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EFFECT OF THYMINE RIBONUCLEOSIDE ON THE METABOLISM OF ^{125}I-5-IODO-2′-DEOXYURIDINE*

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Abstract—Thymine ribonucleoside affects the urinary excretion pattern and tissue distribution of ^{125}I-labeled 5-iodo-2′-deoxyuridine (IUdR) and its metabolic derivatives in mice. In the absence of thymine ribonucleoside, 90–95 per cent of the radioactivity administered is excreted in the urine as iodide.

A tenfold molar excess of thymine ribonucleoside, administered 30 min before the injection of ^{125}I-IUdR does not alter the pattern of excretion but retards the amount of radioactivity excreted in the first 2 hr. A slight inhibition of the incorporation of IUdR into DNA also occurred at this level.

On the other hand, a 100-fold excess of thymine ribonucleoside increased, during the first 2 hr, the urinary excretion of total radioactivity, which was composed mainly of ^{125}I-iodouracil, as well as unchanged ^{125}I-IUdR. The distribution of radioactivity among various tissues after the administration of a 100-fold excess of thymine ribonucleoside indicated a 2·5- to 3-fold greater uptake of radioactivity into the liver, heart, kidney, and muscle. The uptake was altered little or not at all in adrenals or in tissues that show a normal high rate of division, such as small intestine, spleen, and bone marrow. There was no increase in the utilization of IUdR for the biosynthesis of DNA.

5-IODO-2′-DEOXYURIDINE (IUdR)[1] is incorporated readily into the DNA of mammalian cell, bacteria, and viruses (cited in Ref. 2). Conditions that permit extensive incorporation of IUdR into DNA result in greater effectiveness of this compound in inhibition of neoplastic and viral reproduction, in sensitization of cells to the lethal effects of ultraviolet and X-radiations, and in affording a marker for studies of the biosynthesis of DNA, cell turnover, and localization of neoplastic growth. Investigations designed to attain these objectives have been reviewed.[2] These include increasing the amount and frequency of administered IUdR;[3] co-administration of amethopterin,[4] 5-fluoro-2′-deoxyuridine (FUdR)[5] or 5-iodouridine;[4] use of an oil emulsion of IUdR to provide a depot;[4] and alteration of the structure to the corresponding 2′-deoxycytidine derivative.[6, 7] The rapid cleavage of IUdR to 5-iodouracil limits in part the utilization of IUdR for the biosynthesis of DNA. Inhibition of the catabolic enzyme responsible would afford an opportunity to minimize this degradative influence.

The inhibitory action of a number of sugar-containing analogs of thymidine on preparations of nucleoside phosphorylase has been examined previously, and, of these, the most effective found *in vitro* is thymine ribonucleoside.[8, 9]

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The present report is an investigation of the influence of thymine ribonucleoside on the tissue distribution and uptake of ^{125}I-IUdR into the DNA, cold acid-soluble. and protein fractions of liver, spleen, small intestine, and a transplanted hepatoma, The pattern of excretion in urine of radioactive metabolites of IUdR, after the administration of various amounts of thymine ribonucleoside, also is described.

MATERIAL AND METHODS

Mice

Female mice of C3H strain, weighing approximately 20 g and fed Purina chow and water *ad libitum*, were used.

Materials used

^{125}I-IUdR prepared by methods previously described[1, 9] had a specific activity of 1·58 mc/m-mole. Thymine ribonucleoside was prepared by Dr. P. K. Chang of this department, to whom the authors are grateful.

