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SUBSTRATE PROPERTIES OF 5-FLUOROURIDINE DIPHOSPHO SUGARS DETECTED IN HEPATOMA CELLS

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Abstract—Nucleotide sugars derived from 5-fluorouridine were studied in cultured AS-30D hepatoma cells as well as in kinetic enzyme assays *in vitro* in comparison with the physiologic uridine diphospho sugars. Hepatoma cells converted 5-fluoro [^{14}C]uridine to 5-fluorouridine diphospho (FUDP) glucose, FUDP-galactose, FUDP-*N*-acetylglucosamine, FUDP-*N*-acetylgalactosamine, and trace amounts of FUDP-glucuronate, as analyzed by different systems of high-performance liquid chromatography. 5-Fluoro[^{14}C]uridine and [^{14}C]uridine, at concentrations of 5 μM in the culture medium, were phosphorylated by the cells during 60 min to similar amounts of FUTP and UTP, respectively, while the synthesis of [^{14}]FUDP-sugars was reduced to 14% as compared to that of [^{14}C]UDP-sugars.

FUDP-sugars, synthesized by chemical and enzymatic procedures, were assayed *in vitro* as substrates for enzymes of UDP-sugar metabolism. K_m and V values in a range comparable to that of the respective UDP-sugars were determined for FUDP-sugars in the reactions catalyzed by UDP-glucose pyrophosphorylase, galactose-1-phosphate uridylyltransferase, UDP-glucose 4-epimerase, UDP-*N*-acetylglucosamine 2-epimerase, glycogen synthase, and UDP-glucose dehydrogenase.

Our experiments in hepatoma cells and with enzymes *in vitro* have revealed additional reactions of FUDP-sugar metabolism demonstrating a metabolite pattern analogous to that of UDP-sugars. The amounts of FUDP-sugars formed relative to UDP-sugars in intact cells were smaller than suggested on the basis of their kinetic comparison *in vitro*.

The chemotherapeutic agents 5-fluorouracil and 5-fluorouridine (FUrd)* have to be metabolized to their respective nucleotide and deoxynucleotide derivatives to interfere with DNA synthesis and RNA processing [1]. Further metabolism by the anabolic route involves the formation of FUrd nucleotide sugars [2–8]. FUDP-hexoses were detected in liver [8] and tumour cells [7]. Treatment with 5-fluorouracil or FUrd leads to the synthesis of FUDP-*N*-acetylhexosamines in bacteria [2, 3] and in mammalian tumours [5, 6]. In the presence of FUrd and D-galactosamine the intracellular accumulation of FUDP-hexosamines was observed [5]. Quantification of FUDP-sugars demonstrated cellular contents up to those of their non-fluorinated physiologic counterparts [5, 8].

In spite of the established formation of FUDP-sugars, the metabolic consequences of their presence in mammalian cells and tissues have not been assessed so far. We have prepared several FUDP-sugars by chemical and enzymatic procedures to test *in vitro* their substrate properties with enzymes of UDP-sugar metabolism. Moreover, we have separated and identified FUDP-sugars and their 4-epimeric derivatives in intact hepatoma cells cultured in the presence of FUrd.

*Abbreviations used: FUrd, 5-fluorouridine; FUMP, FUDP, FUTP, 5-fluorouridine 5′-mono-,-di-, and -triphosphate, respectively; FUDP-Hex, FUDP-hexoses, FUDP-glucose + FUDP-galactose; FUDP-HexNAc, FUDP-*N*-acetylhexosamines, FUDP-*N*-acetylglucosamine + FUDP-*N*-acetylgalactosamine; GlcNAc, *N*-acetyl-D-glucosamine or 2-acetamido-2-deoxy-D-glucose; GlcN, D-glucosamine or 2-amino-2-deoxy-D-glucose; HPLC, high-performance liquid chromatography; UDP-GlcA, UDP-glucuronic acid; Urd, uridine.

Enzymes: UDP-glucose pyrophosphorylase, UTP, α-D-glucose-1-phosphate uridylyltransferase (EC 2.7.7.9); galactose-1-phosphate uridylyltransferase, UDP-glucose; α-D-galactose-1-phosphate uridylyltransferase (EC 2.7.7.12); UDP-glucose 4-epimerase (EC 5.1.3.2); glycogen synthase, UDP-glucose; glycogen 4-α-glucosyltransferase (EC 2.4.1.11); UDP-glucose dehydrogenase, UDP-glucose; NAD$^+$ 6-oxidoreductase (EC 1.1.1.22); UDP-*N*-acetylglucosamine 2-epimerase, UDP-2-acetamido-2-deoxy-D-glucose 2-epimerase (EC 5.1.3.14).

MATERIALS AND METHODS

Chemicals and isotopes

5-Fluorouridine was obtained from Calbiochem-Behring (La Jolla, CA, U.S.A.) and 5-fluoro[2-^{14}C]-uridine was from Moravek Biochemicals (City of Industry, CA, U.S.A.). Trimethyl phosphate and 2-ethoxy-1 (2H)-quinoline-carboxylic acid ethyl ester (EEDQ) were purchased from EGA-Chemie (Steinheim, F.R.G.). Phosphorus oxychloride, hexamethylphosphoric triamide, 1,1′-carbonyldiimidazole, $Na_4P_2O_7 \cdot 1O\ H_2O$, tributylamine, EDTA, Tris, magnesium acetate and cysteine were from E. Merck (Darmstadt, F.R.G.). Glycogen (rabbit liver), triethanolamine, nucleotide sugars, nucleotides and sugar phosphates were obtained from Boehringer Mannheim (Mannheim, F.R.G.). Glucosamine 1-phosphate and all proteinase inhibitors were from Sigma Chemical Co. (St Louis,

MO, U.S.A.). Sodium [1-^{14}C]acetate (specific activity 52.5 Ci/mole) was from Zinsser Analytic (Frankfurt, F.R.G.).

