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Determination of CTP synthetase activity in crude cell homogenates by a fast and sensitive non-radiochemical assay using anion-exchange high-performance liquid chromatography

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Abstract

A non-radiochemical assay procedure for CTP synthetase was developed in which CTP is detected at 280 nm after separation with anion-exchange HPLC. A complete separation of all nucleoside triphosphates was achieved within 11 min and the minimum amount of CTP which could be accurately determined proved to be 5 pmol. Therefore, our assay procedure is ten-fold more sensitive compared to the frequently used radiochemical assays. The assay was linear with time and protein concentration, although at low protein concentration a lag phase was observed. An amount of 2×10^6 cells was already sufficient to determine the specific activity of CTP synthetase in HL-60 cells, lymphocytes and in lymphoblasts obtained from pediatric patients suffering from acute lymphoblastic leukemia.

Keywords: Leukemia; CTP synthetase; Enzymes; Pyrimidine

1. Introduction

CTP synthetase (EC 6.3.4.2) catalyses the formation of CTP from UTP with the concomitant deamination of glutamine to glutamate:

$$\text{UTP} + \text{ATP} + \text{glutamine} \xrightarrow{\text{GTP, } Mg^{2+}} \text{CTP} + \text{ADP} + P_i + \text{glutamate}$$

CTP synthetase is generally regarded as the rate-limiting enzyme in the synthesis of cytosine nucleotides from both de novo and uridine-salvage pathways. The activity of CTP synthetase is normally regulated by its product CTP and mutations which eliminate the allosteric regulation of CTP synthetase by CTP caused a form of multidrug resistance to cultured Chinese hamster ovary cells [1]. Increased activity of CTP synthetase has been observed in a variety of malignant cells [2–5]. Furthermore, the increased activity of CTP synthetase is often associated with the state of progression of tumours and hence provides a biochemical marker for the clinical aggressiveness of tumours [3,4]. In human lymphoblastic leukemia cells, we showed that the synthesis of CTP occurs predominantly via CTP synthetase, whereas in proliferating normal human T lymphocytes the salvage of cytidine is preferred [6]. There-

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fore, CTP synthetase might be an attractive target for selective chemotherapy. Recently, a phase I clinical trial has been performed with cyclopentenyl cytosine (CPEC), a carbocyclic analogue of cytidine, in adults with solid tumours [7]. CPEC is phosphorylated intracellularly to CPEC-triphosphate which is a powerful inhibitor of CTP synthetase [8,9].

In this respect, the measurement of the activity of CTP synthetase in patient cells has been hampered by assay procedures that are insensitive or time consuming [4,10–14]. The most frequently used assays of CTP synthetase are those in which the amount of CTP is measured spectrophotometrically [10,11] or radiochemically in case radiolabeled UTP is used as a substrate for CTP synthetase [4,10,12,14]. The spectrophotometric assay is insensitive and therefore not suitable to measure low activities of CTP synthetase in crude cell homogenates. The radiochemical method is usually based on the separation of [^{14}C]UTP from [^{14}C]CTP with thin-layer chromatography, exposure of the plates to X-ray films and subsequent counting of the identified spots with a liquid scintillation counter [12,14]. This procedure requires extensive manipulations and exposure times of 48 h are required to detect low amounts of radiolabeled CTP [12].

Up to now, no fast and sensitive assay procedures are available in which the activity of CTP synthetase can be measured in crude cell homogenates through the detection of non-radiolabeled CTP. Therefore, we developed a sensitive and fast assay for CTP synthetase which is based on the detection of non-radiolabeled CTP with anion-exchange HPLC. The usefulness of the method was demonstrated by the analysis of the CTP synthetase activity in lymphoblasts from pediatric patients suffering from acute lymphoblastic leukemia as well as in lymphocytes obtained from healthy donors.

2. Experimental

2.1. Chemicals

All chemicals were of analytical grade. NaH_2PO_4, K_2CO_3, $HClO_4$, Tris, L-glutamine, EGTA and $MgCl_2$ were purchased from Merck (Darmstadt, Germany); phosphoenolpyruvate (PEP), pyruvate kinase (2 mg/ml glycerol, 200 U/mg) and dithiothreitol were obtained from Boehringer Mannheim (Mannheim, Germany); phenylmethylsulphonyl fluoride, ATP, UTP, GTP and CTP were obtained from Sigma (St. Louis, MO, USA); fetal calf serum and RPMI-1640 medium with 20 m*M* HEPES were obtained from Flow Laboratories (Irvine, UK); penicillin and streptomycin were obtained from Imperial Laboratories (Wiltshire, UK).

