Studies on Fluorinated Pyrimidines

III. The Metabolism of 5-Fluorouracil-2-C^{14} and 5-Fluoroorotic-2-C^{14} Acid *in Vivo**

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As one of the initial steps towards the determination of the metabolism and mechanism of action of fluorinated pyrimidines, 5-fluorouracil and 5-fluoroorotic acid were labeled with radiocarbon in the 2-carbon atom. We have previously reported briefly that fluorouracil is incorporated, as such, into the combined nucleic acids of mouse liver, spleen, and Ehrlich ascites cells (9). The present report describes the distribution, excretion, and metabolism of these compounds in mice, with and without tumors, and in a human cancer patient.

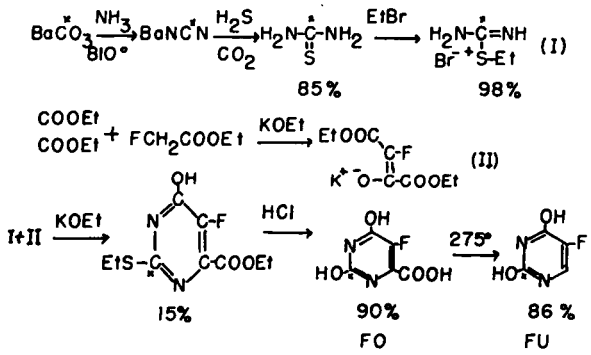

Chart 1.—Synthesis of labeled 5-fluorouracil and 5-fluoroorotic acid.

The incorporation of several purine and pyrimidine analogs into nucleic acids is now well established. 8-Azaguanine has been shown to be incorporated into the RNA of tobacco mosaic virus by Smith and Matthews (26) and into the nucleic acids of animal tissues by Mandel *et al.* (16). Purine and purine riboside conversion into acid-soluble nucleotides in rats has been studied by Brown and his co-workers (7). 5-Bromouracil has been shown by Weygand (28) and by Zamenhof *et al.* (29) to be incorporated into the DNA of bacteria, where it replaces up to 50 per cent of the thymine. Handschumacher has found that 6-azauracil is incorporated to a small extent into RNA, but not into DNA of *S. faecalis* (8). Prusoff (21) has demonstrated the incorporation of 6-azathymine-5-C^{14} into the DNA of *S. faecalis*. The status of the incorporation of 6-mercaptopurine into nucleic acids has not yet been conclusively established (cf. 6). It will be demonstrated in the present report that 5-fluorouracil and 5-fluoroorotic acid are incorporated into RNA, but not into DNA, in mouse tissues and a human tumor.

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MATERIALS AND METHODS

Synthesis of 5-fluoroorotic-2-C^{14} acid and 5-fluorouracil-2-C^{14}.—The syntheses of these compounds were carried out in three batches from radioactive barium carbonate, as shown in Chart 1. The barium carbonate was obtained from the Oak Ridge National Laboratory on allocation from the U.S. Atomic Energy Commission. We are greatly indebted to Dr. Robert Duschinsky of Hoffmann-LaRoche, Inc., who provided many experimental details for the syntheses.

S-Ethylisothiouronium bromide (I).—Barium carbonate, 2.1 gm., containing 100 mc. of C^{14}, was converted into barium cyanamide as described by Murray and Ronzio (19); this was then converted into thiourea (675 mg., 85 per cent) according to the method of Bills and Ronzio (1). The thiourea was refluxed with 2.5 gm. of ethyl bromide and 1 ml. of absolute ethanol on a steam bath for 12 hours. The excess reagents were removed from the salt by three extractions with dry ether, and 1.6 gm. (98 per cent) of the isothiouronium bromide was obtained.

Potassium diethylfluorooxalacetate (II).—Potassium, 3.9 gm., was powdered in hot toluene, and 10 ml. of absolute ethanol was added dropwise with cooling and stirring; 25 ml. of solvent was distilled, and the mixture was cooled. The potassium ethoxide crystallized, and 29 gm. of diethyl oxalate was added with stirring to give a clear yellow solution. To this, 10.6 gm. of ethyl fluoroacetate was added over ½ hour, stirring and cooling was continued for 1 hour, and the mixture was left overnight at room temperature. The colorless crystalline mass was filtered, washed with ether, and dried to give 22 gm. (90 per cent) of the potassium salt.

Ethyl 2-methylmercapto-4-hydroxy-5-fluoro-6-pyrimidine car-

boxylate (III).—To a solution of 0.34 gm. (8.7 millimoles) of potassium in 40 ml. of absolute ethanol was added with stirring 4.0 gm. (16.4 millimoles) of the potassium enolate and 1.6 gm. (8.7 millimoles) of the labeled ethyl isothiouronium bromide. The mixture was refluxed and stirred for 2 hours and evaporated to dryness in vacuum. The residue was dissolved in 8 ml. of ice-cold water, and the solution was extracted 3 times with 10 ml. of ether. The crystalline pyrimidine was obtained on acidification of the aqueous solution with dilute HCl in a yield of 310 mg. (15 per cent), m.p. 166°–168° C., and was recrystallized from toluene to give 290 mg., m.p. 168°–169° C. The aqueous mother liquor contained a great amount of radioactivity and was evaporated to dryness. The resulting mass was extracted with ethanol, and after evaporation it was converted to barium carbonate in a combustion furnace and re-cycled for the second batch synthesis. The same recovery was made at this stage of the second batch and reutilized for the third batch synthesis.

5-Fluoroorotic-2-C^{14} acid.—The above pyrimidine, 285 mg., was refluxed with 10 ml. of conc. HCl for 4 hours. The mixture was cooled in ice, and the crystalline fluoroorotic acid was filtered and dried; yield, 200 mg. (90 per cent). The compound was shown to be pure by ion-exchange chromatography.