Enzymes

UDP-glucose pyrophoshorylase (100 U/mg, beef liver), galactose-1-phosphate uridylyltransferase (1.5 U/mg, calf liver) some batches of which contained UDP-glucose 4-epimerase (6 mU/mg), UDP-glucose dehydrogenase (0.6 U/mg, beef liver), phosphoglucomutase (200 U/mg, rabbit muscle), glucose-6-phosphate dehydrogenase (350 U/mg, yeast) and alkaline phosphatase (1900 U/mg, calf intestine) were supplied by Boehringer Mannheim (Mannheim, F.R.G.). Glycogen synthase (2 U/mg, rabbit muscle) was from Sigma Chemical Co. (St Louis, MO, U.S.A.). Specific activities were defined by the suppliers.

Incorporation of [^{14}C]fluorouridine or [^{14}C]uridine into acid-soluble nucleotides

The transplantable rat ascites hepatoma AS-30D [9] was carried in female Sprague–Dawley rats (Voss, Tuttlingen, F.R.G.). Cells were collected on the seventh day after transplantation, washed and suspended in standard medium [10] at 2 mM phosphate and 20 mM hydrogen carbonate. Cells (10–15 g/l) were incubated in closed Erlenmeyer flasks on a gyratory shaker at 37° under CO_2/air (1/20) at pH 7.4. Preincubation for 30 min was followed by an incubation in the presence of [^{14}C]fluorouridine or [^{14}C]uridine at final concentrations of 5 as well as 500 μM. Cell wet weights and freeze pellets were obtained as described earlier [10]. The perchloric acid extracts (0.6 M, 900 μl) were centrifuged and the supernatants neutralized by addition of 50 μl of a mixture of KOH (8 M) and K_3PO_4 (1 M); $KClO_4$ was spun down and the supernatant analyzed by anion-exchange HPLC using phosphate buffer and flow gradients [11].

Analysis of 4-epimeric nucleotide sugars

In order to eliminate any interference of nucleoside phosphates in the chromatographic analysis of nucleotide sugars, the cell extracts (100 μl) were adjusted to pH 9 by addition of 80 μl of Tris-buffer (150 mM, pH 9.2) containing 0.2 mM magnesium acetate and 2.5 U of alkaline phosphatase. Hydrolysis of nucleoside phosphates (3 hr, 30°), was quantitative, while nucleotide sugars were unaffected. The phosphatase-treated samples were readjusted to pH 6.5 and subjected to HPLC on a Partisil-10 SAX column (4.6 × 250 mm) using the volatile triethylammonium acetate buffer, which was obtained by mixing acetic acid and triethylamine in aqueous solution. Triethylammonium acetate (pH 4.35) was used as the starting buffer (0.17 M) and as the limit buffer (1.5 M); the latter was added 5 min after sample injection by a linear gradient reaching 100% at 35 min. Flow rate was 2.0 ml/min. Complete separation of UDP-*N*-acetylhexosamines from UDP-hexoses or of FUDP-*N*-acetylhexosamines from FUDP-hexoses was achieved with retention times of 12.4 and 15.0 or 12.9 and 16.0 min, respectively. The HPLC equipment has been described earlier [12]. The labeled FUDP-sugars or UDP-sugars obtained by collection of the respective peaks and desalting by lyophilization, were rechromatographed by anion-exchange HPLC using borate buffers [12] to separate FUDP-glucose from FUDP-galactose, UDP-glucose from UDP-galactose, FUDP-*N*-acetylglucosamine from FUDP-*N*-acetylgalactosamine, and UDP-*N*-acetylglucosamine from UDP-*N*-acetylgalactosamine. The HPLC effluent was collected in 30 sec fractions and the radioactivity of the metabolites was determined in a liquid scintillation counter.

Synthesis of 5-fluorouridine nucleotides and nucleotide sugars

Synthesis of 5-fluorouridine 5′-triphosphate. FUrd was phosphorylated by phosphorus oxychloride in trimethyl phosphate to give 5-fluorouridine 5′-monophosphate in 85% yield [13]. Subsequent purification on an Amberlite XAD 4 column using triethylammonium hydrogen carbonate buffer [14] was followed by activation of the thoroughly dried triethylammonium salt of FUMP (0.1 mmole) by 1,1′-carbonyldiimidazole (0.6 mmole) in 4 ml of hexamethylphosphoric triamide. The resulting FUMP imidazolidate, which had been formed in 99% yield within a 12-hr incubation at room temperature, was converted to FUTP by addition of tributylammonium pyrophosphate (0.5 mmole) after degradation of unreacted 1,1-carbonyldiimidazole with 40 μl of methanol [15, 16]. FUTP obtained in 90% yield was purified by DEAE-Sephadex chromatography and converted to the sodium salt [14].

