ABSTRACT

Mercurated nucleic acids are converted to the corresponding tritiated, brominated, and iodinated derivatives by treatment with sodium borotritide, N-bromosuccinimide, and elemental iodine, respectively. All three reactions occur under mild conditions in neutral aqueous solutions. Mercury-halogen conversions are essentially quantitative at both the mononucleotide and polynucleotide levels. Tritiation reactions also proceed efficiently with mononucleotides, although polymers undergo incomplete demercuration. In spite of the latter limitation, these reactions provide novel and efficient synthetic routes to radiolabeled nucleic acid derivatives.

INTRODUCTION

The synthesis and characterization of covalently mercurated nucleotides and polynucleotides has been previously reported.1,2 These reactions, which occur under mild aqueous conditions, attach mercury substituents to the 5 position in the pyrimidine ring; little or no reaction occurs with purine bases. 5-Mercuri-nucleoside triphosphates were found to be excellent substrates for a variety of nucleic acid polymerases,1 and the mercuri-polymers formed, either enzymatically3 or by direct chemical modification,2 were found to possess structural and biochemical properties very similar to those of non-mercurated controls.3 By virtue of their affinity, as organomercurials (R-Hg+), for sulfhydryl groups, mercuri-polynucleotides were selectively retained when chromatographed on columns of sulfhydryl-sepharose. This property has been exploited to develop a new method of selective polynucleotide fractionation.3 During these studies it became apparent that mercuri-nucleic acids might also serve as useful intermediates in the synthesis of other nucleic acid derivatives. To that end, three different reactions were examined: (1) reductive demercuration with sodium borotritide; (2) bromodemercuration with N-bromosuccinimide; and (3) iododemercuration with elemental iodine. Presented below is an evaluation of these procedures.
with respect to the efficiency of the conversion processes and the specificity of the reaction.

**MATERIALS**

Non-radioactive nucleotides, the 5-iodo- and 5-bromo- derivatives of cytidine and uridine, and poly U were purchased from Sigma. Mercuric acetate-$^{203}$Hg, sodium borotritide, $^{125}$I$_2$ (in ethanol) and Aquasol were products of New England Nuclear Corporation. Dialysis tubing was purchased from Rochester Scientific Company. N-bromosuccinimide was bought from K & K Laboratories. Mercuric salts, sodium borohydride, iodine, chromatographic resins and other reagents were obtained from regular commercial sources.

**METHODS**

Ultraviolet absorption spectra were recorded on Cary 15 and Beckman 25K recording spectrophotometers.

Thin layer electrophoreses were run on a Brinkman-Desaga TLC apparatus using Eastman-Kodak cellulose sheets (#13255) without fluorescent indicator. All ascending thin layer chromatography was done using the same sheets. Elemental analyses were performed by Baron Consulting Corporation, Orange, Connecticut.

Sulfhydryl sepharose was prepared according to the procedure of Cautrecasas with the modifications previously described. The resin contained 1.2 μm of sulfhydryl groups per ml, as determined by titration with 5,5'-dithiobis (2-nitrobenzoic acid).

To measure radioactivity, samples were either spotted on GF/A filters, dried, and placed in a toluene based cocktail of PPO and POPOP, or put directly into Aquasol. All samples were counted in a Packard Scintillation Counter.

**Mercuration.** Mononucleotides were treated with a 5-fold molar excess of $^{203}$Hg-mercuric acetate in 0.1 M sodium acetate, pH 7.5, at 50°C for 3 hours and chromatographically purified as previously described. Poly U was mercurated in 0.005 M sodium acetate, pH 7.5, at 50°C with a 10-fold molar excess of $^{203}$Hg-mercuric acetate for periods from 0.2 to 6.0 hours and then subjected to exhaustive dialysis in TNE buffer (0.01 M Tris-HCl buffer pH 7.5, 0.02 M NaCl, and 0.002 M EDTA).