5-Fluorouracil-2-C^{14}.—The 5-fluoroorotic acid (125 mg.) was heated in a test tube to 275° C. until the evolution of CO_2 ceased. On cooling, a brownish solid was obtained, which was sublimed at 200° C./0.5 mm to give a colorless solid, 73 mg. (86 per cent). On paper chromatography in *n*-butanol: formic acid: water (77:10:13) a radioactive impurity was found, and the compound was purified by ion-exchange chromatography on Dowex-1 formate. Since the 5-fluoroorotic acid was pure, the impurity in the 5-fluorouracil was formed during the decarboxylation. The specific activities were obtained by determining the radio activity of solutions in the Packard Tri-Carb liquid scintillation counter, and the quantities were determined spectrophotometrically with the use of the constants at pH 1.1 for fluorouracil of E_{266} (μmoles/ml) of 7.0, and E_{284} (μmoles/ml) of 6.8 for fluoroorotic acid. These specific activities in μc/mg were as follows:

Batch no.	5-Fluorouracil	5-Fluoroorotic acid
1	45	33
2	23	17
3	4.1	3.0

Tissue and nucleic acid isolations.—All mice were albino Swiss female mice, sometimes bearing Sarcoma 180 or the Ehrlich ascites carcinoma, and were obtained from Taconic Farms, Germantown, N.Y. The labeled compounds were given by intraperitoneal injection, and at various time intervals the mice were sacrificed and the tissues dissected.

In the experiments on tissue distribution, the organs were kept frozen until used and were minced fine with scissors, spread evenly on aluminum discs, dried under an infra-red lamp, and radioactivity measured. The specific activities were expressed as counts/min/mg. In certain experiments, the mice were kept in metabolism cages, and the respiratory CO_2 was trapped at various intervals and precipitated as barium carbonate; urine and feces were collected.

The livers, spleens, and ascites cells were processed as described previously, with the omission of the treatment with 10 per cent NaCl at room temperature (27). The acid-soluble fraction was subjected to extended gradient elution chromatography on Dowex-1 formate (13). The combined dialyzed sodium nucleates were hydrolyzed with hot 70 per cent perchloric acid (18) and the bases chromatographed on Dowex-1 chloride with NH_4OH,NH_4Cl according to the procedure of Cohn (4). The separations obtained are shown in Chart 2. The combined nucleic acids were hydrolyzed with 0.1 N NaOH, the DNA was separated, and the RNA nucelotides were chromatographed with gradient elution on Dowex-1 formate; the elution pattern is shown in Chart 2. The DNA obtained on acid precipitation was routinely dissolved and reprecipitated 3 times. When the nuclei were separated from the cytoplasm, the tissues were homogenized in 0.25 M sucrose containing 0.002 M $CaCl_2$, centrifuged at 900 × *g* (27), and washed twice with the medium. The nucleic acids were isolated from these fractions as usual. All RNA analyses were carried out by the orcinol reaction (24), with an RNA solution that had been calibrated by its phosphorus content used as a standard.

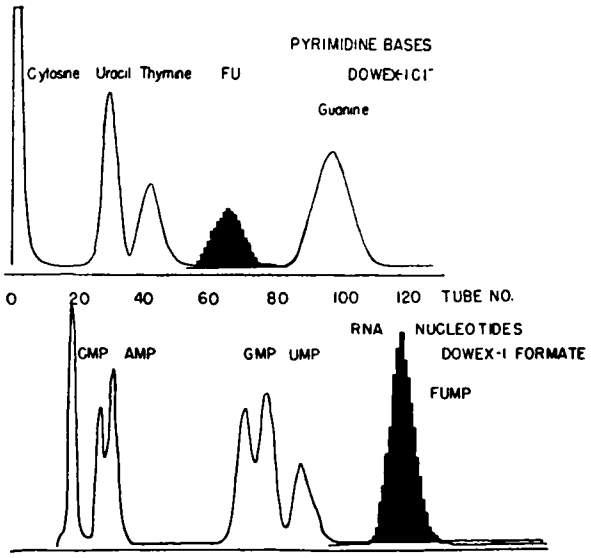

CHART 2.—Chromatographic separation of fluorinated pyrimidines and nucleotides from nucleic acid hydrolysis. The open peaks represent readings taken by ultraviolet spectrophotometry at 260 mμ; the solid peaks represent radioactivity measurements. Upper curve: Dowex-1 chloride column (1 × 20 cm.), 10-ml. fractions, eluted with buffer of 2.67 gm. NH_4Cl and 50 ml. conc. NH_4OH made up to 2 liters, pH, 10.4. These are schematic representations of a number of experiments, so no specific units are given.

Lower curve: Dowex-1 formate column (1 × 20 cm.), 10-ml. fractions; gradient elution with 500 ml. mixer and 2.5 M formic acid.

RESULTS

EXCRETION IN MICE

The excretion of radioactivity in the respiratory carbon dioxide and urine of mice given intraperitoneally doses of uracil-2-C^{14}, 5-fluorouracil-2-C^{14}, and 5-fluoroorotic-2-C^{14} acid is shown in Chart 3. The doses of FU and FO are given in Tables 1 and 2; 3.7 mg. of uracil, specific activity = 0.25 μc/mg was used. These results represent the mean values from a series of experiments ranging from four to 24 mice. The amount of radioactivity in the feces was negligible. With uracil and 5-fluorouracil there was a rapid rate of metabolism to respiratory CO_2 for the first 4

hours, which then decreased; with 5-fluoroorotic acid there was a very slow rate of conversion to carbon dioxide. The total urinary excretion of radioactivity over 24 hours is given in the bar graphs, with the sum of the urine and CO_2 radioactivities also shown. The total amount excreted in 24 hours decreased in this order: uracil, 5-fluorouracil, and 5-fluoroorotic acid, and represents 98, 88, and 73 per cent of the administered dose, respectively. The doses given here were equimolar equivalents of 200 mg/kg of 5-fluorouracil.

