during hypoxia, which impairs, stimulates or does not alter the release of acetylcholine, glutamate or norepinephrine respectively ([9]; J. A. Hirsch and G. E. Gibson, *Neuchem. Res.*, in press). The ineffectiveness of the thiamin antagonists on the potassium-stimulated release of norepinephrine at either concentration suggests that thiamin is integrally involved in acetylcholine release and metabolism [15].

These *in vitro* responses to thiamin antagonists contrast with those observed in chronically treated animals. Thus, acetylcholine release decreases in superior cervical ganglia of thiamin-deprived rats [16], and whole brain acetylcholine synthesis *in vivo* declines [17]. Similarly, the lack of an effect on potassium-stimulated norepinephrine release is not directly comparable to the increase in catecholamine levels in brain cortex during thiamin deficiency [18]. Whether these *in vitro* pharmacological effects are distinct from their *in vivo* actions or if these apparent differences are a matter of acute versus chronic treatment remains unclear.

The elevation of electrically-stimulated norepinephrine release by pyrithiamin was unexpected. The increase in release may reflect the method of stimulation (i.e. electrical vs potassium), the duration of exposure of the tissue to the drug (i.e. treatment before or only during stimulation), or the tissue preparation (rat cortical slices vs whole brain prisms from mice). Other drugs also alter the electrically- or potassium-induced stimulation of the calcium-dependent norepinephrine release differently [12, 14]. At higher concentrations, pyrithiamin may have more general effects such as inhibition of norepinephrine reuptake, which would enhance the electrically-stimulated, but not potassium-stimulated, release of norephinephrine from brain slices [12]. Nevertheless, pyrithiamin may be an effective tool to distinguish between release mechanisms during electrical and potassium stimulation.

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Effect of an inhibitor of nucleoside transport on the disposition of uridine in mice*

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p-Nitrobenzylmercaptopurine ribonucleoside (NBMPR) is a potent inhibitor of nucleoside transport in various animal cells *in vitro* [1] and alters the disposition and toxicology of some nucleoside analogs *in vivo* [2, 3]. Kolassa and Paterson [4] have shown that prior treatment of mice with NBMPR-P, the 5′-monophosphate of NBMPR, effectively blocks the uptake of cytidine into mouse liver during perfusion *in situ*. NBMPR-P has been employed as a readily soluble, prodrug form of NBMPR from which the latter is released by phosphohydrolase activity; NBMPR-P *per se* appears to have little activity as an inhibitor of nucleoside transport.† On the basis of these results, it seemed possible that NBMPR-P could be used to prevent the utilization of circulating uridine by various tissues and tumors. The current report examines the effect of NBMPR-P on the metabolism of intravenously injected [^{3}H]uridine.

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† P. O. J. Ogbunude, W. P. Gati and A. R. P. Paterson, manuscript submitted for publication.

Materials and methods

NBMPR-P was prepared as the disodium salt [3] by the laboratory of the Yamasa Shoyu Co., Chosi, Japan. [^{3}H]Uridine, 25 Ci/mmole, was purchased from Moravek Biochemicals, Brea, CA, and phosphodiesterase I, type VII, from the Sigma Chemical Co., St. Louis, MO.

To measure the distribution of radioactivity after injection of [^{3}H]uridine, tissues were rapidly removed and frozen in liquid nitrogen until processed further. The remaining organs along with the skin were also frozen and

processed as the "carcass". The tissues were weighed and homogenized in 8–10 vol. of cold 0.3 M perchloric acid, using a motor driven glass–Teflon homogenizer. The frozen "carcass" was pulverized and then homogenized with a Polytron homogenizer. The acid-soluble fraction was neutralized with a (1:3) solution of tricaprylyl tertiary amine (Alamine 336) in 1,1,2-trichlorotrifluoroethane (Freon) [5] and then stored at −20° until analyzed by chromatography. The acid-insoluble fraction was washed twice with cold 0.3 M perchloric acid, then suspended in 0.3 M KOH and incubated at 37° overnight to hydrolyze quantitatively RNA to acid-soluble nucleoside monophosphates [6]. The sample was then chilled, made 0.6 M in perchloric acid, and the precipitate removed by centrifugation; radioactivity in the soluble fraction was determined as a measure of label in RNA.