**Reductive Demercuration.** Mercurated compounds were treated with a 10-10,000-fold molar excess of NaBH$_4$ or NaB$_3$H$_4$ (relative to mercury content) at pH 7.0 for times varying from 10 minutes to 8 hours. The
extent of mononucleotide demercuration was analyzed by spotting samples directly on TLE sheets and electrophoresing them for 2-2.5 hours at 350 volts in 0.05 M ammonium bicarbonate, pH 7.0-7.5. The sheets were cut into 2 cm by 1 cm strips and counted in the toluene based scintillation fluid described above. Alternatively, reaction mixtures were treated with acetone for 30 minutes, which reacted with residual borohydride to form 2-propanol, and then chromatographed on DEAE-cellulose, eluting with a linear gradient of triethylammonium bicarbonate. Mercuri-polynucleotides, following borohydride and acetone treatments, were dialyzed exhaustively in TNE buffer until 1.0 ml of the dialysis solution gave no more than 50 cpm, when counted in Aquasol. Aliquots (100 μl) of the polymer solutions were counted in Aquasol and their spectra recorded. The percentage of 203Hg remaining was calculated by comparison with untreated samples which had been dialyzed in parallel.

Bromination. Mercurated nucleotides and polynucleotides were treated with a 5 to 20-fold excess of N-bromosuccinimide at pH's 4 to 8.3. The N-bromosuccinimide solutions were prepared immediately before use. Sodium acetate buffers were used at pH's 4, 5, 6, and 7.4 while Tris-HCl buffers were used at pH's 7, 8, 8.3, and 9. The progress of the reaction was monitored spectrophotometrically by following the increase in OD at 300 nm (for UTP-Hg) or 305 nm (for CTP-Hg), where neither the mercurated nor parent pyrimidine bases absorb significantly. When a plateau was reached, the pH was adjusted to 5.0 (for UTP) or 1.0 (for CTP), a full spectrum was recorded (Figures 1a and 1b) and spectral ratios compared with authentic samples. The plateau values were found to correspond to 100% conversion. As further verification of the product, the pH of a solution which had reached a limit reaction was adjusted to 7.5, with Tris-HCl, and either electrophoresed to verify removal of mercury (which was found to be 100%) or treated with bacterial alkaline phosphatase and chromatographed with appropriate nucleoside markers. Samples co-migrated with the brominated standards without evidence of either mercurated or unmodified nucleosides. Thus, for routine studies, the increase in OD was used in conjunction with spectral ratio analysis to evaluate the extent of reactions. The observed decrease in OD in extended reactions was attributed to the formation of 5-bromo-6-hydroxyuracil and/or 5,5'-dibromo-6-hydroxyhydrouracil derivatives.

Iodination. Mercurated mononucleotides were treated with a 4-fold excess of I2 (in ethanol) at pH's 4-9 for 60 minutes at 20°C. The maximum
percentage of ethanol in the reaction mixture never exceeded 20%. Solutions were extracted with CHCl₃, the pH adjusted to 5.0 (for UTP) or 1.0 (for CTP), and the spectra recorded (Figures 1A and 1B). The extent of reaction was determined by analysis of the spectral ratios in comparison with authentic samples. Selected samples were subjected to electrophoretic and chromatographic analysis to verify spectral results. The percentage of iodination as determined by the different techniques agreed to within 3%.

Polymers were treated with an 8-fold excess of I₂ at pH's 4-9 for 60 minutes at 20°C. Solutions were extracted with CHCl₃, treated with quench buffer (QB) (1.0 M NaCl, 0.01 M Tris-HCl buffer pH 7.5, 0.05 M EDTA) and dialyzed against TNE. Aliquots were counted in Aquasol and spectra recorded. The percentage of ²⁰³Hg remaining was calculated by comparison with untreated samples.