Excretion in a Cancer Patient

Subsequently, a metabolism study with labeled 5-fluorouracil was carried out in a human cancer patient. This subject was a 43-year-old male, weighing 66 kg., who had a primary anaplastic

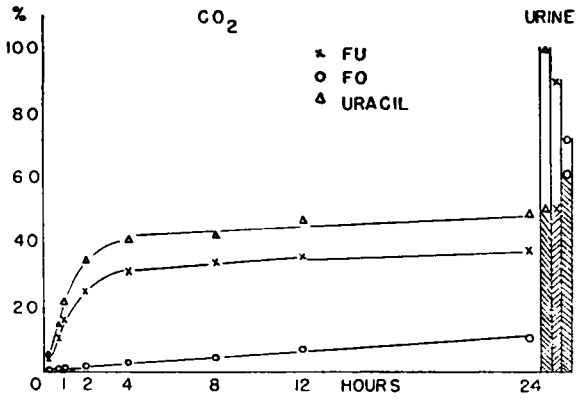

Chart 3.—Per cent excretion of radioactivity in mice against time after injection of labeled uracil, 5-fluorouracil, and 5-fluoroorotic acid. For the urines, the cross-hatched areas represent the 24-hour total excretion. The areas above the cross-hatching represent the sum of the urinary and respiratory carbon dioxide excretions.

carcinoma of the lung with multiple metastatic nodules. He was given a single intravenous injection of 935 mg., 14 mg/kg, 177 μc., of 5-fluorouracil-2-C^{14}. Blood samples were collected frequently, urine samples were obtained as voided, and tissue biopsies were obtained at 4, 12, and 24 hours after injection of the drug. The respiratory carbon dioxide was sampled frequently by collection in a Douglass bag, the specific activity of the precipitated barium carbonate was determined, and the total amount expired was calculated from the patient's basal metabolic rate and the integrated specific activity-time curve. The pattern of excretion of radioactivity is shown in Chart 4. The respiratory CO_2 had its highest specific activity at 60 minutes after injection, and the shape of the curve closely resembled that of the mouse experiments except that in the patient a calculated total of 25 per cent of the dose was expired in 24 hours as compared with 48 per cent determined in the mouse. In this patient, 12.5 per cent of the dose was found in the sample of urine collected 40 minutes after injection, and a total of 45 per cent was excreted at 24 hours, compared with 90 per cent in the mouse. Thus, in this patient, there appeared to be a greater retention of drug than in the mouse, although the radioactivity in the feces was not determined.

The blood-level of radioactivity in the plasma of this patient is shown in Chart 5. There was a rapid drop from a radioactivity equivalent to 28 μg/ml at 10 minutes to 2.8 μg/ml at 2 hours; it then decreased slowly to 0.72 μg/ml at 24 hours. When the curve is extrapolated to zero time, a value of the blood level is obtained which is consistent with a rapid equilibration of the drug with total body water. There was no significant isotope present in the blood cells. We do not have similar data in mice.

Tissue Distribution in Mice

A series of mice bearing bilateral subcutaneous 10-day-old implants of Sarcoma 180 was given 4.5 mg. of 5-fluorouracil-2-C^{14} intraperitoneally, their tissues were dissected at various time intervals, plated and dried, and the specific activities of the dried tissue minces were determined. The results shown in Table 1 represent the mean values of four mice for each value. The specific activities of the tissues are listed, together with their specific activities relative to the tumor, which has beeen assigned the number of 1.00. It will be noted that, at all times, the tumor had the highest specific activity of any of the tissues measured, with bone marrow and small intestine as the next highest tissues. Thus, in the case of 5-fluorouracil some measure of selective localization of radioactivity in the tumor has been achieved. When an equivalent dose was given by stomach tube, the specific activity of the tumor at 24 hours was 68 per cent of that following intraperitoneal injection, which is in accord with the decreased effectiveness of 5-fluorouracil against Sarcoma 180 when given by gavage (10).

Similar information for mice receiving 5-fluoroorotic-2-C^{14} acid by intraperitoneal injection is given in Table 2. It is evident that, in contrast to 5-fluorouracil, there was no selective localization of radioactivity from 5-fluoroorotic acid in the tumors of these mice, since several organs have specific activities considerably higher than those of the tumor. This is similar to the observations of Hurlbert and Potter (12), who found more radioactivity from labeled orotic acid in liver than in tumor. These results possibly explain the fact that

5-fluoroorotic acid is more toxic and less effective than 5-fluorouracil against Sarcoma 180 (10).

TISSUE DISTRIBUTION IN CANCER PATIENT

The specific activities of the dried tissue biopsy samples from the patient who received labeled 5-fluorouracil are given in Table 3. The muscle, skin, and fat were collected from the region surrounding the metastatic tumor nodules, and a single liver biopsy was obtained. It will be noted that the maximum specific activity of the tumor was at 12 hours, that it was much higher than adjacent muscle, fat, and skin, and somewhat higher than the liver. In this respect the tissue distribution in the patient paralleled the pattern observed in mice and indicates some selective localization of FU and/or its metabolites in the tumor. In the mice, the drug was given intraperitoneally at a level of 200 mg/kg, and the tumor specific activity was equivalent to 0.45 μg/mg dry weight. In the patient, 5-fluorouracil was injected intravenously at a dose of 14 mg/kg, and the specific activity of the tumor corresponds to 0.033 μg/mg. Thus, the human dose was 7 per cent of the mouse dose, and the tissue concentration was 7.2 per cent that of the mouse, suggesting that, if the two species may be compared, the tissue concentration of drug may be proportional to the dose. This conclusion is supported by a mouse experiment, in which at 4 hours, after an injection of 200 mg/kg of labeled 5-fluorouracil, the tumor had a tissue equivalent of 0.59 μg/mg, whereas, at the same time, following a dose of 20 mg/kg, the tumor had a specific activity corresponding to 0.022 μg/mg (cf. Table 1).

