Selective Uridine Triphosphate Deficiency Induced by D-Galactosamine in Liver and Reversed by Pyrimidine Nucleotide Precursors

EFFECT ON RIBONUCLEIC ACID SYNTHESIS*

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SUMMARY

1. D-Galactosamine induces a selective deficiency of UTP without reducing the pools of ATP, GTP, or CTP. This has been shown by means of enzymatic analyses in freeze-clamped rat livers and confirmed by column chromatography.

2. UDP-amino sugars derived from galactosamine increase by 0.85 pmole per g of liver while the UTP content drops from 0.26 to 0.02 pmole per g during 30 min after injection of D-galactosamine in a dose of 1.85 mmoles per kg of body weight.

3. Pyrimidine nucleotide precursors administered intraperitoneally in a dose of 4 mmoles per kg of body weight increase the sum of acid-soluble uracil nucleotides in liver during 1 hour to the following percentages as compared to the control value of 1.24 pmole per g of liver (100%): uridine, 210%; orotate, 144%; ureidosuccinate, 120%; carbamylphosphate, 122%. Uridine is also the most efficient precursor to increase the pool sizes of UTP, UDP, and UMP rapidly.

4. D-Galactosamine-induced UTP deficiency is reversed completely within 90 min after uridine administration. This offers the possibility to inhibit UTP-dependent processes in vivo for periods corresponding to the time interval between galactosamine and uridine injection.

5. RNA synthesis, as measured by incorporation of [14C]-guanosine into liver RNA, is depressed to 21% of the controls when the UTP content is reduced to 0.02 µmole per g of liver. Uridine promptly reverses this inhibition of RNA synthesis.

6. Depression of the concentration of UTP as substrate for RNA polymerases and its reversal by uridine provides a new means to inhibit RNA synthesis in vivo for defined time periods.

The amino sugars D-galactosamine and D-glucosamine have been found to deplete the uridine phosphate pools of several normal and malignant tissues and cell lines (1-8). The decrease of cellular uridine phosphate concentrations is a consequence of the rapid accumulation of UDP-amino sugars derived from galactosamine (5, 9) or glucosamine (3, 4). Hepatic UTP deficiency has been suggested as the cause for an inhibition of RNA and protein synthesis induced by galactosamine (9-13).

Studies on the consequences of UTP deficiency require an establishment of its selectivity with regard to the other ribonucleoside triphosphates. Glucosamine, for instance, depletes the ATP pool in Sarcoma 180 (3) and in Novikoff hepatoma cells (8) to a similar percentage as the UTP pool. Furthermore, a specific measurement for UTP, separate from UDP was required in order to correlate the alterations of UTP concentrations with changes in UTP-dependent processes such as RNA synthesis. It was a major purpose of this investigation to define the conditions that would allow the rapid reversal of a severe hepatic UTP deficiency. Thereby a reversible inhibition of UTP-dependent biosyntheses for defined periods of time would become available. Our previous studies had indicated that orotate (14, 15) and uridine (16) counteract the trapping of uridine phosphate by galactosamine.

Preliminary reports on part of this work were presented earlier (17, 18).

EXPERIMENTAL PROCEDURE

Animals—Female rats of the Wistar strain (Ivanovas, Kisslegg, Germany), aged 9 to 11 weeks of age, weighing 145 to 165 g, had free access to water and a carbohydrate-rich, 20% protein diet (Altromin R from Altromin GmbH, Lage, Germany). Animal experiments were initiated between 8 and 10 a.m.; neutral solutions of the respective compounds were administered by intraperitoneal injection. All liver samples were obtained by freeze-clamping in situ (19) under light thiopental anesthesia; the frozen tissue was wrapped in aluminum foil and kept under liquid nitrogen until it was homogenized in 5 volumes of cold, 0.6 M perchloric acid.

