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Note

Method for the determination of the specific activities of UTP and CTP in mouse kidney by high-performance liquid chromatography

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Hormonal effects, specifically of estrogen and testosterone, on gene regulation have been studied by measuring rates of mRNA synthesis in vivo [1,2] A labelled RNA precursor is injected into an animal and after a short period of time, usually 1–3 h, RNA is isolated The mRNA to be studied can be separated from the total RNA by hybridization and the amount of label incorporated into both can be measured to give a relative rate of synthesis for the mRNA [2] To calculate the actual amount of mRNA synthesized, and in turn the absolute transcription rate of a given gene, the specific activities of the labelled nucleoside triphosphates available for incorporation into RNA must be determined [1] Anion-exchange high-performance liquid chromatography (HPLC) allows one to separate the small quantities of nucleotides found in tissues [3] and measure their quantity and radioactivity, the factors in specific activity calculations.

Studies of this type have been done on vitellogenin mRNA using [^{3}H]uridine as the labelled RNA precursor in *Xenopus* liver with subsequent measurement of the specific activity of UTP [1]. The present paper describes modifications to that procedure which were necessary when the source of mRNA is mouse kidney. [^{3}H]Orotic acid was used for pulse-labelling, because it is taken up in the kidney and incorporated into RNA at least ten times more efficiently than is [^{3}H]uridine [2]. The nucleotide extraction procedure which gave a large yield of UTP from *Xenopus* liver was unsuitable for the mouse kidney, because

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of increased action of degradative enzymes [4], so an optimized procedure employing direct homogenization in a perchloric acid solution is described Tritium was incorporated into UTP, but not significantly into CTP during 45 min of pulse-labelling in *Xenopus* liver [1], therefore, the specific activity of UTP could be determined by anion-exchange HPLC using a 0.1–0.3 *M* ammonium phosphate gradient system at one pH, 5 2 In the case of mouse kidney, tritium is incorporated significantly into CTP as well After 30 min of labelling CTP has about half the specific activity of UTP and the two are approximately equal after 1 h. But CTP coelutes with ATP at pH 5 2 Separation of both CTP and UTP could be accomplished by chromatography at pH 4.3. In addition, lengthening the gradient at pH 5 2 allowed the presence of the other potentially labelled pyrimidines, dCTP and dUTP, to be determined to rule out the possibility of interference with specific activity measurements at pH 4 3

EXPERIMENTAL

Reagents

HPLC-grade acetonitrile and ammonium phosphate monobasic were obtained from Fisher Scientific (Springfield, NJ, U.S A) and were prepared using Technic Central System (Seattle, WA, U.S.A) purified water. Nucleotide standards were obtained from Sigma (St. Louis, MO, U.S A.), *Ecolume* scintillation fluid from ICN (Irvine, CA, U.S A) and 5-[^{3}H]orotic acid, with a specific activity of 20 Ci/mmol, from NEN (Boston, MA, U.S.A)

Extraction of nucleotides

Kidneys were removed from mice (*Mus musculus*), each of which had been injected intraperitoneally with 1 mCi of 5-[^{3}H]orotic acid and killed by carbon dioxide asphyxiation after various time intervals The kidneys were demembranated and frozen immediately by immersion in liquid nitrogen for 2 min They were then stored at $-70°$C until use.

A solution of perchloric acid-containing buffer salts was prepared by adding the concentrated acid to stock 10 m*M* Tris–1 m*M* EDTA pH 8.0 to give a final perchlorate concentration of 0.5 *M*. In a typical experiment, a frozen kidney weighing 140 mg was placed in 1.12 ml (8 volumes) of the perchloric acid–Tris–EDTA solution at 0°C and homogenized with a Brinkman polytron at medium speed until a uniform suspension formed (approximately 5 s). The homogenate was centrifuged immediately at 16 500 *g* at 4°C for 5 min. To the supernatant were then added 67 μl of 9 *M* potassium hydroxide (0.06 volume), and the pH of the solution was adjusted with potassium hydroxide or perchloric acid to between 5.5. and 7.0. The solution was maintained at 0°C for 5 min to maximize the precipitation of potassium perchlorate, followed by recentrifugation at 16 500 *g* at 4°C for 5 min The supernatant was quick-frozen by im-

mersion in a dry ice–acetone bath for 1–2 min and was then lyophilized until dry The freeze-dried powder was stored at $-70°C$ until use.

