or orotic acid-C^{14} as labeled precursors. Selective depression of the utilization of these precursors for DNA thymine synthesis was observed *in vivo* as well as *in vitro* with amethopterin and *in vitro* with several 5-fluoro-substituted pyrimidine derivatives.

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MATERIALS AND METHODS

C^{14}-labeled compounds.—Ureidosuccinic acid, labeled in the ureido carbon and having a specific activity of 0.5 $\mu c/\mu mole$, was synthesized by an

adaptation of the method of Nyc and Mitchell (9, 16). Orotic acid-6-C^{14} (1.1 $\mu c/\mu mole$) was obtained from the Bio-Rad Co. Thymidine-2-C^{14} (0.5 $\mu c/\mu mole$) was prepared by a modification of the method of Reichard (17).

Compounds with antitumor activity.—Amethopterin, urethan, N-methylformamide, 5-fluorouracil, 5-fluoroorotic acid, 5-fluorouridine, and 5-fluorodeoxyuridine were supplied to our laboratory through the Chemotherapy Division, Sloan-Kettering Institute. The 5-fluorinated pyrimidines and their derivatives were synthesized in the laboratory of R. Duschinsky of the Hoffman-La Roche Co.

Experimental procedures.—Procedures for the *in vivo* and *in vitro* experiments, as well as the analysis for nucleic acid pyrimidines and measurement of their specific activity, were essentially similar to those described previously (9, 10). The implantation of the tumor tissue and care of the animals throughout the period of conditioning with x-radiation and cortisone were carried out by the Division of Human Tumor Experimental Chemotherapy of this Institute (22–24). Experiments with isotopically labeled precursors were performed 9 days after tumor implantation in young female rats weighing approximately 60 gm. (Carworth Farms Wistar and Charles River Breeding Labs., Wistar). One-half of the total dose of amethopterin or some other compound of chemotherapeutic interest was injected intraperitoneally into a group of five or six rats followed in one-half hour by injection of the remaining dose, together with the C^{14}-labeled ureidosuccinic or orotic acid. A control group received only the labeled pyrimidine precursor. All animals were allowed food and water *ad libitum* until sacrifice 7 hours after administration of labeled compound. To minimize the effect of biological variation, tissue from each group of animals was pooled.

In the tissue slice experiments, orotic acid-C^{14} was added 15 minutes after incubation with amethopterin or one of several other antitumor agents had started.

RESULTS

Utilization of ureidosuccinic acid for synthesis of nucleic acid pyrimidines.—The relative specific activity of the nucleic acid pyrimidines after 7 hours relative to that for the administered ureidosuccinic acid was used as a measure of the extent of utilization. Each column in Table 1 refers to one experiment with a group of rats bearing H.S. #1 tumors. These groups of rats were made available for this study at intervals of approximately 2 months. Unless otherwise specified, each group of animals in any experiment received the standardized conditioning treatment with x-radiation and cortisone (22, 23). The last two columns in Table 1 refer to groups of animals receiving either x-radiation only or not subjected to any conditioning. In all six groups the liver RNA pyrimidines had the largest relative activity (up to 2.7 per cent of the administered compound). Since only half of the administered label was present in the natural stereoisomer, the extent of utilization of the ureidosuccinic acid may be considered appreciable in this case. The ratio of uracil to cytosine activity for the liver RNA pyrimidines ranged from 1.8 to 3. In all four tumor-bearing groups the order of decreasing activity for DNA thymine was tumor, intestine, and liver. In all six groups the specific activity of RNA cytosine was greater than that for DNA cytosine.

The differences observed for any one pyrimidine along a line of Table 1 reflects the use of different animals at various times and implanted with different tumor sources. The reproducibility of the precursor was tested in an experiment in which animals from the same shipment and implanted with the same tumor were sorted into two groups of six animals (Table 2). The largest spread in results was 24 per cent.

Amethopterin studies in vivo.—The effect of a single dose of amethopterin on two groups of tumor-bearing rats (one group receiving cortisone, but not x-radiation) is shown in Table 3. The utilization of the labeled precursor was generally reduced in the presence of amethopterin except for liver DNA cytosine and RNA uracil. In any one tissue the depression of the thymine specific activity was greater than that of the other nucleic acid pyrimidines. The largest factors were 9 and 20 for intestine and spleen of the second group of rats. These data suggest a selective effect of amethopterin on the *in vivo* synthesis of the DNA thymine moiety.

