Mercurated Polynucleotides: New Probes for Hybridization and Selective Polymer Fractionation†

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ABSTRACT: Polynucleotides containing covalently bound mercury atoms have been prepared by chemical or enzymatic syntheses and some of their physical and biochemical properties studied. The mercury substituents do not appear to alter significantly normal polynucleotide structure. Mercurated polymers function efficiently as templates for nucleic acid polymerases, they are fully susceptible to degradation by standard nucleases, and their denaturation and reannealing properties resemble those of the corresponding nonmercurated polymers. While the T_m's of DNA duplexes are lowered by extensive mercuration, the T_m's of DNA–RNA hybrids and RNA duplexes are either unaffected or elevated. Mercuration, as would be expected, greatly increases the buoyant density of both DNA and RNA. The introduction of as few as one mercury atom per 200 bases permits the selective and quantitative retention of the mercurated polymer probe (and associated nucleotide sequences) on columns of sulfhydryl-agarose. The use of mercurated nucleotides (as polymerase substrates) and oligonucleotides (as primers) in conjunction with sulfhydryl-agarose chromatography provides a simple and efficient method for the isolation of selected polynucleotide sequences, such as specific in vitro transcription products or terminal fragments of duplex DNA. Products adsorbed to the affinity resin are readily recovered for further analysis by eluting with buffers containing mercaptoethanol. Although the mercury–carbon bond is somewhat thermolabile, mercurated polynucleotides are suitable as probes in low temperature hybridization studies.

Specific nucleotide sequences and genome fragments are currently being used to probe the structural organization of complex nucleic acids. This approach would be augmented considerably by a simple procedure for selectively recovering such probes and their associated "target" sequences. We have previously described (Dale et al., 1973, 1975) procedures for the introduction of covalently bound mercury atoms into polynucleotides. Mercurinucleotides, being organomercurials, exhibit an extremely high affinity for mercaptans. Polynucleotide sequences containing even a low level of mercury substitution should, therefore, be readily separated from unmodified nucleic acids by chromatography on resins containing sulfhydryl groups. The utility of the mercury atom as a molecular "tag" for sequence or hybridization probes depends upon a number of factors: (1) the mercury atoms must neither seriously perturb the structure (or biochemical properties) of polymer duplexes nor interfere with the annealing of single strands; (2) the mercury–carbon bond must be reasonably stable; and (3) the in vitro fidelity of mercurinucleotides as polymerase substrates should be equivalent to that of their natural counterparts. Examination of the chemical, physical, and biochemical properties of mercurated polynucleotides shows that these criteria can be satisfied. In addition, the results described here demonstrate that mercurated and nonmercurated nucleotide sequences can be rapidly and selectively resolved by chromatography on sulfhydryl-agarose.

Materials and Methods

The 5′-triphosphates of 5-mercuriuridine and 5-mercurideoxyuridine were prepared and purified as previously described (Dale et al., 1975). Standard nonradioactive nucleotides were obtained from P-L Laboratories and Sigma Biochemicals. Radioactive nucleotides and [^{203}Hg]mercuric acetate were products of New England Nuclear Corporation. [^{35}S]Mercaptoethanol was obtained from Amersham Radiochemicals; [^{14}C]cysteine was from Schwarz/Mann.

Escherichia coli DNA polymerase I (fraction VII) was purified by the method of Jovin et al. (1969). *E. coli* RNA polymerase was prepared and assayed as described by Burgess (1969). Purified T4 DNA polymerase and *E. coli* exonuclease III were the generous gifts of Drs. P. Englund and L. Enquist, respectively. Pancreatic DNase, pancreatic RNase, RNase T_1, RNase T_2, micrococcal nuclease, and spleen and venom phosphodiesterases were obtained from Worthington and Sigma.

Calf thymus DNA (Grade V), poly (A), and poly(U) were purchased from Sigma; poly(dT) was from Miles Laboratories and bulk *E. coli* tRNA was from Schwarz/Mann. Yeast phenylalanyl-tRNA was the kind gift of Dr. A. Rich. Synthetic polydeoxynucleotides were prepared according to published procedures: poly[d(A-T)], Richardson et al. (1964); poly(dG)·poly(dC), Radding et al. (1962).

Ultraviolet absorption spectra were recorded using a Cary 15 or a Beckman 25k spectrophotometer. Thermal denaturation profiles were done in water-jacketed cuvets. The temperature of the circulating water was controlled using a Lauda K4R (or Haake FE) circulating water bath: temperature changes were programmed at approximately 1.0°/min. The temperature in the sample cuvets was measured using a Yellow Springs 42SC thermister probe. Absorption and thermal changes were continuously monitored on two synchronized recorders.