Incorporation into nucleic acids.—It has been

TABLE 1

SPECIFIC RADIOACTIVITIES OF TISSUES OF MICE BEARING SARCOMA 180

Dose: 4.5 mg. of 5-fluorouracil-2-C^{14} intraperitoneally. Four mice per group.
Specific activity = 4.1 μc/mg.

	COUNTS/MIN/MG DRY WT. TISSUE				
TISSUE	1 Hour	4 Hours	12 Hours	24 Hours	48 Hours
Tumor	2950 (1.00)*	2714† (1.00)	2050 (1.00)	1120‡ (1.00)	322 (1.00)
Bone marrow	2530 (0.86)	1500 (0.55)		371 (0.33)	247 (0.73)
Intestine	1600 (0.54)	804 (0.30)		321 (0.38)	10 (0.03)
Spleen	1220 (0.41)	736 (0.27)	434 (0.21)	260 (0.23)	35 (0.11)
Kidney	1630 (0.55)	403 (0.15)		128 (0.11)	70 (0.22)
Liver	1180 (0.40)	560 (0.21)	271 (0.13)	150 (0.13)	52 (0.16)
Lungs	318 (0.11)	180 (0.07)		60 (0.05)	23 (0.07)
Heart	192 (0.07)	40 (0.01)		28 (0.01)	17 (0.05)
Muscle	146 (0.05)	33 (0.01)		17 (0.01)	7 (0.00)
Fat	518 (0.18)	234 (0.09)		35 (0.03)	25 (0.08)
Ovaries	189 (0.06)	131 (0.05)		21 (0.02)	14 (0.04)
Brain	307 (0.10)	85 (0.03)		35 (0.03)	15 (0.05)

* Numbers in parentheses give relative radioactivity of the various tissues compared with tumor tissue, which is given an arbitrary value of 1.00.

† At 4 hours, following a dose of 0.1 times the amount of FU* given intraperitoneally, the tumor specific activity was 106 counts/min/mg.

‡ At 24 hours, following an equivalent dose of FU* given by stomach tube, the tumor specific activity was 766 counts/min/mg.

TABLE 2

SPECIFIC RADIOACTIVITIES OF TISSUES OF MICE BEARING SARCOMA 180

Dose: 2.6 mg. of 5-fluoroorotic acid-2-C^{14} intraperitoneally. Four mice per group.
Specific activity = 3.0 μc/mg.

	COUNTS/MIN/MG/DRY WT TISSUE			
TISSUE	1 Hour	4 Hours	12 Hours	24 Hours
Tumor	182 (1.00)*	163 (1.00)	82 (1.00)	76 (1.00)
Liver	865 (4.75)	638 (3.92)	216 (2.64)	147 (1.94)
Kidney	1130 (6.20)	1290 (7.94)		574 (7.55)
Spleen	247 (1.36)	214 (1.31)	97 (1.18)	116 (1.52)
Bone marrow	272 (1.49)	180 (1.10)		72 (0.94)
Intestine	198 (1.09)	229 (1.40)		93 (1.22)
Lungs	118 (0.65)	93 (0.57)		47 (0.62)
Heart	54 (0.30)	50 (0.31)		35 (0.46)
Ovaries	93 (0.51)	72 (0.44)		28 (0.37)
Muscle	26 (0.14)	17 (0.10)		12 (0.16)
Fat	17 (0.09)	23 (0.14)		23 (0.30)
Brain	7 (0.04)	8 (0.05)		4 (0.05)

* See footnote *, Table 1.

previously reported (9) that radioactivity from 5-fluorouracil-2-C^{14} was incorporated into the combined nucleic acids of liver, spleen, and ascites cells. Following perchloric acid hydrolysis and chromatography of the bases, most of the radioactivity was contained in the 5-fluorouracil peak. That the radioactivity actually belonged to 5-fluorouracil was established by carrier chromatography in three different systems (9). Such experiments have now been repeated with the addition of 5-fluorocytosine carrier (which is found between the cytosine and uracil peaks in the Dowex-1 chloride system, shown in Chart 2). No label was found in the 5-fluorocytosine peak, which shows that it is not formed metabolically from 5-fluorouracil.

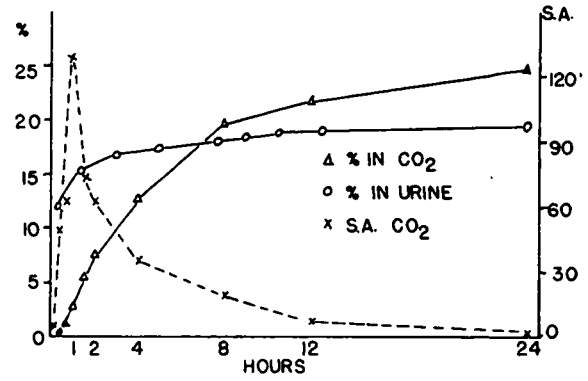

CHART 4.—Specific activity and calculated per cent excretion of respiratory carbon dioxide and per cent urinary excretion of radioactivity against time after intravenous injection of labeled 5-fluorouracil to a cancer patient.

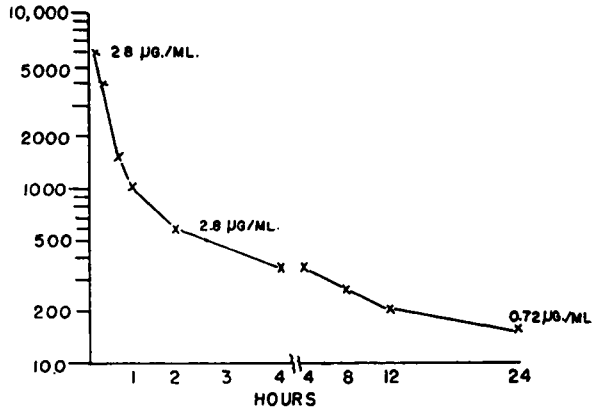

CHART 5.—Plasma level of radioactivity in the cancer patient following intravenous injection of 5-fluorouracil-2-C^{14}.