RESULTS

Reductive Demercuration of UTP-Hg and CTP-Hg. Sodium borohydride is a mild, water-soluble reducing agent. Unlike other reducing agents, such as LiAlH₄, it does not modify the major purine and pyrimidine bases, although it will reduce minor nucleic acid components, such as 5,6-dihydouridine and 4-thiouridine. It has been used successfully to reduce a variety of organomercurials in both non-aqueous and aqueous environments. Experiments with NaBH₄ in D₂O and NaBD₄ in H₂O have shown that the hydrogen replacing the mercury comes from the borohydride and not from the

Figure 1. Ultraviolet spectra of UTP-Hg (1A, —) and CTP-Hg (1B, —) after treatment with sodium borohydride (---), N-bromosuccinimide (1A, — —) (1B, — —), and elemental iodine (1A, — — —) (1B, ...), respectively (see Methods).
water as has been suggested for LiAlH$_4$, an important point in terms of synthesizing tritiated molecules. Also, reduction with NaBH$_4$ proceeds, at least in the case of alkyl mercurials, directly to the hydrocarbon (a) and not through a symmetrical intermediate (b) as has been observed with sodium stannite and hydrazine catalyzed demercurations. Once formed, such symmetrical mercurials undergo reduction only slowly, if at all, thus greatly reducing the yield of potential tritiated product. Since mercur-

\[
a) \quad R-\text{Hg-X} \xrightarrow{[\text{H}]} R-\text{H} + \text{Hg} \\
b) \quad R-\text{Hg-X} \xrightarrow{[\text{H}]} R-\text{Hg-R} \xrightarrow{\text{very slow}} R-\text{H} + \text{Hg}
\]

nucleotides can be easily prepared, it was thought that NaBH$_4$ demercuration might provide a simple and efficient method of synthesizing tritiated nucleic acid derivatives. The data presented below describes the reductive demercuration of UTP-\text{-Hg} and CTP-\text{-Hg}. The results apply equally well to other mercurated nucleosides and nucleotides.

Treatment of UTP-$^{203}$Hg with a 20-fold excess of sodium borohydride at pH 7.0 removed 98% of the $^{203}$Hg within 30 minutes at room temperature. In contrast, sodium borohydride treatment of phenylmercuric acetate produced principally the diarylmercurial. The reduction of UTP-$^{203}$Hg was fast, independent of temperature on the time scale studied, and proceeded best at neutral to alkaline pH. Increasing the borohydride excess to 200-fold did not remove the residual 1-2% of $^{203}$Hg (Table 1). Electrophoretic analysis of the reaction mixture showed that the resistant mercurinucleotide now co-migrated with UTP and not with UTP-$^{203}$Hg (Figure 2). The remaining $^{203}$Hg-mercurinucleotide was also no longer retained on a sulfhydryl sepharose column, in sharp contrast to its behavior before exposure to borohydride. Chromatography of borohydride treated UTP-Hg on DEAE-cellulose resolved two peaks: the first corresponded to UTP; while the second contained all of the remaining $^{203}$Hg (Figure 3). Elemental analysis of this late eluting fraction yielded a Hg/nucleotide ratio of 0.5, exactly 50% of that expected for UTP-Hg. It thus appears that in the reduction of UTP-Hg a small percentage of the molecules undergo symmertization to form UTP-Hg-UTP. These dimers, once formed, are resistant to further reduction even when the borohydride is present in a 1000-fold molar excess.

The reduction of CTP-Hg by sodium borohydride exhibited the same time, pH, and temperature profiles as did UTP-Hg reactions. However,
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Table 1
Reductive Demercurization of UTP-Hg with NaBH₄: Reaction Parameters

<table>
<thead>
<tr>
<th>Variable</th>
<th>NaBH₄ Molar Excess</th>
<th>Time</th>
<th>Buffer pH</th>
<th>Temp.</th>
<th>% Hg Remaining</th>
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<tr>
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<td>20°C</td>
<td>9.1</td>
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<td></td>
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<td>40x</td>
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<td>1.0</td>
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<td></td>
<td>100x</td>
<td></td>
<td></td>
<td></td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>200x</td>
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<td></td>
<td></td>
<td>1.3</td>
</tr>
<tr>
<td>Time</td>
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<td>15 min.</td>
<td>7</td>
<td>20°C</td>
<td>2.2</td>
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<tr>
<td>Temp.</td>
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<td>30 min.</td>
<td>7</td>
<td>20°C</td>
<td>1.3</td>
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<td></td>
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</tr>
<tr>
<td>pH</td>
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<td>30 min.</td>
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<td>20°C</td>
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</table>

