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The reactions of mercurated pyrimidine nucleotides with thiols and with hydrogen sulfide

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ABSTRACT

In the presence of thiols, 5-mercuripyrimidine nucleotides are quantitatively converted to 5-thiomercuri derivatives, but these compounds are unstable and decompose at a rate dependent on the nature of the thiol. The decomposition involves three different reactions and proceeds via a symmetrical mercury derivative of the nucleotide. The end product is the unmodified nucleotide. Similar reactions occur in the presence of hydrogen sulfide. Since mercurated nucleoside triphosphates are substrates for RNA- and DNA polymerase only in the form of thiomercuri derivatives, this implies that when DNA is replicated or transcribed in vitro with a mercurated substrate, the latter is rapidly demercurated to the unmodified substrate which is incorporated as well. Hence the product of the in vitro synthesis can only be partially mercurated in any one pyrimidine. Also, formation of cross-links in the resulting polymer is possible.

INTRODUCTION

The synthesis and properties of 5-mercuripyrimidine nucleotides, as well as the preparation of mercury-containing polynucleotides by chemical modification and by enzymatic polymerization of mercurated substrates, have been described by Dale, Ward and coworkers[1,2,3]. Since then, several investigators have used pppUHgSR[4] as an RNA polymerase substrate allowing convenient isolation of the in vitro transcription product of chromatin and nuclei[6,7,8], although recently attention has been drawn to possible artefacts of this technique[9,10,11].

We have attempted the enzymatic synthesis of poly (UHg) and poly (A-UHg), our purpose being to use polynucleotides with a regular pattern of mercury labeling as model compounds for experimenting the electron microscopic observation of heavy element-labeled nucleic acids. Our attempts to polymerize ppUHgSR with polynucleotide phosphorylase were unsuccessful. With poly d(A-T) as template, RNA polymerase did polymerize pppUHgSR and pppA. However, we found that in the resulting polynucleotide only part of the uridylic acid residues are mercurated. This is because thiols must be added[1] in order to convert pppUHgX to a thiomercuri derivative pppUHgSR acceptable as a substrate for RNA polymerase, but in the presence of thiols the mercurated substrate is converted fairly rapidly to pppU, which of course is also incorporated.

We have searched for, but been unable to find, any stable nucleotide derivative of the form (pp)pUHgSR. In the course of this work, we have elucidated the reactions responsible for the demercuration of pUHgX in the presence of thiols and of hydrogen sulfide, and identified some intermediate compounds formed in the course of this demercuration. The structural formulas of the different types of mercurated nucleotides discussed are drawn in chart 1.

MATERIALS AND METHODS

Chemicals.

Uridine-5'-mono-, di-, and triphosphate and L-cysteine were obtained from Sigma, poly d(A-T) from Boehringer. Mercaptoethanol, thiophenol, 2-thiouracil, thioacetic acid and thioacetamide were from Aldrich, ethylmercaptan from Merck (Darmstadt). The gases methylmercaptan and hydrogen sulfide were purchased from Matheson gas products.

Labeled compounds.

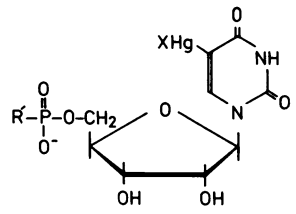
$^{203}Hg(OAc)_2$ with a mean specific activity of 140 mCi/mmole, [^{35}S] L-cysteine at 66 mCi/mmole and [^{14}C] ATP at 1.03 mCi/mmole were obtained from the Radiochemical Centre (Amersham). The activity of ^{203}Hg was determined in a Berthold Gammaszint BF 5300 scintillation counter, those of ^{35}S and ^{14}C in a Packard Tricarb model 2450 liquid scintillation counter. In double-label experiments with ^{203}Hg + ^{35}S or ^{203}Hg + ^{14}C, the samples were counted in both instruments. The contribution of ^{203}Hg to the liquid scintillation counts can be computed from the activity measured in the γ-counter where ^{35}S and ^{14}C are not detected.

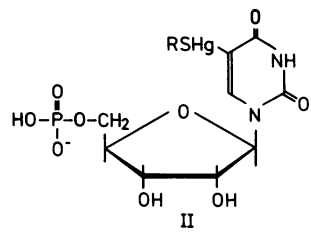
Preparation of mercurinucleotides and thiomercurinucleotides.

Mercuration of uridine-5'-phosphates was usually carried out on 100 µmole of nucleotide according to the reaction conditions prescribed by Dale et al[2]. Ten ml of a mixture containing 0.01 M nucleotide, 0.05 M $^{203}Hg(OAc)_2$ with a specific activity of 1.5 x 10^8 to 3 x 10^8 cpm per mmole, and 0.05 M NaOAc, pH 6, was incubated at 50° C for 3 hours. Excess mercuric ions were removed by passing the mixture over a small column containing 5 equivalents of Chelex 100 (Bio-Rad) with respect to the amount of mercury in the mixture and equilibrated with 0.1 M acetate buffer pH 6. The column was then washed with 100 ml of water. Measurement of the activity and optical density of the eluate showed that this procedure efficiently removed non-covalently bound mercuric ions.

The eluate was diluted to an acetate concentration of 0.01 M and loaded on a 50 ml DEAE-cellulose column (Serva, capacity 0.82 meq/g), equilibrated with 0.01 M NH_4HCO_3. The column was washed with 10 volumes of the same buffer and eluted with 800 ml of a linear gradient from 0.01 to 0.3 M NH_4HCO_3. A small peak of unreacted nucleotide usually preceded the main ^{203}Hg- containing peak,

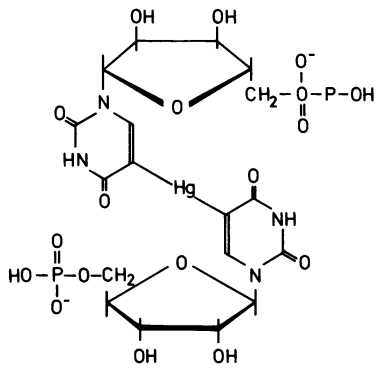
Chart 1. Structure of mercurated uridine nucleotides.