A series of experiments was performed in which labeled 5-fluorouracil and 5-fluoroorotic acid were administered to groups of mice, some with and others without the Ehrlich ascites carcinoma. The animals were sacrificed 12 hours later, and the nucleic acids were isolated from liver, spleen, and tumor, and dialyzed to remove adsorbed impurities. The RNA was hydrolyzed with alkali, and the ribonucleotides were separated on Dowex-1 formate columns, as indicated in Chart 2. The DNA was precipitated with acid, dissolved, and reprecipitated. As shown in Table 4, there was a small amount of radioactivity in the DNA samples. To determine whether this was true incorporation or contamination by small amounts of RNA, a sample of DNA, containing a total of 2000 counts/min, was dissolved and reprecipitated 3 times. There was no detectable radioactivity in the DNA after this degree of purification, and it must therefore be concluded that the small amounts of label in the DNA given in Table 4 are due to contamination, and that 5-fluorouracil and 5-fluororoorotic acid are incorporated into the RNA and not into DNA of these mammalian tissues. The total radioactivities given in Table 4 are meaningless in absolute terms, because different amounts of tissues and different aliquots were used in each case. However, they may be used as a frame of reference to judge the amounts of radioactivity encountered in the nucleotide separations, the recoveries as 5-fluorouridylic acid, and the lack of isotope in the other nucleotides. The specific activities, on the other hand, were comparable in all cases, and are based on radioactivity/μg RNA as determined by the orcinol reaction. The results (Table 4) for the RNA specific activities parallel the tissue distribution of radioactivity (Tables 1 and 2), in that the highest specific activity was found in the ascites cells from 5-fluorouracil as the precursor. With 5-fluoroorotic acid, the specific activities of the liver and tumor RNA's were the same. In the case of both precursors the livers of tumor-bearing mice had higher specific activities than the livers of

TABLE 3

SPECIFIC RADIOACTIVITIES OF TISSUES OF HUMAN CANCER PATIENT

Human male, age 43 years, weight 66 kg.
Anaplastic lung carcinoma with multiple skin nodules.
Dose = 14 mg/kg = 935 mg. 5-fluorouracil-2-C^{14} = 177 μc.
Specific activity = 209,000 counts/min/mg

	Counts/min/mg dry weight		
Tissue	4 Hours	12 Hours	24 Hours
Tumor	4.8	7.0	4.7
Liver		5.8	
Muscle	0	1.1	0
Fat	0.6	0.3	0
Skin	0.8	0.6	0.3

normal mice, a commonplace phenomenon (cf. 11, 16, 27).

The chromatography of the nucleotides from the alkaline hydrolysis of RNA shows clearly that all the radioactivity in the RNA was present as 5-fluorouridylic acid, which was completely separated from the other nucleotides as shown in Chart 2. Although there was not sufficient material in these peaks to be detected by ultraviolet spectrophotometry, it was characterized by perchloric acid hydrolysis in the presence of 5-fluorouracil carrier, and the resulting mixture was chromatographed on Dowex-1 chloride columns. As shown by a typical example in Table 5, the 5-fluorouridylic acid (FUMP) samples from liver and ascites cells, when subjected to this treatment, gave peaks of 5-fluorouracil with constant specific activities throughout. This served as a rigid criterion of purity and established the identity of the labeled material, which before hydrolysis showed the expected chromatographic characteristics of a fluorinated uridylic acid, as 5-fluorouracil. It is noteworthy that 5-fluorouridylic acid was obtained following injection of both 5-fluorouracil and 5-fluoroorotic acid, showing that the latter drug is decarboxylated before conversion into nucleotides. Thus, the behavior, with respect to tissue distribution and incorporation into RNA, of the fluorinated pyrimidines is analogous to their nonfluorinated congeners, uracil and orotic acid.

TABLE 4

DISTRIBUTION OF RADIOACTIVITY IN NUCLEIC ACIDS AND RIBONUCLEOTIDES

Mice killed 12 hours after injection.

	Total counts/min/sample			
Fraction	Normal liver	Liver from ascites mice	Spleen	Ascites cells
	4.5 Mg. 5-fluorouracil-2-C^{14}, specific activity = 23 μc/mg			
DNA	600	1,300	2,800	7,600
RNA	70,000	570,000	32,300	720,000
RNA, counts/min/μg	17.5	43	17	200
" , CMP	0	0	0	0
" , AMP	0	0	0	0
" , GMP	0	0	0	0
" , UMP	0	0	0	0
" , FUMP	63,500	466,000	23,500	540,000
	6.0 Mg. 5-fluoroorotic acid-2-C^{14}, specific activity = 17 μc/mg			
DNA	300	2,000	0	4,000
RNA	82,000	635,000	25,000	575,000
RNA, counts/min/μg	30	57	19	59
" , CMP	0	0	0	0
" , AMP	0	0	0	0
" , GMP	0	0	0	0
" , UMP	0	0	0	0
" , FUMP	63,500	684,000	28,000	560,000

Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; CMP, cytidylic acid; AMP, adenylic acid; GMP, guanylic acid; UMP, uridylic acid; FUMP, 5-fluorouridylic acid.