To prepare the sample for HPLC analysis, the lyophilized powder was dissolved in 100 μl of buffer A (described below) and centrifuged at 16 500 g and 4°C for 2 min, followed by filtration through a 0 2-μm Nylon 66 membrane

High-performance liquid chromatography

The apparatus used was a Water Assoc. (Milford, MA, U S.A) Model 660 solvent programmer with two Model 6000A pumps, a Rheodyne (Cotati, CA, U S.A) Model 7120 injector with a 500-μl sample loop, and a Waters Model 440 absorbance detector at 254 nm. Nucleotides were separated on a 250 mm×4 6 mm I D Whatman (Clifton, NJ, U S.A) Partisil 10 SAX anion-exchange column equipped with a 250 mm×4 6 mm I D. silica saturator pre-column and a 10 mm×4 6 mm I.D 5-μm SAX guard column

Buffer A was a solution of 0 1 M $NH_4H_2PO_4$, pH 4 3–acetonitrile (85 15, v/v) Buffer B was a solution of 0.3 M $NH_4H_2PO_4$, pH 4 3–acetonitrile (85 15, v/v)

The sample (100–200 μl) was injected and buffer A was run at 1 5 ml/min for 5 min followed by a linear gradient of 0–100% buffer B over 10 min at the same flow-rate The column was reequilibrated with buffer A at 2.0 ml/min for 15 min This procedure was carried out at room temperature

Quantitation

Peak areas were determined by multiplying peak height×width at half height×attenuation. Molar amounts for the nucleotide peaks from kidney were determined by plotting peak areas on calibration curves made with standard nucleotides of known concentrations Calibration curves were made for UTP and CTP in the range 100–5000 pmol.

To determine the ^3H content, each peak of the kidney chromatogram (1–3 ml) was collected in a scintillation vial and mixed with 15 ml of *Ecolume* for counting.

Specific activity was defined as cpm/pmol, this result was obtained by dividing the ^3H content of the UTP or CTP peak by the quantity of the triphosphate in pmol as determined from the standard curve The peaks were identified by retention times in comparison to those of standard nucleotides and by addition of known amounts of standard to the kidney extract.

The protein content of the kidney extract was measured by performing the Lowry et al [5] protein assay on the supernatant of the spin after homogenization (before potassium hydroxide was added)

RESULTS AND DISCUSSION

For maximum yield of nucleoside triphosphates from mouse kidney, the action of degradative enzymes must be avoided [4] The kidney should be pro-

cessed immediately after removal from the mouse It can be frozen in liquid nitrogen and stored at $-70\,^{\circ}C$ for later extraction, but homogenization directly after dissection increases the yield of UTP by about 55% The most important factor in nucleotide yield is to homogenize the kidney directly in a perchloric acid solution to precipitate protein. However, the nucleotides are unstable in acid solution [4,6], so a minimum time for the protein precipitation step before neutralization of the solution is advantageous We found that allowing the homogenate to remain in perchloric acid for extended periods of time does not increase the amount of protein precipitated, therefore the homogenate can be centrifuged and neutralized immediately after homogenization Fig 1 shows that the perchloric acid concentration needed for maximum precipitation of protein can be as low as 0 3 *M*. The intermediate concentration of 0 5 *M* was used to maximize protein removal and nucleotide stability A known amount of standard UTP was carried through the extraction procedure without mouse kidney present to determine the amount of recovery About 75% of the UTP was recovered

The kidney extract was quick-frozen and lyophilized for concentration before injection, however, the extract could also be injected directly A final spin and filtration was necessary to remove residual potassium perchlorate precipitate before injection [3]

