Heterocycles Related to Nucleotides. XI. \(^1\) An Organomericurial Column Chromatography for Thiouridine

Eisuke SATO,*,** Atsuko SUZUKI,** Yuichi KANAOKA,** and Koichi KIMURA***

Faculty of Pharmaceutical Sciences, Hokkaido University,** Kita-12, Nishi-6, Kita-ku, Sapporo 060, Japan and Department of Applied Biological Sciences, Faculty of Science and Technology, Science University of Tokyo,** Noda, Chiba 278, Japan

(Received February 7, 1990)

By the organomericurial column: chromatogel or Sephadex coupled to p-aminophenylmercury chloride, 4- and 2-thiouridine were selectively separated from the mixtures with major nucleotides. Oligonucleotides containing 4-thiouridinic acid were also separated from the ribonuclease-T, digests of unfraccionated transfer ribonucleic acid of E. coli.

Keywords — 4-thiouridine; 2-thiouridine; transfer RNA; p-chloromercuriobenzoic acid; p-chloromercurianilino; organomericurial column chromatography

During studies of biopolymers, purification of biopolymers to the single component is an important as well as a tedious step. Since Cuatrecasas reported an elegant method of affinity chromatography,\(^2\) it has been very useful for enzyme purification. We have reported several methods of chemical modification of transfer ribonucleic acid (t-RNA), such as fluorescence labelling,\(^3\) which is effective for thiouridine, one of the minor components of t-RNA. To study the function of a specific t-RNA, a fractionation of amino acid specific t-RNA is essential. For this purpose, DEAE-sephadex or benzoylated-cellulose columns have been used. On the other hand, mercuric chloride, which reacts reversibly with thiopyrimidinone, has been used as a protecting group of the sulfur atom in the synthesis of thiopyrimidinone nucleosides.\(^4\) Recently several examples of a reaction of t-RNA\(^{Tyr}\), t-RNA\(^{Val}\), t-RNA\(^{Phe}\), t-RNA\(^{Met}\), 4-thiouridylic acid and 2-thiouridylic acid with mercurials, have been reported.\(^5\) In addition there are several examples of immobilized organomericurials in protein chemistry for purification of SH-proteins,\(^6a\) bovine serum albumin,\(^6b\) horse hemoglobin,\(^6c\) papain,\(^6d\) creatine phosphokinase,\(^6e\) and human plasma factor XIII.\(^6f\) However, there are no examples of application of an organomericurial column chromatography for t-RNA study, although studies on 6-thioguanosine in deoxyribonucleic acid (DNA) have been reported during our work,\(^7\) and studies on interaction of t-RNA with organomericurial by affinity electrophoresis was recently reported.\(^8\)

In this paper, by virtue of this characteristic of mercurial for thiouridine, we would like to illustrate a new type of column chromatography, which is available for handy and selective separation of the thiouridines from their mixture with major nucleotides. Oligonucleotides containing 4-thiouridinic acid can also be separated from the

\[
\text{R: ribosyl}
\]

* To whom correspondence should be addressed.
mixtures of the ribonuclease-T₁ digests of the unfractionated E. coli t-RNA.

**Experimental**

**Instruments** — Ultraviolet spectra were recorded on a Shimazu UV-200 spectrophotometer. pH was measured with a Hitachi-Horiba M-5 pH meter. Melting points were measured with a Yamato MP-21 apparatus and are uncorrected.

**Materials** — p-Chloromercuribenzoic acid (PCMB) is a product of Wako Pure Chemical (Japan). p-Aminophenylmercuric acetate was obtained from aniline and mercuric acetate by Dimroth’s procedure, mp 165—168 °C (lit., mp 166—167 °C), and p-chloromercuriobaniline (PCMA) was prepared from p-aminophenylmercuric acetate by treating with aqueous sodium chloride, mp 187—188 °C (lit., mp 188 °C). Aminoethyl cellulose and carboxymethylcellulose are from Serva. Chromagel A-4 is a product of Dojindo Co. (Japan). Sephadex G-25 is from Pharmacia. Baker Yeast t-RNA was a kind gift from Professor T. Ueda.

**Preparation of Adsorbents** — Coupling of PCMB to aminoethylcellulose (AE-PCMB): PCMB (36 mg, 0.10 mmol) was coupled to aminoethylcellulose (74 mg, 0.012 m eq.) with water-soluble carbodiimide (EDC-HCl) (193 mg, 1.0 mmol) at pH 7.0 by Cuatrecasas’ procedure. Binding capacity for 4-thiouracil is $6.7 \times 10^{-3}$ meq.

