Pyrimidine nucleotide metabolism in rat hepatocytes: evidence for compartmentation of nucleotide pools

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Pyrimidine nucleotide metabolism in rat hepatocytes was studied by measurement of the labelling kinetics of the various intermediates after double labelling with [^{14}C]orotic acid and [^{3}H]cytidine, the precursors for the *de novo* and the salvage pathways respectively. For the uridine nucleotides, differences were found for the ^{14}C/^{3}H ratios in the UDP-sugars, in UMP (of RNA) and in their precursor UTP, suggesting the existence of separated flows of the radioactive precursors through the *de novo* and the salvage pathways. Higher ratios in the UDP-sugars, which are synthesized in the cytoplasm, and a lower ratio in UMP (of RNA) relative to the ^{14}C/^{3}H ratio in UTP indicated that UTP derived from orotic acid is preferentially used for the cytoplasmic biosynthesis of the UDP-sugars. Uridine, derived from cytidine, is preferentially used for the nuclear-localized synthesis of RNA. In contrast to these findings, the ^{14}C/^{3}H ratios in the cytidine derivatives CMP-NeuAc and CMP (of RNA), and in the liponucleotides CDP-choline and CDP-ethanolamine, were all lower than that in the precursor CTP. This indicates a preferential utilization of the salvage-derived CTP for the synthesis of the liponucleotides as well as for RNA and CMP-NeuAc. Similar conclusions could be drawn from experiments in which the intracellular amounts of several uridine- and cytidine-nucleotide-containing derivatives were increased by preincubating the hepatocytes with unlabelled pyrimidine nucleotides or ethanolamine. Based on these data, we propose a refined model for the intracellular compartmentation of pyrimidine nucleotide biosynthesis in which three pools of UTP are distinguished: a pool of *de novo*-derived molecules and a pool of salvage-derived molecules, both of which are channelled to the site of utilization; in addition an 'overflow' pool exists, consisting of molecules having escaped from channelling. An overflow pool could also be distinguished for CTP, but no discrimination between *de novo* and salvage-derived molecules could be made.

INTRODUCTION

Glycosylation is a co- and post-translational modification of proteins and lipids, which mainly takes place in the lumen of the endoplasmic reticulum and the Golgi apparatus [1–3]. It is a process which, unlike protein or nucleic acid synthesis, is independent of a template and consists of a set of reactions carried out by a multitude of enzymes. The attachment of sugars to glycoconjugates appears to be strictly regulated [4]. Factors such as expression levels, acceptor-substrate specificities of glycosyltransferases and the availability of nucleotide sugar substrates all play a role in the control of synthesis of the oligosaccharide chains. The role of glycosyltransferases has been studied extensively [5]; much less work has been done to elucidate the possible regulatory role of the nucleotide sugars.

Nucleotide sugars are synthesized predominantly in the cytosolic compartment and are translocated across the membranes of the endoplasmic reticulum and Golgi to become available as sugar donors for glycosylation [6–8]. The location and regulation of the synthesis and transport of nucleotide sugars are of importance when studying their role in glycosylation.

UTP and CTP are the common precursors for the activation of sugars and phospholipid precursors, and for RNA synthesis. RNA synthesis takes place almost exclusively in the cell nucleus, whereas most activation processes that require pyrimidine ribonucleotides occur in the cytoplasm [9–12]. It is not known whether the distinct cellular locations at which these processes take place require a similar compartmentation of the nucleoside triphosphates, although many studies are in support of such a concept for pyrimidine nucleotides (for reviews, see [13–16]). This compartmentation is not the result of membrane barriers, but is probably brought about by separate flows of metabolites through *de novo* and salvage pathways [17]. Multienzyme channelling of metabolites and the presence of similar sets of enzymes that reside at separate locations in the cell are likely possibilities [18].

The problem of preferential utilization of a particular nucleotide is particularly important when radioactive precursors are used for studying metabolism. For example, with the use of radioactive precursors it was found that UTP generated by the salvage pathway is preferentially utilized for synthesis of RNA in the nucleus, and is separate from a large pool of UTP, located in the cytoplasm, which can be increased [19].

In contrast to the cytosolic localization of other nucleotide sugar synthesizing enzymes, CMP-neuraminate (CMP-NeuAc) synthase (EC 2.7.7.43) is located in the nucleus {9,10,20–22], although it is debated whether this is the actual site of CMP-NeuAc synthesis [10,23]. Elucidation of metabolic channelling in cytidine metabolism is therefore of particular relevance. We have previously reported our findings on the compartmentation of cytidine and uridine metabolism in rat liver *in vivo* [24,25]. In the present study we substantiate our findings using rat hepatocytes cultured in monolayer. The cells were incubated with labelled pyrimidine precursors, [^{14}C]orotic acid and [^{3}H]cytidine or [^{3}H]uridine, which are metabolized via the *de novo* and the salvage pathway respectively. The isotope ratios in all pyrimidine nucleotides were analysed to obtain information about the relative flows of the radioactive molecules through the *de novo* and the salvage pathways. Compartmentation of both CTP and UTP could be studied with [^{3}H]cytidine as precursor, because the

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^3H label also ends up in uridine derivatives as a result of deamination of cytidine.

By adding unlabelled pyrimidine precursors to the culture medium, we could increase the pool sizes of several pyrimidine nucleotides and influence the incorporation of radioactivity. Our data indicate that there is extensive compartmentation of pyrimidine nucleotides in different intracellular pools.

Part of this work has been presented earlier in abstract form [25].