Metabolic measurements

The animals were placed in a common cage with ample water but without food for a period of 2–3 hr before the experiment, to allow defecation due to stress, etc. A simplified procedure for use with three mice was employed for the collection of urine during periods of 1 hr. The animals were injected i.p. with a solution of ^{125}I-IUdR (0·33 mg/ml) in 0·9% NaCl (0·5 ml) and placed immediately in metabolic beakers (standard 800-ml Pyrex beakers with a hole in the edge of the base to facilitate removal of urine). Two sections of wire mesh were arranged so that the lower one was flat and about 1 cm from the base, and the upper was convex upwardly. The latter allowed the animals to urinate through the mesh, but any fecal matter rolled down the side to the edge, thereby reducing contamination of the urine; the continuous movement of the animals facilitated this process. The inside of the beaker and the whole mesh were siliconized by immersion in a solution of 5% silicone in chloroform followed by drying at room temperature. In our experience, the fecal matter was separate and dry at the end of each hour, while the urine formed discrete globules of clear liquid that could be decanted through the orifice of the beaker or taken up in a Pasteur pipette. By slight abdominal pressure, the mice could be stimulated to void any urine accumulated in the bladder. The beaker and mesh were washed with distilled water at the end of 1 hr, while the animals were transferred to a new metabolic beaker. The samples of urine were frozen in a dry ice–alcohol bath and kept at $-20°$ until analyzed.

Urine analysis

The total radioactivity excreted in the urine of each mouse was measured by taking an aliquot of the urine, diluting to 3·0 ml, and counting in a well-type gamma scintillation spectrometer (NaI, Tl). The distribution of radioactive components was assessed by thin-layer chromatography with silica gel HF as the absorbent and isopropanol : chloroform (20 : 80 v/v) as the solvent.* This system permits an excellent separation of iodide, iodouracil, and IUdR, with R_f values of 0·08, 0·37, and 0·69 respectively.*

* We are grateful to Dr. J. W. Shell of the Allergan Laboratories for making available to us this excellent chromatographic method.

Tissue distribution

Animals were killed with chloroform 24 hr after the injection of ^{125}I-IUdR and the tissues were removed, dried on filter paper, and placed in preweighed tubes. After reweighing, 2 ml of 10 N NaOH was added, and the tissues were dissolved by heating in a boiling water bath. After most of the tissue had dissolved, the volume was adjusted to 3 ml, and the content of radioactivity was determined as described above.

RESULTS

Rate of excretion

The results of seven control experiments performed concurrently with the treated series discussed below are shown in Table 1.

TABLE 1. URINARY EXCRETION OF ^{125}I AFTER THE INTRAPERITONEAL ADMINISTRATION TO MICE OF ^{125}I-IODODEOXYURIDINE

Time (hr)	1	2	3	4	5
Per cent excreted	21 ± 4·3*	34 ± 5·7	45 ± 4·6	54 ± 1·6	56 ± 1·6

* Standard deviation. Values are expressed as percentage of administered dose of ^{125}I-iododeoxyuridine (8·3 mg/kg).

Effect of thymine ribonucleoside

Various amounts of thymine ribonucleoside in 0·9% NaCl were administered parenterally to mice 30 min before a single injection of ^{125}I-IUdR. Samples of urine were taken hourly and the total content of radioactivity determined; the results are

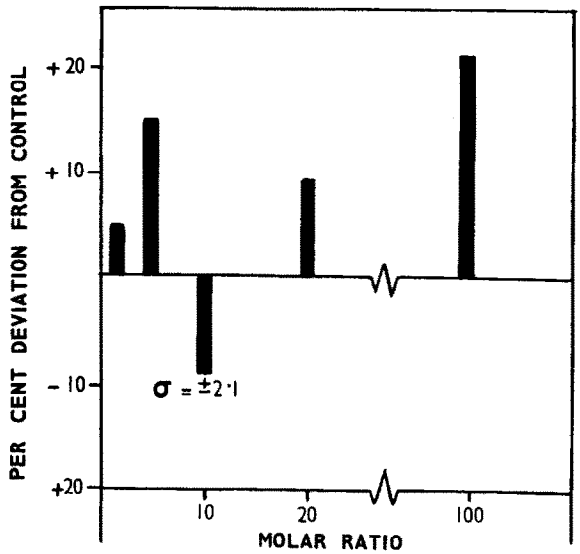

FIG. 1. The effect of various molar ratios of thymine ribonucleoside to IUdR on the excretion of radioactivity in the urine within 1 hr after the i.p. administration to mice of ^{125}I-IUdR (0·3 μmole).