Coupling of PCMA to Carboxymethylcellulose (CM-PCMA): PCMA (33 mg, 0.10 mmol) was coupled to carboxymethyl cellulose (32 mg, 0.015 m eq.) with EDC-HCl (193 mg, 1.0 mmol) at pH 5.0 by the same procedure with the preparation of AE-PCMB. Binding capacity for 4-thiouracil is $9.5 \times 10^{-3}$ meq.

Coupling of PCMA to Chromagel A-4 (Chromagel-PCMA): Column material was prepared as described by Sluyterman. About 11 ml of chromagel A-4 was suspended in 50 ml water, and 4.5 g (42 mmol) of BrCN in 50 ml water was added slowly at pH 11—11.5 maintaining with 5 N aq. NaOH for 30 min at 0 °C with gentle stirring. At the end of the reaction, the chromagel was washed with 100 ml of 0.1 N aq. sodium bicarbonate on a glass filter and

![Fig. 1. Fractionation of 4- and 2-Thiouridine and Major Nucleotides with Chromagel-PCMA Column](attachment:image.png)

a, charged the mixture of 5'-UMP, 4- and 2-thiouridine; b, charged the mixture of 5'-CMP, 4- and 2-thiouridine; c, charged the mixture of 5'-AMP, 4- and 2-thiouridine; d, charged the mixture of 5'-GMP, 4- and 2-thiouridine; e, eluted with 0.001 N HCl aq.; f, eluted with 0.01 N HCl aq.
resuspended in 20 ml of 10% aq. DMSO. To this suspension, 800 mg (2.4 mmol) of PCMA in 10 ml dimethylsulfoxide (DMSO) was added drop-wise at 0 °C and stirred at 5 °C for 26 h, then warmed at 30 °C for 5 min. The chromagel was washed with 3.0 l of 20% aq. DMSO, 0.5 l of 0.001 N aq. HCl and 1.0 l of water. Binding capacity for 4-thiouracil is \(2.2 \times 10^{-3}\) meq/ml.

Coupling of PCMA to Sephadex G-25 (Sephadex-PCMA): 10 g of Sephadex G-25 was treated by the same procedure with Chromagel-PCMA above. Binding capacity for 4-thiouracil is 0.10 meq./g dried gel.

**Separation of 4- and 2-Thiouridine from Major Nucleosides or Nucleotides with an Organomercurial Column** — Chromagel-PCMA Column: Column size 1.0 × 8.0 cm, binding capacity is 0.014 meq. The combined solution of (1.0 ml, 0.05 M NaCl aq.), each of 5'-uridylic acid, 4-thiouridine and 2-thiouridine (1 mM solution each) was placed on the column, and every 20 drops (2.0 ml) was collected. The optical density (OD) was recorded at 262, 300 and 330 nm. OD increased at 262 nm only to release 5'-uridylic acid alone. Mixture of 5'-cytidylic acid, 4-thiouridine and 2-thiouridine was applied, and treated in the same way with the 5'-uridylic acid mixture. OD was recorded at 272, 300 and 330 nm releasing 5'-cytidylic acid only. In the case of mixture with 5'-adenylic acid, only 5'-adenylic acid was eluted monitoring OD at 260, 300 and 330 nm. OD of eluate from the mixture with 5'-guanylic acid only. Then the column was treated with 0.001 N HCl aq., OD at 300 and 330 nm were recorded. The increase at 330 nm was negligible, so only 2-thiouridine was released. The column was finally eluted with 0.01 N HCl aq., and OD at 330 nm was recorded. 4-Thiouridine was eluted as shown in Fig. 1.

**AE-PCMB Column**: Column size 0.9 × 2.0 cm, binding capacity is 0.02 meq. By the same procedure with Chromagel-PCMA column, major nucleosides were separated from the mixture with 4- and 2-thiouridine, by eluting with water, 0.001 N HCl aq. and 0.01 N HCl aq. for major nucleoside, 2-thiouridine and 4-thiouridine, respectively.

**CM-PCMA Column**: Column size 0.7 × 2.0 cm, binding capacity for 4-thiouracil is 0.02 meq. By the same procedure with chromagel-PCMA column, major nucleosides were separated from the mixture with 4- and 2-thiouridine eluting with 0.02 M NaCl aq. (uridine monophosphate (5'-UMP), cytidine monophosphate (5'-CMP), adenosine monophosphate (5'-AMP)), 0.05 M NaCl aq. (guanosine monophosphate (5'-GMP)), 0.005 N HCl aq. (2-thiouridine) and 0.05 N HCl aq. (4-thiouridine).