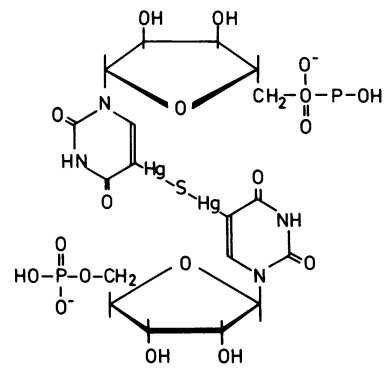


II

	Ia	Ib	Ic	Id	Ie
R´ =	OH	OH	OH	HPO_4^-	$H_2P_2O_7^{--}$
X =	CH_3COO	Cl	X	X	X



III



IV

Ia	pUHgOAc	5-acetoxymercuriuridine-5'-monophosphate
b	pUHgCl	5-chloromercuriuridine-5'-monophosphate
c	pUHgX	unspecified salt of 5-mercuriuridine-5'-monophosphate
d	ppUHgX	" " " 5-mercuriuridine-5'-diphosphate
e	pppUHgX	" " " 5-mercuriuridine-5'-triphosphate
II	pUHgSR	thiol derivative of 5-mercuriuridine-5'-phosphate, e.g. 5-hydroxyethylthiomercuriuridine-5'-phosphate (R=CH_2CH_2OH), the mercaptoethanol derivative.
III	$(pU)_2Hg$	5,5'-bis (uridine-5'-monophosphate) mercury
IV	$(pUHg)_2S$	bis (5-uridine-5'-monophosphate mercuri) sulfide

which was concentrated by rotary evaporation, the residue being desalted by repeated dissolution in 50% methanol and evaporation.

For the preparation of 5-thiomercuriuridine-5'-phosphates (pUHgSR), 100 µmole of pU was converted to [^{203}Hg]pUHgOAc and freed from excess mercuric ions as described above. To the eluate of the Chelex 100 column were added 1 to 5 equivalents of the desired thiol RSH either in the pure form if it was a liquid, or in aqueous solution if it was a solid. In the case of 5-thiouracil, which is insoluble in water, one equivalent of the solid was added and it dissolved by

reaction with the mercurial. Methylmercaptan, which is a gas, was led through the solution. In several cases the conversion of pUHgOAc to pUHgSR was accompanied by formation of a precipitate containing mercury but no nucleotide material, and identified as $(RS)_2Hg$ (see Results). This was removed by centrifugation and the supernatant was separated on a DEAE-cellulose column as described above, except that elution was with an 800 ml gradient from 0.01 M to 0.5 M NH_4HCO_3.

The preparation of [^{203}Hg]$(pU)_2Hg$ also started by mercuration of pU and Chelex 100 treatment as described above. Hydrogen sulfide was then led through the solution for 10 to 20 minutes, and excess H_2S was chased by a current of nitrogen. The solution was allowed to stand overnight and the finely divided precipitate of HgS was partly removed by centrifugation, some of it remaining in suspension. The supernatant was fractionated on DEAE-cellulose under the conditions used for pUHgSR derivatives. $(pU)_2Hg$ was obtained in 60% yield.

Characterization of reaction products.

The products obtained by reaction of pUHgOAc with RSH were characterized mainly by their mobility when chromatographed on ion exchange paper, and by measuring the ratio of ^{203}Hg-radioactivity to UV-absorbance.

The molar extinction coefficient of pUHgX at 267 nm is 10,100.[2] The extinction increases only a few percent upon addition of a thiol not absorbing at this wavelenght, and the ε_{267} of pU is 9,500. Because of these small differences in molar extinction coefficient, we have taken ε_{267} = 10,100 as a mean value for computing the uridine content of the compounds to be identified, the mercury content being known from the radioactivity. All spectra were taken in 0.1 M Tris chloride pH 7.5. The mean Hg/U ratio, determined in this way for several preparations of pUHgOAc, was 0.92. As judged from the paper chromatographic homogeneity however, purity was considerably better than 92%.

Paper chromatography was ascending, on DEAE-cellulose paper (Whatman DE81), with 0.4 M Tris carbonate as a solvent, prepared by saturating an 0.8 M Tris solution with CO_2 and diluting appropriately. Spots were detected by autoradiography and by inspection of the chromatogram in UV-light. This allows a convenient distinction between mercury compounds, nucleotide material, and mercurated nucleotides. The following ^{203}Hg-labeled marker substances were used for identification of unknown compounds: pUHgOAc and $(pU)_2Hg$ were prepared as described above; thiomercurinucleotide markers, pUHgSR, were obtained by directly spotting an equimolar mixture of pUHgOAc and the relevant thiol on the paper; mercury mercaptide markers, $(RS)_2Hg$, were obtained by mixing equivalent amounts of $Hg(OAc)_2$ and RSH solutions, and spotting a saturated solution of the resul-

ting precipitate, formed according to the reaction:

$$Hg(OAc)_2 + 2\ RSH \longrightarrow (RS)_2Hg \downarrow + 2\ HOAc$$

Decomposition reactions of mercurated nucleotides.

Some of the mercurated nucleotides studied decompose spontaneously, others only in the presence of thiols and of H_2S. The reactions were followed by incubating a few µmole of the compound under study, labeled with ^{203}Hg to an activity of 10^5 to 10^6 cpm, in 100 µl of buffer. Most reactions were studied in 0.1 M Tris acetate, pH 5, 7, or 9, at 37°C, during 2 to 120 hours, depending on the reaction rate. Samples of 5 µl were withdrawn periodically, separated in the paper chromatographic system described above, and the Hg-containing reaction products were detected by autoradiography and quantified by scintillation counting. If a precipitate formed during the reaction, the mixture was centrifuged before samples were taken form the supernatant. HgS was not removed in this way because it forms a colloidal solution.

Enzymatic polymerization.

Polynucleotide phosphorylase from Micrococcus luteus was obtained from Boehringer, the enzyme from Escherichia coli was prepared according to Kimhi and Littauer[12]. Polymerization of ppUHgSR was attempted by incubation, at 37°C, of 100 µl of a mixture containing 60 mM [^{203}Hg]ppUHgX at 1.5 x 10^8 cpm/mmole, 0.2 mM UpU, 150 mM Tris acetate pH 8.8, 5 mM $Mg(OAc)_2$, 60 mM mercaptoethanol and 1 to 3 enzyme units. In other experiments the mercaptoethanol concentration was doubled, or L-cysteine was used instead. The reaction was followed by periodically withdrawing 5 µl samples and chromatographing them on DEAE-cellulose paper with 0.6 M Tris carbonate as a solvent.