Large-plaque SV40 virus was used to infect confluent monolayers of secondary African green monkey kidney cells at an input multiplicity of 2–5 plaque forming units/cell. Tritiated thymidine was added 24 hr post infection (10 μCi/

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ml, 5×10^{-6} *M*) and progeny virus isolated 3–4 days later. The virions were purified by isopycnic centrifugation in CsCl and the viral DNA was released by treatment at 50° with 1% sodium dodecyl sulfate–1 m*M* EDTA (Trilling and Axelrod, 1970). Form I and form II SV40 DNA was prepared by isopycnic centrifugation in CsCl containing ethidium bromide (Radloff et al., 1967).

SV40 CRNA was prepared from form I SV40 DNA as described by Westphal (1970). The reaction mixture (2.0 ml) contained: Tris-HCl (pH 7.8), 0.05 *M;* $MgCl_2$, 4 m*M;* $MnCl_2$, 1 m*M;* CTP, GTP, UTP, and [^{14}C]ATP (3×10^3 cpm/nmol), 1 m*M* each; 2-mercaptoethanol, 5 m*M;* form I SV40 DNA, 45 μg; RNA polymerase, 20 units. The reaction was incubated at 37° for 1 hr. Pancreatic DNase was added to a final concentration of 50 μg/ml and the mixture incubated at room temperature for an additional 20 min. The solution was extracted twice with an equal volume of phenol and the RNA in the aqueous phase precipitated by the addition of 3 volumes of ethanol. After standing at −20° overnight the solution was centrifuged for 15 min at 27,000*g* and the RNA pellet dissolved in 0.5 ml of 0.01 *M* Tris-HCl buffer (pH 7.4). The sample was dialyzed for 24 hr against two 1-l. changes of 0.05 *M* sodium acetate buffer (pH 6.0). 60 μg of [^{14}C]SV40 CRNA (1.9×10^3 cpm/μg) was recovered after dialysis.

[^{14}C]SV40 CRNA was mercurated as follows: [^{203}Hg]mercuric acetate (1.1×10^4 cpm/nmol) was added at a final concentration of 2×10^{-3} *M* to a 0.5-ml solution of SV40 CRNA (24 μg) in 0.05 *M* sodium acetate buffer (pH 6.0). This solution was heated, in parallel with an identical sample lacking mercuric acetate, for 30 min at 50°. The reactions were terminated by the addition of 0.2 ml of quench buffer (0.01 *M* Tris-HCl (pH 7.5)–1.0 *M* NaCl–0.1 *M* EDTA) and the samples dialyzed against three 1-l. changes of TNE buffer (0.01 *M* Tris-HCl (pH 7.5)–0.02 *M* NaCl–0.001 *M* EDTA). The second dialysis solution also contained 10^{-5} *M* mercaptoethanol to provide an appropriate mercury ligand for hybridization. As determined from the ^{203}Hg cpm/OD_{260} ratio, 3.2% of the total bases in the SV40 CRNA was mercurated.

Sulfhydryl-agarose was prepared following the procedure of Cuatrecasas (1970). Both Sepharose 6B (Pharmacia) and Bio-Gel A5M (Bio-Rad) have been used as the basic resin support with equally satisfactory results; 100 g of resin, after extensive washing, was suspended in an equal volume of distilled water. Finely powdered cyanagen bromide (250 mg/ml of packed resin) was added to the stirred slurry in a well-ventilated hood. The pH was maintained as near as possible to pH 11 by the dropwise addition of 10 *N* NaOH while the temperature was held at 20° by the addition of crushed ice. After proton release had subsided (15–30 min), the suspension was quickly transferred to a course sintered glass funnel and washed under suction with 1500 ml of water (adjusted to pH 10 with sodium hydroxide); 100 ml of a 2 *M* solution of ethylenediamine (adjusted to pH 10 with 6 *N* HCl) was added immediately to the washed beads and the reaction allowed to proceed, with continual stirring, for 20 hr at 4°. The resin was then washed with 4 l. of water and suspended in 100 ml of 1.0 *M* $NaHCO_3$, adjusted to pH 9.7; 16 g of *N*-acetylhomocysteine thiolactone was added and the solution, which had a pinkish hue, was stirred at 4° for an additional 24 hr. Since incomplete substitution of the aminoethyl-agarose intermediate could possibly elevate the level of nonspecific resin adsorption (see text), a large excess of *N*-acetylhomocysteine thiolactone was used in these reactions. The sulfhydryl-agarose product was washed extensively with 6–8 l. of 0.1 *M* NaCl containing 1 m*M* mercaptoethanol followed by 1–2 l. of 0.1 *M* NaCl.