Azaserine, urethan and N-methylformamide, in vivo *studies.*—In the case of azaserine (Table 4) the utilization of ureidosuccinic acid-C^{14} for nucleic acid pyrimidines was generally slightly depressed in the case of tumor, liver, and intestine (with no effect in the case of RNA cytosine of intestine). The spleen assays indicated a substantial depression of activity in DNA pyrimidines. A selective effect in the case of a particular pyrimidine was not evident.

Urethan (1 gm/kg) had relatively little effect on the specific activities of nucleic acid pyrimidines. A twofold enhancement of activity was ob-

TABLE 1

UTILIZATION OF UREIDOSUCCINIC ACID (UREIDO-C^{14}) FOR SYNTHESIS OF NUCLEIC ACID PYRIMIDINES IN RATS BEARING H.S. #1 TUMORS

TISSUE	NUCLEIC ACID	PYRIMIDINE	RELATIVE ACTIVITY (PER CENT)*					
			Tumor-bearing conditioned rats (x-ray and cortisone)				NORMAL RATS (NO TREATMENT)	NORMAL RATS (X-RAY ONLY)
			Exp. 1	Exp. 2	Exp. 3	Exp. 4		
Tumor	DNA	Thymine	0.067	0.094	0.031	0.025		
		Cytosine	0.100	0.101	0.064	0.041		
	RNA	Uracil	0.13	0.182	0.099	0.086		
		Cytosine	0.19	0.14	0.12	0.115		
Liver	DNA	Thymine	0.0052	0.013	0.0043	0.0091	0.014	
		Cytosine	0.137	0.068	0.034	0.043	0.043	
	RNA	Uracil	2.6	2.7	1.63	1.25	0.93	1.2
		Cytosine	1.1	1.5	0.68	0.60	0.51	0.40
Intestine	DNA	Thymine	0.027	0.053	0.018	0.016	0.016	0.015
		Cytosine	0.050	0.050	0.029	0.021	0.023	0.032
	RNA	Uracil	0.118	0.079	0.099	0.065	0.049	0.22
		Cytosine	0.10	0.086	0.064	0.055	0.039	0.12
Spleen	DNA	Thymine	0.016					0.0061
		Cytosine	0.039					0.017
	RNA	Uracil	0.048					
		Cytosine	0.072					

* Relative activity $= \frac{100 \times \text{counting rate per } \mu\text{mole of nucleic acid pyrimidine}}{\text{Counting rate per } \mu\text{mole of ureidosuccinic acid-}C^{14}}$.

In each experiment five or six rats were used, and the tissue from each group was pooled for analysis.

served in the case of liver DNA cytosine. A selective effect in the case of any one pyrimidine was not observed.

In the case of N-methylformamide (500 mg/kg) there was a slight increase in the utilization of the precursor radiocarbon with the exception of tumor RNA pyrimidines. The largest increases (factor of 2.7) were found for liver DNA cytosine and RNA uracil of intestine.

Amethopterin studies in vitro.—After the 2-hour incubations of tumor slices in medium containing orotic acid-C^{14} and described in Tables 5, 6, and 7, the specific activity of RNA uracil was considerably greater than that of the other nucleic acid pyrimidines. Addition of amethopterin to the incubation medium resulted in a selective depression of the specific activity of DNA thymine in Experiments 1 and 2 of Table 5. In these experiments the utilization of orotic acid for RNA pyrimidines was not appreciably altered by addition of amethopterin. The 43-fold reduction in thymine specific activity in Exp. 2 was accompanied by relatively little change in the specific activities of DNA cyto-

TABLE 2

UTILIZATION OF UREIDOSUCCINIC ACID FOR THE SYNTHESIS OF NUCLEIC ACID PYRIMIDINES WITH RATS FROM ONE TRANSPLANT SOURCE

TISSUE	NUCLEIC ACID	PYRIMIDINE	RELATIVE ACTIVITY (PER CENT)*		DIFFERENCE (PER CENT)†
			Group 1	Group 2	
Liver	DNA	Cytosine	0.034	0.033	3
	RNA	Uracil	1.86	1.41	24
		Cytosine	0.71	0.65	8
Tumor	DNA	Thymine	0.034	0.029	15
		Cytosine	0.064	0.065	1.5

* Relative activity $= \frac{100 \times \text{counting rate per } \mu\text{mole of nucleic acid pyrimidine}}{\text{Counting rate per } \mu\text{mole of ureidosuccinic acid-}C^{14}}$.