Transcription of poly d(A-T) with mercurated substrates was carried out with E. coli RNA polymerase from Boehringer under the reaction conditions used by Dale and Ward[3]. The 500 µl reaction mixture contained 1 mM [^{14}C]pppA AT 10^9 cpm/mmole, 1 mM [^{203}Hg]pppUHgX at 10^8 cpm/mmole, 0.01 mM poly d(A-T), 50 mM Tris chloride pH 8, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 20 mM mercaptoethanol and 10 units of RNA polymerase. From the mixture, incubated at 37°C, 50 µl aliqouts were withdrawn periodically and chromatographed on DEAE-cellulose paper with 0.6 M Tris carbonate as a solvent. The chromatogram was autoradiographed, and the amount of pppA and pppUHgSR polymerized was calculated from the ^{14}C- and ^{203}Hg activity of the spots remaining at the start.

RESULTS

Separation of the reaction products of pUHgX with thiols.

In the course of preliminary attempts to polymerize ppUHgSR in the presence of polynucleotide phosphorylase, we had obtained evidence that the mercurated

substrates lose their mercury fairly rapidly in the presence of 1 to 2 equivalents of mercaptoethanol. In order to examine the stability of different compounds of the type (pp)pUHgSR, and to discover the reactions responsible for the loss of mercury, we converted [^{203}Hg]pUHgOAc to a series of thiomercuri derivatives by reaction with one to five equivalents of SH-compounds :

$$pUHgX + RSH \longrightarrow pUHgSR + HX \qquad \text{(reaction 1)}$$

The conversion is quantitative since equilibrium constants of the order of 10^{16} prevail[13] for reactions of the type :

$$RHg^{+} + R'S^{-} \longrightarrow RHgSR'$$

For studying the reaction with thiols we preferred the monophosphate pUHgX over the tri- or diphosphate because the latter preparations often contain small amounts of di- and/or monophosphates, a fact which renders the analysis of reaction products more complicated.

The column elution profile for the reaction products of pUHgOAc with L-cysteine is shown in figure 1a. In this case, as in most others, the following nu-

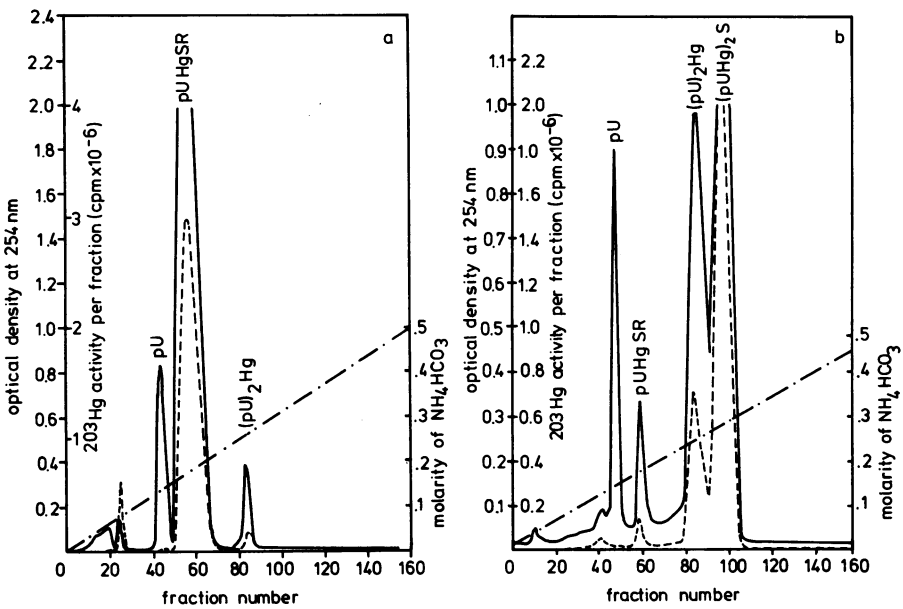


Figure 1. Chromatography of the reaction products of pUHgX with thiols.
a. reaction products obtained by mixing 100 µmole of pUHgX containing 2.3 x 10^{7} cpm ^{203}Hg with 200 µmole of L-cysteine.
b. reaction products obtained by mixing 200 µmole of pUHgX containing 3.8 x 10^{7} cpm ^{203}Hg with 200 µmole of thioacetamide.
Separation on a 50 ml DEAE-cellulose column was as described in methods and 10 ml fractions were collected.
——— optical density at 254 nm, light path 3 mm
– – – – – ^{203}Hg-activity per fraction
—·—·— molarity of NH_4HCO_3

cleotide-containing compounds were eluted : pU, pUHgSR, and $(pU)_2Hg$.

In the case of the reactions with thioacetic acid and with thioacetamide addition of the reactant to pUHgOAc made the solution turn brown or black due to formation of HgS, while the column elution profile showed a major additional peak of $(pUHg)_2S$. This is illustrated for the thioacetamide case in figure 1b.

Although pUHgSR is undoubtedly the first reaction product formed when an SH-compound is added to pUHgX, the data of table 1 show that the time needed to mix the reactants and to load them on a column is sufficient for an appreciable amount of pUHgSR to be converted to other compounds. In this process pUHgSR loses mercury, which precipitates under the form of $(RS)_2Hg$, and is converted to nucleotides containing less than one Hg atom per U residue, viz. pU and $(pU)_2Hg$.