Synthesis of FUDP-glucose, FUDP-glucosamine and FUDP-galactose. Glucose 1-phosphate or glucosamine 1-phosphate were reacted with FUTP in the presence of UDP-glucose pyrophosphorylase to form FUDP-Glc or FUDP-GlcN in quantitative yield [14]. Analogous to the synthesis of FUTP, FUDP-galactose was prepared in 25% yield by reaction of FUMP imidazolidate (0.1 mmole) with the tributylammonium salt of galactose 1-phosphate (0.3 mmole) in 2 ml of hexamethylphosphoric triamide. The FUDP-sugars were purified by DEAE-Sephadex and Amberlite XAD 4 chromatography and converted to their sodium salts [14].

Synthesis of FUDP-N-acetylglucosamine and UDP-N-acetylglucosamine. FUDP-glucosamine or UDP-glucosamine (4 μmoles each) were *N*-acetylated with sodium [^{14}C]acetate (3.1 μmoles) in the presence of 2-ethoxy-1(2H)-quinoline-carboxylic acid ethyl ester (4.9 μmoles) in 1.3 ml of 70% aqueous ethanol [17]. After 5 hr at 56° the acetylated nucleotide sugars were isolated by DEAE-Sephadex chromatography, preparative HPLC and desalting on a Sep-Pak C_{18} cartridge [12]. Unlabeled FUDP-GlcNAc was synthesized from FUDP-GlcN and acetic anhydride [12]. Identification and analysis of FUMP, FUDP, FUTP, FUDP-Glc and FUDP-Gal was achieved by using an enzymatic spectrophotometric assay [18]; reaction velocity was speeded up by performing the assay at pH 7.5. FUDP-GlcN and FUDP-GlcNAc were analyzed by HPLC [11, 12] and coeluted with the respective fluorinated nucleotide sugars formed *in vivo* [5]. All FUrd derivatives synthesized gave peaks of 96–100% purity upon HPLC analysis.

Assay conditions for kinetic analysis of 5-fluorouridine derivatives

Determination of K_m and V values was carried out at 37° and pH 7.4. Kinetic parameters ± S.D. were obtained by direct curve fitting to the Michaelis–Menten equation by the method of least squares using a BASIC program [19].

UDP-glucose pyrophosphorylase [20, 21]. K_m and V values for FUDP-Glc or UDP-Glc and pyrophosphate (PP_i) were determined spectrophotometrically. The assay solution (0.3 ml) contained Tris (100 mM), UDP-glucose pyrophosphorylase (0.2 mg/l), phosphoglucomutase (16 mg/l), glucose-6-phosphate dehydrogenase (5 mg/l), NADP (0.4 mM), magnesium acetate (2 mM), cysteine (1.5 mM) and Glc-1,6-P_2 (2 μM). Nucleotide sugars ranged from 0.04 to 1 mM with PP_i fixed at 2 mM; PP_i ranged from 0.2 to 0.9 mM with the nucleotide sugars fixed at 0.5 mM. K_m and V values for FUTP or UTP and glucose 1-phosphate were determined using stop-time assays lacking the indicator enzymes. FUTP or UTP ranged from 0.2 to 1.3 mM at fixed Glc-1-P levels (2 mM); Glc-1-P was varied between 0.02 and 0.5 mM with the nucleotides fixed at 1 mM. Reactions were terminated after 4 min by addition of EDTA (50 mM) and the UDP-Glc or FUDP-Glc formed was quantitated enzymatically [18].

Galactose-1-phosphate uridylyltransferase [22]. K_m and V values for FUDP-Glc or UDP-Glc and galactose 1-phosphate were measured spectrophotometrically. The assay mixture (0.3 ml) contained triethanolamine (150 mM), uridylytransferase (28 mg/l) phosphoglucomutase (40 mg/l), glucose-6-phosphate dehydrogenase (3 mg/l), NADP (1.6 mM), magnesium acetate (1.6 mM), cysteine (10 mM) and Glc-1,6-P_2(5 μM). Nucleotide sugars varied between 0.06 and 3.6 mM with Gal-1-P fixed at 1.4 mM; Gal-1-P ranged from 0.2 to 2.7 mM in the presence of FUDP-Glc or UDP-Glc (0.5 mM). K_m and V values for FUDP-Gal or UDP-Gal and glucose 1-phosphate were obtained from stop-time assays devoid of indicator enzymes. FUDP-Gal or UDP-Gal ranged from 0.35 to 3.5 mM with Glc-1-P fixed at 2 mM, Glc-1-P was varied between 0.2 and 2.0 mM in the presence of FUDP-Gal or UDP-Gal (1.2 mM). The incubation was stopped after 4 min by addition of $HClO_4$, and FUDP-Glc or UDP-Glc were measured enzymatically in the neutralized supernatant [18].

Glycogen synthase [23]. Kinetic parameters were measured by stop-time assays. The assay solution (0.5 ml) contained Tris (50 mM), glycogen synthase (4.5 mg/l), EDTA (5 mM), mercaptoethanol (5 mM) and Glc-6-P (10 mM). FUDP-Glc or UDP-Glc were varied between 0.05 and 0.5 mM with glycogen fixed at 6 mg/l, glycogen ranged from 3 to 24 mg/l in the presence of FUDP-Glc or UDP-Glc (0.24 mM). After 20 min the reactions were stopped (95°, 3 min) and the FUDP or UDP formed was determined enzymatically [24].