Unless otherwise indicated, the standard reaction conditions were: 20°C; UTP-Hg, 1 mM (²⁰³Hg specific activity 10,900 cpm/nmole); 0.1 M Tris-HCl buffer, pH 7.0, and NaBH₄, 20 mM, in a final reaction volume of 50 µl. At the times indicated, 5 µl samples were withdrawn and subjected to electrophoretic analysis (see Methods).
Figure 2. Electrophoretic separation of UTP-Hg and UTP-Hg-UTP.
UTP-203Hg, 1 mM (203Hg specific activity - 12,000 cpm/nmole), in 0.1 ml of 0.1 M Tris-HCl buffer, pH 7.0, was treated with a 25-fold molar excess of NaB%4 (specific activity, 49,400 cpm/nmole of 3H) for 30 minutes. A 5 µl aliquot was then analyzed electrophoretically, as described in Methods.

Figure 3. Chromatographic separation of UTP and UTP-Hg-UTP.
Five ml of a 1 mM solution of UTP-203Hg in 0.01 M Tris-HCl, pH 7.0, was treated with a 10-fold excess of NaBH4 for 20 minutes. The solution was filtered to remove precipitated Hg metal and 100 µl of acetone added to decompose residual NaBH4. The sample was then diluted to 100 ml with water and applied, at 4°C, to a 10 ml DEAE-cellulose column in the bicarbonate form. The column was washed with two litres of water prior to eluting the reaction products with a linear 600 ml gradient of ammonium bicarbonate (0-0.5 M). Five ml fractions were collected and the absorbance at 260 nm was measured on a Beckman 25 spectrophotometer. 100 µl aliquots of each fraction were added to 4 ml of Aquasol and counted in a Packard scintillation counter.

unlike UTP-Hg, it was possible to avoid symmertization completely if the CTP-Hg was treated with a sufficiently large excess of borohydride (250-fold). Under such conditions no detectable 203Hg remained (Table 2).
Reductive Demercuration of CTP-Hg with NaBH₄

<table>
<thead>
<tr>
<th>NaBH₄ Molar Excess</th>
<th>% ²⁰³Hg Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x</td>
<td>13</td>
</tr>
<tr>
<td>20x</td>
<td>5</td>
</tr>
<tr>
<td>40x</td>
<td>2</td>
</tr>
<tr>
<td>100x</td>
<td>.5</td>
</tr>
<tr>
<td>250x</td>
<td>0</td>
</tr>
</tbody>
</table>

Reaction solutions of 50 μl contained CTP-Hg, 1 mM (²⁰³Hg specific activity 12,100 cpm/nm), 0.1 M Tris-HCl buffer pH 7.0 and NaBH₄ at the indicated excess. Samples were treated as in Table 1.

Reductive demercuration of UTP-Hg and CTP-Hg with sodium borotritide resulted in the rapid formation of ³H-UTP or ³H-CTP. For example, using NaB³H₄ with a specific activity of 45 μc per μmole of H, tritiated UTP at a specific activity of 9 μc per μmole was obtained. The specific activity observed in different preparations, however, has varied from 5-20% of the theoretical. A rapid exchange (³H ↔ H) between the borotritide and water could account for these observations. Possible procedures for lowering or avoiding such exchange reactions are outlined in the Discussion. It is apparent, nevertheless, that tritiated nucleotides of high specific activity can be obtained by utilizing the commercially available NaB³H₄ (10-15 C/mmole).

The tritiation reaction is totally dependent upon prior mercuration. Treatment of non-mercurated UTP, CTP, GTP, ATP, either singly or in combination, did not result in the formation of detectable amounts of any tritiated nucleotides.

Reductive Demercuration of Poly U-Hg. Poly U-Hg was considerably more resistant to reductive demercuration than either UTP-Hg or CTP-Hg. Whereas a 20-fold excess of borohydride removed all but 1% of the mercury from UTP-Hg, the same excess left almost 34% of the mercury remaining in poly U-Hg. Up to 93% of the mercury could be removed, however, by using higher levels of borohydride (500-1000-fold excess) (Figure 4). The residual mercury was non-dialyzable, insensitive to additional borohydride, and not retained on a sulfhydryl-sepharose column. These facts suggested that symmetrical products were being formed. The following experiments, which utilize the observation (see below) that iodination of mercuri-
Figure 4. Reductive demercuratlon of $^{203}$Hg-poly U-Hg using NaBH$_4$.