**Fractionation of Oligonucleotides with an Organomercurial Column Chromatography** — Digestion of Unfractionated t-RNA with

![Fig. 2. Fractionation of RNase-T1 Digest of t-RNA with Sephadex-PCMA Column](image-url)

- a, charged the digest mixture; b, eluted with potassium cyanide solution (5 mm).
- - - - - , OD at 260 nm; — — — , OD at 315 nm.
Ribonuclease-T<sub>1</sub>: The oligonucleotides mixture of RNase-T<sub>1</sub> digests was obtained from 3.0 ml of unfraccionated t-RNA (OD = 0.99 × 10<sup>2</sup> at 260 nm) according to Inoue’s procedure.<sup>10</sup>

Separation of Oligonucleotides Containing 4-Thiouridylic Acid with Sephadex-PCMA Column: Column size 0.6 × 2.0 cm, binding capacity is 0.017 meq. Oligonucleotides mixture of RNase-T<sub>1</sub> digests above were loaded on a column, every 3.0 ml of eluate (5 M NaCl aq.) were collected, monitoring OD at 260 nm. The column was then eluted with 5 mM KCN aq. OD at 260 and 315 nm were recorded as illustrated in Fig. 2. Eluates with KCN aq. shows maximum wave length at 257 and 318 nm, and shifts to 257 and 330 at pH 4.0 as shown in Fig. 3.

**Results and Discussion**

In a previous report,<sup>1</sup> it was illustrated that mercuric chloride and 2-chloromercurio-4-nitrophenol react reversibly with 4- and 2-thiouridine. It can be expected to separate thiouridine from the mixture with major nucleic acid components by using an organomercurial column. First of all, the AE-PCMB adsorbent was used for column chromatography to separate 4- and 2-thiouridine from major nucleosides. Major nucleoside can be eluted with water, 2-thiouridine with 0.001 N HCl aq., and 4-thiouridine with 0.01 N HCl aq. It was also possible to separate not only major nucleoside from thiouridine but also 4-thiouridine from 2-thiouridine. Aminoethyl group of cellulose can not be coupled completely with PCMB, so adsorbent with uncoupled aminoethyl group is not desirable to nucleotides which possess the negative charge of phosphate, although there is no problem with separation among nucleosides. Using AE-PCMA column, we attempted to separate major nucleotide from the mixture with thiouridine, but major nucleotides could not be eluted smoothly with water or phosphate buffer. 5’-UMP, 5’-CMP, 5’-AMP could be eluted with 0.02 M NaCl aq. and 5’-GMP with 0.05 M NaCl aq. From these results, there might be very weak interaction between nucleotide and mercury, and 5’-GMP may interact with mercury most strongly among major nucleotides. In the aqueous solution of sodium chloride, interaction between phosphate and mercury might be overcome with large amount of chloride anion. By this reason, nucleotides can be released from column with aqueous sodium chloride. Thiouridine may bind tightly with mercury even in aqueous sodium chloride, if so, thiouridine can not be eluted with aqueous sodium chloride.

As described above, neither amino group nor carboxy group of cellulose can be coupled completely with mercurials, so as carrier of column chromatography, a neutral carrier might be most desirable. PCMA was coupled to chromagel A-4 activated with cyanogen bromide. As shown in Fig. 1, by using the Chromagel-PCMA column, major nucleotides can be eluted smoothly with 0.05 M aqueous sodium chloride, 2-thiouridine with 0.01 N HCl aq. and 4-thiouridine with 0.01 N HCl aq. It had become apparent that this kind of organomercurial column chromatography is useful to separate thiouridine from the mixture with major nucleotides.