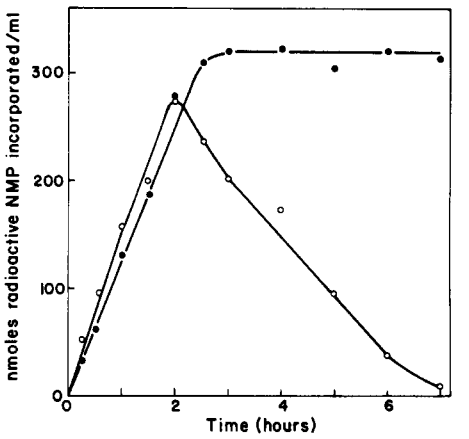


FIGURE 1: Time course of poly[d(A-T)] (○) and poly[d(A-HgU)] (●) synthesis with *E. coli* DNA polymerase. I. Reactions (1.0 ml) contained Tris-HCl buffer (pH 7.4), 50 m*M;* $MgCl_2$, 10 m*M;* [^{3}H]dATP (4.2×10^3 cpm/nmol), 1 m*M;* TTP or Hg-dUTP, 1 m*M;* 2-mercaptoethanol, 10 m*M;* poly[d(A-T)], 0.07 m*M;* and 3 units of DNA polymerase I (Jovin et al., 1969). The reactions were incubated at 37°. At the indicated times 50-μl aliquots were removed and 4 ml of cold Cl_3CCOOH solution (100% Cl_3CCOOH, saturated sodium pyrophosphate, saturated trisodium phosphate, and water, 1:1:1:7) was added and the samples, after standing for 10 min on ice, were filtered onto Whatman GF/A filters. The filters were washed with 50 ml of Cl_3CCOOH solution, dried, and counted in a Packard scintillation counter.

The degree of sulfhydryl substitution was determined spectrophotometrically using 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959) and by quantitating the binding of [^{203}Hg]mercuric acetate. The sulfhydryl content, which varied from preparation to preparation, ranged from 1.0 to 8.8 μmol/ml of resin. The trinitrobenzenesulfonate color test, described by Cuatrecasas (1970), confirmed that in all cases complete amino group substitution had occurred.

Results

The mercurated analogs of the alternating copolymers poly[d(A-T)] and poly[r(A-U)] were studied in some detail because of their structural simplicity and their facile enzymatic synthesis. As shown in Figures 1 and 2, when poly[d(A-T)] is used as a template, dUTP-HgSE[1] and UTP-HgSE are readily polymerized by *E. coli* DNA and RNA polymerases, respectively. The incorporation of the mercurinucleotides is totally dependent on the presence of a mercaptan (or cyanide ion) to act as a mercury ligand. The chloride or acetate salts of Hg-dUTP or Hg-UTP are not polymerized (Figure 2, Dale et al., 1973) and completely inactivate RNA polymerase, an enzyme sensitive to thiol modification. The Hg–S bond is essentially covalent, with an association constant of about 10^{16}, in contrast to the

[1] Abbreviations used are: Hg-U, 5-mercuriuracil; Hg-C, 5-mercuricytosine; UTP-HgSE and dUTP-HgSE, the 5-mercuri derivatives of UTP and dUTP in which the mercury ligand is 2-mercaptoethanol; Hg-UTP and Hg-dUTP, the 5-mercuri derivatives of UTP and dUTP in which the mercury counterion is chloride, acetate, or bicarbonate; poly(Hg-U), the homopolymer of 5-mercuriuridine; poly[d(A-HgU)], an alternating copolymer of deoxyadenosine and 5-mercurideoxyuridine; poly[r(A-U)], an alternating copolymer of adenosine and 5-mercuriuridine. Unless otherwise specified the mercury ligand in poly[d(A-HgU)] and poly[r(A-HgU)] is mercaptoethanol.

Table I: The Polymerization of [^{35}S]Mercaptoethanol and [^{14}C]Cysteine by *E. coli* DNA Polymerase I when Hg-dUTP is used as a Substrate.[a]

Expt No.	Substrates	Radioactive Mercaptan	Nanomoles of Radiolabel Polymerized			
			[^{3}H]dAMP	[^{35}S]Mercapto-ethanol	[^{203}Hg]-dUMP	[^{14}C]Cysteine
1	[^{3}H]dATP + [^{203}Hg]dUTP-HgSE		4.70		4.65	
2	[^{3}H]dATP + dUTP	Mercaptoethanol	4.85	<0.001		
3	[^{3}H]dATP + Hg-dUTP	Mercaptoethanol	4.55	4.45		
4	[^{3}H]dATP + [^{203}Hg]dUTP-HgCys		1.53		1.59	
5	[^{3}H]dATP + dUTP	Cysteine	16.2			<0.001
6	[^{3}H]dATP + Hg-dUTP	Cysteine	1.49			1.51

[a] All reactions (0.125 ml) were run under the conditions given in Figure 1. Experiments 1–3 were incubated at 37° for 20 min while experiments 4–6 were incubated for 1.0 hr. Specific activities (cpm/nmol) were: [^{3}H]dATP (No. 1–3, 7252; No. 4–6, 4500); [^{35}S]mercaptoethanol, 1236; [^{203}Hg]dUTP-HgSE, 7700; [^{203}Hg]dUTP-HgCys, 11,300; [^{14}C]cysteine, 3100.