Poly U-Hg ($^{203}$Hg specific activity 10,530 cpm/nm) at a final concentration of 2.5 OD$_{260}$/ml in 0.1 M Tris-HCl, buffer pH 7.0, was treated at 20°C with the specified molar excess of NaBH$_4$ in a final volume of 8 mls. One ml aliquots were withdrawn at the indicated times, 200 µl of acetone added, and the samples processed as described in Methods.

polynucleotide is a quantitative process, strongly supported this idea.

Two poly U-Hg samples, which after borohydride treatment had 15 and 34 nmoles of Hg remaining per 100 nmoles of polymer respectively, were heated at 37°C with a 200-fold excess of I$_2$ in 0.01 M Tris-HCl, pH 7.0 for 30 minutes. After dialysis it was found that not only was all of the mercury removed, but now there were 29 and 67 nmoles, respectively, of I-UMP per 100 nmoles of polymer; i.e. 1 n mole of mercury had been replaced with 2 nmoles of iodide. In contrast, I$_2$ treatment of poly U-Hg samples (containing 21 and 42 nmoles of mercury per 100 nmoles of polymer) not previously exposed to borohydride had only 21 nm and 41 nm of I-UMP per 0.1 µm of polymer; i.e. 1 nmole of Hg has been replaced by 1 nmole of I.

The introduction of 2 moles of iodide per mole of mercury in the borohydride treated poly U-Hg can be easily understood in terms of the following reaction.13

$$R-\text{Hg}-R + 2X_2 \rightarrow 2RX + \text{HgX}_2$$

Formation of fully substituted organomercurials (R-Hg-R) may also occur in naturally occurring polynucleotides. A high level of resistant mercury was found after borohydride treatment of chemically mercurated calf thymus and fd DNAs, in which only cytidine bases are modified$^3$ (see discussion). Similar results were observed with ribosomal RNA, mercurated in
both pyrimidine bases. After borohydride treatment these polymers, like poly U-Hg, were no longer retained on sulfhydryl-sepharose. Experiments to elucidate the nature of this resistant mercury are currently underway.

Treatment of poly U-Hg with sodium borotritide produced poly U-\(^{3}\)H which contains a significant residual level of \(^{203}\)Hg. The specific activities obtained were, as for UTP-Hg and CTP-Hg, variable and no greater than 20% of theoretical. Although the remaining mercury could be removed via iodination (as above) the method does not appear to be highly suitable for polynucleotide tritiation.

Bromination. Bromination of mercurated nucleic acids could be achieved with either Br\(_2\) or N-bromosuccinimide. In practice, however, it was easier to control reactions with the N-haloimide, which was used in all the studies described below.

Bromination of UTP-Hg at room temperature in 0.05 M sodium acetate buffer, pH 6.0, with a 5-fold excess of N-bromosuccinimide gave quantitative yields of Br-UTP within 25 minutes (Figure 5). A faster rate of reaction could be obtained at pH 4.0 or 5.0 (3-10 minutes) but a slow addition across the 5,6 double bond also occurred, as can be observed by the decrease in OD. The use of Tris-HCl buffers decreased the reaction rate to about one-third that observed in sodium acetate buffers of the same pH. Higher levels of N-bromosuccinimide accelerated the replacement of the mercury but also increased the rate of addition across the double bond. The rate of bromination was also markedly enhanced by the presence of NaCl (10-15 fold by 0.1 M) however, as before, the rate of addition

![Figure 5. Bromination of UTP-Hg with N-bromosuccinimide: pH dependence of Reaction. Reaction mixtures (1.5 ml) containing UTP-Hg (0.066 mM), buffer (0.05 M) and N-bromosuccinimide (0.33 mM) were run in a Beckman 25K spectrophotometer at 20°C. Reaction rates were monitored by following the absorbance increase at 300 nm.](image)
across the double bond was also increased. The optimum conditions for controlled bromination, therefore, appear to occur in 0.05 M sodium acetate buffer pH 5.0-6.0, in the presence of a 4-5-fold molar excess of N-bromo-succinimide.