2.2. Isolation of lymphoblasts from pediatric patients with acute lymphoblastic leukemia

Lymphoblasts were isolated from 20 ml EDTA-anticoagulated whole blood or 2 ml EDTA anticoagulated bone marrow obtained at diagnosis before the start of the chemotherapy. After centrifugation (200 *g*, 10 min, room temperature) the platelet-enriched plasma samples were removed. The remaining cell pellets of whole blood or bone marrow were resuspended in "supplemented phosphate-buffered saline" (supplemented PBS) (9.2 m*M* Na_2HPO_4, 1.3 m*M* NaH_2PO_4, 140 m*M* NaCl, 0.2% (w/v) bovine serum albumin, 13 m*M* sodiumcitrate, 5 m*M* glucose and 5 m*M* EDTA, pH 7.4) to a final volume of 25 ml and 8 ml, respectively. These cell suspensions were layered on 12.5 ml or 8 ml Percoll (1.077 g/cm^3 at 20°C, 290 mosM) and centrifuged at 800 *g* at room temperature for 20 min. The interfaces containing the lymphoblasts were removed, diluted with supplemented PBS to a final volume of approximately 12 ml and centrifuged at 800 *g* for 8 min. To lyse the erythrocytes, the pellets were resuspended in 5 ml ice-cold ammoniumchloride solution (8.29 g/l NH_4Cl, 1.00 g/l $KHCO_3$ and 37.2 mg/l EDTA) and kept on ice for 5 min. After addition of 7 ml ice-cold supplemented PBS, the solutions were centrifuged at 220 *g* at 4°C for 8 min. The cell pellets were washed once more with supplemented PBS and the final cell pellets were resuspended in a buffer containing PBS (9.2 m*M* Na_2HPO_4, 1.3 m*M* NaH_2PO_4 and 140 m*M* NaCl, pH 7.4) and 5 m*M* glucose. The final cell suspensions contained >90% lymphoblasts. The viability of the lymphoblasts as measured by the trypan blue dye exclusion method proved to be >95%. The cell suspensions were centrifuged (220 *g*, 8 min) and the cell pellets were frozen in liquid nitrogen and stored at −80°C until further analysis.

2.3. Isolation of lymphocytes

Peripheral blood mononuclear cells were isolated from 20 ml EDTA-anticoagulated whole blood obtained from healthy donors and from a patient in remission, as described in Section 2.2. The lymphocytes were purified from these mononuclear cells by magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) using monoclonal anti CD11b magnetic beads. The mononuclear cells were suspended in 100 μl of a buffer containing PBS, 0.5% (w/v) bovine serum albumin and 2 m*M* EDTA and incubated with 14 μl CD11b Microbeads (Miltenyi Biotec) at room temperature for 15 min. The CD11b positive cells (human monocytes and granulocytes) were retained on a MiniMACS column. The lymphocytes purified in this way were collected by centrifugation and washed twice in PBS supplemented with 5 m*M* glucose. The final cell pellets were frozen in liquid nitrogen and stored at −80°C until further analysis.

2.4. Culturing and harvesting of HL-60 cells

Cultures of HL-60 cells were maintained in humidified air with 5% CO_2 at 37°C in RPMI-1640 medium supplemented with 2 m*M* glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) heat-inactivated fetal calf serum. Cells were kept in exponential growth by diluting the cells to a density of 3×10^5 cells/ml every 2–3 days. HL-60 cells were collected by centrifugation (223 *g* for 10 min) and washed once with PBS and the cell pellet was frozen in liquid nitrogen and stored at −80°C until further analysis.