Chemicals, Isotopes, and Enzymes—D-Galactosamine·HCl was purchased from C. Roth (Karlsruhe, Germany), orotic acid, and ureidosuccinic acid from Sigma (St. Louis, Mo.), uridine and carbamylphosphate, dinitium salt, from Boehringer Mannheim (Mannheim, Germany). [U-14C]Guanosine (601 µCi per µmole) and [8-14C]Guanosine triphosphate (36 µCi per µmole) were from The Radiochemical Centre (Amersham, England). All enzymes...
and coenzymes were purchased in the highest specific activity and purity available from Boehringer Mannheim (Mannheim, Germany).

**Enzymatic Measurement of Uracil Nucleotides**—UDP-glucose, UTP + UDP, UMP, and the sum of all acid-soluble uracil 5'-nucleotides (ΣUMP) were assayed as described previously (20). For the separate enzymatic determination of UTP and UDP, respectively, an additional assay was performed (21). UDP-hexosamines and UDP-N-acetylhexosamines were determined by an isotope dilution procedure with chromatographic separation and enzymatic measurement of the UMP moiety (5).

**Determination of Guanine Nucleotides**—The sum of all acid-soluble guanine 5'-nucleotides (ΣGMP) was measured after a quantitative hydrolysis of the nucleotides by means of snake venom phosphodiesterase (20), yielding GMP. GMP was assayed specifically with guanylate kinase, pyruvate kinase, and lactate dehydrogenase (22, 23). The hepatic content of GTP was calculated from the specific enzymatic measurement of GMP (22, 23) and from the recovered radioactivity.

**Measurements of ATP and CTP**—ATP was assayed with yeast hexokinase and glucose-6-P dehydrogenase (24). Chromatographic isolation followed by enzymatic analysis of the CMP moiety was used for the determination of CTP (25).

**Column Chromatography of Nucleoside Triphosphates**—Anion exchange chromatography of the acid-soluble fraction of liver on a Dowex 1 (formate) resin was performed according to Hurlbert et al. (26) and used for a complete separation of GTP, UTP, and ATP. The neutralized perchloric acid extract from 1 g of liver was chromatographed at 4° on a column, 1 X 17 cm (Whatman), of Dowex 1-X8 (formate), 200 to 400 mesh. Formic acid (2 M) together with a linear gradient of ammonium formate (0 to 1.5 M) was used as eluent. Fractions of 10 ml were collected; the nucleotide-containing peaks were adsorbed on charcoal, eluted, concentrated under reduced pressure, and analyzed enzymatically.

**Isolation of RNA labeled with [14C]Guanosine**—The incorporation of [U-14C]guanosine (400 µCi per kg of body weight) into liver RNA in vivo was measured 30 min after intraperitoneal administration of the precursor. RNA was isolated from freeze-clamped livers and hydrolyzed by the steps described by Bresnick (27). The optical density of the RNA hydrolysates was read in 0.1 M perchloric acid at 260 nm; an extinction of 1.00 was taken as equivalent to 32 µg of RNA per ml (28). The radioactivity of the neutralized RNA hydrolysates was measured by liquid scintillation counting (Packard Tri-Carb 3380) in Bray's solution (29), containing 4% (w/v) silica powder; the counting efficiency was 92%.

**RESULTS**

**Mechanism of UTP Depletion**—The major changes in the acid-soluble uracil nucleotide pattern following galactosamine administration are shown in Figs. 1 and 2. The metabolism of galactosamine in liver (2, 9, 10, 30) leads to the formation (30) and accumulation (6) of UDP-galactosamine, UDP-glucosamine,
The hepatic UTP content dropped to 20 and 7% of the control at 15 and 30 min after galactosamine injection, respectively. With a single dose of 1.85 mmoles (≈400 mg of galactosamine·HCl) per kg of body weight UTP was below 0.03 μmole per g of liver for at least 3 hours and returned to normal (0.26 μmole per g) after about 20 hours. An extension of the deficiency period was achieved best by repeated injections. The UTP:UDP ratio was 4.4 ± 0.5 (S.D., n = 18) in controls, a decrease to 3.0 ± 0.6 (S.D., n = 6) was observed 1 hour after galactosamine administration. The induction of UTP deficiency was associated with a rapid depletion of the UDP-glucose pool (Fig. 2). It has been shown earlier that UDP-galactose is decreasing together with UDP-glucose, and UMP together with UTP + UDP (5).