The absolute rate of synthesis of mRNA in vivo can be measured through the uptake of radiolabelled precursors provided that the specific activities of the precursor pools are known [1] (Relative rates of mRNA synthesis previously have been measured in kidney [2], but until now the in vivo pools of labelled nucleotide precursors were not readily measured) For the experi-

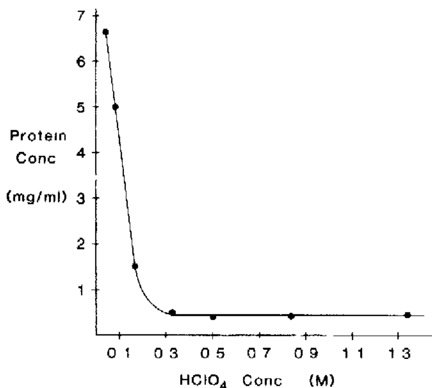

Fig 1 Effect of perchloric acid concentration on protein precipitation from kidney extracts The concentration of protein left in solution after homogenization of the kidney in varying concentrations of perchloric acid was measured by the Lowry et al [5] protein assay, as described in the Experimental section Each point represents the mean of kidney homogenates from three different mice

ments presented here, the label was introduced via 5-[^{3}H]orotic acid which was injected into the animal [2] It can follow several pathways in the pyrimidine biosynthesis system [7,8], the main one being production of UTP and CTP which become incorporated into RNA (The other immediate precursors of RNA, ATP and GTP, are synthesized by a different pathway, in the purine system, and do not become labelled) Addition of a ribose phosphate group to orotic acid followed by decarboxylation yields UMP which is phosphorylated to UTP CTP is formed from UTP by amination The specific activities (cpm/pmol) of CTP and UTP can be measured as described in the Experimental section Labelled pyrimidine may also be incorporated into the deoxynucleotides, dUTP and dCTP, via the thioredoxin system [7] All of the deoxynucleotides are present in cells only in small amounts, especially dUTP, which is an extremely transient intermediate, because they are rapidly incorporated into DNA [8] Nevertheless, the presence of dUTP and dCTP must be accounted for to exclude the possibility that they might contribute radioactivity to the UTP or CTP peaks by coelution The remaining pyrimidine-derived deoxynucleotide, TTP, is not labelled, because it is formed when a methyl group replaces the tritium at position 5 of dUMP

Several buffer systems for separating nucleotides on a SAX column have been described [3,9–11] The one employed here has the advantage of using buffers at one pH, thereby shortening column reequilibration time, and does not use potassium chloride to establish a gradient which can be corrosive to stainless-steel components over time [12]

With buffers A and B at pH 4 3, UTP, CTP, and dCTP are separated from each other, but dUTP and UTP coelute (Fig 2) Raising the pH of buffers A and B to 5 2 and lengthening the gradient to 30 min will adequately separate dUTP and UTP, but CTP coelutes with ATP under these conditions (Fig 3) Therefore, both systems were needed initially to evaluate the purity of the UTP and CTP peaks for specific activity measurements

The results for a mouse kidney extract are shown in Fig 4 Nucleotides were

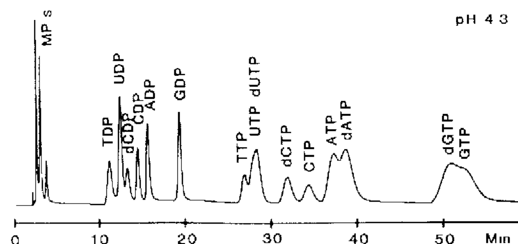

Fig 2 Chromatogram of nucleotide standards at pH 4 3 A 20-μl sample of water containing about 500 pmol of each standard was injected at time zero At 1 5 ml/min buffer A was run for 5 min, followed by a linear gradient to 100% buffer B over 10 min (0 05 A_{254} units full scale) MP's = monophosphates