The amount of pUHgSR remaining after column chromatography is mentioned in table 1 and varies between wide limits: 87% for the L-cysteine derivative to less than 2% for the thioacetic acid derivative. However, this amount cannot be considered as a reliable measure of the stability of the different pUHgSR derivatives, because no precautions were taken to keep conditions, such as temperature, or time between reaction and column loading, constant. Moreover, the amount of pUHgSR remaining depends on the ratio of RSH to pUHgX in the reaction. The variability of the pUHgSR yield is illustrated (table 1) by the three experiments where L-cysteine was used as the thiol. When the column fractions containing pUHgSR were desalted by evaporation, poorly soluble $(RS)_2Hg$ formed again, together with pU and $(pU)_2Hg$. When the residue was dissolved, we obtained a mixture of pUHgSR, pU and $(pU)_2Hg$, as demonstrated by paper chromatography and by the fact that the Hg/U ratio in the redissolved residue was less than 1. This ratio is mentioned in the last column of table 1 and can be considered as a qualitative measure of pUHgSR stability, the L-cysteine derivative being the most stable of those examined.

Identification of reaction products.

Below we summarize the identification as pUHgSR, $(pU)_2Hg$, $(pUHg)_2S$, and $(RS)_2Hg$ of the products obtained by reaction of pUHgOAc with several thiols and separated by column chromatography. The yields of these compounds are mentioned in table 1, their paper chromatographic mobilities in table 2.

pUHgSR : These derivatives formed the first peak containing both nucleotide material and radioactivity in the column profiles (see figure 1). When desalted by evaporation of the volatile buffer, they gave rise to residues which only partly redissolved in water. The poorly soluble fractions were identified as $(RS)_2Hg$, the soluble fractions as mixtures of pUHgX, pU, pUHgSR and $(pU)_2Hg$, by paper chromatography in the presence of marker substances. In the case of the

Table 1. Yield of products formed by reaction of pUHgX with RSH.
Reaction products were separated by DEAE-cellulose column chromatography and the yields calculated by measuring the UV-absorption of the fractions. Not included is the insoluble $(RS)_2Hg$ which was removed before loading the column, and usually not quantified. The total yield is a measure of column recovery. The Hg/U ratio of pUHgSR (last column) is a qualitative measure of the stability of these compounds, as explained in the text. For the reactions with methyl-and ethylmercaptan, ppUHgX was used instead of pUHgX. The number of equivalents of methylmercaptan added cannot be specified since it was led through the solution as a gas.

RSH		% yield of reaction products					Hg/U ratio in pUHgSR after desalting
compound used	equivalents added	pU	pUHgSR	$(pU)_2Hg$	$(pUHg)_2S$	total	
methylmercaptan	undefined	0.3	86.4	1.4	1.4	91.5	0.55
ethylmercaptan	5	0	82.7	10.7	0	93.4	0.56
mercaptoethanol	2	20.2	6.7	51.8	0	78.7	0.69
L-cysteine	2	7.0	87.2	5.9	0	100.1	
L-cysteine	2	8.0	62.2	24.6	0	94.8	0.86
L-cysteine	1	0	20.0	69.5	0	89.5	
thiophenol	2	7.2	2.4	83.4	0	93.0	0.47(*)
2-thiouracil	2	0	2.3	96.5	0	98.8	0.33(*)
thioacetic acid	1	8.4	1.2	31.0	47.0	87.6	0.54
thioacetamide	1	7.1	2.6	25.8	50.5	86.0	0.41(*)

(*) These ratios are lower than the true Hg/U ratio because the R group contributes to the UV-absorbance at 267 nm, taken as a measure for U concentration.

Table 2. Rf values of uridine-5'-phosphates and mercury derivatives. Paper chromatography was ascending on DEAE-cellulose paper.

compound		solvent	
		0.4 M Tris carbonate	0.6 M Tris carbonate
pU		0.34	0.46
ppU		0.21	0.35
pppU		-	0.27
$pUHg^+$		0.56	0.61
$ppUHg^+$		0.42	0.53
$pppUHg^+$		-	0.44
$(pU)_2Hg$		0.11	0.32
$(ppU)_2Hg$		-	0.25
$(pUHg)_2S$		0.09	0.27
$(ppUHg)_2S$		-	0.21
pUHgSR	RSH=CH_3SH	0.34	-
"	C_2H_5SH	0.34	-
"	$HOCH_2CH_2SH$	0.29	-
"	CH_3COSH	0.26	-
"	CH_3CSNH_2	0.25	-
"	C_6H_5SH	0.27	-
"	L-cysteine	0.22	-
ppUHgSR	$HOCH_2CH_2SH$	-	0.31
pppUHgSR	$HOCH_2CH_2SH$	-	0.20
$(RS)_2Hg$	$HOCH_2CH_2SH$	0.74	0.82
"	L-cysteine	0.28	-

L-cysteine derivative, the pUHgSR column fraction was found to contain 1.19 equivalent S per equivalent Hg in a $^{35}S/^{203}Hg$ double label experiment.

From these observations, and from others described below, we inferred that the pUHgSR fraction isolated by column chromatography decomposes, upon desalting, into $(RS)_2Hg$, $(pU)_2Hg$, pUHgX, and pU.

$(pU)_2Hg$: In the column profiles, this derivative formed the second peak of mercurated nucleotide material. The identification was based mainly on the Hg/U ratio of the product, which gave a mean value of 0.43 for 9 experiments. Also, the low mobility on DEAE-celllulose paper fits in with the presence of two phosphate groups. Finally, the compound is also formed by decomposition of $(pUHg)_2S$ (identification discussed below) with formation of a black percipitate of HgS, and this reaction is a general feature of organomercury sulfides[14] :

$$(RHg)_2S \cdot \rightarrow R_2Hg + HgS \downarrow$$

$(pUHg)_2S$: This was the main product of the reaction of pUHgOAc with thioacetic acid and with thioacetamide, and a minor product when pUHgOAc was treated with methylmercaptan. It was the last compound eluted from the DEAE-cellulose columns and on ion exchange paper its Rf value was just slightly lower than that of $(pU)_2Hg$, indicating a similar negative charge. Column fractions containing this compound turned brown and then black upon standing due to HgS formation. This decomposition went further during desalting. The fact that a mean Hg/U ratio of 0.78 was measured for this compound, while the theoretical ratio is 1, can be attributed to this decomposition: in fact the ratio is always measured on a mixture of $(pUHg)_2S$ and $(pU)_2Hg$, due to the reaction:

$$(pUHg)_2S \longrightarrow (pU)_2Hg + HgS \downarrow$$

Finally, the compound had exactly the same mobility on paper as $(pUHg)_2S$ obtained by briefly leading H_2S through a solution of pUHgX:

$$2\ pUHgX + H_2S \longrightarrow (pUHg)_2S + 2\ HX$$

The formation of $(pUHg)_2S$ by the reaction of pUHgOAc with thioacetic acid and thioacetamide can be explained by the hydrolysis of these compounds in aqueous solution, with formation of H_2S[15] :

$$CH_3COSH + H_2O \longrightarrow CH_3COOH + H_2S$$
$$CH_3CSNH_2 + H_2O \longrightarrow CH_3CONH_2 + H_2S$$

H_2S is also present as an 0.2% impurity in the methylmercaptan gas used, a fact which accounts for the presence of a small $(pUHg)_2S$ peak in the CH_3SH derivative preparation (table 1).