2.5. Preparation of cell homogenates

The HL-60 cells, lymphoblasts and lymphocytes were solubilised at a concentration of 20×10^6 cells/ml, 40×10^6 cells/ml and 40×10^6 cells/ml, respectively in a buffer containing 35 m*M* Tris–Mops (pH 7.9), 1 m*M* EGTA, 2.5 m*M* phenylmethylsulphonyl fluoride and 10 m*M* dithiothreitol. The HL-60 cells were lysed by rapid freeze–thawing twice in liquid nitrogen. The lymphoblasts and lymphocytes were sonicated three times at 40 W (Vibra-cell Sonificator, output control 20%) for 10 s with intervals of 30 s under constant cooling in ice-water. The samples were incubated on ice for 15 min and centrifuged in a microfuge at 11 000 *g* for 15 min at 4°C. The resulting supernatants were collected and used to determine the activity of CTP synthetase. Protein concentrations in the supernatants were determined with a modified Lowry method using human serum albumine as a standard [15].

2.6. Standard assay method

The reaction mixture contained an aliquot of cell sample, 35 m*M* Tris–Mops (pH 7.9), 4 m*M* ATP, 1 m*M* UTP, 1 m*M* GTP, 10 m*M* L-glutamine, 20 m*M* $MgCl_2$, 10 m*M* KCl, 1 m*M* EGTA, 10 m*M* dithiothreitol, 2 m*M* PEP and pyruvate kinase (17 U/ml) in a total volume of 50 μl. The reaction solution and the sample were equilibrated separately at 37°C for 10 min and the reaction was started by addition of the sample. We observed that the sample itself or the nucleotide standards occasionally contained small amounts of CTP. Furthermore, in the absence of UTP small amounts of CTP were produced during the time of incubation. Therefore, each CTP synthetase assay was also performed in the absence of UTP in order to obtain the proper blank values.

After an appropriate time of incubation (usually 1 h) the reaction mixtures were placed on ice and the reaction catalysed by CTP synthetase was terminated by addition of ice-cold 11.8 *M* $HClO_4$ to a final concentration of 0.56 *M* and kept on ice. After 10 min, the samples were centrifuged (11 000 *g* at 4°C for 5 min) and the resulting supernatants were removed and neutralised to pH 7.0 by addition of 5 *M* K_2CO_3.

2.7. HPLC analysis

Prior to the analyses by HPLC, the supernatants were briefly centrifuged and diluted 10-fold with 35 m*M* Tris–Mops (pH 7.9). The separation of the nucleotides UTP, CTP, ATP and GTP was accomplished by HPLC after injecting 100 μl of a diluted supernatant into the HPLC system. HPLC was performed on an anion-exchange column (type Partisphere SAX, 5 μm particle size, 125×4.6 mm I.D., Whatman International, Maidstone, UK) and an anion-exchange guard column. The HPLC system

consisted of a Perkin-Elmer Series 410 LC pump (Perkin-Elmer, Norwalk, CT, USA) including a Gilson 231 sample injector with a Gilson 401 diluter (Gilson Medical Electronics, Villiers Le Bel, France) and a Neslab Endocal cooling waterbath (Neslab Instruments, Newington, USA) operating at 6°C. The elution of the nucleotides was performed isocratically with 0.594 *M* NaH_2PO_4 (pH 4.55) at a flow-rate of 1 ml/min. The buffer was prepared with Milli-Q water and filtered before use through a 0.45 μm filter. UV detection was performed at 280 nm with a SPD-10A Shimadzu programmable wavelength detector (Shimadzu, Kyoto, Japan). The integration of the chromatographic peaks was performed with a Nelson 900 Series Interface in combination with Nelson Analytical Model 2600 chromatography software (Perkin Elmer Nelson, Cupertina, CA, USA).

2.8. Statistical analysis

The difference between the mean specific activity of CTP synthetase in normal lymphocytes and in lymphoblasts from patients with acute lymphoblastic leukemia (ALL) was analysed with two sample *t*-tests using the log transformed data. Similar results were obtained with analysis by the non-parametric Mann-Whitney test of the original data. The level of significance was set a priori at $P \leq 0.05$. Analysis were performed with the Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA).

3. Results

3.1. HPLC procedure

In order to determine the amount of CTP in the presence of other nucleotides we separated a mixture of nucleotide standards by anion-exchange HPLC. Fig. 1 shows that the nucleotides CDP, ADP and GDP were separated from each other and that a complete base-line separation of the nucleotides UTP, CTP, ATP and GTP was achieved within 9 min. Under these conditions, the remaining components of the standard mixture NAD, AMP, IMP, GMP, UDP-*N*-acetylglucosamine, UDP-glucose and UDP eluted before CDP and were not separated from each other.