UDP-glucose 4-epimerase [25]. Stop-time assays were performed to obtain the kinetic parameters for FUDP-Glc or UDP-Glc and FUDP-Gal or UDP-Gal. The assay solution (0.3 ml) contained triethanolamine (150 mM), UDP-glucose 4′-epimerase (210 mg/l), NAD (1 mM) and EDTA (10 mM). The substrates ranged from 0.03 to 0.5 mM. After 50 min the reactions were terminated (95°, 2 min) and the products formed were quantitated enzymatically [18, 25].

UDP-GlcNAc 2-epimerase [26]. Stop-time assays were used to measure the K_m and V values for FUDP-GlcNAc and UDP-GlcNAc. The assay solution (0.15 ml) contained Tris (160 mM), 100,000 *g* supernatant from rat liver (1.0 g protein/l), 5′-AMP (4.0 mM), magnesium acetate (1.6 mM) and the proteinase inhibitors chymostatin, pepstatin, antipain and leupeptin. Proteinase inhibitors (0.2 mg/l each) were added prior to homogenization of the liver and increased 2-epimerase activity 2-fold as compared to preparations lacking the inhibitors. FUDP-[^{14}C]-GlcNAc and UDP-[^{14}C]GlcNAc were varied between 0.01 and 1.2 mM. After 15 min the reactions were stopped (95°, 2 min) and the deproteinized supernatant was separated by anion-exchange HPLC using phosphate buffer [11], with [^{14}C]ManNAc and nucleotide sugar eluting at 2.5 min and 10.5 min, respectively.

UDP-glucose dehydrogenase [27, 28]. Determination of K_m and V values for FUDP-Glc or UDP-Glc was carried out spectrophotometrically. The assay solution (0.6 ml) contained Tris (50 mM) (system 1) or triethanolamine (150 mM) (system 2), UDP-glucose dehydrogenase (8 mg/l), NAD (0.8 mM), and FUDP-Glc or UDP-Glc ranging from 0.01 to 0.1 mM.

RESULTS

Incorporation of 5-fluorouridine and uridine into acid-soluble nucleotides

Exposure for 1 hr of AS-30D hepatoma cells to 5 and 500 μM concentrations of [^{14}C]FUrd or [^{14}C]-Urd led to the intracellular synthesis of the respective labeled nucleoside phosphates and nucleotide sugars as determined by radio-HPLC. At 5 μM [^{14}C]FUrd (Fig. 1) 120 μmoles of acid-soluble [^{14}C]Furd nucleotides were formed per kg cell wet weight, including FUTP (88%), FUDP (5%), FUDP-hexoses (2%), FUDP-glucuronate (FUDP-GlcA, <0.05%), and FUDP-*N*-acetylhexosamines (5%); the corresponding amount of ^{14}C-labeled pyrimidine nucleotides derived from 5 μM [^{14}C]Urd was 210 μmoles/kg comprising UTP (58%), CTP (9%), UDP (3%), UDP-hexoses (8%), UDP-GlcA (3%), and UDP-*N*-acetylhexosamines (18%) (Fig. 1). Rechromatography by borate-HPLC allowed the determination of the relative amounts of labeled 4-epimeric FUDP-sugars and UDP-sugars after their isolation by anion-exchange HPLC. The ratios for FUDP-Glc/FUDP-Gal and UDP-Glc/UDP-Gal were 3.3 each. The ratios for FUDP-GlcNAc/FUDP-GalNAc and UDP-GlcNAc/UDP-GalNAc were 2.8 and 2.5, respectively.

At 500 μM [^{14}C]FUrd the acid-soluble [^{14}C]nucleotides increased in 1 hr to 850 μmoles/kg, consisting of FUTP (76%), FUDP (4%), FUDP-hexoses (9%), FUDP-GlcA (0.2%) and FUDP-*N*-acetylhexosamines (10%); the ^{14}C-labeled pyrimidine nucleotides formed from 500 μM [^{14}C]Urd amounted to 560 μmoles/kg and were composed of UTP (56%), CTP (11%), UDP (2%), UDP-hexoses (10%),