$(RS)_2Hg$: When methylmercaptan, ethylmercaptan, mercaptoethanol, thiophenol or 2-thiouracil were added to pUHgOAc, white precipitates were slowly formed, which were removed before column chromatography. We postulated the formule $(RS)_2Hg$ for these substances since poorly soluble compounds of this type are described for RSH = CH_3SH, C_2H_5SH, HOC_2H_4SH, C_6H_5SH, and cysteine[16]. In the reaction of pUHgOAc with L-cysteine, mercury cysteinate did not precipitate but was eluted from the column during loading, or in the beginning of the gradient. The composition $(RS)_2Hg$ was confirmed by an S/Hg ratio of 2.18 found in a double label experiment. Furthermore, the product had the same mobility on paper as [^{203}Hg] mercury cysteinate prepared from L-cysteine and $^{203}Hg(OAc)_2$.

The formation of $(pU)_2Hg$ and $(RS)_2Hg$ from pUHgSR is to be understood in terms of the known tendency of asymmetric organomercurials to react to their symmetrical counterparts[17] :

$$2\ RHgR' \longrightarrow R_2Hg + R'_2Hg$$

which gives in our case :

$$2\ pUHgSR \longrightarrow (pU)_2Hg + (RS)_2Hg \qquad \text{(reaction 2)}$$

Stability of mercurated nucleotides.

The reactions discussed up to this point explain the presence in pUHgSR preparations of $(RS)_2Hg$ and $(pU)_2Hg$, but not that of pUHgX and pU, which is also noted. In order to gain full insight in the pathway by which pUHgX is demercurated in the presence of thiols, we examined the stability of pUHgX, $(pU)_2Hg$, and pUHgSR, and the appearance of breakdown products as a function of time.

Stability of pUHgX : No appreciable decomposition of this compound was observed. After 150 hours incubation at pH 5, 37°C, 97% of it remained intact, and it was at least equally stable at pH 7 and 9.

Stability of $(pU)_2Hg$: This derivative was fairly stable at pH 9 but decomposed slowly into pU and pUHgX at pH 7 and faster at pH 5. The concentration decreased exponentially, indicating a pseudo first order reaction :

$$(pU)_2Hg + H^+ \longrightarrow pUHg^+ + pU \qquad \text{(reaction 3)}$$

Reaction rates and half lifes at pH 5, 7, and 9 are summarized in table 3.

Stability of pUHgSR : None of these derivatives could be obtained in a pure state since they decompose during isolation. The best preparation available was that of the L-cysteine derivative, which is the most stable of those studied. It still contained about 0.2 equivalents $(pU)_2Hg$. Figure 2 shows the reaction kinetics when the preparation was incubated at pH 5. Mercury cysteinate precipitated during the reaction and was not assayed. The $(pU)_2Hg$ concentration first rose and then decreased, whereas the pUHgX concentration steadily rose. This points to the following reaction scheme :

$$2\ pUHgSR \longrightarrow (pU)_2Hg + (RS)_2Hg \downarrow \qquad \text{(reaction 2)}$$

$$(pU)_2Hg + H^+ \longrightarrow pUHg^+ + pU \qquad \text{(reaction 3)}$$

The same reactions were observed at pH 7 and 9 but the rate slowed down as the pH increased.

Reactions of mercurated nucleotides with thiols.

Reactions 1, 2, and 3 studied up to this point can be arranged into the following scheme :

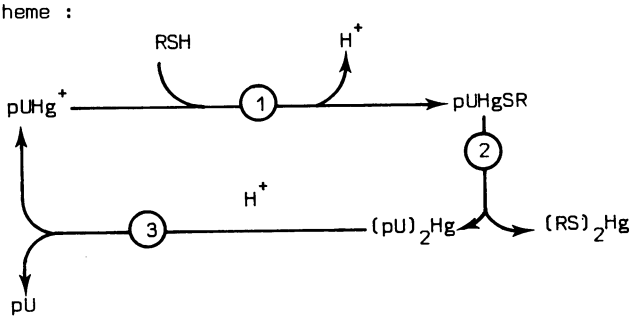


Table 3. Rate constants and half life for the decompostition of $(pU)_2Hg$. Reaction temperature was 37°C and the solutions were buffered with 0.1 M Tris acetate at the pH indicated.

pH	rate constant (sec^{-1})	half life (hours)
5	38.3×10^{-7}	50
7	14.4×10^{-7}	134
9	2.4×10^{-7}	802

Reactions 2 and 3 are responsible for the demercuration of isolated pUHgSR derivatives into the observed reaction products $(RS)_2Hg$, $(pU)_2Hg$, pU, and pUHgX. $(pU)_2Hg$ accumulates, especially in preparations of the least stable derivatives, because reaction 3 is relatively slow at neutral pH. In the presence of an excess thiol, pUHgX is gradually demercurated via a cyclic reaction sequence, 2 equivalents of thiol being necessary to achieve complete demercuration according to the sum reaction :

$$pUHgX + 2\ RSH \longrightarrow pU + (RS)_2Hg + HX$$

With an excess thiol present, no pUHgX is detected during the demercuration because the equilibrium of reaction 1 is entirely to the right.

Further experiments showed, however, that an additional reaction contributes to the demercuration process.