MATERIALS AND METHODS

Chemicals

[6-^{14}C]Orotic acid (sp. radioactivity 61 mCi/mmol) and [5-^{3}H]-uridine (sp. radioactivity 29 Ci/mmol) were products of Amersham International, Amersham, Bucks., U.K. [5-^{3}H]-Cytidine (sp. radioactivity 26 Ci/mmol) was a product of Moravek Biochemicals Inc., Brea, CA, U.S.A. Newborn calf serum (heat-inactivated) was purchased from Gibco BRL. 1,1,2-Trichlorotrifluoroethane, tri-n-octylamine, ethanol and $HClO_4$ were obtained from Merck, Darmstadt, Germany. The nucleotides used as chromatographic standards were from Sigma Chemical Co., St. Louis, MO, U.S.A., or Boehringer Mannheim, Mannheim, Germany. All other chemicals were of the highest quality commercially available.

Hepatocyte preparation and culture

Hepatocytes were isolated from adult male Wistar rats (specific-pathogen-free; 250–300 g body weight; home bred) essentially according to the method of Berry and Friend [26], using retrograde perfusion of the liver. This procedure yields a cell population that consists nearly exclusively of morphologically free hepatic parenchymal cells, as confirmed by microscopic examination. The hepatocytes were suspended at a concentration of 0.75×10^6 viable cells/ml (viability > 85%, based on exclusion of Trypan Blue) in modified Waymouth medium (5 mM glucose final concentration). The medium was supplemented with penicillin (100 units/ml), streptomycin (100 units/ml), amphotericin B (0.25 μg/ml), insulin (20 m-i.u./ml) and 10% (v/v) dialysed heat-inactivated newborn calf serum. A 3 ml portion of the cell suspension was plated on to collagen-precoated dishes (6 cm diameter) and the cells were allowed to settle at 37 °C in a humid CO_2/air (1:19) atmosphere. After 4 h, the medium together with the non-attached cells was removed and each dish was washed twice with Waymouth medium without supplements at 37 °C. In the subsequent experiments 3 ml of the above fresh medium was supplemented, now containing 5% (v/v) calf serum. All incubations were done in duplicate.

The cells were preincubated with non-labelled uridine, cytidine or orotic acid (0, 0.2 and 0.5 mM). After 16 h, the medium was replaced by 3 ml of fresh medium containing 2 μCi of [^{14}C]orotic acid plus 2 μCi of [^{3}H]cytidine or 2 μCi of [^{3}H]uridine. The incubation was continued for 60 min.

In separate experiments the cells were preincubated with 0.2 mM cytidine together with ethanolamine (0.05 mM). The latter substance was also present during the subsequent radioactive labelling.

Extraction of soluble nucleotides

At the end of the incubation the medium was aspirated and the cells were washed twice with 1 ml of ice-cold phosphate-buffered saline (pH 7.4). The cells were extracted by addition of 1 ml of ice-cold ethanol (75%, v/v). The dishes were scraped with a Teflon policeman, and soluble and insoluble material was harvested. The dishes were washed with 2×1 ml of fresh ethanol (75%, v/v). After centrifugation of the combined extract and washes, the pellet was washed twice with 250 μl of ethanol (75%, v/v). The combined supernatant and wash was evaporated to dryness. Lipids were extracted from the residue with 3.6 ml of a mixture of chloroform/methanol/water (10:5:3, by vol.). After centrifugation, the upper phase (approx. 1.2 ml) was taken off, and the chloroform-containing lower phase was washed once with 1.2 ml of methanol/water (1:1, v/v). The upper phase and wash were combined, evaporated and stored at −20 °C until use.

Preparation of the RNA hydrolysate

The pellet obtained after ethanol extraction was washed twice with 250 μl of ice-cold 0.4 M $HClO_4$ and subsequently incubated with 0.5 ml of 0.2 M KOH at 37 °C for 18 h, in order to hydrolyse the RNA. The reaction was stopped by thorough mixing with 150 μl of 2.0 M $HClO_4$ at 0 °C. After 15 min on ice the mixture was centrifuged and the supernatant (RNA hydrolysate) was removed. The pellet was washed twice with 100 μl of 0.4 M $HClO_4$. The RNA hydrolysate and washes were combined and excess $HClO_4$ was removed with 2 vol. of tri-n-octylamine/trichlorotrifluoroethane (1:4, v/v) as described [24]. After centrifugation the aqueous upper phase was removed and the organic lower phase was washed once with 1 vol. of twice-distilled water. The aqueous phases were combined and lyophilized. The residue was stored at −20 °C until use.

Separation of metabolites by h.p.l.c.

H.p.l.c. in a two-step procedure was performed on a Spectroflow 400 solvent delivery system equipped with a Spectroflow 480 injection/valve module from Kratos Analytical Instruments. The absorbance was detected with a Kratos Spectroflow 757, operating at 280 nm, combined with a Hitachi D-2000 chromato-integrator. Separation of the soluble nucleotides was performed on a Whatman Partisphere SAX cartridge (4.6 mm × 125 mm; particle size 5 μm), and was achieved with gradient elution as described [24]. The flow rate was 1.0 ml/min. Two combinations of fractions were collected, i.e. 2.5–7 min and 7–11 min, and lyophilized. These samples were dissolved in 100 μl of Milli-Q water and rechromatographed on a Lichrosorb RP-18 column (4 mm × 250 mm; particle size 4 μm; Merck). Starting with 0.1 M H_3PO_4/KH_2PO_4, pH 4.0, for 5 min, the separation was accomplished with a linear gradient of methanol in the same buffer up to 30% (v/v), with an increase of 4%/min. The flow rate was 0.5 ml/min. After each run the column was equilibrated for at least 15 min with the initial buffer.