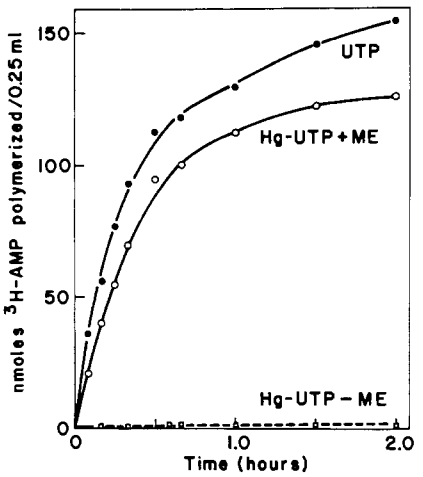


FIGURE 2: Time course of poly[r(A-U)] (●) and poly[r(A-HgU)] (○) synthesis with *E. coli* RNA polymerase. Reactions (1.0 ml) contained Tris-HCl buffer (pH 8.0), 50 m*M*; $MgCl_2$, 5 m*M*; $MnCl_2$, 1 m*M*; poly[d(A-T)] 0.01 m*M*; [^{3}H]ATP (1.5×10^{3} cpm/nmol), 1 m*M*; UTP or Hg-UTP, 1.0 m*M*; 2-mercaptoethanol (ME), where indicated, 20 m*M*; RNA polymerase, 35 units (Burgess, 1969). Aliquots (50 μl) were removed at the indicated times and processed as in the legend to Figure 1.

Hg–Cl or Hg–O (acetate) bonds which possess association constants of 10^{5} and 10^{3}, respectively (Simpson, 1961). The covalent nature of the Hg–S bond would indicate that the mercaptan ligand must also be polymerized. The data in Table I show that this is indeed the case. When [^{35}S]mercaptoethanol or [^{14}C]cysteine are added as the mercury ligand in DNA polymerase reactions approximately 1 nmol of mercaptan radiolabel is polymerized per nmol of [^{3}H]dAMP incorporated. The mercaptan–nucleotide ratio maintains the 1:1 stoichiometry throughout all stages of routine polymer purification: phenolization, gel filtration and dialysis. The radioactive mercaptan is, however, rapidly exchanged if additional thiol compounds are added. The ready exchangeability of the mercury ligand with other sulfhydryls facilitates the selective binding of mercurated polynucleotides to resins or proteins containing free thiol groups (see below). The cyanide ligand, which is polymerized with an efficiency equal to that of mercaptoethanol, is useful when such exchange reactions are desired since the Hg–CN association constant (10^{14}, Simpson, 1961) favors exchange to form Hg–S bonds.

The mercaptoethanol derivatives of Hg-dUTP and Hg-UTP are excellent analogs of TTP and UTP, respectively, and exhibit K_m values which are only slightly higher than the natural substrates (Table II). As previously noted (Dale et al., 1973), the *E. coli* polymerases will efficiently utilize mercurinucleotides containing mercaptan substituents as large as 1-thioglucose.

Table II: K_m Values of dUTP-HgSE and UTP-HgSE.[a]

Enzyme	NTP	K_m Value (*M*)
DNA polymerase I	TTP	6.1×10^{-6}
DNA polymerase I	dUTP-HgSE	7.7×10^{-6}
RNA polymerase	UTP	4.2×10^{-5}
RNA polymerase	UTP-HgSE	5.4×10^{-5}

[a] Reactions were incubated at 37° for 10 min with poly[d(A-T)] template under the conditions given in Figures 1 and 2 using 0.1 and 3.5 units/ml of DNA or RNA polymerases, respectively.

In addition to its polymerase function, *E. coli* DNA polymerase I contains two exonuclease activities; one degrading in the 3′ → 5′ direction, the other in the 5′ → 3′ direction (Kornberg, 1969). Proteolysis of the intact enzyme yields two fragments: one (76,000 daltons) retains polymerase and 3′ → 5′ nuclease activity while the other (36,000 daltons) retains only the 5′ → 3′ exonuclease activity (Setlow et al., 1972). The poly[d(A-T)] synthesized in holoenzyme reactions normally undergoes extensive degradation after a significant proportion of the input nucleoside 5′-triphosphates are utilized. Removal of the 5′ → 3′ exonuclease permits extensive synthesis without degradation (Setlow et al., 1972). Poly[d(A-T)] production (Figure 1) exhibits the typical holoenzyme synthesis–degradation profile. In contrast, when dUTP-HgSE is used as a substrate, no late degradation occurs. Poly[d(A-HgU)] appears to be more resistant to the polymerase associated 5′ → 3′ nuclease than poly[d(A-T)], a property which permitted the preparation of poly[d(A-HgU)] in milligram quantities.