Decomposition of pUHgSR in the presence of excess thiol: When pUHgX was incubated at pH 7 with 4, 20, or 100 equivalents of mercaptoethanol, the pUHgSR concentration dropped very fast, reflecting the low stability of this derivative as compared to the L-cysteine analog. However, as illustrated in figure 3, when the excess thiol is larger pUHgSR disappears faster, and the $(pU)_2Hg$ concentration is lowered. This cannot be explained by the aforementioned reaction sheme. Also surprising is the fact that small amounts of pUHgSR remain present after 10 hours in spite of a very fast initial decomposition.

Reaction of $(pU)_2Hg$ with thiols : When $(pU)_2Hg$ was incubated with 2 equivalents of mercaptoethanol at pH 7 and 9, formation of pUHgSR and pU was observed as expected. However, the $(pU)_2Hg$ concentration dropped much faster than observed in the absence of thiols (table 3), the decrease was non-exponential, and the pH dependance of the reaction rate was reversed, breakdown being faster at pH 9 than at pH 7. If the decomposition of $(pU)_2Hg$ were merely the result of reactions 3 and 1 in sequence the rate would be limited by reaction 3 and addition of thiol would not increase it. Hence, we have to assume that $(pU)_2Hg$ disappears via a second pathway, viz. direct reaction with the thiol :

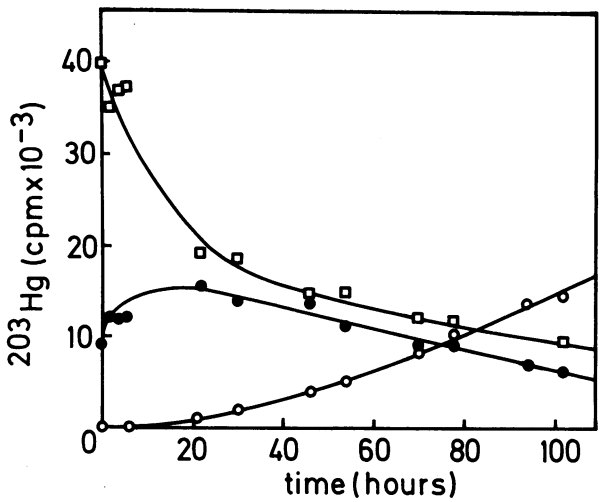


Figure 2. Stability of pUHgSR (RSH=L-cysteine).
6 µmole of pUHgSR with a ^{203}Hg activity of 6 x 10^5 cpm were incubated at 37°C in 100 µl 0.1 M Tris acetate buffer, pH 5. Samples of 5 µl were withdrawn periodically and analyzed by paper chromatography as described under methods. □ pUHgSR; ● $(pU)_2Hg$; ○ pUHgX.

$$(pU)_2Hg + RSH \longrightarrow pU + pUHgSR \qquad \text{(reaction 4)}$$

Reaction of $(pU)_2Hg$ with $(RS)_2Hg$: [^{203}Hg] $(HOCH_2CH_2S)_2Hg$ was brought in suspension in a solution of [^{203}Hg] $(pU)_2Hg$ in 0.1 M sodium acetate, pH 5, and after 1 hour the supernatant was subjected to paper chromatography. Autoradiography demonstrated the presence of pUHgSR, together with the starting products $(pU)_2Hg$ and $(RS)_2Hg$, in the approximate ratio 2 : 1 : 1. We conclude that reaction 2 is an equilibrium reaction :

$$2\ pUHgSR \rightleftharpoons (pU)_2Hg + (RS)_2Hg$$

This explains the non-exponential decrease of pUHgSR concentration noticeable in figure 2. As decomposition proceeds, $(pU)_2Hg$ and $(RS)_2Hg$ accumulate, slowing down the rate of reaction 2. The kinetics of this reaction are further complicated because one reaction product, $(pU)_2Hg$, further decomposes, whereas the other, $(RS)_2Hg$, reaches a maximum concentration when it starts precipitating from solution.

Reactions of mercurated nucleotides with H_2S.

These reactions were studied because H_2S was the reacting species in the experiments with thioacetic acid and thioacetamide, and also because the reaction of H_2S with pUHgX was exploited for the preparation of $(pU)_2Hg$, which is a key intermediate in the demercuration process.

Reaction of pUHg with H_2S : When H_2S was led through a solution of pUHgX in 0.01 M NH_4HCO_3 (pH 7.8) the solution turned brown and then black after a

