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The Trapping of Uridine Phosphates by D-Galactosamine, D-Glucosamine, and 2-Deoxy-D-galactose

A Study on the Mechanism of Galactosamine Hepatitis

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The effects of D-galactosamine, D-glucosamine, and 2-deoxy-D-galactose on rat liver uracil nucleotides were studied *in vivo*. Enzymic and isotope dilution analyses of the UDP-sugars and of uridine phosphates revealed three major, related changes: an accumulation of the respective UDP-sugar derivatives, a marked decrease of UTP, UDP, and UMP, and a subsequent increase of the sum of hepatic uracil nucleotides. The decrease of uridine phosphates was accompanied by diminished contents of UDPG and UDP-galactose. UMP of total liver RNA was not altered significantly. Inhibition of uridylate synthesis by use of 6-azauridine resulted in a suppression of the D-galactosamine-induced stimulation of uridine phosphate synthesis and of the increase in total acid soluble uracil nucleotides. The trapping of uridine phosphates by formation of UDP-sugar derivatives was most pronounced and most prolonged after administration of D-galactosamine. The uridine phosphate content was lowered to less than 10% of normal within three hours, while the sum of uracil nucleotides increased by 0.35 μmole × g liver^{-1} × hour^{-1}, from an initial value of 1.24 μmole/g. The quantitative analysis of the time dependent changes of UDP-hexosamines, UDP-*N*-acetylhexosamines, UDPG, and UDP-galactose revealed a pronounced alteration by D-galactosamine of the UDP-sugar pattern.

Corresponding changes in the distribution of liver uracil nucleotides were obtained after administration of D-glucosamine and 2-deoxy-D-galactose. Both, however, are ineffective in provoking hepatitis. In contrast to D-galactosamine, D-glucosamine and 2-deoxy-D-galactose do not lead to the formation of UDP-hexosamines; furthermore they are less efficient in trapping uridine phosphates *in vivo*. These observations contribute to an understanding of the orotate-mediated prevention of the galactosamine-induced liver damage, and of the role of pyrimidine nucleotides in this experimental hepatitis.

The synthesis of UDP-sugar derivatives from D-galactose [1,2], D-galactosamine [3,4], and D-glucosamine [5–7] in the liver is well established. The formation and accumulation of large amounts of UDP-sugar derivatives within the cells has been suggested to strain the biosynthesis of uridine phosphates with a subsequent decrease of their levels [7]. The trapping of uridine phosphates by formation of UDP-sugar derivatives must be considered as one possible mechanism for cytotoxic effects of D-galactosamine [4] and D-glucosamine [8]. An inhibitory effect of these amino sugars on the biosyntheses of ribonucleic acid, deoxyribonucleic acid, and protein in normal and especially in tumor cells has been shown *in vitro* [9].

Among a variety of sugar analogs D-galactosamine has been found to induce specifically a liver damage which closely resembles human viral

Enzymes. Alkaline phosphatase, or orthophosphoric monoester phosphohydrolase (EC 3.1.3.1); aspartate carbamoyltransferase, or carbamoylphosphate : L-aspartate carbamoyltransferase (EC 2.1.3.2); dihydroorotase, or L-4,5-dihydro-orotate amidohydrolase (EC 3.5.2.3); galactokinase, or ATP: D-galactose-1-phosphotransferase (EC 2.7.1.6); galactose dehydrogenase, or D-galactose: NAD oxidoreductase (EC 1.1.1.48); nucleoside diphosphokinase, or ATP: nucleosidediphosphate phosphotransferase (EC 2.7.4.6); nucleoside monophosphokinase, or ATP: nucleosidemonophosphate phosphotransferase (EC 2.7.4.4); orotidine-5′-phosphate decarboxylase, or orotidine-5-phosphate carboxylyase (EC 4.1.1.23); phosphodiesterase, or orthophosphoric diester phosphohydrolase (EC 3.1.4.1); phosphoglucomutase, or α-D-glucose-1,6-diphosphate: α-D-glucose-1-phosphate phosphotransferase (EC 2.7.5.1); UDP-galactose 4-epimerase, or uridine diphosphoglucose 4′-epimerase (EC 5.1.3.2); UDPG-dehydrogenase, or uridine diphosphoglucose: NAD oxidoreductase (EC 1.1.1.22); UDPG: galactose-1-phosphate uridylyltransferase, or uridine diphosphoglucose: α-D-galactose-1-phosphate uridylyltransferase (EC 2.7.7.12); UDPG-pyrophosphorylase, or UTP: α-D-glucose-1-phosphate uridylyltransferase (EC 2.7.7.9).

hepatitis [10,11]. The studies described here examine the effects of D-galactosamine, D-glucosamine, and 2-deoxy-D-galactose on total and individual uracil nucleotides in rat liver *in vivo*. All three sugar analogs cause a trapping of uridine phosphates and an alteration of the UDP-sugar pattern. Only D-galactosamine, however, provokes a hepatitis.