The nucleotides in the neutralized acid-soluble extracts were hydrolyzed to 5′-monophosphates by incubation at 37° for 10 min with an equal volume of phosphodiesterase solution containing 0.05 units/ml in 0.05 M Tris–acetate (pH 7.8) and 2 mM magnesium chloride. The samples were then placed in a boiling water bath for 1 min, chilled to 0°, and then centrifuged to remove the protein. Control experiments indicated that this procedure resulted in essentially complete (>98%) conversion of nucleoside triphosphates to the corresponding 5′-monophosphates. Radiolabeled uracil nucleotides (as 5′-UMP) were determined by chromatography of the hydrolyzed extracts on a Whatman SAX column, 25 cm × 4.6 mm. The column was eluted at 2 ml/min with 0.03 M ammonium phosphate adjusted to pH 3.3 with phosphoric acid. Under these conditions, UMP had a retention time of 4.0 min and was clearly resolved from all other detectable radioactive or ultraviolet-absorbing compounds. Fractions (1 ml) were collected and their radioactivities were measured by liquid scintillation counting at an efficiency of 28%.

Results and discussion

Tracer amounts of uridine are metabolized quickly by the mouse, as reported earlier for the rat [7]. Preliminary experiments revealed that no labeled free nucleoside could be detected in plasma or tissues 30 min after i.v. injection of [^{3}H]uridine; all injected material had been either catabolized or converted to nucleotides. Thus, in evaluating the effect of NBMPR-P on the metabolism and distribution of uridine in the mouse, a 30-min post-injection interval was chosen.

Kolassa and Paterson have shown previously that NBMPR-P treatment of mice, the livers of which would later be perfused *in situ*, reduces cytidine uptake in the perfused organ. Intraperitoneal NBMPR-P doses of *ca.* 1 mg/kg result in maximal inhibition of cytidine uptake during perfusion [4]. The optimal dose, however, in terms of therapeutic effect, is about 25 mg/kg when mice bearing leukemia L1210 are treated with high doses of nebularine, coadministered with doses of NBMPR-P that protect the host [3]. In the present experiments, an NBMPR dose of 25 mg/kg was chosen to evaluate the effect of this drug on the uptake of uridine *in vivo*.

The results shown in Table 1 indicate that in control animals appreciable amounts of radioactive nucleotides were formed in all tissues examined, with label in free nucleotides plus nucleotides in RNA constituting 47–65% of the total tissue radioactivity. Thus, the efficiency of salvage, i.e. nucleotide formation, from uridine was substantially higher in the mouse than in the rat [7]. This difference is conspicuous when salvage by the liver is compared in the two species; thus, after infusion of [^{3}H]uridine in rats only <2% of the label in liver was present as nucleotides. A more detailed analysis of the metabolism of uridine in the normal mouse will be published elsewhere.

NBMPR-P treatment produced only modest changes in the disposition of intravenously injected [^{3}H]uridine. Formation of labeled uracil nucleotides by liver was reduced 53% but was increased by 63% in kidney (Table 1). Total acid-soluble radioactivity was also higher in the kidneys of mice pretreated with NBMPR-P. No significant effect on the formation of nucleotides was observed in the spleen or the combined other tissues. Although NBMPR-P has been reported to increase $T_{1/2}$ of uridine in the plasma [8], it has only minor effects on its ultimate disposition.

Even though the administration of NBMPR-P did not greatly change the overall efficiency of uridine salvage, the tissue-selective effects of NBMPR-P administration on the salvage of uridine demonstrated here suggest that the inhibitors of nucleoside transport may be useful in modifying the selective toxicity of nucleoside analogs.

Factors that might contribute to tissue selectivity of NBMPR-P effects on nucleoside salvage include the following: (a) NBMPR-P distribution and rates of dephosphorylation in different tissues, (b) changes in tissue blood-flow resulting from vasodilatory effects of NBMPR [9, 10], (c) possible differences among cell types in the extent to which the mechanisms of nucleoside transport participate

Table 1. Effect of a transport inhibitor on salvage of [^{3}H]uridine by mouse tissues*

Treatment	Tissue	Radioactivity (10^{-4} × cpm/g tissue wet weight)		
		Total acid-solubles	Acid-soluble uracil nucleotides	RNA
Control	Liver	53 ± 4.9	32 ± 4.8	6.8 ± 0.9
Control	Kidney	77 ± 5.0	43 ± 4.4	19 ± 1.3
Control	Spleen	37 ± 2.4	19 ± 2.1	15 ± 1.1
Control	"Carcass"	23 ± 1.9	8.5 ± 1.3	4.4 ± 0.3
NBMPR-P	Liver	47 ± 2.7	15 ± 1.1†	3.3 ± 2.5†
NBMPR-P	Kidney	120 ± 15†	70 ± 7.1†	18 ± 2.1
NBMPR-P	Spleen	48 ± 3.6	17 ± 0.3	11 ± 1.9
NBMPR-P	"Carcass"	22 ± 0.7	6.7 ± 0.6	3.1 ± 0.1†

* ICR mice (28 g) received saline (0.9% NaCl) or NBMPR-P (25 mg/kg) by i.p. injection 1 hr prior to i.v. injection of 20 μCi (0.8 nmole) of [^{3}H]uridine. Radiolabel incorporated into nucleotides or RNA 30 min after injection was determined as described in the text. Data are given as means ± S.E.M. The values are from seven mice for liver, kidney and carcass and from four mice for the spleen.