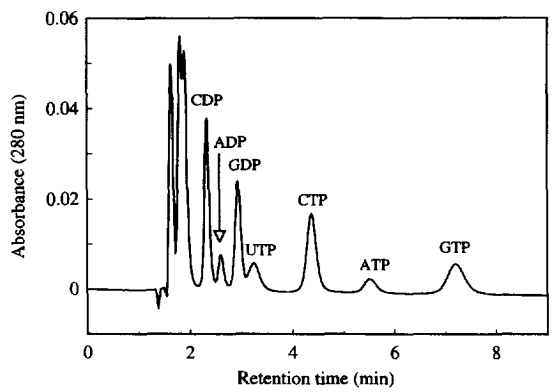

Fig. 1. Elution profile of nucleotide standards. The elution profile was obtained by injecting 100 μl of a standard containing 5.0 μ*M* NAD, 5.5 μ*M* AMP, 5.4 μ*M* IMP, 5.8 μ*M* GMP, 4.9 μ*M* UDP-*N*-acetylglucosamine, 4.8 μ*M* UDP-glucose, 5.6 μ*M* UDP, 5.7 μ*M* CDP, 5.1 μ*M* ADP, 5.6 μ*M* GDP, 5.8 μ*M* UTP, 5.2 μ*M* CTP, 5.5 μ*M* ATP and 5.1 μ*M* GTP into the HPLC system. The different nucleotide peaks in the chromatogram of the standard mixture were identified by comparison of the retention times with authentic nucleotides.

In a reaction mixture, only the nucleotides UTP, CTP, ATP and GTP are normally present and at much higher concentrations compared to the concentrations of the nucleotides in our standard mixture. In this respect, the optimal detection wavelength for CTP in the presence of a large concentration of ATP proved to be 280 nm. At this wavelength, which is near the absorbance maximum of CTP (275 nm), the interference of the broad ATP peak is minimised. Fig. 2 shows the elution profile of nucleotide standards (Fig. 2A) and that of a reaction solution prior to the addition of sample (Fig. 2B). The amount of CTP produced by a homogenate of HL-60 cells is shown in Fig. 2C and the identification of the CTP peak was confirmed by spiking the nucleotide extract with CTP (Fig. 2D). A complete separation of UTP, CTP, ATP and GTP was achieved within 11 min. This figure also clearly demonstrates that low amounts of CTP can adequately be separated and analysed in the presence of large concentrations of UTP, ATP and GTP. Under these conditions, PEP and pyruvate kinase were able to maintain the nucleotides in their triphosphate form and no significant amounts of nucleoside diphosphates were observed in the reaction mixture (results not shown).