† The numbers in this column were obtained by dividing the difference between Groups 1 and 2 by the larger value of the relative activity and multiplying by 100.

In this experiment, the six rats used in each group were chosen at random from a larger pool in which the same H.S. #1 transplant tissue was used.

sine and the RNA pyrimidines. A secondary objective of Experiments 2 and 3 in Table 5 was to determine the effect of using both amethopterin and 5-bromouridine in the same incubation medium. Previous studies had shown that 5-bromouridine depressed the utilization of orotic acid-C^{14} for the synthesis of DNA thymine (10). A substantially larger depression of the specific activity of DNA thymine was observed for the combination than for each compound alone. In Experiment 4, the compounds were administered *in vivo* during the standard chemotherapy test period (22, 23). Twenty-four hours following the last injection the tumor tissue was removed, minced, and used for incubation experiments without further addition of amethopterin or 5-bromouridine. Under these conditions a depression in the utilization of the precursor for DNA thymine synthesis was not observed.

Some fluoropyrimidines and derivatives in vitro. —Addition of 5-fluoroorotic acid depressed the utilization of orotic acid-C^{14} for the synthesis of

TABLE 3

EFFECT OF AMETHOPTERIN ON THE UTILIZATION OF UREIDOSUCCINIC ACID-C^{14} FOR SYNTHESIS OF NUCLEIC ACID PYRIMIDINES IN RATS BEARING H.S. #1 TUMORS

Conditioning of animals	Total dose (mg/kg)	Tissue	Ratio of activities (counts/min/μmole) (control/treated)			
			DNA		RNA	
			Thymine	Cytosine	Uracil	Cytosine
X-radiation plus cortisone	3	Tumor	3.7	2.3	1.6	1.7
		Liver	6.6	1.0	1.1	1.5
		Intestine	5.9	2.8	1.4	2.2
Cortisone only	5	Tumor	7.7	2.2	1.9	2.1
		Liver	3.1	0.62	1.0	0.90
		Intestine	9.1	3.6	1.5	2.0
		Spleen	20	3.5		

Two injections of amethopterin ½ hour apart. Ureidosuccinic acid-C^{14} was administered with the second injection. Animals were sacrificed after 7 hours. Six rats were used in each group.

TABLE 4

EFFECT OF AZASERINE, URETHAN, AND N-METHYLFORMAMIDE ON THE UTILIZATION OF UREIDOSUCCINIC ACID-C^{14} FOR THE SYNTHESIS OF NUCLEIC ACID PYRIMIDINES IN RATS BEARING H.S. #1 TUMORS

Compound	Dose (mg/kg)	Tissue	Ratio of activities (counts/min/μmole) (control/treated)			
			DNA		RNA	
			Thymine	Cytosine	Uracil	Cytosine
Azaserine	2	Tumor	2.1	2.6	1.5	2.8
		Liver	1.8	1.8	1.7	1.9
		Intestine	2.3	2.5	1.2	1.0
		Spleen	11	11	1.7	2.3
Urethan	1000	Tumor	0.71	0.77	1.1	1.4
		Liver	1.1	0.47	1.0	0.90
		Intestine	0.90	0.90	1.4	1.0
		Spleen	0.77	0.67	1.0	0.71
N-methyl-formamide	500	Tumor	0.83	0.83	1.1	1.3
		Liver	0.90	0.37	0.77	0.67
		Intestine	0.77	0.62	0.37	0.52
		Spleen	0.71	0.52	1.0	0.56

Six rats were used in each group.

TABLE 5

EFFECT OF AMETHOPTERIN AND 5-BROMOURIDINE ON THE UTILIZATION OF OROTIC ACID-C^{14} FOR SYNTHESIS OF NUCLEIC ACID PYRIMIDINES IN TUMOR SLICES