Selection of Galactosamine-induced UTP Deficiency—Two independent methods were used to establish the selectivity with regard to other ribonucleoside triphosphates of the depletion of the hepatic UTP pool by galactosamine. Separation of ATP, UTP, and GTP by anion exchange chromatography indicated unchanged ATP and GTP contents and an apparently complete loss of UTP at 1 hour after galactosamine administration (Fig. 3). A similar elution pattern was obtained with samples freeze-clamped 4 hours after galactosamine.

It was confirmed by means of enzymatic analyses that no significant changes in ATP, GTP, and the sum of guanine nucleotides (ΣGMP) occur during 8 hours after galactosamine administration (Figs. 1, 2, and Table I). Only with higher doses and at later times a moderate depression of adenosine phosphates can be observed (2). Selective UTP deficiency is associated with an increase of the CTP content (Table I); furthermore a 3-fold increase of the sum of cytosine nucleotides has been measured after galactosamine administration (15).

Reversal of UTP Deficiency by Pyrimidine Nucleotide Precursors—A rapid reversal of galactosamine-induced uridine phosphate deficiency requires agents or mechanisms that increase the uridine phosphate pool at a rate faster than the rate of uridine phosphate trapping by formation of UDP-amino sugars. According to Fig. 1 only agents that increase the hepatic uracil nucleotide content by more than 0.85 μmole per g within 90 min can be effective during the initial period after galactosamine administration. This rate of 1.7 μmole per g × hour was exceeded exclusively by uridine (Table II). Other pyrimidine nucleotide precursors, although leading to significantly higher levels of hepatic uracil nucleotides, were comparatively inefficient (Tables II and III). Unlike the other precursors, uridine caused a linear increase with time of total uracil nucleotides for only about 1 hour (Fig. 1). The rise of uracil nucleotides with time was consistently higher when the precursor was administered combined with or following a dose of galactosamine (Table II). As shown in Fig. 1 and Table II galactosamine itself caused an increase of total uracil nucleotides. This increase is due to a relief of feedback inhibition of de novo pyrimidine biosynthesis (5, 9). This mechanism, however, does not account for the entire effect of precursor plus galactosamine administration on the sum of acid-soluble uracil nucleotides.

Corresponding to its effects on total uracil nucleotides, uridine proved most effective in enlargement of the hepatic pools of UTP, UDP, and UMP within short periods of time (Table III). Furthermore, uridine was the only precursor to restore completely the depleted UTP, UDP, and UMP pools within 90 min (Table III, Fig. 4). The duration of this uridine effect was de-

### Table I

| Nucleotide measured | Control | Galactosamine |
|---------------------|---------|---------------|
| UTP                 | 0.26 ± 0.04 (18) | 0.02 ± 0.005 (14) |
| CTP                 | 0.08 ± 0.01 (4)  | 0.17 ± 0.02 (5)   |
| ATP                 | 2.44 ± 0.11 (8)  | 2.24 ± 0.13 (5)   |
| GTP                 | 0.31 ± 0.03 (9)  | 0.57 ± 0.07 (4)   |

### Table II

Increase of total uracil nucleotides caused by exogenous pyrimidine nucleotide precursors and by galactosamine