shown in Fig. 1. At low molar ratios of thymine ribonucleoside to IUdR, increased excretion of radioactivity during the first 1-hr period was observed. A 10-fold molar excess of thymine riboside resulted in a decreased excretion of radioactivity during the first hour in two experiments, giving excretion values of 11 and 13 per cent of the administered dose, respectively. A third experiment in which nine animals were used instead of three, confirmed this initial low rate of excretion. The amount of administered radioactivity excreted in the urine within 2 hr was 19 $\pm$ 2·1% for the animals that received the 10-fold molar excess of thymine ribonucleoside, and 34 $\pm$ 5·7% for the control series. A 20-fold and to a much greater extent a 100-fold molar excess of thymine ribonucleoside increased the excretion of radioiodine.

The results of the chromatographic analysis of some of these urine samples are shown in Fig. 2. The major component excreted during the first hour in the urine of

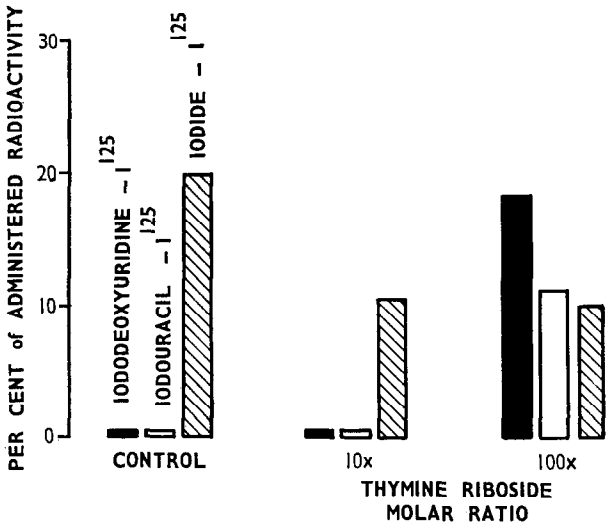

FIG. 2. The effect of a 10- and 100-fold molar excess of thymine ribonucleoside on the distribution of radioactive components in urine within 1 hr after i.p. administration to mice of ^{125}I-IUdR (0·3 μmole).

the control series is iodide. A 10-fold molar excess of thymine ribonucleoside produced no appreciable change in the composition of the urine, even though the amount of radioiodide excreted in the first hour is markedly less. This accounts for the difference observed in the total amount of activity excreted (Fig. 1). A 100-fold molar excess of thymine ribonucleoside resulted, however, in a marked change in the amount of IUdR and iodouracil excreted. Thus, the increased radioactivity excreted in the 1-hr sample in the latter situation is composed of nonmetabolized IUdR and the first catabolic product—iodouracil.

The effect of a 100-fold molar excess of thymine ribonucleoside administered 30 min before the injection of ^{125}I-IUdR on the distribution of radioactivity in various tissues is shown in Table 2. Thymine ribonucleoside resulted in a greater uptake of radioactivity in most tissues, particularly the lung and body fat. The uptake in liver,

heart, kidney, and muscle was 2·5 – 3·0 times greater than the control after pretreatment with thymine ribonucleoside, whereas the uptake in adrenals, small intestine, spleen, and bone marrow was essentially unchanged.

TABLE 2. THE EFFECT OF A 100-FOLD MOLAR EXCESS OF THYMINE RIBONUCLEOSIDE ON THE DISTRIBUTION IN THE TISSUES OF MICE OF RADIOACTIVITY DERIVED FROM ^{125}I-IODODEOXYURIDINE INJECTED INTRAPERITONEALLY

Tissue	Control	Treated	Ratio treated/control
Liver	778*	1930	2·5
Heart	563	1510	2·7
Lung	1195	5340	4·5
Adrenal	1055	1365	1·3
Kidney	795	2390	3·0
Body fat	349	1750	5·0
Small intestine	9000	12,900	1·5
Muscle	270	800	3·0
Spleen	7100	8490	1·3
Bone marrow	34,700	30,900	0·9

* Counts per minute per gram wet weight of tissue. Thymine ribonucleoside (557 mg/kg) was given i.p. 30 min before the administration of ^{125}I-iododeoxyuridine (8·3 mg/kg). Distribution of radioactivity was determined 23 hr later.