Exp.	Tumor	Compound	Ratio of activities (counts/min/μmole) (control/treated)			
			DNA		RNA	
			Thymine	Cytosine	Uracil	Cytosine
1	H.S. #1	Amethopterin	3.0 (115)*	0.27 (8)*	1.3 (6050)*	0.9 (90)*
2	H.Ep. #3	Amethopterin	43.0 (130)	0.9 (25)	1.1 (3440)	1.1 (137)
		5-Bromouridine	10.0	5.0	2.4	2.8
		Amethopterin plus 5-bromouridine	>100	6.0	2.7	3.5
3	H.S. #1	Amethopterin	11.0 (321)	0.8 (31)		
		5-Bromouridine	6.3	2.1		
		Amethopterin plus 5-bromouridine	36	3.1		
4†	H.S. #1	Amethopterin	1.05 (358)	0.93 (106)		
		5-Bromouridine	0.98	2.5		
		Amethopterin plus 5-bromouridine	0.73	2.0		

* Number in parentheses represent radiocarbon activity in control experiments, counts/min/μmole.

† Compounds administered *in vivo*.

Experiments 1, 2, and 3 were carried out in the normal procedure for slice experiments described above. The concentration of amethopterin and 5-bromouridine in the incubation medium were 4 and 300 μg/ml respectively.

In Exp. 4, amethopterin and/or 5-bromouridine had been administered to rats bearing H.S. #1 tumors following the standardized protocol (22, 23). This involved eight injections, each corresponding to 0.2 mg/kg amethopterin and/or 500 mg/kg 5-bromouridine. The animals were sacrificed 24 hours after the last injection, and slices were incubated in the normal procedure in the presence of orotic acid-C^{14}. Amethopterin and/or 5-bromouridine were not added to the incubation mixture. The chemotherapy test was supervised by M. N. Teller and P. C. Merker, Division of Human Tumor Experimental Chemotherapy.

TABLE 6

EFFECT OF SEVERAL 5-FLUOROPYRIMIDINE DERIVATIVES ON THE UTILIZATION OF OROTIC ACID-C^{14} FOR THE SYNTHESIS OF NUCLEIC ACID PYRIMIDINES IN TUMOR SLICES

Compound	Concentration (μg/ml)	Ratio of activities (counts/min/μmole) (control/treated)			
		DNA		RNA	
		Thymine	Cytosine	Uracil	Cytosine
5-Fluoroorotic acid	6.7	2.01 (515)*	2.54 (674)*	1.14 (11,000)*	1.84 (2470)*
	67	4.40	2.57	1.46	1.84
5-Fluorouridine	10	1.21 (179)	0.17 (11)	1.32 (12,100)	0.85 (210)
	100	11	0.066	1.27	0.49
5-Fluorodeoxyuridine	10	60 (179)	0.22 (11)	1.2 (12,100)	0.68 (210)

* Numbers in parentheses represent activity in control experiments, counts/min/μmole.

H.Ep. #3 tumor slices were used in the experiment with 5-fluoroorotic acid. H.S. #1 tumor slices were used in the other experiments.

TABLE 7

EFFECT OF 5-FLUOROURIDINE AND 5-FLUORODEOXYURIDINE ON THE UTILIZATION OF THYMIDINE-C^{14} FOR DNA THYMINE SYNTHESIS IN H.S. #1 TUMOR SLICES

Compound	Concentration (μg/ml)	Specific activity (counts/min/μmole) of DNA thymine		Relative activity (control/treated)
		Control	Treated	
5-Fluorodeoxyuridine	10	890	2240	0.40
5-Fluorouridine	10	890	1910	0.47
	100	890	2060	0.43

DNA pyrimidines to a larger extent than for RNA pyrimidines (Table 6). The specific activity of DNA thymine was depressed by the large factors of 11 and 60 in the presence of 5-fluorouridine (100 μg/ml) and 5-fluorodeoxyuridine (10 μg/ml). This was accompanied by enhancement of DNA cytosine activity and relatively little change in the activity of the RNA pyrimidines. Addition of 5-fluorouridine and 5-fluorodeoxyuridine was found to enhance the utilization of thymidine-C^{14} for DNA thymine synthesis by a factor of 2–2.5 (Table 7).