Chromatography of the RNA hydrolysate was also done on the Lichrosorb RP-18 column under the same conditions as described above. The 2′ and 3′ isomers of UMP and CMP present in this hydrolysate are denoted UMP_{RNA} and CMP_{RNA} respectively.

For measurement of the radioactivity, 0.5 min fractions were collected and counted in a Berthold BF 8000 liquid scintillation counter.

The concentrations of the nucleotides were determined by comparison of their u.v. absorbances with those of references of known concentrations.

Table 1 Pool sizes of soluble nucleotides and RNA, and their increase in rat hepatocytes after incubation with pyrimidine nucleotide precursors in the medium

The experiments were performed as described in the Materials and methods section. The increase factors of the pool sizes after incubation with pyrimidine precursors are calculated relative to controls. The data are presented as the means ± S.D. of n incubations; for the columns where $n = 2$, the variation was always less than 15%. *CDP-ethanolamine and CDP-choline could not be determined accurately in all instances except when the hepatocytes were preincubated with 0.2 mM cytidine + 50 μM ethanolamine (last column). For this reason only absolute concentrations are given, in nmol/10^6 cells. nd, not determinable accurately.

		Pool-size increase (-fold) upon incubation with:						
Nucleotide	Pool size in control cells (nmol/10^6 cells) ($n = 8$)	Orotic acid (0.2 mM) ($n = 2$)	Orotic acid (0.5 mM) ($n = 2$)	Uridine (0.2 mM) ($n = 2$)	Uridine (0.5 mM) ($n = 2$)	Cytidine (0.2 mM) ($n = 2$)	Cytidine (0.5 mM) ($n = 2$)	Cytidine (0.2 mM) + 50 μM ethanolamine ($n = 8$)
UDP-HexNAc	2.96 ± 0.21	3.1	2.6	1.2	1.9	1.3	1.6	1.2
UDP-hexose	3.95 ± 0.54	2.2	2.7	1.2	1.7	1.2	1.7	1.2
UDP-GlcA	2.52 ± 0.23	1.8	2.2	1.2	1.5	1.1	1.3	1.1
UTP	1.68 ± 0.24	5.7	6.0	1.8	3.6	2.0	2.7	1.5
UMP_{RNA}	9.4 ± 1.2	1.0	1.0	1.0	1.0	1.0	1.0	1.0
CMP-NeuAc	0.24 ± 0.02	1.2	1.0	1.0	1.0	1.1	1.1	1.0
CTP	0.36 ± 0.04	1.6	1.8	1.0	1.4	1.3	1.7	1.2
CMP_{RNA}	17.0 ± 1.8	1.0	1.0	1.0	1.0	1.0	1.0	1.0
CDP-ethanolamine	nd							0.28 ± 0.03*
CDP-choline	nd							0.20 ± 0.08*
NAD^+	5.25 ± 0.07	1.0	1.0	1.0	1.0	1.0	1.0	1.0
ATP	9.83 ± 0.44	1.0	0.8	0.9	0.8	1.0	0.8	1.0

* Pool-size, expressed in nmol/10^6 cells (mean ± S.D.).

RESULTS

Nucleotide extraction

By extraction of hepatocytes with ethanol, more than 95% of the nucleotides were recovered, as measured using labelled standards. This method also prevented the hydrolysis of the acid-labile CMP-NeuAc [27]. Analysis of the extracts by h.p.l.c. allowed the identification of all pyrimidine ribonucleotides of interest. Moreover, the concentrations of most of these compounds appeared to be measurable accurately (Table 1, first column), with the exception of the two liponucleotides. The total concentration of the soluble uridine nucleotides was approx. 12 nmol/10^6 cells, of which more than 75% were UDP-sugars. The total concentration of the soluble cytidine nucleotides was less than 1 nmol/10^6 cells.

Manipulation of cellular nucleotide concentrations

Incubation of hepatocytes with increasing concentrations of unlabelled pyrimidine nucleotide precursors resulted in a concentration-dependent increase in the amounts of most of the soluble pyrimidine nucleotides (Table 1). Preincubation with orotic acid produced a much greater increase in UTP than in the other uridine derivatives. A similar but less pronounced effect was seen after preincubation with uridine and cytidine. The increase in the concentration of CTP was relatively small, even with cytidine in the medium. The presence of cytidine also caused significant increases in the concentrations of uridine nucleotides. These increases were hardly different from those observed with similar concentrations of uridine in the medium. Elevated cellular concentrations of CTP were not accompanied by an increase in the CMP-NeuAc concentration (Table 1).

Identification and quantification of the liponucleotides CDP-ethanolamine and CDP-choline was achieved using a previously described two-step h.p.l.c. procedure [24], which allows complete separation of these cytidine nucleotides from interfering purines and (radioactive) pyrimidine metabolites. Briefly, this method consists of a first separation on an anion-exchange column, followed by reverse-phase chromatography. Figure 1(a) shows the liponucleotide-containing fractions in untreated control cells. CDP-ethanolamine (peak 1) and CDP-choline (peak 3) could be easily detected. However, accurate quantification was difficult, due to their low concentrations (less than 10 pmol/10^6 cells) and the high background (see Figure 1). When cells were grown in the presence of both ethanolamine and cytidine, the amounts of the liponucleotides increased in a concentration-dependent manner (results not shown). As an example, incubation for 16 h with 50 mM ethanolamine, the lowest concentration used, resulted in a CDP-ethanolamine concentration of 0.28 nmol/10^6 cells. For CDP-choline this value was 0.20 nmol/10^6 cells (Fig. 1b; Table 1). Addition of choline did not have any effect on the pool sizes of the liponucleotides (results not shown).