Bromination of CTP-Hg proceeded much more rapidly than with UTP-Hg. Quantitative conversions occurred within 6 minutes in 0.05 M sodium acetate, pH 6.0, with only a 5-fold molar excess of N-bromosuccinimide. The rate of reaction, unlike UTP-Hg, showed no pH effect in sodium acetate buffers (pH 4-6) but was completely inhibited by Tris-HCl buffers even at pH 7.0 (Figure 6). As for UTP-Hg, larger excesses of borohydride or the addition of NaCl accelerated both replacement of Hg and addition across the double bond.

Figure 6. Bromination of CTP-Hg with N-bromosuccinimide. Reaction mixtures (1.5 ml) contained CTP-Hg (0.1 mM), buffer (0.05 M), and N-bromosuccinimide (0.5 mM). The reaction was monitored spectrophotometrically at 305 nm.

Bromination of poly U-Hg, other than proceeding much more slowly at pH's above 4, (Figure 7), exhibited the same borohydride concentration and salt effects as UTP-Hg. Examination of the polymer after exposure to N-bromosuccinimide and dialysis indicated that complete demercuration had indeed occurred.

Treatment of non-mercurated UTP, CTP, or poly U with N-bromosuccinimide under the conditions described led to an immediate and rapid loss of OD, presumably corresponding to the formation of hydrates and dibromoderivatives. Introduction of the mercury substituent considerably lowers the rate of double-bond reduction and provides a means of obtaining controlled bromination reactions in aqueous solutions. Although guanosine residues are also known to react with N-bromosuccinimide, they were not examined in this study.
Figure 7. Bromination of poly U-Hg. Reaction mixtures (1.5 ml) contained poly U-Hg (0.12 mM), N-bromosuccinimide (0.6 mM) and sodium acetate buffers (0.01 M) at the indicated pH. Reaction rates were monitored spectrophotometrically.

Iodination. Iodination of UTP-Hg and CTP-Hg, like bromination, could be carried out with either the elemental form of the halogen or the N-haloimide. However, unlike bromination, elemental iodine proved preferable to N-iodoacetic acid since with it no addition products formed and the rate of reaction with non-mercurated bases was negligible. Otherwise, the conditions used for iodination were essentially the same as those for bromination.

Iodination of UTP-Hg proceeded rapidly at room temperature in 0.01 M buffer from pH's 4 to 7 with only a 4-fold excess of iodine (Figure 8). Quantitative conversion was achieved within 60 minutes with all but the more basic solutions. If a 20-fold excess of iodine were used, quantitative conversions could be achieved within 15 minutes. Addition of NaCl did not have any noticeable effect on the rate of the reaction.

Iodination of CTP-Hg proved to be even faster than UTP-Hg. Quantitative yields were obtained within 5-10 minutes at pH 4-7, 20°C and 4-fold excess of I₂. Even at 0°C a 100% conversion of CTP-Hg to I-CTP occurred within 10 minutes.

Poly U-Hg also reacted smoothly with I₂ at room temperature (Figure 9). Complete loss of ²⁰³Hg from the polymer occurred within 60 minutes over the pH range from 4 to 7. Increasing the I₂ excess to 30-fold led to quantitative conversion within 30 minutes. Examination of spectral characteristic, or the use of ¹²⁵I₂, indicated that the degree of iodination corresponded directly with the loss of ²⁰³Hg.
Figure 8. Iodination of UTP-Hg. Reaction mixtures (10 ml) containing UTP-Hg, 1 mM; buffer, 0.01 M; elemental Iodine, 4 mM; and 10% (V/V) ethanol, were incubated at 20°C. One ml samples were removed at the indicated times and processed as described in Methods.

Figure 9. Iodination of Tlg-poly U-Hg. Reaction mixtures (10 ml) containing 0.1 mM poly U-Hg (specific activity, 27,300cpm/nmole), 0.1 M buffer, 0.8 mM elemental iodine and 10% (V/V ethanol), were incubated at 20°C. At the times noted, 1 ml samples were removed and processed as described in Methods.