Stability of binding of thiouridines to Chromagel-PCMA against various buffer or salt solutions, was examined and shown in Table 1. Thiouridine can be eluted with hydrochloric acid or high concentration of sodium hydroxide solution, but neither with buffer nor with salt solution used for biopolymer studies. Thiouridine can also be eluted very smoothly with mercaptoethanol, and a binding ability of mercurial column can be restored by treatment with aqueous mercuric chloride solution to exclude the bound mercaptoethanol, so this organomeru-
Table I. Stability for Binding of Thiouridine to Mercurial Column against Buffer or Salt Solution

<table>
<thead>
<tr>
<th>Eluent</th>
<th>4-Thiouridine</th>
<th>2-Thiouridine</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCl</td>
<td>Released (0.01 N)</td>
<td>Released (0.001 N)</td>
</tr>
<tr>
<td>0.01 N AcOH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AcOH aq.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 M H₃PO₄-KH₂PO₄</td>
<td>(pH 2)</td>
<td>(pH 3)</td>
</tr>
<tr>
<td>0.1 M KCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 M NaCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaOH</td>
<td>Released (0.1 N)</td>
<td>Released (0.01 N)</td>
</tr>
<tr>
<td>Tris-HCl (0.02 M pH 7.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cacodylate-HCl (0.05 M, pH 7.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphate buffer (0.1 M, pH 7.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgCl₂ (0.01 M)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSCH₂CH₂OH (0.001 M)</td>
<td>Released</td>
<td>Released</td>
</tr>
<tr>
<td>KCN (0.001 M)</td>
<td>Released</td>
<td>Released</td>
</tr>
</tbody>
</table>

1.0 ml each of 4-thiouridine and 2-thiouridine (1.0 mm) were charged on column, and then eluted with buffer or salt solution. Column adsorbent: Chromagel-PCMA (1.0 x 8.0 cm), binding capacity: 0.014 m eq. — , not released.

Mercurial column can be used repeatedly. Compared with hydrochloric acid, the acetic acid solution of the same concentration does not elute thiouridine as shown in Table I. This suggests that a release of thiouridine with hydrochloric acid solution may be caused mainly with the protonation at N-3 position of thiopyrimidinone and as auxiliary with the interaction of chloride anion with mercury. Neither 1,3-dimethyl-4-thiouracil nor 1,4-dimethyl-4-thiouracil nor 1,2-dimethyl-2-thiouracil bind to this organomercurial column at all. It might be that the thiopyrimidinone possessing a dissociable proton can bind to this kind of column.

Since some of E. coli t-RNA contain 4-thiouridylic acid, unfractionated t-RNA (Q-13 stamp) was applied on a Chromagel-PCMA column. Unexpectedly t-RNA could not be eluted at all with Tris-HCl buffer (10 mM, pH 7.6, MgCl₂ 0.01 mM) containing 0.5 M aq. sodium chloride, or 1.0 M aq. potassium chloride, or 10 mM aq. potassium bromide, or 1.0 mM aq. potassium iodide, or 0.1 M aq. sodium acetate or 0.1 M sodium perchlorate. It can therefore be assumed that the interaction between the phosphate group of polynucleotide and mercury can not be neglected. To compete the interaction with phosphate group of nucleotide, phosphate buffer (0.1 M, pH 6.5) was used, but it could not elute nucleotides from an organomercurial column. As an example of polyanion, an aqueous solution of chondroitin sulfate was tried, but it failed to elute. An aqueous potassium cyanide (1 mM) or mercaptoethanol (1 mM) can elute the whole t-RNA without separation. A Baker Yeast t-RNA which does not contain 4-thiouridylic acid, can not be eluted from an organomercurial column with Tris-HCl buffer, but it can be eluted with 1 mM potassium cyanide or with 1 mM mercaptoethanol similarly to E. coli t-RNA. An E. coli t-RNA can be eluted smoothly through a Sephadex column with Tris-HCl buffer. It is now certain that there are significant interactions between polynucleotides and mercury. By linear gradient of potassium cyanide concentration, the expected separation does not occur with a Chromagel-PCMA column, or with a carrier inserted with 6-aminocaproic acid or succinic acid between carrier and mercury.

From the above experimental results, there is significant interaction between polynucleotide and mercury, though mono-nucleotide can be eluted smoothly with 0.05 M sodium chloride. Oligonucleotide was then examined. RNase-T₁ digests were charged on Sephadex G-25 PCMA column. After elution with 5 M sodium chloride, the column was eluted with 5 mM of potassium cyanide. As shown in Fig. 3, ultraviolet spectrum of potassium cyanide eluate has an absorption maximum at 330 nm beside 257 nm at pH 4.0,
and this indicates that the potassium cyanide eluate contains oligonucleotides containing 4-thio uridylic acid. This kind of organomercurial column might be applicable for a study of sulfur containing nucleic acids.

Acknowledgement We are grateful to Professor Tohru Ueda (Faculty of Pharmaceutical Sciences, Hokkaido University) for helpful advice.

References