Some fluoropyrimidines and derivatives in vivo. —The numbers in parentheses in Table 8 represent the specific activity of the untreated (control)

TABLE 8

EFFECT OF SEVERAL 5-FLUOROPYRIMIDINES AND DERIVATIVES ON UTILIZATION OF OROTIC ACID-C^{14} FOR SYNTHESIS OF NUCLEIC ACID PYRIMIDINES IN TUMOR-BEARING RATS

Compound	Dose (mg/kg)	Tissue	Ratio of activities (counts/min/μmole) (control/treated)			
			DNA		RNA	
			Thymine	Cytosine	Uracil	Cytosine
5-Fluorouracil	50	Tumor	1.14 (884)*	1.38 (1380)*	0.79 (1526)*	0.81 (1808)*
		Intestine	1.53 (391)	0.90 (520)	0.52 (3250)	0.80 (2280)
		Liver	18 (143)	2.70 (965)	0.97 (55,800)	1.20 (20,210)
	150	Tumor	2.60 (578)	0.67 (920)	0.77 (2148)	1.24 (3030)
		Intestine	2.13 (565)	0.79 (824)	0.76 (6650)	1.08 (3830)
		Liver	14 (112)	1.6 (832)	1.23 (43,840)	1.76 (20,500)
		Kidney	2.3 (43)	2.2 (343)	—† (33,800)	— (11,400)
5-Fluoroorotic acid	67	Tumor	4.02 (884)	2.10 (1380)	0.86 (1526)	1.21 (1808)
		Intestine	2.64 (391)	0.90 (520)	0.96 (3250)	1.20 (2280)
		Liver	24 (143)	5.81 (965)	3.50 (55,800)	2.82 (20,210)
5-Fluorouridine	0.5	Tumor	1.20 (525)	0.85 (383)	0.93 (913)	0.75 (795)
		Intestine	0.93 (1040)	0.78 (1150)	0.71 (4500)	0.52 (2700)
		Liver	1.14 (1460)	1.05 (2760)	0.49 (35,000)	0.51 (20,800)
		Kidney	2.04 (81)	0.44 (168)	0.61 (24,900)	0.44 (6700)
	5	Tumor	0.80 (652)	1.17 (1770)	0.96 (3360)	0.89 (3390)
		Intestine	0.66 (246)	0.94 (660)	1.30 (5530)	1.00 (2825)
		Liver	1.3 (56)	0.63 (348)	0.59 (62,000)	0.77 (36,600)
		Kidney	2.7 (40)	0.53 (154)	1.25 (37,200)	0.75 (10,200)
5-Fluorodeoxyuridine	2	Tumor	0.62 (578)	0.52 (920)	0.38 (2148)	0.79 (3030)
		Intestine	1.44 (565)	1.00 (824)	1.15 (6650)	1.80 (3830)
		Liver	1.75 (112)	0.58 (832)	0.61 (43,840)	0.71 (20,500)
		Kidney	1.48 (43)	0.91 (343)	0.94 (33,800)	1.13 (11,400)
	10	Tumor	1.65 (652)	1.54 (1770)	1.24 (3360)	1.20 (3390)
		Intestine	0.86 (246)	0.80 (660)	1.02 (5530)	1.40 (2825)
		Liver	2.54 (56)	1.20 (348)	1.20 (62,000)	1.40 (36,600)
		Kidney	0.89 (40)	0.75 (154)	— (37,200)	1.24 (10,200)

* Numbers in parentheses represent activity in control experiments, counts/min/μmole.

† Dash indicates samples lost in analysis.
Five or six animals were in each group.

animals 7 hours following administration of orotic acid-C^{14}. As in the case of ureidosuccinic acid-C^{14} (Table 1), liver RNA pyrimidines have the highest specific activities. After the administration of 50 and 150 mg/kg of 5-fluorouracil and 67 mg/kg 5-fluoroorotic acid, the specific activity of DNA thymine was depressed by a substantially greater factor in liver (14, 18, and 24) than in the case of tumor and intestine. Relative to these large factors, the specific activity of the liver RNA pyrimidines was not appreciably altered.

The specific activity of DNA thymine was not appreciably depressed following the administration of 5-fluorouridine at 0.5 and 5 mg/kg. At the latter concentration, the specific activity was decreased by a threefold factor in kidney tissue accompanied by a twofold increase in the specific activity of DNA cytosine. At 0.5 mg/kg the specific activity of the RNA pyrimidines was slightly increased in all tissues tested.

In general, relatively small changes in the specific activities of the nucleic acid pyrimidines were observed following administration of 5-fluorodeoxyuridine at 2 and 10 mg/kg. In the case of liver DNA thymine, the specific activity was reduced by a factor of 2.5.