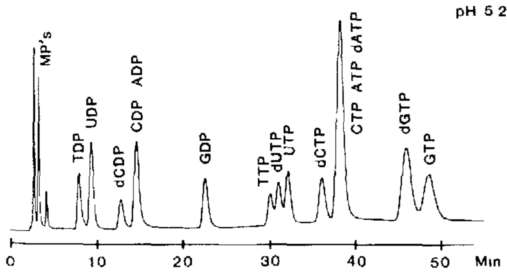

Fig 3 Chromatogram of nucleotide standards at pH 5 2 A 20-μl aliquot of the same nucleotide standard mix that was used in Fig 2 was injected at time zero At 1 5 ml/min buffer A was run for 5 min, followed by a linear gradient to 100% buffer B over 30 min (0 05 A_{254} units full scale) MP's = monophosphates

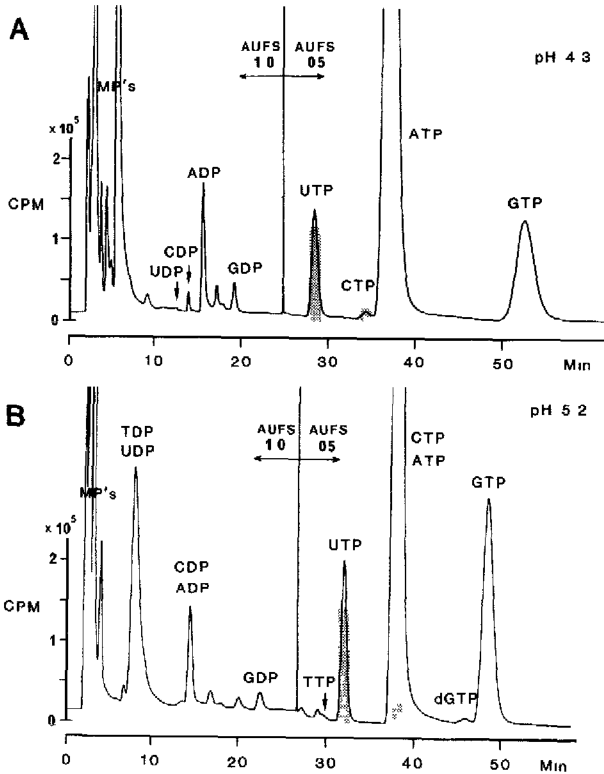

Fig 4 Chromatograms of kidney extract The pulse-labelled kidney extract described in the text was injected at time zero The solid line is the absorbance profile at 254 nm Full scale changes from 1 0 to 0 05 at the vertical line at approximately 25 min (AUFS = absorbance units full scale) The amount of radioactivity in each peak, measured in cpm, is shown for the triphosphates by the bars (A) Chromatographed at pH 4 3 as described in Fig 2 (B) Chromatographed at pH 5 2 as described in Fig 3

extracted from one kidney of a mouse pulse-labelled with [^{3}H]orotic acid for 3 h and the amount injected for each chromatogram was equivalent to one fourth of the kidney. Fig. 4A, at pH 4 3, shows that UTP and CTP are both labelled and that ATP and GTP are not, as expected There is no dCTP peak At pH 5 2 (Fig 4B), the same peaks are labelled, but in this case CTP and ATP coelute There is no dCTP present and also no dUTP peak We have observed these results for several kidney extracts from various pulse-labelling times between 30 min and 96 h Therefore, in the present study the buffer system at pH 4 3 can be used exclusively to measure the specific activities of CTP and UTP, because no contaminating radioactive components are present

The specific activities of UTP and CTP in the kidney extract shown in Fig 4 were 100 and 107 cpm/pmol, respectively Note that although relatively small amounts of CTP (190 pmol) were observed, its specific activity was similar to that of UTP (1300 pmol) after 3 h of labelling We have found that the specific activities of the UTP and CTP pools appear to reach maximum values by 3 h after injection of the labelled orotic acid

The in vivo labelling of nucleoside triphosphate pools can be followed by the HPLC methods described Combining specific activity measurements of the RNA precursors UTP and CTP, as described here, with measurements of relative rates of mRNA synthesis, as described in Watson and Paigen [2], will allow the calculation of transcription rates in terms of mRNA molecules made per gene

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