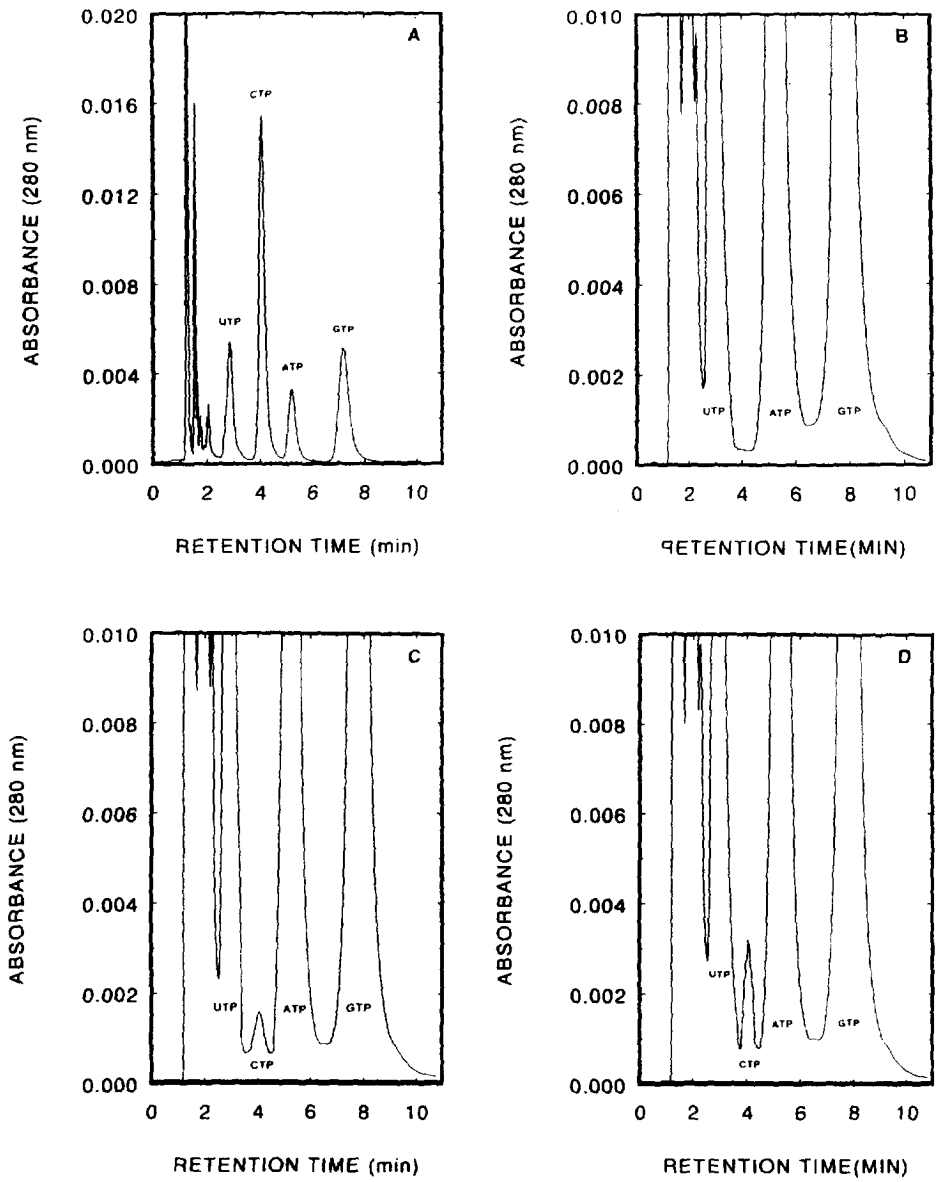

Fig. 2. Identification of CTP in a reaction mixture. Panel A shows the elution profile of a nucleotide standard composed of 5.8 μ*M* UTP, 5.0 μ*M* CTP, 5.4 μ*M* ATP and 5.2 μ*M* GTP, obtained after injecting 100 μl into the HPLC system. Panel B shows the elution profile of the nucleotides in a reaction mixture prior to the addition of a sample. The amount of CTP (5.7 μ*M*) produced by a homogenate from HL-60 cells (0.41 mg/ml) after 1 h incubation at 37°C is shown in panel C. Panel D shows the identification of the CTP peak by spiking the nucleotide extract of panel C with 5.2 μ*M* CTP.

The intra-assay C.V.s and inter-assay C.V.s for the detection response-factors at 280 nm of the nucleotide standards were less than 1.0% ($n=15$) and 3.7% ($n=10$), respectively. The separation of the nucleotide standards and the nucleotides obtained after extraction of a reaction solution supplemented with 4 μ*M* CTP proved to be highly reproducible with intra-assay C.V.s and inter-assay C.V.s for retention times of less than 1.3% ($n=15$) and 9.2% ($n=10$), respectively. Under these latter conditions the re-

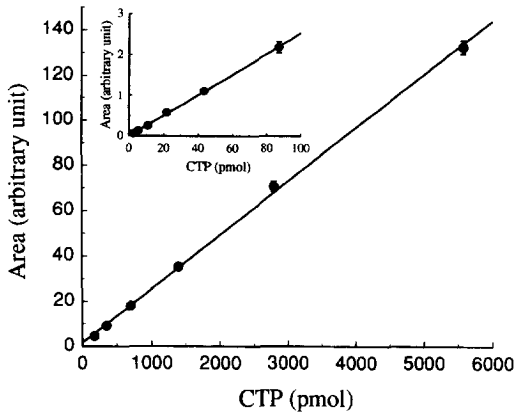

Fig. 3. Relationship between the injected amount of CTP and the peak area. CTP was added to a reaction solution to final concentrations of 0.5 μ*M*–625 μ*M*. Nucleotides were extracted with $HClO_4$ and analysed by HPLC. The insert shows the data points obtained for CTP amounts between 5–100 pmol. Each data point represents the mean±S.D. of 3–6 experiments. In those cases where no S.D. values (bars) are given, the bar sizes do not exceed the symbol size.