The metabolism of D-galactosamine in rat liver follows in its first steps the pathway of D-galactose, and enters the pathway of D-glucosamine [3,4,12]:

$$\text{galactosamine} \xrightarrow{\text{ATP}} \text{galactosamine-1-phosphate} \overset{\text{UDPG}}{\rightleftharpoons} \text{UDP-galactosamine} \rightleftharpoons \text{UDP-glucosamine}.$$

UDP-*N*-acetylglucosamine, UDP-*N*-acetylgalactosamine, sialic acid, and *N*-acetylhexosamine monophosphates were identified as further metabolites of D-galactosamine in rat liver [3]. D-Glucosamine is both acetylated and phosphorylated before its uridylylation to UDP-*N*-acetylglucosamine; therefore detectable amounts of UDP-hexosamines are not formed in rat liver after glucosamine administration [5,6,13]. 2-Deoxy-D-galactose has been shown to be phosphorylated by rat liver galactokinase [12]. The formation of UDP-2-deoxy-D-galactose and UDP-2-deoxy-D-glucose from 2-deoxy-D-galactose has been demonstrated in yeast cells [14].

EXPERIMENTAL PROCEDURE

Materials

D-Galactosamine · HCl (puriss.) was obtained from C. Roth (Karlsruhe), D-glucosamine · HCl (puriss.) from Sigma Chemical Co. (St. Louis), 2-deoxy-D-galactose (puriss.) from Baker Chemicals (Gross-Gerau), 6-azauridine from Boehringer Mannheim GmbH (Mannheim), 6-azauridine-5′-monophosphate from Byk-Fermotest (Hamburg), D-[1-^{3}H]galactose, D-[1-^{14}C]galactosamine, and UDP-D-[U-^{14}C]glucuronate from The Radiochemical Centre (Amersham).

The following enzymes were supplied by Boehringer Mannheim GmbH (Mannheim): UDPG-dehydrogenase (1 U/mg), UDPG: galactose-1-phosphate uridylyltransferase (1 U/mg), UDPG-pyrophosphorylase (20 U/mg), nucleoside diphosphokinase (20 U/mg), nucleoside monophosphokinase (0.5 U/mg), galactose dehydrogenase (50 U/mg), phosphodiesterase from *Crotalus terr.terr.* (45 U/mg with UDPG as substrate), phosphoglucomutase (200 U/mg), alkaline phosphatase (300 U/mg). Some of the commercial preparations of UDPG:galactose-1-phosphate uridylyltransferase could be used as a source of calf liver galactokinase (0.3 U/mg). UDP-galactose 4-epimerase was prepared from fresh calf liver by 6 steps as described by Maxwell [15]. All other reagents used were analytical grade.

Methods

Fed, female Wistar rats (140—165 g) were injected intraperitoneally with neutralized solutions of the respective compounds. All experiments were initiated between 8 and 10 a. m. Galactosamine hepatitis was induced either by a single dose of 400 mg galactosamine · HCl/kg [16] or by 6 injections of 250 mg/kg within 24 h as described earlier [10]. The livers were removed by freeze-stop technique [17] under pentothal anaesthesia (45 mg/kg).

Glycogen and UDPG were determined enzymically [4]. Uridine phosphates were assayed by measurement of the sum of UTP + UDP [4,18] and of UMP [18,19]. The sum of all acid soluble uracil nucleotides was determined after quantitative hydrolysis of the nucleotides by use of snake venom phosphodiesterase followed by specific measurement of UMP [19]. The 6-aza derivative of UMP did not react in this assay. UDP-galactose and UDP-galactosamine were measured after conversion to UDPG by UDPG:galactose-1-phosphate uridylyltransferase with UDPG-dehydrogenase as indicator enzyme [19,20]. UDP-galactose was measured without interference by UDP-galactosamine using UDP-galactose 4-epimerase. Phosphorylation and subsequent uridylylation of D-galactosamine, 2-deoxy-D-galactose, and D-galactose by calf liver galactokinase and UDPG:galactose-1-phosphate uridylyltransferase, respectively, was followed by the assay system described earlier [4] supplemented with ATP in a final concentration of 5 mM. 2-Deoxy-D-galactose was assayed with galactose dehydrogenase in the presence of NAD [21].

Determination of UMP from RNA

The freeze-clamped liver tissue (1 g) was homogenized in 5 ml of 0.6 N perchloric acid; after centrifugation (30000 × *g*, 15 min, 0°) the pellet was twice homogenized in 5 ml of 0.2 N perchloric acid and centrifuged. Finally the pellet was rehomogenized in 5 ml of 0.2 M Tris-Cl buffer pH 8.9. 50 µl of this homogenate were incubated at 37° in 500 µl of 0.2 M Tris-Cl, pH 8.9, containing 0.5 mM magnesium acetate and 50 µg of snake venom phosphodiesterase. After 60 min, when no further increase of UMP and AMP was obtained, the reaction was discontinued by boiling the stoppered tubes for 5 min. All steps were performed without delay. UMP was assayed specifically in the hydrolysate [19].