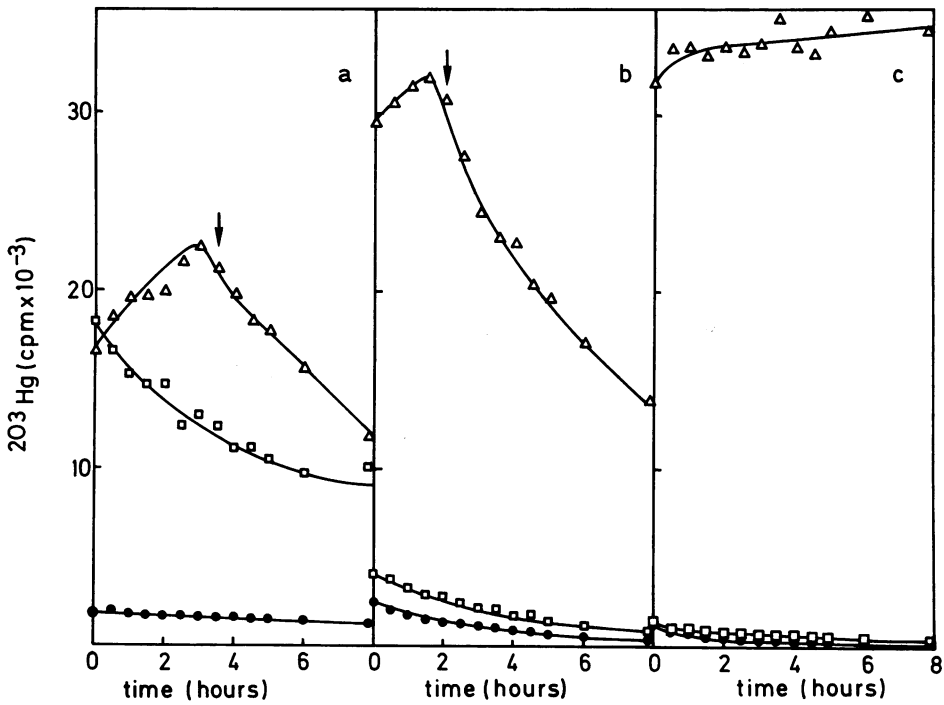


Figure 3. Reaction of pUHgSR with RSH (RSH=mercaptoethanol).
5 µmole of pUHgOAc with a ^{203}Hg-activity of 8 x 10^5 cpm were incubated at 37°C in 200 µl 0.1 M Tris acetate pH 7 in the presence of the following amounts of mercaptoethanol :
a. 20 µmole (4 equivalents)
b. 100 µmole (20 equivalents)
c. 500 µmole (100 equivalents)
Samples of 10 µl were withdrawn periodically and analyzed by paper chromatography. Total radioactivity in the samples is not constant in (a) and (b) because $(RS)_2Hg$ started to precipitate at the time marked by an arrow, and only the dissolved compounds were analyzed. In the presence of a large excess of mercaptoethanol (c) $(RS)_2Hg$ remained in solution.
□ pUHgSR; ● $(pU)_2Hg$; △ $(RS)_2Hg$

few minutes, due to formation of HgS which remained in colloidal solution. Paper chromatographic analysis of the reaction products as a function of time showed that the H_2S immediately and quantitatively converts pUHgX into $(pUHg)_2S$. After a few minutes, the latter product starts decomposing into $(pU)_2Hg$ and HgS. This reaction sequence is analogous to that deduced for the reaction of pUHgX with thiols :

$$2\ pUHgX + H_2S \longrightarrow (pUHg)_2S + 2\ HX \qquad \text{(reaction 1')}$$

$$(pUHg)_2S \longrightarrow (pU)_2Hg + HgS\downarrow \qquad \text{(reaction 2')}$$

Initially $(pU)_2Hg$ and HgS are formed at the same rate, but the concentration of the former compound levels off after about 10 min, while that of HgS continues to rise and pU appears in the mixture. This points to breakdown of

$(pU)_2Hg$ in the presence of an excess H_2S, as confirmed in the next experiment.

Reaction of $(pU)_2Hg$ with H_2S : H_2S was led through a $(pU)_2Hg$ solution in 0.01 M NH_4HCO_3 (pH 7.8), and the mixture analyzed as usual for 2 hours. The $(pU)_2Hg$ concentration decreased exponentially and the mercury was found quantitatively as HgS, the only other reaction product being pU. We conclude that $(pU)_2Hg$ reacts directly with H_2S, in analogy with reaction 4 :

$$(pU)_2Hg + H_2S \longrightarrow 2\ pU + HgS \downarrow \qquad \text{(reaction 4')}$$

Since the solution was saturated with H_2S, this is a pseudo first order reaction. The rate constant was 1.92×10^{-5} sec^{-1} at pH 7.8 and 20°C, which is considerably faster than the rate for $(pU)_2Hg$ decomposition in the absence of H_2S (table 3).

Polymerization of mercurated nucleotides.

When [^{203}Hg]ppUHgOAc was incubated with Micrococcus luteus polynucleotide phosphorylase in the presence of 1 equivalent mercaptoethanol, a precipitate of $(HOCH_2CH_2S)_2Hg$ gradually appeared in the reaction mixture. Samples of the supernatant were taken for up to 7 hours and analyzed by paper chromatography, but no polymerization took place, as judged from the absence of any radioactive or UV-absorbing material at the start of the chromatogram. When the experiment was repeated with 2 equivalents of mercaptoethanol the formation of a non radioactive polymer was detected.

Similar results were obtained when mercaptoethanol was substituted by L-cysteine, and when E. coli polynucleotide phosphorylase was used instead of the M. luteus enzyme. This shows that ppUHgSR is not a substrate for polynucleotide phosphorylase. When equimolar amounts of ppUHgX and RSH are used, they are quantitatively converted to ppUHgSR, which in turn yields $(RS)_2Hg$ and $(ppU)_2Hg$. The latter product is quite stable at pH 8.8. When an excess RSH is present, however, it is rapidly degraded by reaction 4, yielding ppU which is polymerized to poly U by the enzyme.

[^{14}C]pppA and [^{203}Hg]pppUHgX were incubated with E. coli RNA polymerase and poly d(A-T) as template under the conditions used by Dale and Ward[3], i.e. in the presence of 20 equivalents of mercaptoethanol relative to pppUHgX. A precipitate of $(HOCH_2CH_2S)_2Hg$ appeared in the mixture but, as shown in figure 4, the paper chromatographic assay demonstrated the formation of a polymer containing both ^{14}C and ^{203}Hg. The incorporation of pppUHgSR, which initially paralleled that of pppA, slowed down after about an hour and completely ceased after 2 hours. This can be interpreted as an initial incorporation of pppUHgSR, which is a substrate for RNA polymerase but is at the same time degraded to pppU in the presence of excess thiol. The pppU, being a better substrate and rising in concentration, gradually supersedes pppUHgSR in the polymerization.

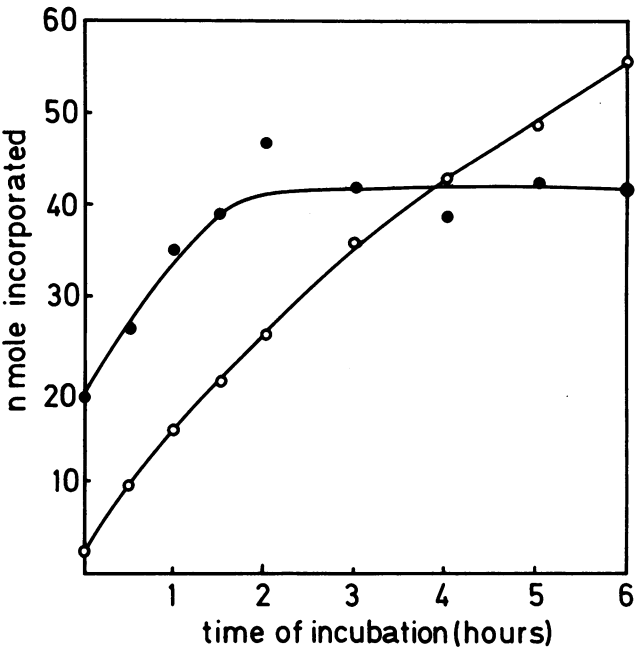
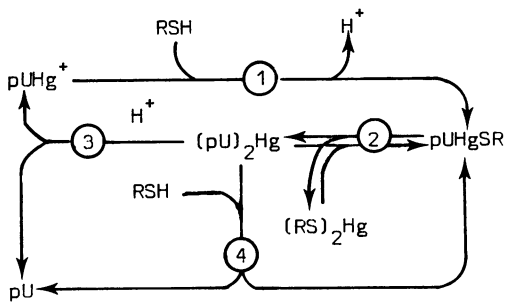