Although the hydrogen bonding characteristics of mercuripyrimidine nucleotides should be similar to those of the parent compounds (the p*K* values of the ionizable ring protons are essentially identical, Dale et al., 1975) the mercurinucleotides could possibly exhibit grossly abnormal base-pairing properties, like the nucleotides of 7-deazapurine (Ward and Reich, 1972). However, nearest neighbor analysis of poly[d(A-HgU)] and poly[r(A-HgU)] (Table III) demonstrate that the polymers contain a perfectly alternating sequence of A and Hg-U bases.[2] In addition, attempts

[2] The observed low level of ^{32}P transfer from [^{32}P]AMP and [^{32}P]dAMP to UMP or dUMP likely results from slight impurities in the Hg-dUTP and Hg-UTP preparations or from demercuration that occurred during the phenol extraction and subsequent polymer work-up.

Table III: Nearest Neighbor Analysis of Poly[d(A-HgU)] and Poly[r(A-HgU)].[a]

Polymer	Substrates	Radioactivity in Isolated Mononucleotides		
		3′-dAMP	3′-dUMP-HgSE	3′-dUMP
Poly[d(A-HgU)]	[^{32}P]dATP + dUTP-HgSE	25	73,560	1475
		3′-AMP	3′UMP-HgSE	3′UMP
Poly[r(A-HgU)]	[^{32}P]ATP + UTP-HgSE	40	47,312	660
	ATP + [^{32}P]UTP-HgSE	63,205	36	20

[a] Reactions (1.0 ml) were run as described in Figures 1 and 2 for 15 min. The reaction mixtures were extracted twice with an equal volume of freshly distilled phenol and the aqueous phase was dialyzed against TNE buffer (0.01 *M* Tris-HCl buffer (pH 7.5)–0.02 *M* NaCl–0.001 *M* EDTA) for 36 hr, the buffer being changed every 12 hr. The solutions were then concentrated to 0.1–0.15 ml by dialysis against a 50% solution of polyethylene glycol 6000 (dialyzed briefly in 0.01 *M* Tris-HCl buffer (pH 7.5) prior to use). The poly[d(A-HgU)] was digested in a 0.2-ml reaction which contained: 25 μg of micrococcal nuclease, 25 μg of spleen phosphodiesterase, 3 m*M* $CaCl_2$, 1 m*M* mercaptoethanol, and 10 m*M* Tris-HCl buffer (pH 7.5). The poly[r(A-HgU)] digestion mixture (0.2 ml) contained 20 μg of micrococcal nuclease, 10 μg of ribonuclease T_2, 1 m*M* mercaptoethanol, 3 m*M* $CaCl_2$, and 10 m*M* Tris-HCl buffer (pH 7.5). Enzyme digestions were incubated at 37° for 1 hr. Aliquots were removed and directly applied to a Kodak cellulose-thin-layer electrophoresis plate with appropriate cold marker compounds. The nucleotides were separated by electrophoresis for 3 hr at 300 V in 0.05 *M* sodium citrate buffer (pH 3.5). The spots, located by uv absorption, were scraped and counted in a Packard scintillation counter.

Table IV: The Effect of Mercury Substitution on the T_m of Polynucleotides.

Polymer	U or C Bases Mercurated (%)	T_m[a] Value (°C)	Complete Range of Thermal Transition (°C)	Polymer Hyperchromicity (%)
Poly[r(A-U)]	0	44	41–46	48
Poly[r(A-HgU)]	100	44	40–47	46
Calf thymus DNA	0	73	65–85	35
	3.8	71	64–83	31
	7.4	71	63–81	27
	17	70	58–81	28
	37	61	48–72	18
Poly(U)·poly(A)	0	46	38–48	44
	18	47	38–51	46
	45	48	39–53	45
	61	51	42–57	30
	85	57	46–68	24
	100	58	46–68	22
Poly(U)·poly(dA)	0	46	42–49	38
	22	46	41–50	35
	100	45	40–51	26

[a] T_m values of the poly[r(A-U)], C.T. DNA, and poly(U)·poly(A) polymers were determined in 0.01 *M* Tris-HCl buffer (pH 7.5)–0.02 *M* NaCl–0.001 *M* EDTA. Poly(U)·poly(dA) complexes were denatured in 0.01 *M* Tris-HCl (pH 7.5)–0.11 *M* NaCl–0.001 *M* EDTA.

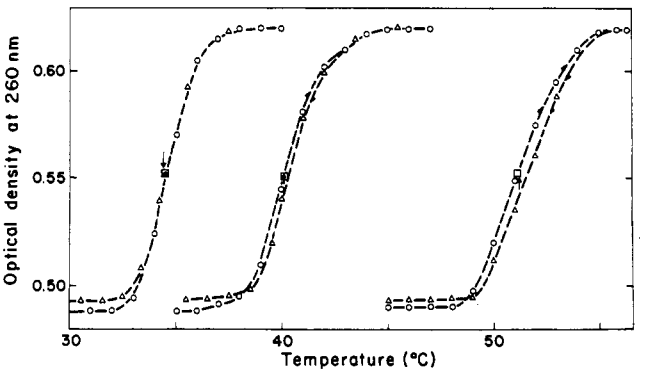


FIGURE 3: Thermal denaturation profiles of poly[d(A-HgU)] in 0.001 *M* Tris-HCl buffer (pH 7.5)–0.001 *M* EDTA containing 0.02, 0.035, and 0.10 *M* NaCl (T_m's, 34, 40, and 51°, respectively). Temperature changes were programmed at 0.8°/min. After denaturation, the solutions were held at approximately 10° above the T_m for 30 min prior to renaturation. Heating profile (○); renaturation (△).