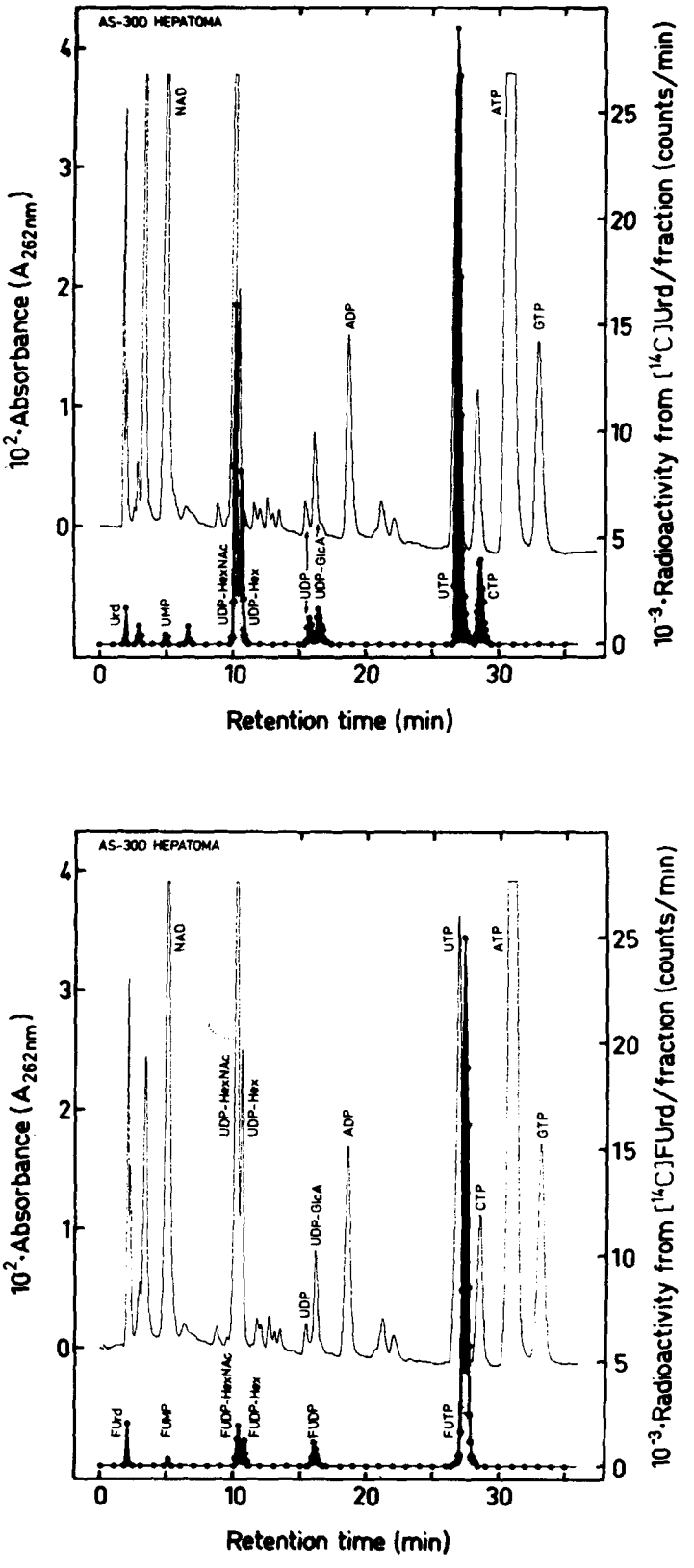

Fig. 1. Nucleotide derivatives of 5-fluorouridine and uridine in AS-30D hepatoma cells. The cells were incubated in the presence of [^{14}C]Urd (5 μM, 53 Ci/mole; upper panel) or [^{14}C]FUrd (5 μM, 52 Ci/mole; lower panel) for 1 hr. Extracts were separated on a Partisil-10 SAX anion-exchange HPLC column [11]. Nucleotide absorbance is indicated by the upper profiles; radioactivity derived from [^{14}C]FUrd or [^{14}C]-Urd is represented by dotted lines.

UDP-GlcA (3%) and UDP-*N*-acetylhexosamines (18%). Thus, in 1 hr FUDP-hexoses plus FUDP-*N*-acetylhexosamines reached intracellular levels of 9 and 160 μmoles per kg cell wet weight at 5 and 500 μM [^{14}C]FUrd, respectively. The disappearance of radioactivity from the culture medium indicated that the uptake of both [^{14}C]FUrd and [^{14}C]Urd by the cells increased 12-fold by elevation of the nucleoside concentration in the medium from 5 to 500 μM. At both concentrations the ATP/ADP ratio was unaffected as compared to normal, averaging 14.

FUDP-GlcA could be detected only in the presence of 500 μM [^{14}C]FUrd (Fig. 2) and amounted to 5 μmoles per kg after an incubation of the cells for 3 hr. Cellular [^{14}C]FUDP-GlcA coeluted with synthetic FUDP-GlcA, was resistant to treatment with alkaline phosphatase and was separated from endogenous UDP-GlcA as well as from FUDP which is present prior to the phosphatase treatment.

Substrate properties of 5-fluorouridine (5′)diphospho sugars

Kinetic studies *in vitro*, using the FUDP-sugars prepared by chemical and enzymatic methods, revealed that FUDP-Glc, FUDP-Gal, FUDP-GlcNAc and FUTP function as substrates for enzymes of UDP-sugar metabolism. These FUrd derivatives were converted at rates similar to or slower than those of their natural counterparts (Tables 1–4).

UDP-Glc pyrophosphorylase (Table 1) catalyzes the uridylylation of glucose 1-phosphate by UTP. UTP and FUTP acted as equivalent substrates for this enzyme. In the back reaction the maximal velocities with FUDP-Glc and with pyrophosphate at fixed FUDP-Glc levels were reduced by 26% and