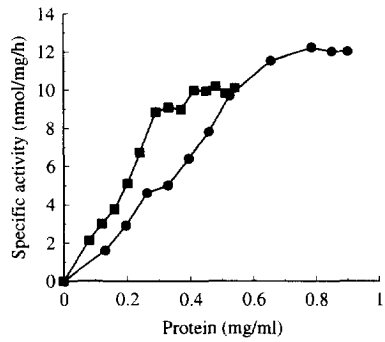

Fig. 5. Relationship between the specific activity of CTP synthetase and the protein concentration. The data points to calculate the specific activity of CTP synthetase from HL-60 cells (●) and lymphoblasts (■) were obtained from Fig. 4.

covery of all nucleotides proved to be more than 93% with an inter-assay C.V. of 3.7% ($n=9$).

To investigate the range of CTP concentrations that can be accurately determined in a reaction solution, CTP was added to a reaction solution resulting in final concentrations of 5 n*M*–5 m*M*. Fig. 3 shows that a linear correlation exists between the injected amount of CTP from 5 pmol up to 5500 pmol and the area of the chromatographic peak ($r \geq 0.999$). These amounts of CTP correspond to a CTP concentration range of 0.5 μ*M* up to 625 μ*M* in the reaction solution. Within this range of CTP concentrations the recovery of the amount of CTP added to the reaction solution was approximately 93±3%. At CTP concentrations above 625 μ*M*, a deviation from linearity occurred due to a decreased response of the UV detector to such high absorbance values (results not shown). The detection limit of CTP in the HPLC system, defined as three times the value of the baseline noise, is approximately 2 pmol.

3.2. Reaction conditions

The rapid freeze–thaw procedure was used for disruption of HL-60 cells. Although this technique

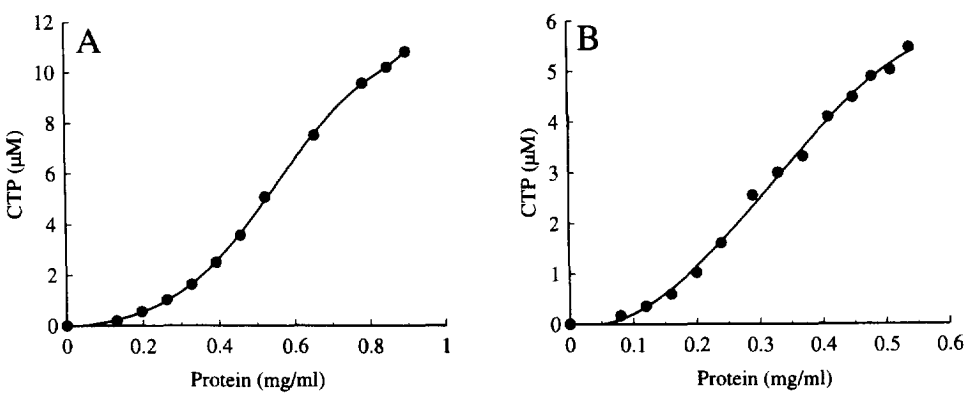

Fig. 4. Protein dependence of the CTP synthetase reaction. Panel A shows the amount of CTP produced by CTP synthetase from HL-60 cells. In panel B the amount of CTP produced by CTP synthetase from lymphoblasts is depicted. The reactions were allowed to proceed for 1 h at 37°C. Each data point represents the mean of three experiments.

has also been used to prepare cell extracts of normal lymphocytes and leukemic lymphoblasts [4], we frequently observed an increase in viscosity of homogenates of lymphoblasts due to disruption of nuclei with the concomitant liberation of the nucleic acids. In contrast, this phenomenon was not encountered when disruption of the lymphoblasts was performed by sonication. Solubilisation of HL-60 cells at a concentration of 20×10^6 cells/ml and the lymphoblasts and lymphocytes at a concentration of 40×10^6 cells/ml, yielded homogenates with protein concentrations of 1.3 mg/ml, 0.9 mg/ml and 0.9 mg/ml, respectively. These protein concentrations correlate nicely with those obtained by De Korte and coworkers who showed that HL-60 cells contained approximately 2–4 times as much protein per cell than normal lymphocytes from healthy volunteers and lymphoblasts obtained from patients suffering from acute lymphoblastic leukemia [16,17].