Synthesis of Labelled UDP-galactosamine and UDP-N-acetylgalactosamine

The unspecificity of galactokinase and UDPG: galactose-1-phosphate uridylyltransferase with regard to D-galactosamine and D-galactosamine-1-phosphate, respectively, as substrates [4,12,22,23]

was used for a one-step synthesis of labelled UDP-galactosamine from D-[1-^{14}C]galactosamine. In a final volume of 0.70 ml the following components were incubated at 37°: 150 mM triethanolamine pH 7.9, 3 mM magnesium acetate, 1 mM EDTA, 20 mM ATP, 28 mM UDPG, 40 mM mercaptoethanol, 1 mM NADH, 4 mM D-[1-^{14}C]galactosamine (3.5 μC/μmole), 0.3 U galactokinase, 1 U UDPG: galactose-1-phosphate uridylyltransferase, 10 U phosphoglucomutase. After 12 h the mixture was adjusted to pH 1.2 by addition of 1 N HCl and boiled for 6 min to destroy the acid labile nucleotides. The remaining monophosphate esters were hydrolyzed with alkaline phosphatase (30 U) at pH 9.0 for 2 h. After heat inactivation and centrifugation the mixture was passed through a Dowex-50-H$^+$ column (6×1 cm). UDP-galactosamine was eluted with water (15 ml). The neutralized eluate was concentrated under reduced pressure and spotted on Whatman 3 paper. After elution of salts with abs. ethanol, UDP-galactosamine was eluted with water in a final yield of 70%. Measurement of UMP after venom phosphodiesterase hydrolysis of UDP-galactosamine [19] and determination of radioactivity by liquid scintillation counting resulted in a specific radioactivity of 3.51 μC/μmole.

Acetylation of UDP-[1-^{14}C]galactosamine [23] with quantitative formation of labelled UDP-*N*-acetylgalactosamine was achieved by addition of 300 μl acetic anhydride (3% in acetone) to 700 μl of an aqueous solution of UDP-galactosamine (0.4 μmole, pH 8.8) and heating (100°, 4 min). Radio paper chromatography of labelled UDP-galactosamine and UDP-*N*-acetylgalactosamine (ethanol—1 M ammonium acetate, 5:2, v/v, pH 7.4, 50 h) resulted in a single, homogeneous peak of the respective nucleotide.

Determination of UDP-glucuronate, UDP-N-acetylhexosamines, and UDP-hexosamines by Isotope Dilution

5×10^4 dis./min of ^{14}C-labelled UDP-glucuronate, UDP-*N*-acetylgalactosamine, or UDP-galactosamine and the acid soluble nucleotides of the supernatant (pH 4) of freeze-clamped liver tissue (1 g) were adsorbed on charcoal (1 g) [8,24]. The charcoal was collected by centrifugation and washed twice with 0.01 N acetic acid. The nucleotides were eluted from charcoal by washing twice with 25 ml of 0.01 M ammonium hydroxide in 50% ethanol [8,24]. The eluates were concentrated under reduced pressure and spotted on Whatman 3 paper. Separation of UDP-*N*-acetylhexosamines, UDP-hexosamines and UDP-glucuronate was obtained by chromatography in ethanol—1 M ammonium acetate (5:2, v/v, pH 7.5, 60 h) [25]. UDP-glucuronate was further separated from interfering uracil nucleotides by a second chromatography in the above system at pH 3.8 (20 h) [26]. The UDP-sugars were localized by scanning of the radioactivity and by their ultraviolet fluorescence. Ammonium acetate was removed from the paper by washing with abs. ethanol, subsequently the UDP-sugars were eluted with water. The UDP-sugars were hydrolyzed with venom phosphodiesterase and assayed by enzymic measurement of UMP [19]. As UDP-hexosamines are not completely separated from UDPG, UDP-galactose, and UMP, the latter were measured in an additional assay [19] before venom phosphodiesterase hydrolysis, and a correction was made. Determination of the recovered radioactivity in the respective sample allowed the calculation of the content of UDP-glucuronate, UDP-hexosamines, and UDP-*N*-acetylhexosamines in the liver.

RESULTS

Trapping of Uridine Phosphates by D-Galactosamine

The intraperitoneal injection of 400 mg/kg of D-galactosamine · HCl produced a rapid and strong decrease of UTP, UDP, and UMP in liver (Fig. 1). The uridine phosphate content was decreased to less than 10% of normal at one and three hours after the injection. After about 25 and 40 h control levels were reached, an overshoot of uridine phosphates up to 150% of normal, however, was measured at 30 h. The ratio (UTP + UDP):(UMP) remained in the normal range throughout. The decrease of uridine phosphates was almost paralleled by a decrease of UDP-galactose and UDPG (Fig. 1, 2). The ratio UDPG:UDP-galactose was 3.06 ± 0.36 (S.D.) 8 h after galactosamine administration; this value is close to the equilibrium of UDP-galactose 4-epimerase [19].

The sum of acid soluble uracil 5′-nucleotides, however, increased strongly (Fig. 1); more than three times the normal value was reached within 8 h (Table 1). These uracil nucleotides consisted mainly of UDP-derivatives of galactosamine:UDP-galactosamine, UDP-glucosamine, and UDP-*N*-acetylhexosamines. The maximal content of UDP-galactosamine was measured within the first 8 h, whereas the highest levels of UDP-*N*-acetylhexosamines were determined 13 and 25 h after galactosamine application (Fig. 2).

Corresponding changes of uracil nucleotides were detected when 6×250 mg/kg galactosamine · HCl was injected within 24 h and the livers removed 1.5 h after the last dose [4,10]. Under these conditions the sum of acid soluble uracil 5′-nucleotides increased about 4-fold (Table 2).