Treatment of non-mercurated nucleic acids with I₂ under the conditions described above did not produce any measurable reaction.
A simple, efficient and inexpensive method of preparing tritiated nucleic acid compounds in vitro would be of considerable utility for many biochemical studies. In an attempt to develop such a procedure we examined a borotritide reduction reaction utilizing mercurated intermediates. This approach seemed to hold promise since both the mercuration and reduction steps could be done under extremely mild reaction conditions. Initial studies with mononucleotides, e.g. UTP-Hg and CTP-Hg, were encouraging. When treated at room temperature with a 20-fold excess of sodium borotritide at pH 7.0, 95-99% of the mercury was removed within 30 minutes with the concomitant formation of $^3$H-UTP and $^3$H-CTP. The specific activities obtained ranged from 5-20% of theoretical. It should, however, be possible to achieve higher specific activity yields by performing the reductions in deuterated or tritiated water or in non-aqueous solvents, e.g. diglyme, where $^3$H $\leftrightarrow$ H exchange should be minimized.

The low level (1-2%) of residual mercurinucleotide left after borohydride reduction of UTP-Hg was shown to be a symmetrical dimer (UTP-Hg-UTP). Although such dimers are extremely resistant to further reduction once formed, even in the presence of vast molar excesses of borohydride, they are readily separated from UTP by ion-exchange chromatography. The tendency toward mercury symmetrization is significantly greater during the reduction of poly U-Hg. Although up to 93% of the mercury covalently bound to the polymer can be removed with a 500-fold excess of borohydride, no reduction condition has been found which yields quantitative demercuration. An even greater resistance to total reductive demercuration has been observed in preliminary experiments with naturally occurring DNA and RNA polymers. Borohydride excesses of up to a 100,000-fold have been required to remove 90% of the mercury, while excesses of a 1000-fold have been observed to leave up to 90% of the mercury in a non-dialyzable form which is not retained on sulfhydryl-sepharose. If, as shown with UTP-Hg and poly U-Hg, mercury crosslinked bases are being formed, their facile synthesis may provide a novel method of introducing interstrand crosslink in duplex DNA (or DNA-RNA hybrids) similar to those induced by treatment with mitomycin C or psoralen. It should be noted, however, that the residual mercury, found in the reactions described above, can be quantitatively removed in all cases via subsequent iododemercuration.

The mercuration-borotritide reduction method of tritiation is probably
most useful with monomers and small oligomers, where the physical separation of product and cross-linked molecules (if present) can be achieved by chromatographic or electrophoretic means. If one is willing to use large borotritide excesses and to accept a low level of mercury or iodo-substituents in the product, then the procedure is applicable to polynucleotides of all chain lengths. It should be kept in mind, however, that certain minor bases are reduced by borotritide in the absence of prior mercuration.

Bromodemercuration of UTP-Hg, CTP-Hg and poly U-Hg gives quantitative conversions to UTP-Br, CTP-Br and poly U-Br. Care must be exercised to avoid formation of bromohydrates or dibromo-compounds but with low N-bromosuccinimide excesses and a pH of 6 or above, monosubstituted derivatives are readily achieved. Although complete replacement of mercury in mercurated natural DNA's and RNA's may also be possible, the extensive modification of non-mercurated UMP, CMP and GMP derivatives which will also occur, limits the usefulness of this technique.

Iododemercuration has considerable potential as a new method of providing radioiodinated polynucleotides. Like bromination, it gives quantitative conversions of UTP-Hg, CTP-Hg, and poly U-Hg, but, unlike bromodemercuration, little if any modification of non-mercurated residues occur. Under the conditions studied, no detectable reaction occurred with U, C, A, G, or T bases, nor is there any evidence of hydrate formation. Preliminary investigations indicate that iododemercuration reactions proceed with equal efficiency on natural mercurated DNA's and RNA's. The combined mercuration-iododemercuration procedure using $^{125}I$-elemental iodine may prove to be a useful alternative to the widely used TiCl$_3$-NaI iodination procedure. A detailed study of iododemercuration is currently underway and will be presented elsewhere.

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