The sum of all acid-soluble uracil nucleotides (ΣUMP) was measured enzymatically as I'UMP after snake venom phosphodiesterase hydrolysis of the nucleotides (20). The increase (Δ) of ΣUMP over the control level of 1.24 ± 0.06 (S.D., n = 36) μmole per g of liver wet weight during 1 hour is given. In the combination experiments galactosamine was administered 1 hour before the respective pyrimidine nucleotide precursor; the control level of ΣUMP under this condition (at the time of precursor injection) was 1.57 ± 0.06 (n = 11) μmole per g. The doses per kg of body weight were 1.85 mmoles for galactosamine and 4 mmoles for each of the precursors. Orotic acid and ureidosuccinic acid were neutralized with Tris prior to the intraperitoneal injection. Mean values ±S.D. are given with the number of animals in parentheses.

| Pyrimidine nucleotide precursor | Control | Galactosamine |
|--------------------------------|---------|---------------|
| None                           | 0 ± 0.06 (12) | 0.33 ± 0.06 (23) |
| Uridine                        | 1.36 ± 0.23 (8) | 1.87 ± 0.27 (7)  |
| Orotate                        | 0.55 ± 0.04 (6) | 0.73 ± 0.10 (8)  |
| Ureidosuccinate                | 0.25 ± 0.05 (4) | 0.44 ± 0.06 (4)  |
| Carbamylphosphate              | 0.28 ± 0.06 (4) | 0.38 ± 0.04 (9)  |

*Fig. 3. Separation of ATP, UTP, and GTP on a Dowex 1 (formate) column. One hour before freeze-clamping of the liver, 1.85 mmoles of galactosamine (GaIN) per kg of body weight were injected. The acid-soluble fractions from normal (---) and galactosamine-treated (-----) livers, corresponding to 1 g of tissue each, were chromatographed as described under "Experimental Procedure."*
The pyrimidine nucleotide precursors in a dose of 4 mmol/kg of body weight and galactosamine (1.85 mmol/kg) were injected at the times indicated before freeze-clamping of the liver. The number of animals (n) and mean values ± S.D. are given.

Table III

| Treatment                      | UTP     | UDP    | UMP     |
|--------------------------------|---------|--------|---------|
| None                           | 0.26 ± 0.04 | 0.06 ± 0.01 | 0.03 ± 0.01 |
| Uridine (1 hr)                 | 0.30 ± 0.08 | 0.17 ± 0.04 | 0.10 ± 0.02 |
| Uridine (2 hrs)                | 0.96 ± 0.05 | 0.24 ± 0.03 | 0.12 ± 0.02 |
| Orotate (2 hrs)                | 0.60 ± 0.04 | 0.18 ± 0.03 | 0.08 ± 0.02 |
| Ureidonicotate (2 hrs)         | 0.42 ± 0.05 | 0.12 ± 0.03 | 0.05 ± 0.01 |
| Carbamylphosphate (2 hrs)      | 0.33 ± 0.01 | 0.07 ± 0.02 | 0.04 ± 0.01 |
| Galactosamine (2 hrs)          | 0.02 ± 0.005 | 0.007 ± 0.002 | <0.005 |
| Galactosamine (2 hrs) + uridine (1 hr) | 0.17 ± 0.03 | 0.05 ± 0.01 | 0.02 ± 0.01 |
| Galactosamine (3 hrs) + orotate (2 hrs) | 0.05 ± 0.01 | 0.02 ± 0.005 | <0.01 |

DISCUSSION

The depression of the intracellular concentration of only one of the ribonucleotide triphosphates to less than 10% of the control value offers a new way to interfere selectively with hepatic nucleotide metabolism. UTP deficiency can be induced for defined periods of time (Fig. 4) and to varying extents by administration at selected times of galactosamine and pyrimidine nucleotide precursors, respectively. By following the time course of the UTP content, it is possible to correlate UTP deficiency with its effects on UTP-dependent processes. The available evidence strongly suggests, that the inhibitory effects of galactosamine on the syntheses of RNA and protein (10-12, Fig. 4), and on the induction of tyrosine aminotransferase (13) are a consequence of UTP deficiency and not related to direct effects of galactosamine metabolites. The hepatitis-like liver injury provoked by galactosamine (10, 31) has also been shown to result from the initial depletion of the pools of UTP, UDP-glucose, and UDP-galactose (5, 10). A significant pathogenetic function of galactosamine metabolites has been ruled out on the basis of uridine reversal studies (10, 16) and imitation of the lesions with 2-deoxy-D-galactose (15, 32).