The possibility, that the increase in acid soluble uracil nucleotides is due in part to an enhanced degradation of RNA was excluded in the following way: UMP from RNA was determined in freeze-

clamped livers from normal ($n = 5$) and galactosamine-treated (6×250 mg/kg; $n = 5$) rats. A slight, but not significant decrease from 2.82 ± 0.32 (S.D.) to 2.55 ± 0.13 (S.D.) μmole UMP from RNA per g fresh liver was observed. Based on an UMP-content of total rat liver RNA of about 18% [27,28] these values are equivalent to 5.1 and 4.6 mg RNA/g, respectively. The values correspond to an almost normal amount of rat liver RNA [29,30].

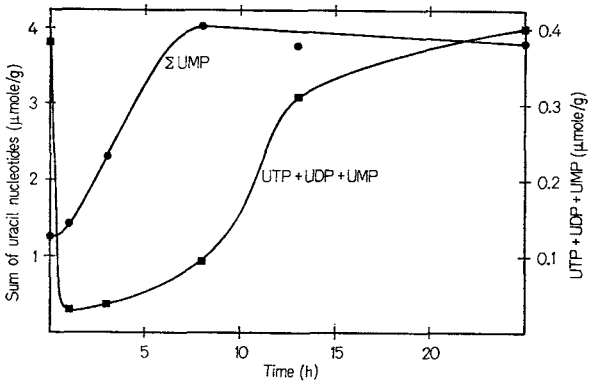

Fig. 1. *Trapping of uridine phosphates by D-galactosamine.* Uridine phosphates and the sum of acid soluble uracil 5′-nucleotides (ΣUMP) were assayed in the extract of freeze-clamped rat livers at different times after a single injection of D-galactosamine · HCl (400 mg/kg). Each point is the mean from 3—8 livers; the data for 8 h after injection are also given in Table 1

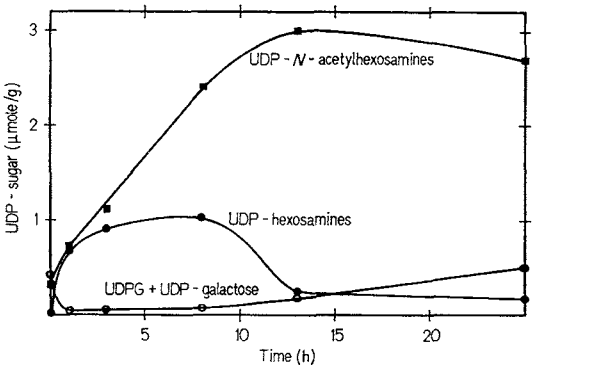

Fig. 2. *D-Galactosamine-induced change of the UDP-sugar pattern.* UDP-sugars were assayed by isotope dilution and enzymic methods at the time indicated after intraperitoneal administration of D-galactosamine · HCl (400 mg/kg). Each point is the mean from 3—6 freeze-clamped rat livers

Inhibition of Uridine Phosphate Synthesis by 6-Azauridine

6-Azauridine-5′-monophosphate, an inhibitor of uridylate synthesis [31—33], which is readily formed from 6-azauridine in liver [31], was used to study whether the increase of the sum of uracil nucleotides is due to a galactosamine-induced stimulation of uridine phosphate synthesis. 6-Azauridine was injected twice intraperitoneally (2×270 mg/kg) 20 min before and 4 h after application of D-galactosamine · HCl (400 mg/kg); this treatment resulted in only a small increase of the sum of uracil nucleotides (Table 1). Thus, the galactosamine-induced increase of uridine phosphate synthesis was effectively suppressed by 6-azauridine.

The inhibition of uridine phosphate synthesis, however, does not appear to be the only effect of 6-azauridine. In rats treated exclusively with this compound (2×270 mg/kg) a significant increase of the sum of uracil nucleotides, including uridine phosphates and UDP-hexoses was observed (Table 1).

Galactosamine-Induced Changes of the UDP-Sugar Pattern

The decrease of UDP-galactose and UDPG was accompanied by an increase of UDP-hexosamines and UDP-*N*-acetylhexosamines (Fig. 2, Table 2). UDP-hexosamines were not detectable in normal livers. Depending on the experimental conditions, the ratio of UDP-hexosamines: UDP-hexoses increased from a value near zero up to 35. The average ratio of UDP-glucosamine: UDP-galactosamine in livers treated with 6×250 mg/kg galactosamine · HCl was 1.85. The UDP-*N*-acetylhexosamines, which reach their maximal levels much later than the UDP-hexosamines, exceed the control levels more than 9-fold.