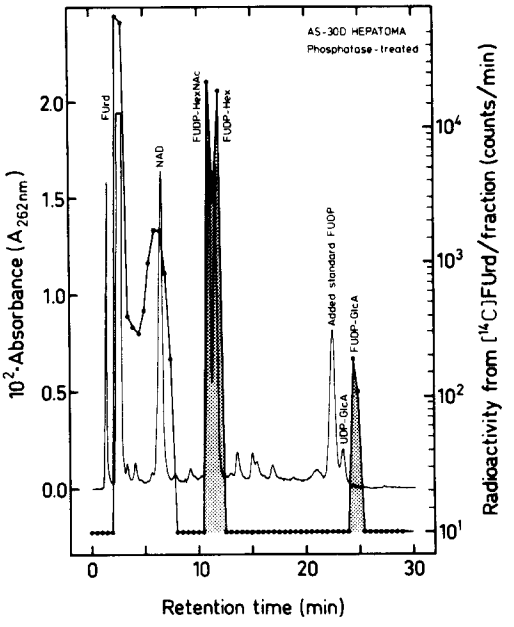

Fig. 2. Formation of labeled FUDP-glucuronate, FUDP-hexoses, and FUDP-*N*-acetylhexosamines from [^{14}C]FUrd in AS-30D hepatoma cells. After exposure of the cells to 500 μM [^{14}C]FUrd (8.3 Ci/mole) for 3 hr, the acid-soluble nucleoside phosphates were hydrolyzed by alkaline phosphatase. The remaining FUDP-sugars and UDP-sugars together with added standard FUDP were separated by anion-exchange radio-HPLC [11] using a modified phosphate buffer gradient with the slope decreased to one-third. The absorbance of the FUDP-HexNAc peak includes UDP-*N*-acetylhexosamines and the FUDP-Hex peak also coelutes with UDP-hexoses. The shaded peak areas represent ^{14}C-labeled FUDP-sugars. The use of a log radioactivity scale emphasizes the relatively small amount of FUDP-GlcA.

Table 1. Kinetic parameters of uridylyltransferases in the presence of fluorouridine derivatives and physiologic substrates

Enzyme	Substrate	K_m ± S.D. (mM)	V ± S.D. (μmole · min^{-1} · mg^{-1})
UDP-glucose pyrophosphorylase*	UTP	0.25 ± 0.02	32.8 ± 0.9
	FUTP	0.24 ± 0.03	32.5 ± 1.9
	Glucose 1-phosphate	0.12 ± 0.01	36.6 ± 1.6
	Glucose 1-phosphate (F)†	0.14 ± 0.02	27.9 ± 2.6
	UDP-glucose	0.07 ± 0.01	83.0 ± 2.3
	FUDP-glucose	0.08 ± 0.01	61.1 ± 2.1
	Pyrophosphate	0.38 ± 0.02	64.9 ± 3.6
	Pyrophosphate (F)†	0.14 ± 0.01	38.8 ± 1.1
Galactose-1-phosphate uridylyltransferase‡	UDP-glucose	0.26 ± 0.03	1.02 ± 0.04
	FUDP-glucose	0.52 ± 0.05	0.51 ± 0.03
	Galactose 1-phosphate	0.37 ± 0.05	0.90 ± 0.04
	Galactose 1-phosphate (F)†	0.23 ± 0.05	0.47 ± 0.03
	UDP-galactose	0.64 ± 0.02	3.23 ± 0.03
	FUDP-galactose	1.45 ± 0.13	3.12 ± 0.14
	Glucose 1-phosphate	0.48 ± 0.02	2.72 ± 0.05
	Glucose 1-phosphate (F)†	0.11 ± 0.01	1.58 ± 0.05

*Beef liver enzyme.
†(F) Denotes the respective fluorinated nucleotide held constant as the second substrate.
‡Calf liver enzyme.

40%, respectively, as compared to the physiologic reactions. Using pyrophosphate as the varied substrate, sigmoidal reaction curves were obtained in the presence of both FUDP-Glc and UDP-Glc requiring a fitting of the data to the Hill equation [29].

Galactose-1-phosphate uridylyltransferase (Table 1) transfers the UMP moiety from a UDP-hexose to hexose 1-phosphate. FUDP-Glc and FUDP-Gal functioned as FUMP donors for galactose 1-phosphate and glucose 1-phosphate, respectively. Since uridylyltransferase displays ping-pong mechanism [30], its action on FUDP-sugars involves the formation of a FUMP-enzyme as an intermediate. FUDP-sugars showed 2-fold increased K_m values and decreased V values in comparison with the UDP-sugars. The K_m as well as V values for galactose 1-phosphate and glucose 1-phosphate were lowered with the respective FUDP-sugars as fixed second substrates.

UDP-Glc 4-epimerase (Table 2) catalyzes the epimerization of the 4-hydroxyl group of UDP-Glc and UDP-Gal. This enzyme was also able to epimerize FUDP-Glc and FUDP-Gal. The K_m values for these analogs were in a range comparable to that for the normal substrates. Maximal velocities with FUDP-sugars were reduced by one-third in comparison with the UDP-sugars.

The dual action of UDP-GlcNAc 2-epimerase (Table 2) involves epimerization and concomitant cleavage of UDP-GlcNAc to yield *N*-acetylmannosamine and UDP. The highly labile enzyme assayed directly in the 100,000 *g* supernatant of rat liver in the presence of a mixture of proteinase inhibitors was characterized by a low K_m value for FUDP-GlcNAc and UDP-GlcNAc. The kinetics for FUDP-GlcNAc showed that fluorination of the uracil ring did not hamper the catalytic activity of 2-epimerase.

Glycogen synthase (Table 3) elongates glycogen chains by attaching the glucose residue from UDP-Glc. When this physiologic glucose donor was replaced by FUDP-Glc the reaction proceeded at half the maximal velocity. The K_m value for FUDP-Glc, however, showed a decrease as compared to UDP-Glc. Variation of glycogen at fixed levels of FUDP-Glc gave similar kinetics in that the K_m and the V value were lower than the respective parameters in the presence of constant UDP-Glc.