The elevated concentrations of UTP and CTP after incubation with all three pyrimidine precursors had no effect on the RNA concentration, as measured by the amounts of UMP and CMP isolated from RNA (UMP_{RNA} and CMP_{RNA}; Table 1). Incubation with high concentrations of all three pyrimidine precursors resulted in a slight decrease in the levels of ATP, but the amount of total soluble adenine nucleotides was not altered (results not shown). Furthermore, it appeared that the levels of NAD^+ were not affected by addition of the pyrimidine precursors.

Uptake and metabolic flow of pyrimidine precursors through the *de novo* and salvage pathways

After preincubation under conditions that were the same as described in Table 1 and above, cells were subsequently incubated with tracer amounts of radioactive pyrimidine precursors whose uptake and metabolic fate were then determined. The results are presented in Table 2 and Figure 2.

The uptake of [^{14}C]orotic acid by the cells was not altered significantly under any of the conditions tested. Uptake after 1 h in 10^6 cells varied between 82×10^3 d.p.m. and 94×10^3 d.p.m.,

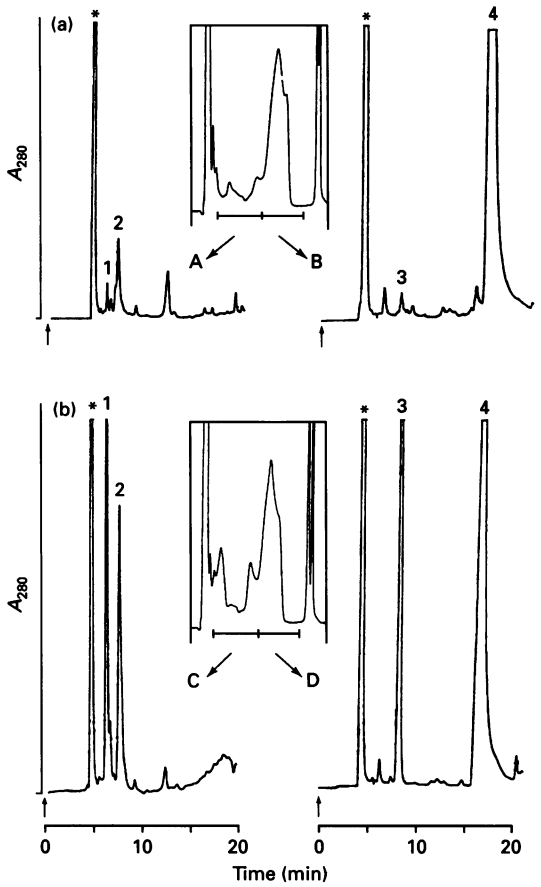

Figure 1 Reverse-phase chromatography of the CDP-ethanolamine- and CDP-choline-containing fractions collected after anion-exchange chromatography of soluble nucleotides extracted from rat hepatocytes

Soluble nucleotides extracted from control cells (**a**) and from cells incubated with 50 μM ethanolamine and 0.2 mM cytidine (**b**) were separated by anion-exchange h.p.l.c. on Partisphere SAX (see insets). Fractions containing CDP-ethanolamine (A, C) or CDP-choline (B, D) were collected and re-chromatographed on Lichrosorb RP18 as described in the Materials and methods section. The peaks were identified as: 1, CDP-ethanolamine; 2, CMP; 3, CDP-choline; 4, NAD^+; *, solvent front containing buffer salts from anion-exchange chromatography.

equivalent to approx. 0.65 nmol (Figure 2a; sum of closed and open bars). In this case, 'uptake' denotes the total intracellular accumulation of radioactivity from labelled exogenous substrate, regardless of metabolic conversions [28]. The amount of ^{14}C label in the soluble fraction (closed bars) was about 6–9 times higher than that in RNA (open bars) for both control and preincubated cells. H.p.l.c. separation of nucleotides in the soluble fraction and quantification revealed that the amounts of ^{14}C label in the various compounds also remained constant (results not shown).

In contrast to the above findings in the *de novo* pathway, large effects of preincubation conditions were found on the uptake of the tracer amounts of [^{3}H]cytidine and [^{3}H]uridine, metabolized via the salvage pathway (Figures 2b and 2c; note that the vertical axes in these panels are broken). Firstly, with [^{3}H]cytidine a decrease in the uptake of ^3H label was observed in the cells that were preincubated with the pyrimidine precursors (Figure 2b). This decrease was more pronounced for cells preincubated with orotic acid than for those that had been pretreated with uridine or cytidine at similar concentrations. The observed decrease appeared to be correlated with the increase in the amounts of UTP and CTP mentioned above (see also Table 1). Secondly, a concentration-dependent increase was found in the ^3H label in the soluble fraction relative to that in the RNA fraction. For example, in control cells the uptake of [^{3}H]cytidine after 1 h was about 5.6 pmol/10^6 cells. Slightly more of the ^3H label was found in RNA (53 %) than in the soluble fraction. For cells preincubated with 0.5 mM orotic acid, a more than 30-fold decrease in [^{3}H]cytidine uptake was found, and in this case more than 90 % of the total resulting intracellular ^3H label ended up in the soluble fraction. These changes in label distribution appeared to be due to an increase in unmetabolized [^{3}H]cytidine and/or catabolites (W. R. Pels Rijcken and G. J. Peters, unpublished work).