The administration of a 10-fold molar excess of thymine ribonucleoside resulted in a slightly diminished uptake of ^{125}I-IUdR into DNA, as measured by the procedure of either Schneider[10] or Kirby,[11, 12] and a slight increase of radioactivity in the low-molecular weight fraction (cold acid-soluble fraction) (Table 3).

TABLE 3. THE EFFECT OF A 10-FOLD MOLAR EXCESS OF THYMINE RIBONUCLEOSIDE ON THE INCORPORATION OF RADIOACTIVITY DERIVED FROM ^{125}I-IODODEOXYURIDINE IN VARIOUS FRACTIONS OF TISSUES FROM MICE

	Control			Treated		
Tissue	DNA	Acid-soluble	Protein	DNA	Acid-soluble	Protein
Liver	80*	2380	185	32	2910	256
Spleen	3900	1500	346	2850	2500	341
Small intestine	2950	645	92	2200	850	94

* Values are expressed as cpm/g wet tissue, corrected to an administered dose of 100,000 cpm per mouse. Thymine ribonucleoside was given i.p. 30 min before the i.p. administration of ^{125}I-iododeoxyuridine (8·3 mg/kg); the distribution of radioactivity was measured 18 hr later.

DISCUSSION

The availability of a greater amount of IUdR for the biosynthesis of DNA in mammalian cells should be obtained by a reduction in the activity of the catabolic enzymes responsible for the cleavage of 5-iodo-2′-deoxyuridine to iodouracil. Preliminary studies[8, 9] have shown that thymine ribonucleoside is an effective inhibitor

of the nucleoside phosphorylase responsible for the conversion of IUdR to iodouracil. The kinetics of this inhibition will be described elsewhere.

Thymine ribonucleoside was first found to occur naturally in various preparations of RNA by Littlefield and Dunn.[13] Subsequent studies by many investigators have shown that methylated bases are found in transfer RNA (s-RNA) and that the methyl group is derived from S-adenosylmethionine and incorporated into the s-RNA by direct methylation of the purine or pyrimidine component by a specific methylating enzyme (cited in Ref. 14).

Thymine ribonucleoside (1-β-D-ribofuranosyl thymine) synthesized by Fox *et al.*[15] was shown to be identical in properties with that prepared enzymically by Lampen.[16] Other synthetic preparations of thymine ribonucleoside have been described.[17, 18] The biosynthesis of the 5'-phosphate of thymine ribonucleoside also has been described,[19, 20] as well as its formation via a pyrophosphorylase reaction.[21]

5-Methyluridine, not identical in properties with thymine ribonucleoside prepared enzymically, was synthesized by Roberts and Visser[22] and shown neither to stimulate nor to inhibit the growth of *Neurospora* 1298 grown in the presence of suboptimal concentrations of uridine, cytidine, or uracil.[23] However, the stimulation of the growth of *Neurospora* by uridine or cytidine in a medium supplemented with uracil was inhibited by 5-methyluridine.[22] Thymine ribonucleoside inhibited the reproduction of *Escherichia coli* 15/r grown either in the presence or the absence of thymidine and, under conditions of normal growth, thymine ribonucleoside was slowly degraded.[24] The cleavage of the nucleoside also has been demonstrated by bacterial enzymes derived from *Thermobactor acido philus*[25] and *Lactobacillus delbrueckii*.[26]

Friedkin and Roberts[27] demonstrated the enzymic formation of thymine ribonucleoside, using pyrimidine nucleoside phosphorylase of horse liver; the compound also has been formed by the incubation of thymine with either a homogenate or slices of rat[28, 29] or mouse[30, 31] liver.