TABLE 5

HOMOGENEITY OF 5-FLUOROURACIL PEAK OBTAINED FROM HYDROLYSIS OF 5-FLUOROURIDYLIC ACID

Tube nos.	Counts/min/ml	μmoles/ml	Counts/min/μmoles	Total counts/min
		Liver		
86– 93	200	.028	7,140	16,000
94– 97	550	.081	6,770	22,000
98–101	590	.080	7,400	23,700
102–110	270	.038	7,050	24,100
				85,800
		Ascites cells		
79– 87	1,400	.057	24,800	110,000
88– 91	2,190	.085	25,900	72,400
92– 95	1,750	.074	23,800	64,800
96–105	650	.029	22,500	51,200
				298,400

It became of interest to compare the specific activities of the nuclear and cytoplasmic RNA's following administration of the labeled drugs, and, because of the difficulty of homogenizing Ehrlich ascites cells, the studies were carried out in mice bearing Sarcoma 180. The relative specific activities of the nuclear and cytoplasmic RNA samples obtained at 2 and 12 hours after administration of labeled 5-fluorouracil and 5-fluoroorotic acid are shown in Table 6. The usual finding that from other precursors at early times the nuclear RNA has a higher specific activity than the cytoplasmic (cf. 27), and that there is less difference at a later time, was also observed with these compounds. Samples of hydrolysates of RNA from Sarcoma 180 were also shown to contain fluorouridylic acid as the only radioactive component.

TABLE 6

RELATIVE SPECIFIC ACTIVITIES OF LIVER AND SARCOMA 180 NUCLEAR AND CYTOPLASMIC RNA

Tissue	Time after admin. (hours)	Compound	Ratio of nuclear/cytoplasmic RNA*
Liver	2	5-Fluorouracil	2.3
"	2	5-Fluoroorotic	2.6
"	12	5-Fluorouracil	1.6
Tumor	2	5-Fluorouracil	4.1
"	2	5-Fluoroorotic	2.5
"	12	5-Fluorouracil	0.63

* $\frac{\text{Counts/min/}\mu\text{g nuclear RNA}}{\text{Counts/min/}\mu\text{g cytoplasmic RNA}}$. The relative specific activity of the cytoplasmic RNA is, therefore, 1.0 in all cases.

Some information appeared to be desirable on the nature of the RNA containing a fluorinated nucleotide. That the abnormal nucleotide was not merely attached to RNA chains in the terminal position is proved by the fact that the radioactivity in the hydrolysate was almost entirely found as the nucleotide; if it were terminal, the label would have been only in the nucleoside fraction (cf. 3, 17). A sample of tumor cytoplasmic RNA from the 12-hour experiment was fractionated on an ECTEOLA column,[1] by elution with increasing concentrations of NaCl. As shown in Chart 6, two peaks, of ultraviolet-absorbing material were obtained. The radioactivity closely followed the nucleic acid, and a constant specific activity was found within each peak. Moreover, the mean specific activities in the first and second peak were 715 and 723 counts/min/E_{260-1}. Similar results were obtained by ECTEOLA fractionation of the liver cytoplasmic RNA. The material eluted from the column in the large peaks was dialyzed extensively against 2 M NaCl, a treatment in which oligonucleotides become dialyzable (17). The recovery of 96 and 74 per cent of the radioactivity within the dialysis bags from tumor and liver shows that the 5-fluorouracil was present in polymerized RNA. Although, with our present state of knowledge, interpretation of experiments such as these is difficult and hazardous, the results suggest that the 5-fluorouracil may be randomly distributed throughout the RNA molecule.

[1] We are indebted to Dr. Aaron Bendich for this sample of resin.

Following the above experiments, which were almost all carried out for an interval of 12 hours, a time at which nucleic acid specific activities following labeled precursors is usually at its maximum (cf. 27), a series of experiments was carried out to determine the time course of the incorporation of 5-fluorouracil and 5-fluoroorotic acid into the RNA of the livers and tumors of mice bearing Sarcoma 180 subcutaneously and the Ehrlich ascites carcinoma. Each mouse was given a dose intraperitoneally, equimolar to 200 mg/kg of fluorouracil (specific activities: FU = 4.1 μc/mg; FO = 3.0 μc/mg), and the groups were sacrificed at 1, 4, 12, and 24 hours after injection. The specific activities of the RNA samples in counts/min/mg RNA (orcinol reaction) are shown in Chart 7. It is evident that, in all tumor samples, the maximum specific activity was reached at 4 hours, after which it decreased. There was a higher specific activity in the RNA from the ascites cells than from Sarcoma 180, which might be expected because of the intraperitoneal injection, and higher specific activities in both tumor RNA's were obtained from 5-fluorouracil than from 5-fluoroorotic acid.

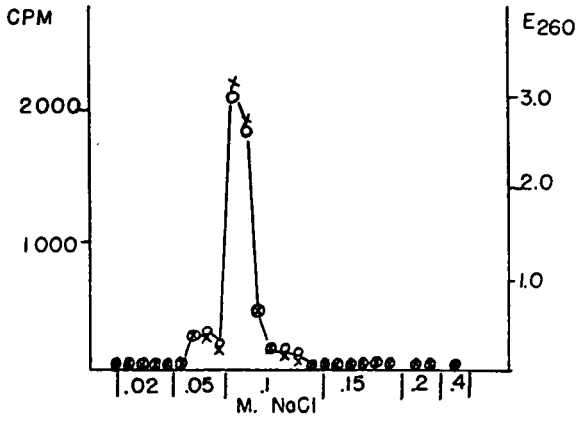

CHART 6.—ECTEOLA fractionation of labeled RNA. Counts/min (X) and E_{260} (O) against concentration of eluting NaCl.

An attempt has been made to calculate the actu-

al amount of 5-fluorouracil incorporated into the RNA, expressed as a percentage of the amount of uracil present. The uracil content of liver and the tumors, per mg. RNA orcinol, was determined and is 0.5 μmoles/mg in liver and 0.6 μmoles/mg in the tumors. The amount of 5-fluorouracil incorporated can be calculated from the radioactivity in the RNA at 4 hours and from the specific activity of the administered dose, since there is no endogenous 5-fluorouracil to dilute the samples. The values turned out to be, in liver, Sarcoma 180, and the Ehrlich ascites tumor: 0.0032, 0.021, and 0.035 μmoles/mg RNA, respectively, which correspond to the following percentages of the uracil content: 0.64, 3.5, and 6.1. It is of course impossible with the information now at hand to determine whether 5-fluorouracil replaced uracil, since the incorporation was so small. This is in contrast to the findings that 5-bromouracil in microbial systems can replace up to 50 per cent of the DNA thymine (29).