Initially [^{3}H]uridine was tested. In control cells the relative amount of label in the RNA fraction was considerably lower than found for [^{3}H]cytidine (19 % versus 53 %). Both the sum of the uptake of label in the soluble and RNA fractions and its distribution over these fractions showed a similar decrease to that seen with cytidine upon preincubation with increasing concentrations of unlabelled uridine or cytidine (Figure 2c). In cells that had been preincubated with 0.2 mM uridine a slight decrease to 17 % was found, further decreasing to 5 % for 0.5 mM uridine. Preincubation with cytidine gave comparable percentages (0.2 mM, 15 %; 0.5 mM, 5 %) (Figure 2c). In contrast to the findings with [^{3}H]cytidine, h.p.l.c. analysis of the soluble fraction of control cells revealed that only low levels of label were found in the uridine and cytidine nucleotides (results not shown). A similar result was found previously for whole rat liver [22]. Incorporation of label from [^{3}H]uridine was decreased even more after preincubation with increasing concentrations of the pyrimidine precursors. As a consequence, determination of the $^{14}C/^3H$ ratios in these nucleotides became inaccurate. Since uridine metabolism could also be studied with cytidine, the subsequent experiments were carried out with the combination of [^{3}H]cytidine and [^{14}C]orotic acid only.

To determine the relative flows through the *de novo* and salvage pathways, we measured the incorporation of ^{14}C and ^3H radioactivity from both orotic acid and cytidine into each nucleotide and calculated the ratios (Table 2). Within the group of uridine nucleotides, we found in untreated control cells that the $^{14}C/^3H$ ratios in the UDP-sugars (3.4–3.7) were higher than those in UMP isolated from RNA (UMP_{RNA}; ratio 2.2) (Table 2). The $^{14}C/^3H$ ratio in UTP, the direct precursor of both the UDP-sugars and UMP_{RNA}, was intermediate (2.9).

Cells that had been preincubated with the various pyrimidine precursors showed higher $^{14}C/^3H$ ratios in the uridine derivatives. These high ratios are due to the fact that the amount of ^{14}C label in each nucleotide remained constant, whereas that of the ^3H label decreased.

A concentration-dependent increase in the $^{14}C/^3H$ ratio for the UDP-sugars was found. This increase was considerably higher than that found in UTP or in UMP_{RNA}. The latter was always found to be lower than that in UTP (Table 2). For the cytidine nucleotides in control cells, the $^{14}C/^3H$ ratio in CTP was higher than that in the end products CMP-NeuAc and CMP_{RNA} (Table 2). As observed for the uridine derivatives, when cells had been preincubated as indicated, a large increase in the $^{14}C/^3H$ ratios of the cytidine derivatives was also found. The ratios in CTP were slightly higher than in the end products, with the exception of preincubation with 0.5 mM cytidine. In this case the ratio in CMP_{RNA} was slightly higher.

Accurate determination of the $^{14}C/^3H$ ratios in the lipo-nucleotides CDP-ethanolamine and CDP-choline was only possible when cells had been preincubated in the presence of

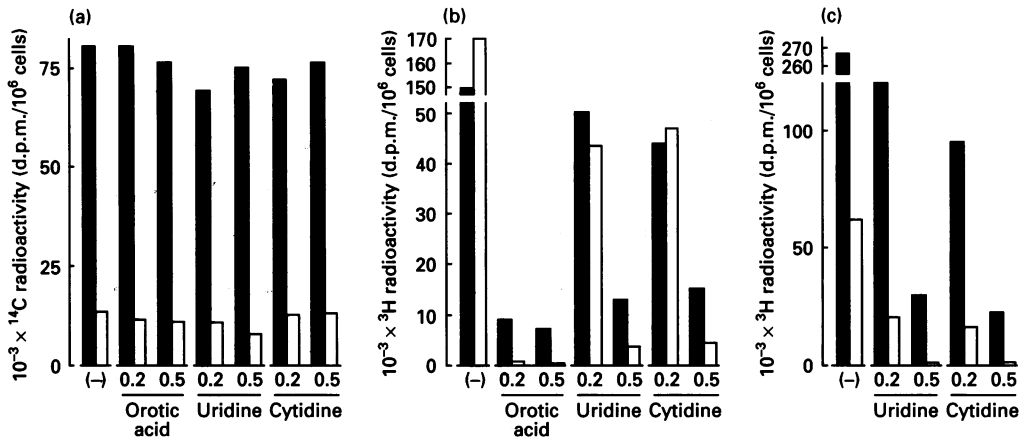

Figure 2 Uptake and incorporation of [^{14}C]orotic acid, [^{3}H]cytidine and [^{3}H]uridine in the soluble fraction and in RNA of rat hepatocytes

Rat hepatocytes were preincubated without (—) or with orotic acid, uridine or cytidine (0.2 or 0.5 mM, as indicated) and subsequently incubated for 1 h in fresh medium containing tracer amounts of a mixture of [^{14}C]orotic acid plus [^{3}H]cytidine or [^{14}C]orotic acid plus [^{3}H]uridine (2 μCi of each label per mixture). The total uptake and incorporation of [^{14}C]orotic acid (**a**), [^{3}H]cytidine (**b**) and [^{3}H]uridine (**c**) in the soluble fraction (closed bars) and in RNA (open bars) was determined.