Fig. 4A shows that the amount of CTP produced by CTP synthetase from HL-60 cells increases linearly with respect to the protein concentration in the range of 0.3 mg/ml to 0.85 mg/ml, corresponding to a cell amount in the reaction mixture of approximately 0.25×10^6 cells and 0.65×10^6 cells, respectively. However, at low protein concentrations (<0.3 mg/ml) a deviation from linearity occurred. In an analogous way, Fig. 4B shows that the amount of CTP produced by CTP synthetase from lymphoblasts increases linearly with the amount of protein in the range of 0.15 mg/ml up to 0.54 mg/ml, corresponding to a cell amount in the reaction mixture of approximately 0.35×10^6 cells and 1.25×10^6 cells, respectively. At low protein concentrations (<0.15 mg/ml) a deviation from linearity was observed. This phenomenon is clearly illustrated when the specific activities of CTP synthetase from HL-60 cells and lymphoblasts are plotted against the protein concentration (Fig. 5). Fig. 5 also shows that the maximum specific activities of CTP synthetase from HL-60 cells and lymphoblasts were obtained at protein concentrations above 0.8 mg/ml and 0.4 mg/ml, respectively. The deviation from linearity of the activity of CTP synthetase at low protein concentrations could not be prevented by inclusion of human serum albumin at a concentration of 1 mg/ml in the reaction mixture. With respect to the time dependence of the CTP synthetase reaction the formation of CTP by CTP synthetase from HL-60 cells and lymphoblasts was linear with reaction times up to at least 120 min (Fig. 6).

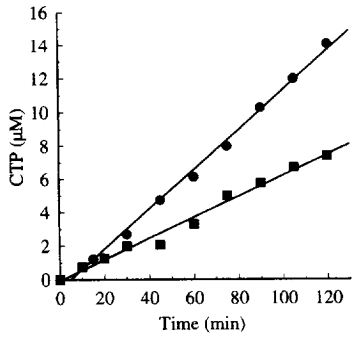

Fig. 6. Time dependence of the CTP synthetase reaction. The activities of CTP synthetase from HL-60 cells (●) and lymphoblasts (■) were measured at a protein concentration of 0.52 mg/ml and 0.43 mg/ml, respectively. Each data point represents the mean of three experiments.

Approximately 2×10^6 cells proved to be sufficient to perform all the necessary assays to determine the maximum specific activity of CTP synthetase in HL-60 cells, lymphoblasts and lymphocytes. With this assay we determined the specific activity of CTP synthetase in lymphoblasts obtained from pediatric patients suffering from acute lymphoblastic leukemia and in lymphocytes obtained from healthy donors (Fig. 7). The mean specific activity of CTP synthetase in lymphoblasts (6.6±2.8 nmol/mg/h, range 3.1–10.0 nmol/mg/h) was approximately 3.5-fold higher than that observed in lymphocytes from healthy donors (1.9±0.8 nmol/mg/h, range 0.44–2.70 nmol/mg/h). Furthermore, we observed that the specific activity of CTP synthetase in lymphoblasts from a patient obtained at diagnosis (10 nmol/mg/h)

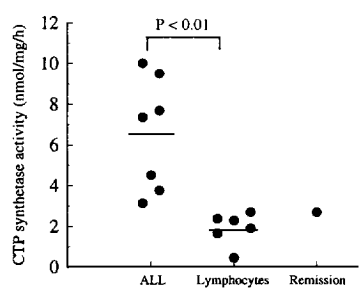

Fig. 7. Specific activity of CTP synthetase. The mean specific activity of CTP synthetase is indicated by solid lines.

was almost 4-fold higher than that observed in the lymphocytes obtained at remission (2.7 nmol/mg/h).