DISCUSSION

Dose of compounds used in these studies.—The toxicity and chemotherapy results for several of these compounds have been reported by Teller *et al.* for nine daily injections into rats bearing H.S. #1 tumors and the seven to eight daily injections into rats with H.Ep. #3 tumors (22). The single dose used in the 7-hour experiments reported here (Tables 3 and 4) was chosen to be at least equal to the toxicity values found. Toxic doses for rats bearing H.S. #1 tumors were found to be 0.4, 0.75, 250, and 500 mg/kg for amethopterin, azaserine, N-methylformamide, and urethan, respectively. N-methylformamide slightly inhibited tumor growth at tolerated doses, urethan inhibited tumor growth only at highly toxic doses, and amethopterin and azaserine were ineffective even at toxic doses (22).

Toxicity data had not yet been measured by the Division of Human Tumor Experimental Chemotherapy when the fluorinated pyrimidines were studied (Table 8). In a personal communication, Teller and Woolley, Division of Human Tumor Experimental Chemotherapy, Sloan-Kettering Institute, reported the following preliminary results with the use of human tumor transplants in the rat: 5-fluorouracil, 5-fluorodeoxyuridine, and 5-fluoroorotic acid did not inhibit either the sarcoma H.S. #1 or the epidermoid carcinoma H.Ep. #3 at maximum tolerated doses. 5-Fluorouracil was tested at 17.5 mg/kg against H.S. #1 and 35 mg/kg against H.Ep #3. The dose for 5-fluorodeoxyuridine was 50 mg/kg for both tumors. 5-Fluoroorotic acid was tested at 35 mg/kg against H.S. #1 and 70 mg/kg against H.Ep. #3. 5-Fluorouridine was inactive against H.S. #1 at 15 mg/kg but had a slight activity against H.Ep. #3 at the same dose.

The single dose of 5-fluoroorotic acid, 5-fluorouracil, and 5-fluorouridine used in the experiments described in Table 8 was greater than the values stated in the preceding paragraph. The single dose (Table 8) for 5-fluorodeoxyuridine was much smaller than the dose used in the chemotherapy test.

Amethopterin.—The substantial selective depression by amethopterin of the utilization of orotic acid-C^{14} for DNA thymine synthesis was observed in Experiment 2, Table 5, under conditions in which the specific activity of the other nucleic acid pyrimidines was not affected appreciably. The "thymine effect" using H.Ep. #1 slices is in accord with the results of Balis and Dancis using spleens of normal and leukemic mice, of Totter using bone marrow suspensions, and of Skipper and co-workers (1, 20, 25). In these reports, the effect of amethopterin on the utilization of formate-C^{14} for the synthesis of DNA thymine and nucleic acid purines was studied.

The *in vivo* studies in Table 3 demonstrate that the selective depression of the utilization of ureidosuccinic acid-C^{14} for DNA thymine synthesis could be detected in the human tumor transplant, intestine, liver, and spleen 7 hours following administration of amethopterin. This effect was observed in groups of rats conditioned with cortisone only as well as x-radiation plus cortisone.

Fluoropyrimidines.—The results in Tables 6 and 7 demonstrate a substantially decreased utilization of orotic acid-C^{14} for DNA thymine synthesis in slice experiments at 100 μg/ml fluorouridine and 10 μg/ml fluorodeoxyuridine. This is accompanied by an approximately twofold increase in the utilization of thymidine-C^{14} for DNA thymine synthesis. This selective effect of the fluorinated pyrimidines on the utilization of precursors for *de novo* synthesis of the thymine moiety in slice experiments with H.S. #1 tumor tissue extends the *in vitro* studies previously reported, with 5-fluorouracil (8). These results are in accord with the extensive *in vitro* studies by Heidelberger and his colleagues using suspensions of Ehrlich ascites cells (4, 12) and with isotopic-tracer studies carried out in this laboratory in which H.Ep. #1 cells

under cell culture conditions in complete growth medium were exposed to 5-fluorodeoxyuridine (6, 18).

Administration of 5-fluoroorotic acid (67 mg/kg) and 5-fluorouracil (50 and 150 mg/kg) resulted in a marked depression after 7 hours in the DNA thymine specific activity of liver tissue, considerably larger than the factor for tumor and intestine (Table 8). When 67 and 150 mg/kg of 5-fluoroorotic acid and 5-fluorouracil, respectively, were used (Table 8), larger factors for the depression of specific activity were observed for DNA thymine than for the other nucleic acid pyrimidines. Danneberg *et al.* observed this substantial effect for liver DNA thymine in mice after 12 hours with the use of 150 mg/kg 5-fluorouracil and 5-fluoroorotic acid (7). However, these investigators reported considerably larger factors (30 and 11) for the DNA thymine of Ehrlich ascites cells *in vivo* than observed here for H.S. #1 tumors.