Table 1. *Uracil nucleotides in 6-azauridine- and D-galactosamine-treated liver*
8 h after administration of D-galactosamine · HCl (400 mg = 1.85 mmole/kg), or 8.3 and 4 h after the injections of 6-azauridine (270 mg = 1.1 mmole/kg each) the livers were freeze-clamped *in situ*. The results are expressed as μmole per g fresh liver ± S.D. The number of animals and experiments (n) is given in parentheses

Nucleotides	D-Galactosamine	D-Galactosamine + 6-Azauridine	6-Azauridine
	μmole/g	μmole/g	μmole/g
ΣUMP[a]	4.01[b,c] ± 0.54 (8)	2.38[c] ± 0.51 (6)	1.88[c] ± 0.32 (7)
UTP + UDP	0.08[c] ± 0.01 (7)	0.06[c] ± 0.01 (6)	0.50[b,c] ± 0.09 (7)
UDPG	0.04[c] ± 0.02 (8)	0.02[c] ± 0.01 (6)	0.46[b,c] ± 0.07 (7)

[a] Sum of acid soluble uracil 5′-nucleotides.
[b] $P < 0.001$ as compared with D-galactosamine + 6-azauridine.
[c] $P < 0.001$ as compared with untreated controls (see Table 2).

Table 2. *Uracil nucleotides in D-galactosamine-treated and normal rat liver*
Doses, 6×250 mg/kg of D-galactosamine · HCl were applied intraperitoneally [10]. The livers were frozen 25.5 h after the first and 1.5 h after the last dose. The nucleotides were determined enzymically [18—20] or by isotope dilution as described in Methods. Mean values (μmole/g fresh liver ± S.D.) and the number of animals (*n*) are given. Experimental and control values differ significantly ($P < 0.001$)

Nucleotides	D-Galactosamine	Control
	μmole/g	μmole/g
UDPG	0.044 ± 0.008 (18)	0.32[a] ± 0.04 (65)
UDP-galactose	0.013 ± 0.005 (6)	0.09[a] ± 0.01 (19)
UDP-hexosamines	2.01 ± 0.15 (3)	<0.01
UDP-glucuronate	0.09 ± 0.02 (4)	0.28 ± 0.06 (7)
UDP-*N*-acetyl-hexosamines	3.01 ± 0.59 (5)	0.32 ± 0.12 (6)
UTP + UDP	0.14 ± 0.02 (9)	0.34[a] ± 0.05 (36)
UMP	0.02 ± 0.01 (4)	0.04[a] ± 0.02 (11)
ΣUMP	4.86 ± 1.08 (12)	1.24[a] ± 0.13 (21)

[a] Data from previous work [19].

term experiment indicates, that the UDP-galactose 4-epimerase reaction is not grossly impaired in galactosamine hepatitis.

UDP-glucuronate decreased to one third of its normal content in rat liver after administration of 6×250 mg/kg D-galactosamine · HCl (Table 2).

Thus D-galactosamine leads to pronounced quantitative changes of all UDP-sugars in the liver as well as to a qualitative alteration of the UDP-sugar pattern by formation of UDP-galactosamine and UDP-glucosamine.

Trapping of Uridine Phosphates by D-Glucosamine

D-Glucosamine · HCl was applied in an equimolar (6×250 mg/kg) and a 3.2-fold dose as compared with D-galactosamine. The ratio (UTP + UDP): (UMP) as well as the ratio UDPG:UDP-galactose remained in the normal range. But a dose-dependent decrease of uridine phosphates and UDP-hexoses, and an increase of the sum of acid soluble uracil nucleotides due to an accumulation of UDP-*N*-acetylhexosamines was observed (Table 3). UDP-hexosamines were not detectable in D-glucosamine treated rat livers. Signs of hepatitis were provoked neither with the low [10,11] nor with the high dose of D-glucosamine.

In comparison with D-galactosamine the administration of D-glucosamine *in vivo* produced significantly less pronounced effects on uracil nucleotide and liver glycogen contents.

Table 3. *Metabolites in D-glucosamine- and 2-deoxy-D-galactose-treated liver*
25.5 h after the first and 1.5 h after the last of 6 injections [10] the rat livers were freeze-clamped *in situ* and homogenized in 5 vol. of 0.9 N $HClO_4$. Doses of 6×250 and 6×800 mg/kg of D-glucosamine · HCl and 6×380 mg/kg of 2-deoxy-D-galactose were applied. The results are expressed as μmole per g fresh liver ± S.D. The number of animals (*n*) is given in parentheses

Metabolites	D-Glucosamine (6×1.16 mmole/kg)	D-Glucosamine (6×3.71 mmole/kg)	2-Deoxy-D-galactose (6×2.33 mmole/kg)
	μmole/g	μmole/g	μmole/g
ΣUMP[a]	1.75[e] ± 0.31 (6)	2.25[e] ± 0.37 (4)	1.99[e] ± 0.08 (3)
UTP + UDP	0.21[e] ± 0.05 (6)	0.11[e] ± 0.02 (4)	[1.12 ± 0.15][c] (3)
UMP	0.02 ± 0.01 (6)	0.01 ± 0.005 (4)	0.015 ± 0.005 (3)
UDPG	0.13[e] ± 0.02 (6)	0.09[e] ± 0.01 (4)	0.09[e] ± 0.03 (3)
UDP-galactose	0.04 ± 0.01 (6)	0.03[e] ± 0.01 (4)	0.02[e] ± <0.01 (3)
UDP-glucuronate	0.26 ± 0.01 (6)	0.38 ± 0.03 (4)	—
UDP-*N*-acetylhexosamines	1.11[d,e] ± 0.27 (6)	1.61[d,e] ± 0.38 (4)	—
Σ 2-Deoxygalactose[b]	—	—	18.8 ± 2.9 (3)
Glycogen (glycosyl units)	153[e] ± 30 (6)	90[e] ± 18 (4)	64[e] ± 21 (3)

[a] Sum of acid soluble uracil 5′-nucleotides.
[b] Sum 2-deoxygalactose-1-phosphate, UDP-2-deoxygalactose, and 2-deoxygalactose.
[c] Sum of UDP-2-deoxyhexoses, UTP, and UDP.
[d] Calculated from the difference of ΣUMP and uracil nucleotides other than UDP-*N*-acetylhexosamines.
[e] $P < 0.001$ as compared with untreated controls (see Table 2).