Table 2 ^{14}C/^{3}H ratios of pyrimidine nucleotides and derivatives in the soluble fraction and in the RNA of rat hepatocytes incubated with [^{14}C]orotic acid and [^{3}H]cytidine: effect of preincubation with pyrimidine precursors

The experiments were performed as described in the Materials and methods section. The data are presented as the means ± S.D. of n incubations; for the columns where $n = 2$, the variation was always less than 12%. ^{14}C/^{3}H ratios > 100 cannot be determined accurately. nd, not determinable accurately.

	^{14}C/^{3}H ratios upon preincubation with:							
Nucleotide	Control ($n = 4$)	Orotic acid (0.2 mM) ($n = 2$)	Oritic acid (0.5 mM) ($n = 2$)	Uridine (0.2 mM) ($n = 2$)	Uridine (0.5 mM) ($n = 2$)	Cytidine (0.2 mM) ($n = 2$)	Cytidine (0.5 mM) ($n = 2$)	Cytidine (0.2 mM) + 50 μM ethanolamine ($n = 8$)
UDP-HexNAc	3.7 ± 0.30	> 100	> 100	7.7	> 100	11.6	> 100	12.6 ± 1.1
UDP-hexose	3.4 ± 0.11	83	> 100	6.3	65	7.8	77	11.1 ± 0.9
UDP-GlcA	3.5 ± 0.20	89	> 100	7.2	> 100	9.2	> 100	11.8 ± 1.0
UTP	2.9 ± 0.15	38	56	5.1	34	4.9	22	8.3 ± 1.1
UMP_{RNA}	2.2 ± 0.24	27	31	3.2	18	3.4	20	7.5 ± 0.8
CMP-NeuAc	0.02 ± 0.0010	0.47	0.54	0.05	0.40	0.05	0.39	0.10 ± 0.009
CTP	0.04 ± 0.0013	0.79	1.30	0.07	0.53	0.07	0.59	0.15 ± 0.009
CMP_{RNA}	0.03 ± 0.0012	0.62	0.80	0.05	0.48	0.06	0.61	0.10 ± 0.010
CDP-ethanolamine	nd	nd	nd	nd	nd	nd	nd	0.10 ± 0.009
CDP-choline	nd	nd	nd	nd	nd	nd	nd	0.07 ± 0.006

ethanolamine (see Table 2). The ratios found were close to those in CMP-NeuAc and CMP_{RNA}. Again, the ^{14}C/^{3}H ratio in CTP was higher than in the other cytidine derivatives mentioned (Table 2, final column).

DISCUSSION

Intracellular compartmentation of pyrimidine nucleotides is often described with the assumption of the existence of two UTP pools, a *de novo* pool and a salvage pool, that can both increase to a varying extent [14,15,18,29,30]. Our results with rat hepatocytes can only be partially explained by such a model. On the basis of our results we propose that, in fact, three separate pools of UTP should be distinguished (see Figure 3). The first two are the pool of *de novo*-derived molecules and the pool of salvage-derived molecules. In addition, there exists an 'overflow' pool, consisting of molecules that have escaped from both pathways. An increase in the intracellular UTP pool reflects an increase in the amount of material present in the overflow pool. The overflow pool does not influence the rate of metabolic flow from orotic acid to UTP, as this is the second part of the *de novo* pathway. Similarly, the existence of an overflow pool can be proposed for the compartmentation of CTP; however, no evidence was obtained for separate *de novo* and salvage pathways.

Our conclusions are based on the following observations. Firstly, the incorporation of [^{14}C]orotic acid into both the UDP-sugars and RNA was not or only slightly decreased after expansion of the total pool of UTP. This indicates that the [^{14}C]UTP, derived from the *de novo* pathway, does not mix with the overflow pool of unlabelled UTP, but rather is strictly channelled to the end products RNA and the UDP-sugars. The incorporation of ^{14}C label into the cytidine nucleotides was also not altered by an increase in pool size. Therefore, channelling of *de novo*-derived CTP to the end products, i.e. the CXP compounds

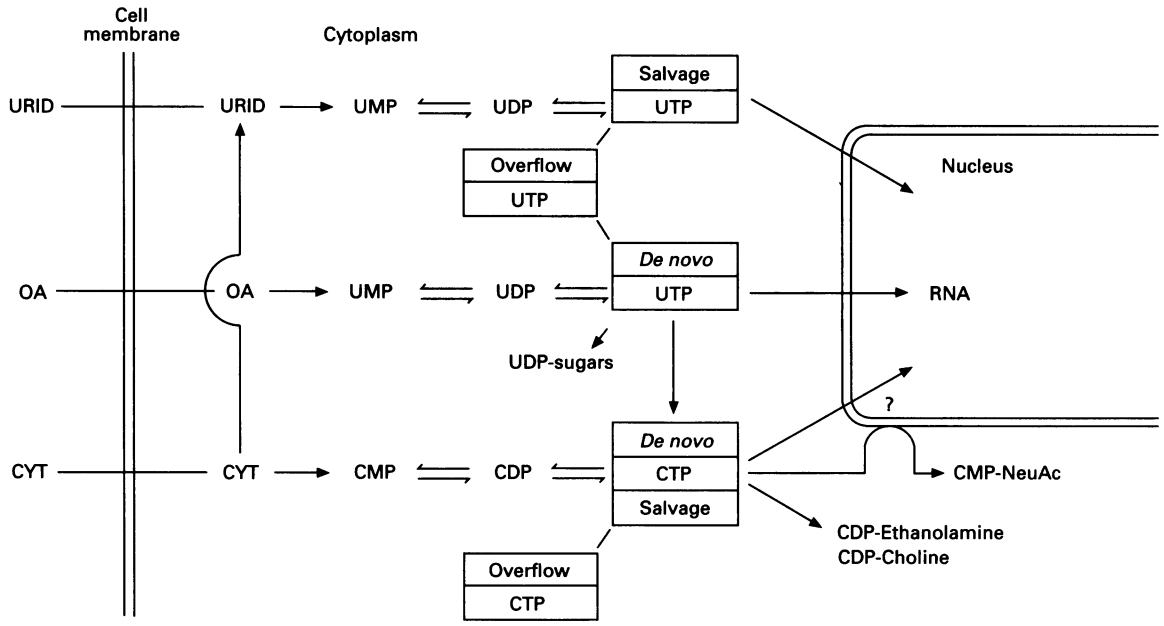

Figure 3 Model for the intracellular compartmentation of pyrimidine nucleotide biosynthesis