to incorporate [^{203}Hg]dUTP-HgSE into poly(dG)·poly(dC) or [^{203}Hg]dCTP-HgSE into poly[d(A-T)], by base mismatching using DNA polymerase I, were without success. The in vitro fidelity of the mercurinucleotides is apparently equivalent to that of the natural pyrimidine substrates, an essential property if mercurated products of in vitro replication or transcription are to be of utility as hybridization probes.

The utility of mercurated polymers for hybridization studies requires that several other criteria be satisfied: (1) incorporation of mercurinucleotides must not significantly distort the structure of polymer duplexes; (2) mercurated single-stranded polymers must be capable of efficient reannealing; and (3) the mercury-carbon bond must be stable under the reannealing conditions. Since poly[d(A-HgU)] and poly[r(A-HgU)] contain one mercury atom per base pair, near the maximum obtainable level of mercuration, it was of interest to examine some of their physical and biochemical properties. Unless otherwise noted, the polymers, prepared and purified as described under Materials and Methods, were studied with 2-mercaptoethanol as the mercury ligand. Thermal denaturation studies on poly[d(A-HgU)] and poly[r(A-HgU)] showed that both polymers possess highly organized secondary structures. The melting profiles are sharp, occur in a highly cooperative manner, and are associated with a high-degree of hyperchromicity (Figure 3). While the T_m of poly[d(A-HgU)] is approximately 9° lower than poly[d(A-T)] under the same ionic conditions (Figure 4), the T_m of poly[r(A-HgU)] is identical with that of poly[r(A-U)] (Table IV). The uv spectra of the native and denatured polymers (Figure 5) strikingly resemble those of poly[d(A-T)].

The apparent differential effect of mercury substituents on the thermal stability of DNA and RNA helices was further examined by studying the melting curves of chemically mercurated polymers. Samples of calf thymus DNA containing from 3 to 37% of the total cytosine bases mercurated were prepared as previously described (Dale et al., 1975), as were samples of mercurated poly(U) (0–100% base substitution). Attempts to anneal poly(Hg-U), the homopolymer of 5-mercuriuridine, with poly(rA) or poly(dA) were unsuccessful when the mercury ligand was chloride ion. However, duplex formation occurred rapidly when the

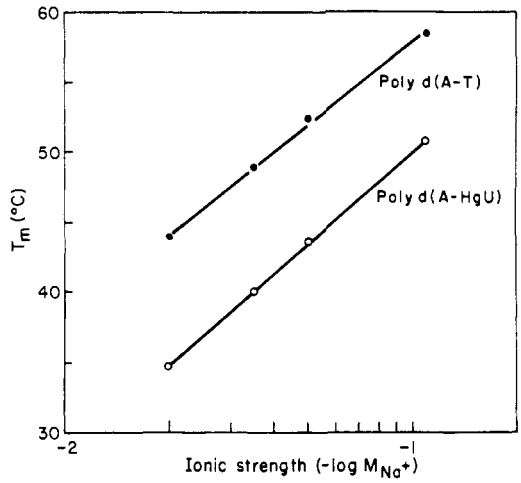


FIGURE 4: The T_m values of poly[d(A-T)] and poly[d(A-HgU)] as a function of sodium ion concentration. Melting curves were done in 0.001 *M* Tris-HCl buffer (pH 7.5)-0.001 *M* EDTA containing the indicated concentration of NaCl. Polymer concentration, 8×10^{-5} *M*.

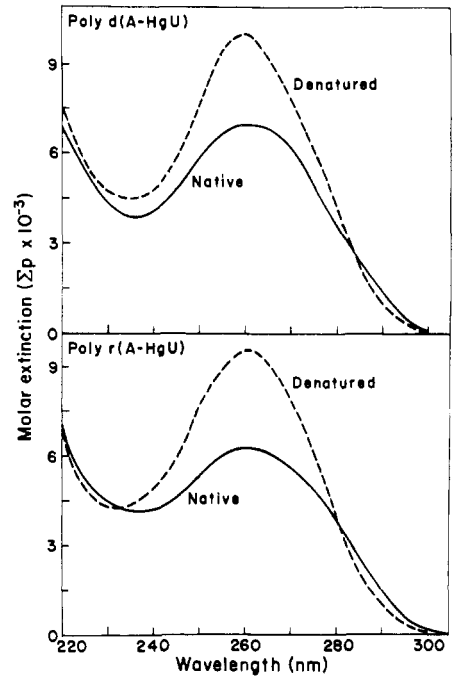


FIGURE 5: The uv spectra of poly[d(A-HgU)] and poly[r(A-HgU)] in 0.01 *M* Tris-HCl buffer (pH 7.5). The spectra of the denatured polymers were run at 50°.