Factors influencing the induction of uridine phosphate deficiency by galactosamine and its reversal are schematically shown in Fig. 5. Furthermore, the activities of the enzymes of uridylylate biosynthesis and of galactosamine metabolism strongly influence both extent and duration of uridine phosphate deficiency (9). A prerequisite for the accumulation of UDP-amino sugars derived from galactosamine is the slow regeneration of the UDP moiety in sugar transferase reactions relative to the rate of formation of UDP-amino sugars. In addition, the rate of transfer of N-acetylhexosamines to glycoproteins and glycolipids may be depressed as a result of the low UDP-glucose and UDP-galactose levels. Upon liver perfusion with galactosamine, a slow incorporation of hexosamine, presumably glucosamine, from UDP-hexosamines into rat liver glycogen has been reported (30, 33).

Uridine in large doses (3 to 4 mmol/kg) increases the hepatic UTP pool faster than any of the other pyrimidine nucleotide precursors (Table III). However, the effect of uridine is
more short-lived. This corresponds to a more than 20 times faster disappearance from the acid-soluble fraction of radioactivity from labeled uridine as compared to orotate (34). The increased uptake of uridine into the acid-soluble nucleotide pool during periods of uridine phosphate depletion (Table II) has been also observed in chick fibroblasts after reduction of the uptake of uridine into the acid-soluble nucleotide pool with glucosamine (35). It may be related to a negative feedback on the pyrimidine nucleoside kinase or on uridine transport (35).

The data of Table III allow calculation of the energy charge (36) for rat liver uridine phosphates. A value 0.83 ± 0.02 is obtained for both control and uridine-treated liver. This corresponds to an adenylate energy charge of 0.82 measured previously under similar conditions (2, 31).

The conclusion that the inhibition of RNA synthesis after galactosamine administration is caused by a deficiency of UTP as substrate for RNA polymerases is based on the following observations. (a) The incorporation of labeled guanosine into RNA from an unaltered pool of GTP is strongly depressed; (b) the hepatic UTP content is reduced to 0.02 pmole per g wet weight, corresponding to an estimated intracellular concentration of about 0.027 μmole per g wet weight, corresponding to an estimated intracellular concentration of about 0.027 μmole per g wet weight; (c) a Kₚ value for UTP of about 0.05 μmole per g liver has been measured in vitro for both types of rat liver nuclear RNA polymerases; (d) the ability of nuclei isolated from rat liver 2 hours after galactosamine administration to incorporate labeled ribonucleoside phosphates into RNA in vitro is perfectly normal (11); (e) the depression of guanosine incorporation into RNA in vivo is completely and promptly abolished when the deficiency of UTP is reversed by administration of uridine (Fig. 4). Hepatic RNA synthesis can thus be inhibited in vivo for defined periods of time by selecting the interval between galactosamine and uridine injection.

UTP deficiency has also been induced by addition of galactosamine at a concentration of 2 mM to the isolated perfused rat liver (1) and to rat ascites hepatoma cells in suspension (37). This argues against an extrahepatic, e.g., hormonal influence on the effects of galactosamine on uracil nucleotide metabolism in vivo. The pronounced liver specificity of galactosamine (10, 31) is in accordance with the predominant uptake of this galactose analog by the hepatic tissue (38). Galactosamine differs in its high organ specificity from most of the other efficient inhibitors of RNA synthesis, including actinomycin D (39) and α-amanitin (40). Although the latter are effective at significantly lower doses per kg of body weight, galactosamine offers the distinct advantage of reversibility by means of uridine administration.

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