The respective pools of UTP and CTP are depicted within boxes. A detailed description of the model is given in the Discussion section. URID, uridine; CYT, cytidine; OA, orotic acid.

and RNA, does occur, and the *de novo* CTP thus formed remains separated from the overflow pool of CTP (see Figure 3).

Secondly, the $^{14}C/^{3}H$ ratios in the pyrimidine nucleotides reveal that UTP and CTP, synthesized via the salvage pathway, are also both channelled to the end products. After an increase in pool size, the incorporation of ^{3}H label into RNA and all soluble nucleotides, including UTP and CTP, was decreased, resulting in an overall increase in all $^{14}C/^{3}H$ ratios. If the newly synthesized ^{3}H-labelled UTP or CTP had entered an enlarged pool and subsequently equilibrated with this pool before being used for end product formation, additional decreases in the ^{3}H specific radioactivities of the end products would have occurred as a result of the increase in the triphosphate pools. As a result, the $^{14}C/^{3}H$ ratios in all end products, including RNA, would have been increased to a level considerably higher than those in UTP and CTP. The $^{14}C/^{3}H$ ratios in UTP and CTP, however, remained intermediate and higher respectively than those of their corresponding end products. This led us to conclude that UTP and CTP, synthesized via the salvage pathway, are also separated from their corresponding overflow pools, and are thus channelled to the end products.

These results also indicate that the observed accumulation of the triphosphates after preincubation of cells with pyrimidine precursors in the medium is a result of the increase in the size of the overflow pool. The concentration of the molecules in the overflow pool determines the extent of uptake and feedback inhibition and thus the flow through the salvage route. Published results by other authors concerning pyrimidine compartmentation fit this model [18,19,29–31].

The $^{14}C/^{3}H$ ratios of the UDP-sugars were higher than that of UTP, whereas that of UMP_{RNA} was lower. This indicates that the relative contributions of the two UTP flows of the ^{14}C-labelled *de novo*-derived molecules and the ^{3}H-labelled salvage-derived molecules respectively were different for the UDP-sugars and for RNA. Either the *de novo*-derived molecules are relatively more used for the cytosol-localized synthesis of UDP-sugars, or the salvage-derived molecules are relatively less used in this reaction. The opposite is true for the nucleus-localized synthesis of RNA. This is in agreement with results found by others in various cell types [26,31–34]. Compartmentation of the *de novo* and salvage pathways has also been described in the nucleus [16,30,35]. However, with our method of cell extraction we were unable to discriminate between different forms of RNA in the nucleus as found by the latter authors.

It seems that, of the two CTP flows, the salvage pathway-derived molecules evolving from [^{3}H]cytidine are used relatively more for the synthesis of the CXP compounds and for RNA synthesis than those evolving from [^{14}C]orotic acid, because the $^{14}C/^{3}H$ ratios in the cytidine end products are always lower than that of the total CTP pool. The fact that no differences were found between the cytosol-synthesized liponucleotides [11,12] and the nuclear-localized RNA does not allow us to arrive at a conclusion about the location of the synthesis of CMP-NeuAc. Similar results for rat liver *in vivo* were found previously [24].

In agreement with the results of others (for a review, see [36]), the enzymes involved in the conversion of [^{14}C]orotic acid to UTP and CTP, the second part of the *de novo* route, showed no feedback inhibition by the increased levels of UTP and CTP in the pools. This can be concluded from the fact that the largest increase of the nucleotide pools was obtained after preincubation of cells with orotic acid. Moreover, the increase in the nucleotide pools had no effect on the subsequent uptake of [^{14}C]orotic acid into the cell.

The effect of an increase in the nucleotide pool on the incorporation of [^{3}H]cytidine in rat hepatocytes is two-fold. It results in inhibition of the uptake of [^{3}H]cytidine and in feedback inhibition of the phosphorylation of ^{3}H-labelled uridine and cytidine. The inhibition of the uptake of [^{3}H]cytidine, as was concluded from the decrease in the total amount of ^{3}H label (Figure 2b, sum of closed and open bars), seems to be correlated with the pool sizes of CTP and UTP (Table 1). However, from our results an inhibition of the uptake due to other expanded

nucleotide pools, such as cytidine/uridine or CMP/UMP, cannot be excluded. Feedback inhibition of the salvage enzyme nucleoside phosphokinase (EC 2.7.1.48) probably occurs via increased concentrations of UTP and/or CTP, as was concluded from the increase in the amounts of unmetabolized [^{3}H]cytidine and [^{3}H]uridine found in the soluble fraction after preincubation (results not shown; see also [37]). The UTP and CTP in the overflow pools might thus exert a specific effect. Once the ^{3}H-labelled cytidine or uridine is phosphorylated to the monophosphate nucleoside, the molecules are effectively channelled to the end products, as no accumulation of ^{3}H label was found in the intermediate mono-, di- and tri-phosphates.