90 min after the last galactosamine injection the contents of UDPG and UDP-galactose, though strongly decreased, were near the UDP-galactose 4-epimerase equilibrium of 3.5 (Table 2). The UDP-galactose ⇌ UDPG epimerisation was studied *in vivo* by intraportal injection of D-[1-^{3}H]galactose (125 μC, 50 nmole). 15 min later the nucleotides of normal and galactosamine-treated (6×250 mg/kg, $n = 3$) rat livers were isolated and chromatographed after addition of unlabelled UDPG and UDP-galactose. UDPG and UDP-galactose were separated by chromatography in ethanol—methylethylketone—0.5 M morpholinium tetraborate (7:2:3, v/v/v, pH 8.7, 43 h) [34]. The ratio of radioactivities in UDPG and UDP-galactose was 2.6 and 1.8 in control and galactosamine-treated livers, respectively. This short-

Effects of 2-Deoxy-D-galactose on Uracil Nucleotides

The phosphorylation of 2-deoxy-D-galactose with ATP by calf liver galactokinase and the subsequent

uridylylation with UDPG by UDPG:galactose-1-phosphate uridylyltransferase was followed by measurement of glucose-1-phosphate formation [4]. The formation of UDP-2-deoxygalactose in this sequence was about 4 times slower than the formation of UDP-galactosamine from D-galactosamine, and about 20 times slower than UDP-galactose formation from D-galactose.

Formation of 2-deoxygalactose-1-phosphate in rat liver after injection of 2-deoxy-D-galactose *in vivo* was observed in the heat inactivated liver extracts by use of UDPG:galactose-1-phosphate uridylyltransferase [4] and with galactose dehydrogenase after addition of alkaline phosphatase [4, 21]. Due to its acid-lability [14] 2-deoxygalactose-1-phosphate was not measurable in perchloric acid extracts. The extreme instability of UDP-2-deoxyhexoses [14] produced highly increased concentrations of UDP in both heat and perchloric acid inactivated liver extracts. The increase of UDP was reflected by an increased concentration of both UTP + UDP (Table 3), and of non-adenosine diphosphates as measured according to Gruber *et al.* [35].

After injection of 2-deoxy-D-galactose strongly decreased contents of UMP, UDPG, and UDP-galactose were determined. An accompanying decrease of UTP and UDP must be concluded, although it was not measurable under the conditions used. Corresponding to the effects of D-galactosamine and D-glucosamine the sum of acid soluble uracil 5′-nucleotides rose to 160% of normal (Table 3).

DISCUSSION

The exogenous supply of several sugars which are phosphorylated and subsequently uridylylated produces a change in the distribution of liver uracil nucleotides. The extent of UDP-sugar accumulation as well as of the decrease of the uridine phosphate pools depends on the turnover of the respective UDP-sugar and on the rate of uridine phosphate synthesis. A rapid decrease of uridine phosphates has been observed after addition of D-galactosamine but not of an equimolar amount of D-galactose to the medium of isolated perfused livers [36]. This indicates that the uridine phosphate moiety is regenerated much more slowly from UDP-galactosamine than from UDP-galactose. Administration *in vivo* of the different sugars mentioned below induces three quantitatively related changes in uracil nucleotides: Accumulation of UDP-sugar derivatives, decrease of UTP, UDP, and UMP, and a subsequent increase of the sum of hepatic uracil nucleotides. The extent of these changes is decreasing in the following order of sugars investigated *in vivo*: D-galactosamine, 2-deoxy-D-galactose, D-glucosamine, D-galactose.

The trapping of uridine phosphates can be used as a means for an experimental stimulation of uridine phosphate synthesis by pull reactions or feedback control mechanisms. Feedback inhibition by UMP was described for carbamoyl phosphate: aspartate carbamoyltransferase and dihydroorotase from Ehrlich ascites cells [37], and for orotidine-5′-phosphate decarboxylase from rat liver [32, 38]. The rate of increase of the sum of acid soluble uracil nucleotides within the first 8 h after administration of D-galactosamine (Fig. 1) was 0.35 $\mu mole \times g^{-1} \times h^{-1}$. As the increase was suppressed by use of 6-azauridine and as UMP of RNA was not altered significantly, this rate can be regarded as a minimum value for the rate of uridine phosphate synthesis under the conditions used.

The change in the distribution of liver uracil nucleotides after D-glucosamine administration (Table 3) is in good agreement with studies in sarcoma 180 ascites cells using labelled uridine [8].