Urethan and N-methylformamide.—Several observations have been reported linking the mechanism of biological action by urethan with nucleic acid synthesis and, in particular, with pyrimidine metabolism (11, 19). Bresnick has recently reported interference by urethan with the ureidosuccinate synthetase system of Ehrlich ascites cells (5). The *in vivo* experiment described in Table 4 did not show any appreciable effect of this compound on the utilization of ureidosuccinic acid (ureido-C^{14}) for nucleic acid pyrimidines. A slight enhancement of utilization of labeled ureidosuccinic acid for nucleic acid pyrimidines of liver and intestine following administration of N-methylformamide is observed in Table 3. This is consistent with speculation that this compound interferes with *de novo* pyrimidine synthesis, possibly at the ureidosuccinic acid stage (21). An increased utilization of formate-C^{14} for synthesis of adenine and guanine of rat liver RNA (but not DNA thymine) has been reported by Barclay *et al.* (2, 3). The intestine nucleic acids were not affected.

Azaserine.—Levenberg *et al.* found 6-diazo-5-oxo-norleucine and azaserine to be inhibitors of glutamine in a specific reaction concerned with inosinic acid synthesis (14, 15). Studies in this laboratory had shown that 6-diazo-5-oxo-norleucine substantially depresses the utilization of both ureidosuccinic as well as orotic acid for the synthesis of the cytosine moiety of nucleic acids in some mammalian tissues including H.S. #1 and H.Ep. #3 tumors (9). Kammen and Hurlbert found 6-diazo-5-oxo-norleucine to inhibit the action of glutamine specifically required in the amination of uridine nucleotides to cytidine nucleotides and found azaserine to suppress the formation of cytosine compounds from orotic acid (13). The *in vivo* experiment described in Table 3 did not reveal a selective "cytosine effect" of azaserine under conditions in which the specific activity of the nucleic acid pyrimidines were depressed in tumor, liver, and spleen.

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REFERENCES

1. Balis, M. E., and Dancis, J. Effects of A-Methopterin on Nucleic Acid Synthesis in Leukemic Spleen Breis. Cancer Research, **15**:603–8, 1955.
2. Barclay, R. K., and Garfinkel, E. The Influence of N-Methylformamide on Formate-C^{14} Incorporation. II. In Nucleic Acids of Tumor-bearing Rats. Cancer Research, **17**:345–48, 1957.
3. Barclay, R. K.; Garfinkel, E.; and Stock, C. C. The Influence of N-Methylformamide on C^{14}-Formate Incorporation. I. In Nucleic Acids of Rat Liver. J. Biol. Chem., **208**:875–82, 1954.
4. Bosch, L.; Harbers, E.; and Heidelberger, C. Studies on Fluorinated Pyrimidines. V. Effects on Nucleic Acid Metabolism *in Vitro*. Cancer Research, **18**:335–43, 1958.
5. Bresnick, E. Effect of Urethane upon Pyrimidine Biosynthesis. Federation Proc., **19**:313, 1960.
6. Cheong, L.; Rich, M. A.; and Eidinoff, M. L. Mechanism of Growth Inhibition of H.Ep. #1 Cells by 5-Fluorodeoxycytidine and 5-Fluorodeoxyuridine. Cancer Research, **20**:1602–7, 1960.
7. Danneberg, P. B.; Montag, B. J.; and Heidelberger, C. Studies on Fluorinated Pyrimidines. IV. Effects on Nucleic Acid Metabolism *in Vivo*. Cancer Research, **18**:329–34, 1958.
8. Eidinoff, M. L.; Knoll, J. E.; and Klein, D. Effect of 5-Fluorouracil on the Incorporation of Precursors into Nucleic Acid Pyrimidines. Arch. Biochem. Biophys., **71**:274–75, 1957.
9. Eidinoff, M. L.; Knoll, J. E.; Marano, B.; and Cheong, L. Pyrimidine Metabolism Studies. I. Effect of DON (6-Diazo-5-oxo-L-norleucine) on Incorporation of Precursors into Nucleic Acid Pyrimidines. Cancer Research, **18**:105–9, 1958.
10. Eidinoff, M. L.; Knoll, J. E.; Marano, B. J.; and Klein, D. Pyrimidine Studies. II. Effect of 5-Bromodeoxyuridine and Related Nucleosides on Incorporation of Precursors into Nucleic Acid Pyrimidines. Cancer Research, **19**:738–45, 1959.
11. Elion, G. B.; Brewer, S ; and Hitchings, G. H. Studies on the Mechanism of Action of Urethane on Mammary Adenocarcinoma 755. Acta Unio Internat. contra cancrum, **XVI**:605–8, 1960.
12. Harbers, E.; Chaudhuri, N. K.; and Heidelberger, C. Studies on Fluorinated Pyrimidines. VIII. Further Biochemical and Metabolic Investigations. J. Biol. Chem., **234**:1255–62, 1959.
13. Kammen, H. O., and Hurlbert, R. H. The Formation of Cytidine Nucleotides and RNA Cytosine from Orotic Acid