UDP-Glc dehydrogenase (Table 4) catalyzes the oxidation of UDP-Glc yielding UDP-glucuronate. FUDP-Glc and UDP-Glc exhibited similar K_m values, the constants in triethanolamine buffer being 3-fold higher than in Tris buffer. FUDP-Glc was oxidized in both buffers at about 50% of the maximal velocity of UDP-Glc. In addition, Table 4 shows that the initial rates with the hexose-modified substrate UDP-glucosamine and the hexose- as well as uracil-modified substrate FUDP-glucosamine were strongly reduced. The K_m for UDP-glucosamine was at least 100-fold higher than that for UDP-Glc.

Table 3. Kinetic parameters of glycogen synthase* in the presence of FUDP-glucose and physiologic substrates

Substrate	$K_m \pm$ S.D. (μM or mg/l)†	$V \pm$ S.D. (μmole · min^{-1} · mg^{-1})
UDP-glucose	85 ± 1	0.27 ± 0.001
FUDP-glucose	43 ± 4	0.13 ± 0.003
Glycogen	16 ± 1	0.72 ± 0.03
Glycogen (F)‡	12 ± 1	0.35 ± 0.02

*Rabbit muscle enzyme.
†The K_m value of glycogen is expressed in mg/l.
‡(F) Denotes FUDP-glucose held constant as the second substrate.

DISCUSSION

The nucleotide derivatives of 5-fluorouridine (FUrd), particularly the fluorinated sugar nucleotides, were studied both in intact cells and as *in vitro* substrates for enzymes of UDP-sugar metabolism (Fig. 3).

Detection of several FUDP-sugars, which were previously unidentified [1, 33], has been achieved by employing three different HPLC systems for separation of the ^{14}C-labeled nucleotide derivatives. In spite of similar pools of ^{14}C-labeled UTP and FUTP, in hepatoma cells at 5 μM of the respective ^{14}C-labeled nucleoside in the culture medium, the level of labeled UDP-sugars exceeded that of FUDP-sugars 7-fold (Fig. 1). The relatively small amount of FUDP-sugars is indicative of their decreased intracellular formation via the reactions 1, 5 and 6 (Fig. 3). This may be a consequence of less favourable substrate properties of FUTP in the presence of a large pool of UTP competing in the reactions catalyzed by UDP-Glc pyrophosphorylase (1) and UDP-GlcNAc pyrophosphorylase (6). Accordingly, intracellular UDP-Glc [10] may interfere with the oxidation of FUDP-Glc in the UDP-Glc dehydrogenase reaction (5). Moreover, the coupling of sequential reactions in the metabolic pathway of fluorinated nucleotides will potentiate minor reductions in re-

Table 2. Kinetic parameters of epimerases in the presence of FUDP-sugars and UDP-sugars

Enzyme	Substrate	$K_m \pm$ S.D. (μM)	$V \pm$ S.D. (nmole · min^{-1} · mg^{-1})
UDP-glucose 4-epimerase*	UDP-glucose	147 ± 12	2.27 ± 0.09
	FUDP-glucose	158 ± 18	1.59 ± 0.08
	UDP-galactose	90 ± 3	5.24 ± 0.06
	FUDP-galactose	101 ± 6	3.54 ± 0.06
UDP-GlcNAc 2-epimerase†	UDP-GlcNAc	9 ± 2	0.89 ± 0.03
	FUDP-GlcNAc	9 ± 1	0.99 ± 0.02

*Calf liver.
†Rat liver.

Table 4. Kinetic parameters of UDP-glucose dehydrogenase* in the presence of base- and sugar-modified nucleotides and UDP-glucose

Substrate	K_m ± S.D. (μM)	V ± S.D. (μmole · min^{-1} · mg^{-1})	Relative rates§ pH 7.4
UDP-glucose†	15 ± 1	0.59 ± 0.02	100‖
FUDP-glucose†	14 ± 2	0.33 ± 0.02	58
UDP-glucosamine†	1599 ± 62	0.13 ± 0.01	1.7
FUDP-glucosamine†	—	—	0.3
UDP-glucose‡	43 ± 2	0.50 ± 0.01	
FUDP-glucose‡	44 ± 4	0.26 ± 0.01	

*Beef liver enzyme.
†Data obtained in Tris buffer.
‡Data obtained in triethanolamine buffer.
§Substrate concentration 100 μM.
‖0.48 μmole · min^{-1} · mg^{-1}.

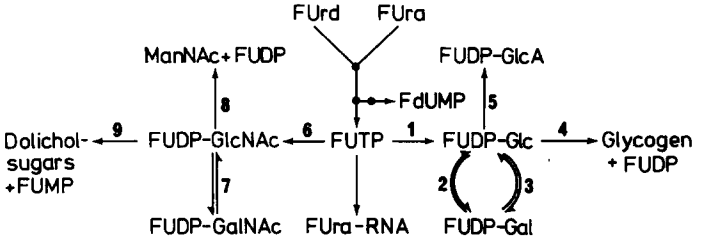

Fig. 3. Formation and function of nucleotide analogs derived from 5-fluorouridine. The reactions analyzed are indicated: 1, UDP-Glc pyrophosphorylase (EC 2.7.7.9); 2, galactose-1-phosphate uridylyltransferase (EC 2.7.7.12); 3, UDP-Glc 4-epimerase (EC 5.1.3.2); 4, glycogen synthase (EC 2.4.1.11); 5, UDP-Glc dehydrogenase (EC 1.1.1.22); 6, UDP-GlcNAc pyrophosphorylase (EC 2.7.7.23); 7, UDP-GlcNAc 4-epimerase (EC 5.1.3.7), probably being identical with UDP-Glc 4-epimerase [31]; 8, UDP-GlcNAc 2-epimerase (EC 5.1.3.14); UDP-GlcNAc: dolicholphosphate acetylglucosamine-phosphotransferase (EC 2.7.8.15) catalyzing the initial step forming GlcNAc-PP-Dol and UMP, or FUMP, which is followed by the synthesis of $(GlcNAc)_2$-PP-Dol and UDP, FUDP [32].

action velocities observed in the single reactions (Tables 1–4).