mercury ligand was a mercaptan or when a twofold molar excess of 2-mercaptoethanol was added to the chloride form of poly(Hg-U) prior to mixing. Formation of the 1:1 homopolymer duplexes took 90–120 sec, as judged from the time required to give the maximum amount of hypochromicity. Under similar conditions, the annealing of poly(U) with poly(rA) was complete within 60–90 sec. The mercury substituents do not appear to significantly reduce the rate of homopolymer annealing provided an appropriate mercaptan is available for the mercury charge. The T_m values of calf thymus DNA, poly(U)·poly(A), and poly(U)·poly(dA) as a function of the percent mercuration are given in Table IV. While the T_m's of mercurated calf thymus DNA decrease as the mercury content is increased, the T_m's of the RNA and the DNA-RNA hybrid duplexes are either unaltered or elevated. A large percentage of the bases in all three polymers can be mercurated, however, without causing a major T_m change. Extensive mercuration is required in order to profoundly influence the T_m; such changes are often, but not always, accompanied by a reduction in the level of hyperchromicity and a decrease in the cooperativity of the melting profile of chemically mercurated polymers (Table IV). Since prolonged heating is required to achieve extensive chemical substitution the chain length of highly substituted polymers is likely to be considerably less than those which are only slightly mercurated. The decrease in hyperchromicity and the reduction in melting cooperativity are likely a reflection of the decreased molecular weight.

Examination of space-filling CPK models of mercurated DNA and RNA duplexes reveal no obvious steric interaction which could account for the enhanced thermal lability of DNA secondary structure. The mercury atom and its 2-mercaptoethanol ligand are readily accommodated within the major groove of the DNA helix, even when every base pair contains one mercurithio substituent (Figure 6). The possibility that mercuration induces a drastic perturbation of the normal DNA structure appears unlikely since such structurally aberant polymers would be expected to function poorly, if at all, in enzymatic reactions. As shown in Table V and Figure 7, poly[d(A-HgU)] and mercurated calf thymus DNA function as templates for *E. coli* DNA and RNA polymerases with an efficiency similar to that of the corresponding nonmercurated polymers. Furthermore,

Table V: Template Properties of Mercurated DNA.[a]

Enzyme	Template	nmoles Total Product Synthesized/0.1 ml 10 min	20 min
DNA polymerase I	Poly[d(A-T)]	2.05	3.96
	Poly[d(A-HgU)]	1.47	3.24
	C.T. DNA	0.53	0.98
	C.T. Hg-DNA	0.39	0.77
RNA polymerase	Poly[d(A-T)]	7.52	14.1
	Poly[d(A-HgU)]	6.01	11.3
	C.T. DNA	1.10	1.93
	C.T. Hg-DNA	0.86	1.51

[a] Polymerase reactions were run under the conditions described in Figures 1 and 2. Where C.T. DNA was used as a template the nucleoside triphosphates of C and G were also added at 1 m*M*. DNA concentrations were: 0.05 m*M* for poly[d(A-T)] and poly[d(A-HgU)]; 0.15 m*M* (as nucleotide phosphate) for C.T. DNA and C.T. Hg-DNA (21% of C bases mercurated).

mercurated DNAs are fully susceptible to degradation by pancreatic DNase, spleen DNase, micrococcal nuclease, as well as spleen and venom phosphodiesterases (Table III, Figure 8). Mercurated RNAs are also readily digested by micrococcal nuclease, pancreatic ribonuclease, and T_1 and T_2 ribonucleases. The 5′ → 3′ nuclease associated with *E. coli* DNA polymerase I is the only enzymatic activity found so far to be essentially inactive with mercurated polymers. A detailed report on the nuclease susceptibility of these polymers will be presented elsewhere; however, two points should be noted. (1) Analysis of the products of nuclease digestion can be complicated by the presence of "split-spots". This problem is a consequence of the fact that the electrophoretic and chromatographic properties of Hg-nucleotides are different in the presence and absence of a mercaptan (Dale et al., 1975). Incomplete ligand substitution or loss of mercaptan during handling and analysis will, therefore,