by the Novikoff Tumor *in Vitro*. Cancer Research, **19**: 654–63, 1959.

14. LEVENBERG, B.; MELNICK, I.; and BUCHANAN, J. M. Biosynthesis of the Purines. XV. The Effect of Aza-L-serine and 6-diazo-5-oxo-L-norleucine on Inosinic Acid Biosynthesis *de Novo*. J. Biol. Chem., **225**: 163–76, 1957.
15. MANDEL, H. G. The Physiological Disposition of Some Anticancer Agents. Pharmacol. Rev., **11**: 743–838, 1959.
16. NYC, J. F., and MITCHELL, H. K. Synthesis of Orotic Acid from Aspartic Acid. J. Am. Chem. Soc., **69**: 1382–84, 1957.
17. REICHARD, P. Utilization of Deoxyuridine and 5-Methyluridine for the Biosynthesis of Thymine by the Rat. Acta Chem. Scand., **9**: 1275–85, 1955.
18. RICH, M. A.; BOLAFFI, J. L.; KNOLL, J. E.; CHEONG, L.; and EIDINOFF, M. L. Growth Inhibition of a Human Tumor Cell Strain by 5-Fluorouracil, 5-Fluorouridine, and 5-Fluoro-2′-deoxyuridine—Reversal Studies. Cancer Research, **18**: 730–35, 1958.
19. ROGERS, S. Studies on the Mechanism of Action of Urethane in Initiating Pulmonary Adenomas in Mice. II. Its Relation to Nucleic Acid Synthesis. J. Exp. Med., **105**: 279–306, 1957.
20. SKIPPER, H. E. *A Review:* On the Mechanism of Action of Certain Temporary Anticancer Agents. Cancer Research, **13**: 545–51, 1953.
21. SKIPPER, H. E., and BENNETT, L. L., JR. Biochemistry of Cancer, *In:* J. M. LUCK, F. W. ALLEN, and G. MACKINNEY (eds.), Annual Review of Biochemistry, **27**: 137–66. Palo Alto, California: Annual Reviews, 1958.
22. TELLER, M. N.; MERKER, P. C.; PALM, J. E.; and WOOLLEY, G. W. Transplantable Human Tumors in Experimental Chemotherapy; A Comparison with Animal Tumor Systems. Cancer Research, **20**: 112–19, 1960.
23. TELLER, M. N.; PALM, J. E.; MERKER, P. C.; HARRIS, J. J.; and WOOLLEY, G. W. The Use of Transplantable Human Tumors in Experimental Chemotherapy. Cancer Research, **18**: 522–26, 1958.
24. TOOLAN, H. W. Transplantable Human Neoplasms Maintained in Cortisone-treated Laboratory Animals: H.S. #1; H.Ep. #1, H.Ep. #2, H.Ep. #3; and H.Emb.Rh. #1. Cancer Research, **14**: 660–66, 1954.
25. TOTTER, J. R. Antimetabolite Studies on Bone Marrow *in Vitro*. *In:* C. P. RHOADS (ed.), Antimetabolites and Cancer, pp. 153–61. Washington, D.C.: Am. Assoc. Adv. Sci., 1955.

Pyrimidine Studies III. Effect of Several Compounds with Antitumor Activity on Utilization of Precursors for Synthesis of Nucleic Acid Pyrimidines

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