The effect of prior administration of thymine ribonucleoside on the metabolism of ^{125}I-IUdR has been studied in mice. The total radioactivity excreted in the urine during the first 2 hr after the injection of ^{125}I-IUdR is dependent on the molar ratio of thymine ribonucleoside to IUdR administered. At the lower molar ratios of 2 or 5, there is an increase in the amount of urinary excretion of radioactivity, whereas at a 10-fold molar excess there is a marked decrease. Nevertheless, the composition of the urine is similar to the control urine in that radioiodide comprises the main fraction of radioactivity (Fig. 2). However, an increase in the molar ratio to 20 or 100 not only results in an increase in the excretion of radioactivity during the first 2 hr but the pattern of excretion is altered in that the urine now contains an appreciable amount of unchanged IUdR as well as the initial catabolic product—iodouracil. These findings suggest that high molar ratios of thymine ribonucleoside to IUdR result in: (a) a block in transport of IUdR into the cell, thereby limiting the susceptibility of IUdR to catabolism; (b) an inhibition of the enzyme responsible for cleavage of IUdR; or (c) a diuretic action of thymine ribonucleoside, causing a more rapid removal of IUdR and iodouracil from the circulation and thereby limiting the amount of IUdR available for cell transport and subsequent phosphorylation. The metabolism of ^{125}I-IUdR was studied in the mouse and, although thymine ribonucleoside increases the uptake of radioactivity into many tissues, the increase is less marked in such normally rapidly dividing tissues as small intestine and bone marrow (Table 2).

More important, however, thymine ribonucleoside has not increased the amount of incorporation of ^{125}I-IUdR into the DNA but merely altered the amount of ^{125}I-IUdR present in the intracellular fluids.

Attempts to inhibit the cleavage of deoxyribonucleoside analogs have been made in several laboratories. Birnie *et al.*[32] observed that a 10-fold excess of fluorouracil inhibited the degradation of 5-fluoro-2′-deoxyuridine by 65 per cent, and a 3-fold greater molar concentration of 6-azathymidine exerted a degree of inhibition similar to fluorouracil.[32] Since deoxyuridine phosphorylase is inhibited by fluorouracil,[33] FUdR[33] and uridine,[34] a study was made by Birnie *et al.*[32] of the effect of uridine on the clinical efficacy of FUdR in mice *in vivo*. A large excess or urdine resulted in an increased toxicity of FUdR, a finding attributed to an inhibition of the metabolic inactivation of fluorouracil by uracil derived from cleavage of the administered uridine.[32] 5-Trifluoromethyl-2′-deoxyuridine[32, 35] and 5-allyl-2′-deoxyuridine[36] have been reported to inhibit pyrimidine nucleoside phosphorylase *in vitro*, and to comparable extents.[37] 5-Bromo-2′-deoxyuridine and IUdR were more effective than 5-trifluoromethyl-2′-deoxyuridine as inhibitors of the phosphorolysis of IUdR by an extract of mouse liver.[38] Cihak and Sorm[39] reported that 5-azauracil inhibited the cleavage of deoxyuridine by an enzyme preparation from mouse liver; however, inhibition of the cleavage of FUdR by even an 8-fold excess of 5-azauracil did not occur.[37] Thyminedeoxyglucoside was observed by Langen and Etzold[40] to inhibit competitively pyrimidine nucleoside phosphorylase derived from Ehrlich ascites tumor cells; however, the enzyme(s) present in horse liver or human tissues were not affected.[41] This latter finding has been attributed to the presence in the insensitive tissues of a single protein possessing a sensitive and an insensitive site, both capable of cleaving deoxyribonucleosides;[33] however, two enzymes possessing deoxyribonucleosidase activity have been reported to be present in mammalian tissues, of which only one is inhibited by thymine deoxyglucoside.[42, 43]

Yamada[44] has observed that either uridine or 6-azauridine can increase uridine and deoxyuridine phosphorylase activities in slices of regenerating rat liver *in vitro*. The ability of puromycin to inhibit this increase[44] implies that new enzyme is being formed. Similar induction of thymidine phosphorylase activity in bacterial systems also has been observed.[45, 46] One wonders, therefore, whether the administration of IUdR also could induce its own destruction by causing an increase in phosphorylase activity. Thus, the finding of an effective inhibitor of this enzyme is still a very important problem.

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