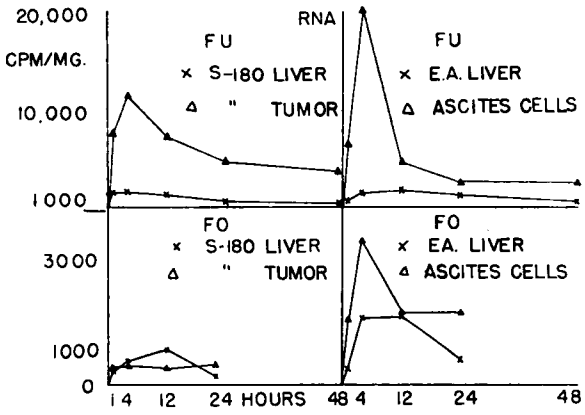

CHART 7.—Specific activities of RNA's from liver and tumors following injection of 5-fluorouracil and 5-fluoroorotic acid against time.

Acid-soluble nucleotides.—The profile of radioactivity and ultraviolet light absorption of an extended gradient ion-exchange chromatogram (13) of the acid-soluble fraction of Ehrlich ascites cells 90 minutes after 5-fluorouracil-2-C^{14} administration is shown in Chart 8. An almost identical chromatogram was obtained by Drs. Bollum and Potter of the McArdle Laboratory following incubation of 5-fluoroorotic-2-C^{14} acid with liver slices (private communication). The first small peak of radioactivity is due to nucleoside material, the second peak is unchanged fluorouracil, and the third peak is as yet uncharacterized. The largest peak of radioactivity is closely analogous to the 5-fluorouridylic acid obtained by alkaline hydrolysis of RNA, but, since it is in the acid-soluble fraction, it is most probably 5-fluorouridine-5'-phosphate, and is labeled FUMP. Insufficient material has been obtained of all these substances to permit quantitative comparisons of base and phosphate, but, by their location on the chromatogram, their identification and degree of phosphorylation are established by their analogous behavior to the uridine nucleotides and the fact that all four compounds gave 5-fluorouracil on perchloric acid hydrolysis. Therefore, these four peaks can be identified with considerable probability as FUMP, 5-fluorouridine diphosphate glucose (or other sugar), FUDP, and FUTP. Thus it is clear that 5-fluorouracil is converted *in vivo* into nucleotides of all three levels of phosphoryla-

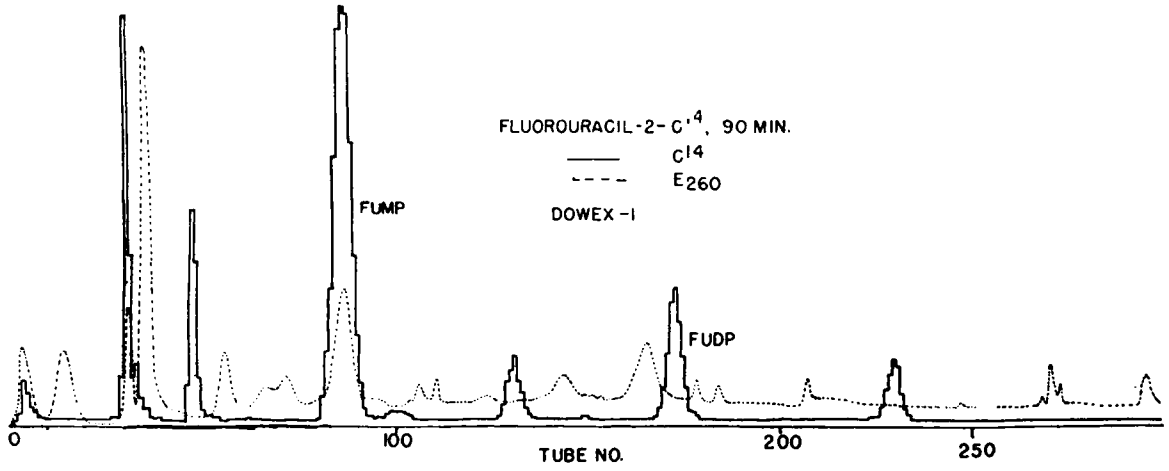

CHART 8.—Acid-soluble chromatograms from Ehrlich ascites cells following administration of labeled 5-fluorouracil. The solid line denotes radioactivity, the dashed line represents ultraviolet absorption at 260 mμ. This is a schematic representation of several experiments, so no specific units are given.

tion, just as is uracil (11). Furthermore, it will be shown (2) that, in suspensions of Ehrlich ascites cells, 5-fluoro-2′-deoxyuridine-5′-phosphate is also produced from 5-fluorouracil.

Quantities of 5-fluorouracil in samples from the human patient.—The samples of urine, plasma, and the 12-hour tumor sample from the cancer patient were analyzed for 5-fluorouracil, and the results are given in Table 7. To each sample was added carrier 5-fluorouracil, and the amount of radioactivity was determined following ion-exchange chromatography on Dowex-1 chloride. It will be noted that in the urine voided 40 minutes after the injection, the majority of the radioactivity was present as 5-fluorouracil. However, no unchanged drug was present in the 24-hour urine. In the 1-hour plasma, one-third of the radioactivity was present as 5-fluorouracil. In the human tumor sample, just as in mouse tumors, 5-fluorouracil was incorporated as such in nucleotide form into the RNA, but not into the DNA. Studies are currently in progress aimed at the elucidation of the degradative pathways of 5-fluorouracil catabolism in various tissues and the indentification of the urinary metabolites.