Figure 4. Transcription of poly d(A-T) with pppA and pppUHgSR as substrates. pppA was ^{14}C-labeled and pppUHgOAc, labeled with ^{203}Hg, was converted to pppUHgSR by the presence of 20 equivalents mercaptoethanol. The detailed composition of the mixture is described in the methods section. Samples were analyzed by paper chromatography and the amount of each substrate incorporated was computed from the ^{14}C- and ^{203}Hg activities at the start.
○ nmole [^{14}C] pppA incorporated; ● nmole [^{203}Hg] pppUHgSR incorporated.

The high initial value of ^{203}Hg-activity at the start of the chromatogram may be due to non-covalent binding of mercury, either in the form of Hg^{++} or as $(RS)_2Hg$, to the poly d(A-T) template.

DISCUSSION.

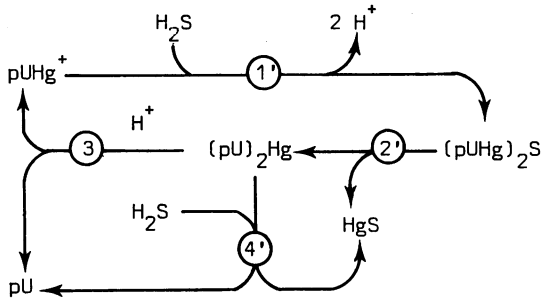
The following reaction scheme emerges from our experiments on the demercuration of pUHgX in the presence of thiols :



In the presence of a thiol, the mercurinucleotide is quantitatively converted to

a thiomercuri derivative (reaction 1). This derivative in turn is converted to $(pU)_2Hg$ and $(RS)_2Hg$ (reaction 2), an observation which is in agreement with the known tendency of asymmetric diorganomercurials to yield symmetrical compounds[17]. $(pU)_2Hg$ slowly decomposes into pUHgX and pU, the reaction rate depending on the acidity of the solution (reaction 3). In the presence of an excess thiol, however, $(pU)_2Hg$ is directly converted into pU and pUHgSR by reaction 4, at a rate much faster than that of reaction 3.

An analogous scheme explains the reactions in the presence of H_2S :



In this case, the mercurinucleotide is quantitatively converted to the organomercurial sulfide (reaction 1'). Compounds of this type are known to decompose spontaneously into diorganomercurials and HgS[14] (reaction 2'). This reaction is not reversible due to the extremely low solubility product of HgS, which is of the order of 10^{-51}. $(pU)_2Hg$ disappears slowly by reaction 3 commented above, but also, at a much faster rate, by reaction 4' in the presence of an excess H_2S. As a result, pUHgX can be nearly quantitatively converted to $(pU)_2Hg$ by a short treatment with H_2S, but the yield is much lower when the reaction is allowed to proceed for several hours.

Our original purpose was to try and use mercurated nucleotides for preparing base-specifically mercurated DNA transcripts or replica's, which could then be useful for attempts at electron microscopic sequence determination of nucleic acids. Transcription of the repetitive template poly d(A-T) was among the first experiments carried out, because it would be easy to assess the fidelity of transcription with mercurated substrates by nearest neighbour analysis of the transcript. It soon became apparent that demercuration occured and that the pppUHgSR initially incorporated is gradually replaced by pppU as the polymerization proceeds. Dale et al.[1] have claimed that ppUHgSR is polymerized to poly (UHg) by polynucleotide phosphorylase, without specifying the experimental conditions employed. We have tried to polymerize ppUHgX with polynucleotide phosphorylase from M. luteus and from E. coli, both in the absence and in the presence of thiols, but the only polymer obtained was poly U, formed in the

presence of mercaptoethanol by polymerization of the demercuration product ppU.

For investigating the demercuration reactions we have chosen pUHgX, which is easy to prepare and purify. Analogous reactions in all likelihood occur with C derivatives, but the preparation of pCHgX presents difficulties because pC forms insoluble mercury complexes when treated with $Hg(OAc)_2$, a phenomenon which has also been noted[2] with poly C.

Although demercuration in the presence of thiols has been observed for mercurated polynucleotides [3,8], it has been said to affect mercurated mononucleotides only after prolonged exposure to a 50 to 1000 fold excess of mercaptoethanol [2]. This is clearly an underestimation. Our results show that demercuration is so rapid that it is impossible to obtain an in vitro DNA replication or transcription product that is fully mercurated in one of the pyrimidine bases. This fact does not seem to have been appreciated in a study[18] of E. coli lac repressor binding to poly [d(A-UHg)], and also to have gone unnoticed by several authors who applied the method for obtaining mercury-labeled in vitro transcripts [6-11]. The reason seems to be that formation of such transcripts is usually observed with the mercury label in one substrate and the radioactive label in a different one. In this way, decomposition of pppUHg goes unnoticed because its breakdown product pppU is incorporated into the radioactive transcript as well. Limited mercuration of in vitro transcripts is not necessarily a drawback for their isolation by affinity chromatography since a low level of mercuration is probably sufficient for quantitative binding to sulfhydryl agarose.

The symmetrical mercurial $(pU)_2Hg$ occupies a central position in the demercuration reaction scheme and it can reach high concentrations when thiol is not present in large excess. By analogy, the reaction of thiols or hydrogen sulfide with mercurated polynucleotides should give rise to mercury bridges forming cross-links between pyrimidines. Formation of this type of cross-links in the presence of thiols was conjectured by Dale et al.[3] and the same type of bond was found to be formed[19] upon reduction in the presence of sodium borohydride of mercurated mono- and polynucleotides.

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