The trapping of uridine phosphates by D-galactosamine, D-glucosamine, and 2-deoxy-D-galactose is accompanied by a decrease of UDPG (Table 2, 3). This is also observed in isolated livers perfused with D-galactosamine [36]. The low content of UDPG, which is in equilibrium with UDP-galactose, could be due to a decreased UTP level, to an inhibition of the UDPG-pyrophosphorylase reaction [4], or to an increased utilisation of UDPG. An inhibition of UDPG-pyrophosphorylase by galactosamine-1-phosphate has been shown [4]. The synthesis of UDPG, however, is sufficient for the uridylylation of galactosamine-1-phosphate with UDPG as substrate [4] (Fig. 2). The activity of UDPG:galactose-1-phosphate uridylyltransferase from rat liver under optimal conditions *in vitro* is less than 5% of the UDPG-pyrophosphorylase activity [39, 40], and further diminished when galactosamine-1-phosphate instead of galactose-1-phosphate is the substrate of the uridylyltransferase [4]. This indicates that the uridylylation of galactosamine-1-phosphate is not impaired in spite of a more than 90% inhibition of the UDPG-pyrophosphorylase. This inhibition of the UDPG-pyrophosphorylase reaction after administration of D-galactosamine is superimposed by the trapping of uridine phosphates. The reduced contents of both glycogen and UDPG after administration of D-glucosamine, 2-deoxy-D-galactose (Table 3) and of D-galactosamine [4] support the view, that the glycogen decrease is mainly a consequence of the low UDPG level [4] and not of a galactosamine induced activation of glycogen phosphorylase.

The measurements of UDP-glucuronate in normal liver by a combined isotope dilution—enzymic method are in good agreement with previous determinations [41, 42]. The UDP-glucuronate content in galactosamine hepatitis is lowered less than the UDPG content (Table 2). Still under these conditions

60—90% of the serum bilirubin is present in a conjugated form [43]. In liver slices, however, a strong inhibition of glucuronide synthesis was measured upon incubation in a D-galactosamine containing medium [44]. From the experiments with D-glucosamine (Table 3) it appears that factors other than the UDPG content influence the UDP-glucuronate content.

Evidence for a conversion of 2-deoxy-D-galactose to UDP-2-deoxyhexoses by enzymes of the D-galactose pathway has been presented with yeast cells and higher plants [14,45]. An inhibition of tumor growth by 2-deoxy-D-galactose and toxic effects in mice have been described [46]. The changes of uracil nucleotides in rat liver indicate a trapping of uridine phosphates by formation of UDP-2-deoxyhexoses. As judged from the sum of acid soluble uracil nucleotides and from the UDP-hexose content, D-galactosamine acts much stronger on uracil nucleotides than 2-deoxy-D-galactose. Attempts to induce hepatitis with 2-deoxy-D-galactose or with D-glucosamine [10] or with high doses of a mixture of both were unsuccessful.

The comparison of the three sugar derivatives used in this study revealed analogous effects on uracil nucleotide metabolism which could explain their cytotoxic and tumor growth inhibitory effects. D-Galactosamine produces the most pronounced and the most prolonged decrease of UTP, UDP, and UMP in liver. Neither the extent of this decrease nor of the subsequent increase of the sum of uracil nucleotides could be imitated by D-glucosamine and 2-deoxy-D-galactose *in vivo*. Furthermore, D-galactosamine is the only one of all sugar derivatives investigated which leads to the formation of UDP-galactosamine and UDP-glucosamine in liver. Besides this qualitative alteration of the UDP-sugar pattern, the high ratio of unphysiological UDP-aminosugar to corresponding UDP-hexose should be considered [47]. An unspecificity of glycosyl transferases for the nucleotide sugar [48] could induce changes of carbohydrate moieties in polysaccharides, glycolipids or carbohydrate-containing membranes with subsequent functional alterations. Furthermore an inhibition of RNA and protein synthesis as a result of strongly depressed UTP levels [49] is considered as a possible effect of D-galactosamine. The beneficial effect of orotate [16] demonstrates, that the disturbance in pyrimidine nucleotide metabolism is related to the induction of hepatitis.