The synthesis of orotic acid, the first part of the *de novo* pathway, is subject to feedback inhibition by UTP. It is possible that UTP in the overflow pool has a specific function in this regulatory process. However, the latter regulatory effect is not of importance in establishing the existence of separate pathways for *de novo*- and salvage-derived UTP and CTP.

In conclusion, in this study we have obtained evidence for the intracellular compartmentation of the *de novo* and salvage pathways for pyrimidine synthesis in rat hepatocytes. By double labelling cells with ^{14}C and ^{3}H we found evidence for the existence of three UTP pools. Furthermore, we were able to discriminate between cytosolic and nuclear processes for the utilization of UTP. For CTP, however, a discrimination between *de novo*- and salvage-derived molecules could not be shown; therefore only two CTP pools can be distinguished, and hence discrimination between cytosolic and nuclear utilization was not possible. Firther studies are required in order to resolve this problem and the related question of the nuclear localization of CMP-NeuAc synthesis.

REFERENCES

1 Hubbard, S. C. and Ivatt, R. J. (1981) Annu. Rev. Biochem. **50**, 555–583
2 Fleischer, B. (1981) J. Cell Biol. **89**, 246–255
3 Carey, D. J. and Hirschberg, C. B. (1981) J. Biol. Chem. **256**, 989–993
4 Schachter, H. (1985) Biochem. Cell. Biol. **64**, 163–181
5 Kornfeld, R. and Kornfeld, S. (1985) Annu. Rev. Biochem. **54**, 631–664
6 Capasso, J. M. and Hirschberg, C. B. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 7051–7055
7 Cacan, R., Cecchelli, R., Hoflack, B. and Verbert, A. (1984) Biochem. J. **224**, 277–284
8 Perez, M. and Hirschberg, C. B. (1986) Biochim. Biophys. Acta **864**, 213–222
9 Coates, S. W., Gurney, T., Sommers, L. W., Yeh, M. and Hirschberg, C. B. (1980) J. Biol. Chem. **255**, 9225–9229
10 Van Rinsum, J., Van Dijk, W., Hooghwinkel, G. J. M. and Ferwerda, W. (1983) Biochem. J. **210**, 21–28
11 Coleman, R. and Bell, R. M. (1978) J. Cell Biol. **76**, 245–253
12 Pelech, S. L. and Vance, D. E. (1984) Biochim. Biophys. Acta **779**, 217–251
13 Hauschka, P. V. (1973) Methods Cell. Biol. **7**, 361–462
14 Keppler, D. and Holstege, A. (1982) in Metabolic Compartmentation (Sies, H., ed.), pp. 147–203, Academic Press, London and New York
15 Moyer, J. D. and Henderson, J. F. (1985) CRC Crit. Rev. Biochem. **19**, 45–61
16 Andersson, M., Lewan, L. and Stenram, U. (1988) Int. J. Biochem. **20**, 1039–1050
17 Sitte, P. (1980) in Cell Compartmentation and Metabolic Channelling (Nover, L., Lynen, F. and Mothes, K., eds.), pp. 5–14, VEB Gustav Fischer Verlag, Jena, and Elsevier/North-Holland Biomedical Press, Amsterdam
18 Losman, M. J. and Harley, E. H. (1978) Biochim. Biophys. Acta **521**, 762–769
19 Plagemann, P. G. W. (1971) J. Cell. Physiol. **77**, 241–258
20 Kean, E. L. (1970) J. Biol. Chem. **245**, 2301–2308
21 van den Eijnden, D. H. (1973) J. Neurochem. **21**, 949–958
22 van Dijk, W., Ferwerda, W. and van den Eijnden, D. H. (1973) Biochim. Biophys. Acta **315**, 162–175
23 Ferwerda, W., Blok, C. M. and Van Rinsum, J. (1986) Glycoconjugate J. **3**, 153–161
24 Pels Rijcken, W. R. and Ferwerda, W. (1990) Biochem. J. **266**, 777–783
25 Pels Rijcken, W. R., Blok, C. M. and Ferwerda, W. (1987) Klin. Wochenschr. **65**, suppl. X, 38
26 Berry, M. N. and Friend, D. S. (1969) J. Cell Biol. **43**, 506–520
27 Ferwerda, W., Blok, C. M. and Heijlman, J. (1981) J. Neurochem. **36**, 1492–1499
28 Plagemann, P. G. W. (1988) Biochim. Biophys. Acta **947**, 405–443
29 Genchev, D. D. (1980) Biochem. J. **188**, 75–83
30 Genchev, D. D., Kermekchiev, M. B. and Hadjiolov, A. A. (1980) Biochem. J. **188**, 85–90
31 Nikolov, E. N. and Dabeva, M. D. (1985) Biochem. J. **228**, 27–33
32 Plagemann, P. G. W. (1971) J. Cell. Physiol. **77**, 213–240
33 Plagemann, P. G. W. (1972) J. Cell Biol. **52**, 131–146
34 Cortes, P., Levin, N. W., Dumler, F., Venkatachalam, K. K., Verghese, C. P. and Bernstein, J. (1979) Biochem. J. **182**, 677–686
35 Bissonnette, R. (1992) Cell Tissue Res. **267**, 131–137
36 Jones, M. E. (1980) Annu. Rev. Biochem. **49**, 253–279
37 Anderson, E. P. and Brockman, R. W. (1964) Biochim. Biophys. Acta **91**, 380–386

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