Elevation of the medium concentration of FUrd and Urd from 5 to 500 μM resulted in a rise of FUDP-sugars from 14% to 95% of labeled UDP-sugars. Under the same condition, the amount of FUTP formed from [^{14}C]FUrd exceeded that of UTP formed from [^{14}C]Urd two-fold. This expansion of the FUTP pool at 500 μM allowed efficient competition of FUTP with UTP in reactions (1) and (6) (Fig. 3). In contrast to the labeling of UDP-GlcA in the presence of both 5 and 500 μM [^{14}C]Urd in the medium, trace amounts of FUDP-GlcA could only be detected at 500 μM [^{14}C]FUrd, a concentration far above that required in chemotherapy. The low cellular content of FUDP-GlcA is not accounted for by breakdown during its isolation since the recovery of standard FUDP-GlcA under the conditions of acid extraction and alkaline phosphatase-treatment was higher than 90%. In addition to the reported formation of FUDP-hexoses and FUDP-*N*-acetylhexosamines *in vivo* [5–8], we demonstrate that these pools comprise the 4-epimeric FUrd sugar nucleotides at ratios similar to those of the respective UDP-sugars.

The marked labeling of the CTP pool from [^{14}C]-Urd (Fig. 1) indicates a major route of utilization of UTP, whereas 5-fluoro-CTP formation from FUrd was not detected.

The demonstration of FUDP-sugar formation *in vivo* is in line with the *in vitro* studies employing enzymes metabolizing UDP-sugars and accepting the fluorinated analogs instead of their physiologic counterparts (Tables 1–4). Fluorination of the uracil ring at position 5 affects the substrate properties for the enzymes tested in different ways. Unaltered kinetics were observed with UDP-GlcNAc 2-epimerase acting on FUDP-GlcNAc (Table 2). A decrease in the maximal velocity was determined when FUDP-sugars were substrates for UDP-Glc pyrophosphorylase, galactose-1-phosphate uridylyltransferase (Table 1), UDP-Glc 4′-epimerase (Table 2), glycogen synthase (Table 3) and UDP-Glc dehydrogenase (Table 4). Accordingly the initial rates of these reactions with fluorinated substrates were slowed down to as much as one-third of the rates in the presence of the natural substrates. K_m values for the FUDP-sugars, however, were similar to those of the respective UDP-sugars in the reactions catalyzed by UDP-Glc pyrophosphorylase UDP-Glc 4-epimerase and UDP-Glc dehydrogenase. In spite of reduced reaction velocities glycogen synthase exhibited a K_m value for FUDP-Glc only one-half that for UDP-Glc. On the other hand, the K_m value of galactose-1-phosphate uridylyltransferase for the FUDP-sugars was significantly higher as compared to that for the UDP-sugars.

Table 4 includes a comparison of sugar nucleotide analogs not only modified in the base moiety but also in the hexose portion. The relatively small effects observed in the UDP-Glc dehydrogenase reaction with FUDP-Glc in comparison with the greatly reduced initial rates with UDP-GlcN and FUDP-GlcN indicate that this sugar modification is more critical

than the base modification for the catalysis of this enzyme. The same substrate specificity is shared by galactose-1-phosphate uridylyltransferase acting on FUDP-sugars (Table 1) better than on UDP-GlcN [14].

UDP-Glc dehydrogenase oxidizes only the undissociated form of FUDP-Glc implying that the K_m values given in Table 4 may be lower by one-third if the pK_a for FUDP-Glc of 7.7 is taken into account whereas the pK_a for UDP-Glc is 9.5 [28].

In a preliminary report [32] FUDP-Glc and FUDP-GlcNAc were shown to serve as substrates for enzymes of the dolichol pathway catalyzing the synthesis of dolichol phospho glucose and dolichol diphospho *N,N'*-diacetylchitobiose($(GlcNAc)_2$-PP-Dol) (Fig. 3). Taken together the data indicate that a series of enzymes forming and converting UDP-sugars or utilizing them as sugar donors also act on the fluorinated derivates.

The kinetic parameters for FUDP-sugars (Tables 1–4) are in line with other enzymological studies on FdUTP as substrate for DNA polymerases α and β [34] and for dUTP pyrophosphorylase [35], FUDP as substrate for ribonucleotide reductase [36], FUTP as substrate for RNA-polymerase [37], or 5-fluorouracil as substrate for pyrimidine phosphoribosyl-transferase [38], all of which were kinetically characterized as functioning *in vitro* with only minor kinetic differences as compared to their uracil counterparts.

Assessing the chemotherapeutic consequences of these findings, the pharmacokinetic qualities rather than the mode of action of 5-fluorouracil and FUrd seem to be affected by their capacity to form FUDP-sugars *in vivo*. FUDP-sugars may account for retarded drug effects by gradually liberating FUMP or FUDP, that are subsequently converted to 5-fluoro-2'-deoxy-UMP inhibiting thymidylate synthase [1] or incorporated into RNA [39].

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