4. Discussion

In this study we developed a fast and sensitive assay of CTP synthetase which is based on the detection of non-radiolabeled CTP with anion-exchange HPLC. One of the essential features of this method is the maintenance of the nucleotides in their triphosphate form through inclusion of PEP and pyruvate kinase in the reaction mixture. In this way, a true reaction velocity of CTP synthetase can be measured. Williams et al. observed that the degradation of nucleotides by enzymes such as ATPase and adenylate kinase could be prevented by NaF in the presence of PEP [12]. Therefore, NaF has been routinely included into the assay mixture by others [2,13,14,18–20]. However, one should bear in mind that Mg^{2+} precipitates in the presence of F^- and that the presence of Mg^{2+} has been shown to be obligatory for the activity of CTP synthetase [19,21,22].

The separation of all nucleoside monophosphates, diphosphates and triphosphates usually requires a gradient of several buffers and long elution times [23,24]. Therefore, an additional advantage of the maintenance of the nucleotides in their triphosphate form is that a complete separation of all nucleoside triphosphates could be performed under isocratic conditions within 11 min. Furthermore, this analysis time is also considerably shorter compared to other HPLC procedures that have been used to detect nucleoside triphosphates [13,24–27]. The HPLC procedure proved to be highly reproducible with inter-assay C.V.s of the retention times and recovery for the different nucleotides in a reaction solution of less than 9.2 and 3.7%, respectively.

At low protein concentrations, both HL-60 cells and lymphoblasts showed a deviation from linearity with respect to the formation of CTP. Such a lag phase or hysteresis is typical for an enzyme existing in a slow association–dissociation equilibrium in which the two polymerisation states differ in their activities [28,29]. For CTP synthetase purified from *Escherichia coli* and rat liver various aggregated states have been observed such as monomeric, dimeric and tetrameric forms with the tetrameric form being the most active one [18,30,31]. The presence of saturated amounts of ATP and UTP as well as high enzyme concentrations have been shown to promote the formation of tetramers [18,31,32]. Our observation that at low protein concentrations no increase in activity of CTP synthetase was observed after pre-incubation in the presence of UTP and ATP when compared to the untreated sample (results not shown) is in line with results obtained for the enzyme from *Escherichia coli* [31]. Deviations from linearity with respect to the protein dependency of the reaction catalysed by CTP synthetase have been reported for the enzyme from *Escherichia coli* [31], Chinese hamster fibroblasts [14] and bovine liver [22] whereas no lag phase was observed for CTP synthetase from other mammals, including man [3,4,12]. A conceivable possibility might be that in those cases the occurrence of a lag phase has been overlooked since this phenomenon occurs only at low protein concentrations.

Within this context, we would like to point out the profound effect of the protein concentration on the specific activity of CTP synthetase. This phenomenon might partially explain the considerable variation in the specific activity of CTP synthetase that has been observed for malignant lymphoid cells from patients suffering from the same type of leukemia [4]. Indeed, the mean specific activities of CTP synthetase of normal lymphocytes and lymphoblasts obtained from patients suffering from acute lymphoblastic leukemia proved to be 2-fold higher than the mean specific activities of CTP synthetase as reported by Ellims et al. [4]. It should be noted that the variation of the specific activity with protein concentration is not a very rare phenomenon since it has been observed for many other enzymes such as purine nucleoside phosphorylase, AMP deaminase and UMP synthase [29].

The minimum amount of CTP which could be accurately determined proved to be 5 pmol CTP. Therefore, our assay is ten-fold more sensitive compared to the radiochemical method in which radiolabeled CTP was separated from radiolabeled UTP with thin-layer chromatography [12]. Due to this high sensitivity this assay is especially suitable for the measurement of low CTP synthetase activities in crude cell homogenates, thus eliminating the necessity to purify the enzyme [33]. Since only a

very low amount of cells is required to determine the activity of CTP synthetase (2×10^6 lymphoblasts), one should be able to monitor the CTP synthetase activity in patient cells during treatment with for example CPEC. With this assay we already observed that the specific activity of CTP synthetase in the lymphoblasts obtained at diagnosis was almost 4-fold higher than that observed in the lymphocytes obtained at remission. The appearance of resistance to CPEC during the treatment due to altered kinetic properties of CTP synthetase [33] or a drug-induced upregulation of enzyme activity might also be discovered at an early stage. The characterisation of CTP synthetase from various malignant cells is in progress in our laboratory.

Acknowledgments

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