TABLE 7

FRACTIONATION OF RADIOACTIVITY IN HUMAN PATIENT GIVEN 5-FLUOROURACIL-2-C^{14}

Sample	Total counts/min in sample	Total counts/min as FU	Per cent FU
Urine, 40 min.	102,000	77,500	76
Urine, 24 hours	90,000	0	0
Plasma, 1 hour	4,700	1,500	32
Tumor, 12-hour acid-soluble	4,700	1,800	38
Tumor, 12-hour DNA	0		
Tumor, 12-hour RNA	3,600	3,500	97

DISCUSSION

The most distinctive observation about the tissue distribution of 5-fluorouracil is that it or its metabolites are, at least to some degree, selectively localized in Sarcoma 180 when injected at a distant site. The literature is replete with attempts to find compounds that are selectively taken up by tumors. Reid and Jones (22) observed no concentration of labeled tyrosine in a transplanted mouse melanosarcoma, and Reid and Weaver found no selective uptake of stilbamidine in mouse lymphoid tissue (23). Likewise, no tumor localization of OPSPA, a typical alkylating agent, was found by Maller *et al.* (14, 15) either in the Flexner-Jobling rat carcinoma or in human metastatic breast carcinomas. Sloviter (25) has studied a radioiodine-labeled dye, Nile blue 2B, and, although there was no selective localization of radioactivity in mouse tumors, nevertheless there was a significantly greater prolongation of life of mice treated with the labeled dye than of those treated with nonlabeled dye, suggesting that there was some measure of radiation therapy delivered to the tumor by the dye. We are not aware of any other demonstration of selective localization of a chemotherapeutic agent in tumors. However, the degree of selectivity is not great, since, at 12 hours following 5-fluorouracil administration, the tumor has only about 3 times the specific activity of the intestine and bone marrow in mice. In the human patient the specific activity of the tumor biopsy was 1.2 times that of liver; in the case of OPSPA the same ratio in the human was only 0.21 (15).

The conversion of 5-fluorouracil and 5-fluoroorotic acid into the acid-soluble fluorouridine nucleotides at the mono-, di-, and triphosphate level has been demonstrated and is entirely analogous to the behavior of uracil and orotic acid. Thus, it is not surprising that both compounds are incorporated into RNA in mouse liver, spleen, Sarcoma 180, Ehrlich ascites carcinoma, and a human metastatic carcinoma. Although it has been shown that 5-fluorouracil is converted *in vitro* in suspensions of Ehrlich ascites carcinoma cells into 5-fluoro-2′-deoxyuridine monophosphate (2), the compound is *not* incorporated into DNA, in contrast to the finding with the other halogenated pyrimidines (29). Whether the lack of incorporation into DNA results from the great difference in size between the fluorine atom and the methyl group of thymine (Van der Waal's radii 1.35 and 2.0 A, respectively [20]) with correspondingly unfavorable steric configuration, or from a lack of production of 5-fluorodeoxyuridine triphosphate, presumably required for synthesis of DNA, is not known.

It will be noted from Chart 7 that the incorporation into the RNA reached a maximum at 4 hours and then rapidly declined. This suggests a rather high turnover rate for the process, although what little data there are also suggest that the 5-fluorouridine nucleotides are randomly distributed throughout the RNA molecule. The actual amount incorporated was small, never exceeding a quantity corresponding to 6 per cent of the uracil content. Whether this relatively small amount of "fraudulent" ribonucleic acid could account for the toxic and tumor-inhibitory properties of these fluorinated pyrimidines cannot be decided at present. Thus, these compounds join the nucleic acid analogs, listed in the introduction, which are incorporated into cellular nucleic acids.

A number of stimulating and persuasive theo-

retical discussions have been published (cf. 26, 29) dealing with possible mechanisms whereby the "fraudulent" nucleic acids resulting from the incorporation of unnatural analogs of the purine or pyrimidine bases are considered to cause the growth inhibition or mutagenesis generally encountered; it would be fruitless as well as presumptive to embellish them here. The uncomfortable fact remains that we do not know in most cases whether unnatural nucleic acids actually produce the observed biological effects, much less how or why they accomplish this.

The effects of various fluorinated pyrimidines on nucleic acid biosynthesis will be described in accompanying papers (2, 5).

SUMMARY

1. The excretion, distribution, and metabolism of 5-fluorouracil-2-C^{14} and 5-fluoroorotic-2-C^{14} acid have been studied in normal and tumor-bearing mice and in a human cancer patient.

2. Both compounds were rapidly excreted in the urine. 5-Fluorouracil was excreted unchanged shortly after injection. At later times several as yet uncharacterized metabolites were excreted. The radiocarbon appeared much more extentively in the respiratory carbon dioxide following 5-fluorouracil injection than after 5-fluoroorotic acid administration.

3. At all times after intraperitoneal injection of 5-fluorouracil into mice bearing Sarcoma 180, a higher specific activity was found in the tumor than in all other tissues investigated. In the cancer patient the tumor biopsy had a higher specific activity than surrounding skin, muscle, fat, and a liver biopsy. No selective localization of radioactivity in tumor occurred with labeled 5 fluoroorotic acid.

4. 5-Fluorouracil and 5-fluoroorotic acid were converted into acid-soluble fluorouridine nucleotides at the mono-, di-, and triphosphate levels, and were incorporated into RNA, but not DNA, in mouse liver, spleen, Sarcoma 180, Ehrlich ascites carcinoma, and a human metastatic carcinoma. The incorporation into RNA at most represented a quantity equivalent to 6 per cent of the uracil content. 5-Fluoroorotic acid was decarboxylated and was present in the RNA as 5-fluorouracil in nucleotide linkage, most probably distributed randomly throughout the macromolecule.

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