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REFERENCES

1. Leloir, L. F., *Phosphorus Metabolism* (edited by W. D. McElroy and B. Glass), Johns Hopkins Press, Baltimore 1951, Vol. I, p. 67.
2. Maxwell, E. S., Kalckar, H. M., and Burton, R. M., *Biochim. Biophys. Acta*, 18 (1955) 444.
3. Maley, F., Tarentino, A. L., McGarrahan, J. F., and Del Giacco, R., *Biochem. J.* 107 (1968) 637.
4. Keppler, D., and Decker, K., *Eur. J. Biochem.* 10 (1969) 219.
5. McGarrahan, J. F., and Maley, F., *J. Biol. Chem.* 237 (1962) 2458.
6. Molnar, J., Robinson, G. B., and Winzler, R. J., *J. Biol. Chem.* 239 (1964) 3157.
7. Kornfeld, S., Kornfeld, R., Neufeld, E. F., and O'Brien, P. J., *Proc. Nat. Acad. Sci. U. S. A.* 52 (1964) 371.
8. Bekesi, J. G., and Winzler, R. J., *J. Biol. Chem.* 244 (1969) 5663.
9. Bekesi, J. G., Bekesi, E., and Winzler, R. J., *J. Biol. Chem.* 244 (1969) 3766.
10. Keppler, D., Lesch, R., Reutter, W., and Decker, K., *Exp. Mol. Pathol.* 9 (1968) 279.
11. Reutter, W., Lesch, R., Keppler, D., and Decker, K., *Naturwissenschaften*, 55 (1968) 497.
12. Walker, D. G., and Khan, H. H., *Biochem. J.* 108 (1968) 169.
13. Del Giacco, R., and Maley, F., *J. Biol. Chem.* 239 (1964) 2400.
14. Fischer, W., and Weidemann, G., *Hoppe-Seyler's Z. Physiol. Chem.* 336 (1964) 206.
15. Maxwell, E. S., *J. Biol. Chem.* 229 (1957) 139.
16. Keppler, D., Rudigier, J., Reutter, W., Lesch, R., and Decker, K., *Hoppe-Seyler's Z. Physiol. Chem.* 351 (1970) 102.
17. Wollenberger, A., Ristau, O., and Schoffa, G., *Pflügers Arch. Ges. Physiol.* 270 (1960) 399.
18. Keppler, D., Gawehn, K., and Decker, K., in *Methoden der enzymatischen Analyse*, 2. Aufl. (herausgegeben von H. U. Bergmeyer), Verlag Chemie, Weinheim 1970, p. 2094.
19. Keppler, D., Rudigier, J., and Decker, K., *Anal. Biochem.* (1970) in press.
20. Keppler, D., and Decker, K., in *Methoden der enzymatischen Analyse*, 2. Aufl. (herausgegeben von H. U. Bergmeyer), Verlag Chemie, Weinheim 1970, p. 2142.
21. Wallenfels, K., and Kurz, G., *Biochem. Z.* 335 (1962) 559.
22. Ballard, F. J., *Biochem. J.* 98 (1966) 347.
23. Maley, F., *Biochem. Biophys. Res. Commun.* 39 (1970) 371.
24. O'Brien, P. J., and Neufeld, E. F., *Biochim. Biophys. Acta*, 83 (1964) 352.
25. Paladini, A. C., and Leloir, L. F., *Biochem. J.* 51 (1962) 426.
26. Bischoff, E., Liersch, M., Keppler, D., and Decker, K., *Hoppe-Seyler's Z. Physiol. Chem.* 351 (1970) 729.
27. Kirby, K. S., *Biochem. J.* 64 (1956) 405.
28. Hirsch, C. A., *Biochim. Biophys. Acta*, 123 (1966) 246.
29. Hohorst, H. J., Kreutz, F., and Bücher, Th., *Biochem. Z.* 332 (1959) 18.
30. Haider, M., and Tarver, H., *J. Nutr.* 99 (1969) 433.
31. Pasternak, C. A., and Handschumacher, R. E., *J. Biol. Chem.* 234 (1959) 2992.
32. Creasey, W. A., and Handschumacher, R. E., *J. Biol. Chem.* 236 (1961) 2058.
33. Rabkin, M. T., Frederick, E. W., Lotz, M., and Smith, L. H., Jr., *J. Clin. Invest.* 41 (1962) 871.
34. Carminatti, H., Passeron, S., Dankert, M., and Recondo, E., *J. Chromatog.* 18 (1962) 342.
35. Gruber, W., Moellering, H., and Bergmeyer, H. U., *Enzymol. Biol. Clin.* 7 (1966) 115.
36. Keppler, D., Fröhlich, J., Reutter, W., Wieland, O., and Decker, K., *FEBS Letters*, 4 (1969) 278.
37. Bresnick, E., and Hitchings, G. H., *Cancer Res.* 21 (1961) 105.

38. Blair, D. G. R., and Potter, V. R., *J. Biol. Chem.* 236 (1961) 2503.
39. Bertoli, D., and Segal, S., *J. Biol. Chem.* 241 (1966) 4023.
40. Maddaiah, V. T., and Madsen, N. B., *Can. J. Biochem.* 46 (1968) 521.
41. Wong, K. P., and Sourkes, T. L., *Anal. Biochem.* 21 (1967) 444.
42. Müller-Oerlinghausen, B., and Künzel, B., *Life Sci.* 7 (1968) 1129.
43. Keppler, D., and Decker, K., unpublished observations.
44. Hänninen, O., and Marniemi, J., *FEBS Letters*, 6 (1970) 177.
45. Fischer, W., and Weidemann, G., *Biochim. Biophys. Acta*, 93 (1964) 677.
46. Laszlo, J., Humphreys, S. R., and Goldin, A., *J. Nat. Cancer Inst.* 24 (1960) 267.
47. Keppler, D., Bischoff, E., and Decker, K., *7th Int. Congr. Clin. Chem.*, Geneva/Evian 1969; Vol. 3, Karger, Basel 1970 (in press).
48. Maley, F., McGarrahan, J. F., and DelGiacco, R., *Biochem. Biophys. Res. Commun.* 23 (1966) 85.
49. Weiss, S. B., *Proc. Nat. Acad. Sci. U. S. A.* 46 (1960) 1020.

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