† $P < 0.05$ by Student's unpaired *t*-test.

in nucleoside permeation, and (d) variation in the sensitivity of the nucleoside-transport mechanisms to NBMPR among different cell types. The existence in particular lines of cultured neoplastic cells of nucleoside-transport mechanisms of low sensitivity to NBMPR has been recognized recently [11, 12]. One instance is known of a line of neoplastic cells, the Walker 256 carcinosarcoma, which lacks NBMPR-binding sites and possesses a nucleoside-transport mechanism that is insensitive to NBMPR [13].

In conclusion, this study indicates that prior i.p. treatment of mice with NBMPR-P, a prodrug form of the potent inhibitor of nucleoside transport, NBMPR, had only a modest inhibitory effect on the salvage of circulating uridine in several tissues and increased uridine salvage by 63% in kidney.

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Uracil enhancement of 5-fluorodeoxyuridine incorporation into human breast carcinoma deoxyribonucleic acid*

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The cytotoxicity of 5-fluorouracil (FUra) and 5-fluorodeoxyuridine (FdUrd) has been ascribed to the inhibition of thymidylate synthetase by 5-fluorodeoxyuridine monophosphate [1, 2]. The fluorinated pyrimidines also incorporate into RNA and disrupt RNA processing [3–7]. The relative importance of each cytotoxic mechanism may be dependent upon varying patterns of intracellular FUra metabolism [8]. The incorporation of FUra into MCF-7 human breast carcinoma cellular RNA, however, correlates with loss of clonogenic survival [9]. This relationship is also maintained when inhibition of DNA synthesis is reversed by thymidine [9]. These observations suggest that the formation of (FUra)RNA is a major mechanism of cytotoxic action.

FUra residues have also been detected in eukaryotic DNA [10–13]. The FUra residues are excised from DNA [14], and this excision may contribute to the cytotoxicity associated with formation of (FUra)DNA [15]. There are at least two mechanisms that limit the incorporation of FUra into eukaryotic DNA. Deoxyuridine-triphosphate nucleotidohydrolase degrades intracellular FdUTP, and uracil-DNA glycosylase removes FUra residues incorporated in the DNA strand [16]. The excision of FUra from DNA by uracil-DNA glycosylase is much less efficient than that of uracil [17], and this enzyme is inhibited by uracil [18–20].

In view of the inhibition of uracil-DNA glycosylase by uracil and the previous demonstration that FUra residues are excised from MCF-7 DNA, we have studied the effects of uracil on the formation of (FUra)DNA. The results demonstrate that uracil enhances the incorporation of FUra into MCF-7 DNA.

Materials and methods

Cell culture. The human breast carcinoma MCF-7 cell line was obtained from the Michigan Cancer Foundation, Detroit. MI. The cells were grown free of Mycoplasma contamination as a monolayer in Dulbecco's Modified Eagle's medium (Grand Island Biological Co., Grand Island, NY) with 10% heat-inactivated dialyzed fetal calf serum, 1% L-glutamine, 100 μg penicillin/ml, 10 μg insulin/ml, and 100 units streptomycin/ml.

Incorporation of FUra into nucleic acids. MCF-7 cells in logarithmic growth phase at a concentration of 1×10^6 cells/100×20 mm tissue culture dish (Costar Plastics, Cambridge, MA) were washed twice with serum-free medium and incubated with 10^{-7}, 10^{-6}, or 10^{-5} M [^{3}H]FdUrd (18 Ci/mmole; Moravek Biochemicals, City of Industry, CA) and 10 μCi $H_3{}^{32}PO_4$ (carrier-free; New England Nuclear Corp., Boston, MA) for 3 or 6 hr. Uracil (Sigma Chemical Co., St. Louis, MO) was added at concentrations of 0.015, 0.15, 1.5 and 15 mM. The total cellular nucleic acids were purified as previously described [9] and analyzed by cesium sulfate gradient centrifugation.

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