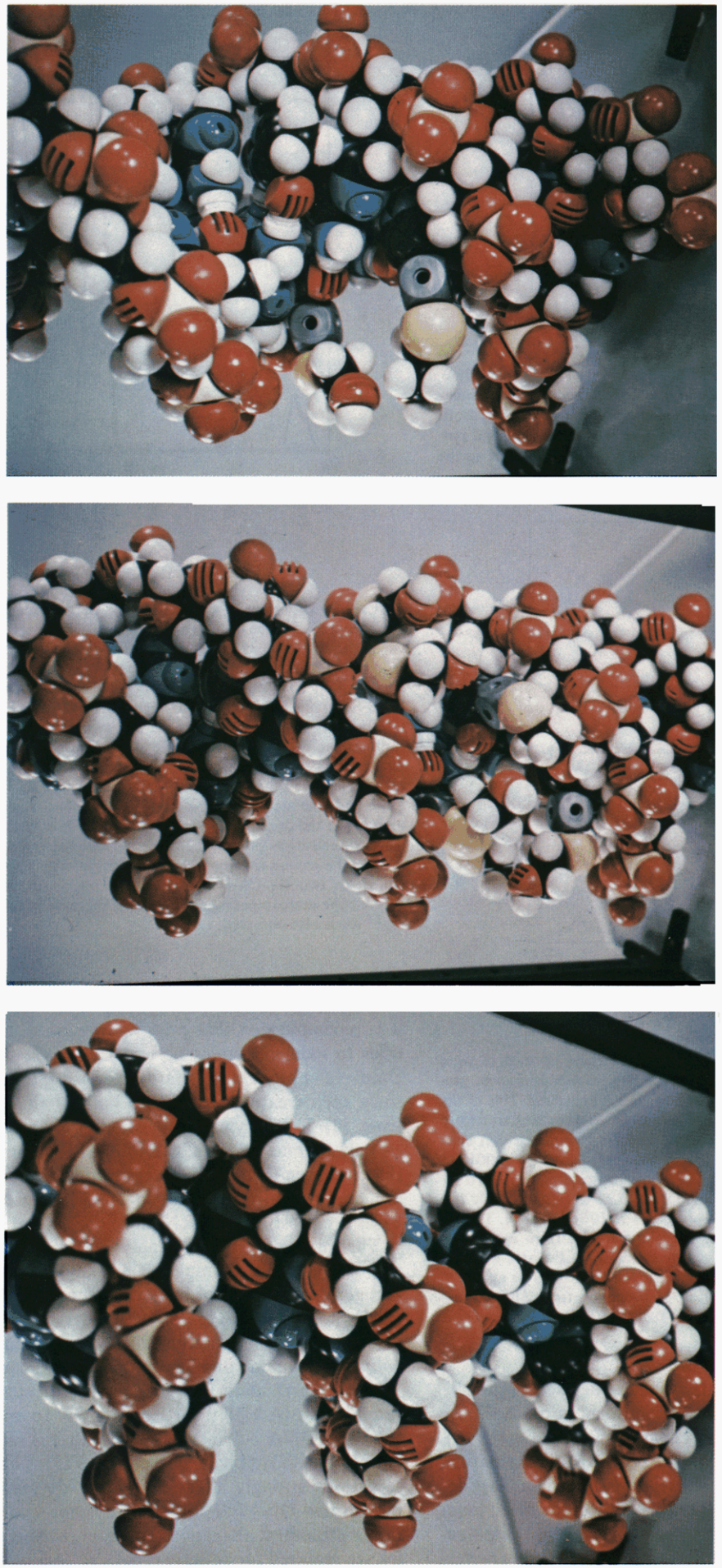


FIGURE 6: Molecular model of a DNA helix containing an alternating sequence of adenine and thymine (or 5-mercuriuracil) bases. In the unmodified poly d(A-T) (Figure 6A), both the major (bottom) and minor (top) grooves of the helix are free of protruding substituents. In Figure 6B, the 5-methyl group of each thymine base has been replaced by a mercuri thioester of 2-mercaptoethanol ($-HgSCH_2CH_2OH$). Six of these groups can be seen, in part, in the photograph; the mercury atom is silver-gray and the sulfur atom yellow. Although the mercurithioester groups fill almost the entire major groove, there is no distortion of the basic helix structure. As shown in Figure 6C, there is no steric interaction either between the individual mercury substituents of adjacent Hg-U bases (on opposite strands) or between the mercury substituents and the phosphodiester backbone.

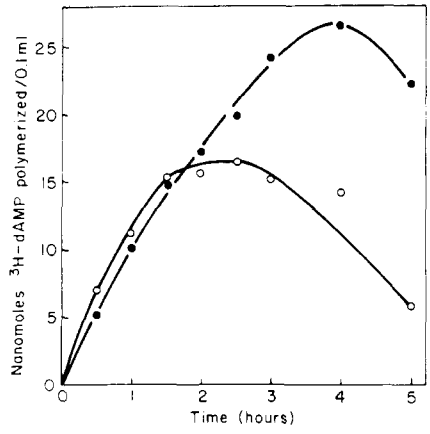


FIGURE 7: The template activity of poly[d(A-T)] (○) and poly[d(A-HgU)] (●) with *E. coli* DNA polymerase I. The reactions were incubated and processed under the conditions described in Figure 1. Both polymers were used at a concentration of 0.04 m*M*. The prolonged synthesis in the presence of poly[d(A-HgU)] reflects its resistance to the 5′ → 3′ exonuclease associated with the polymerase (see text).

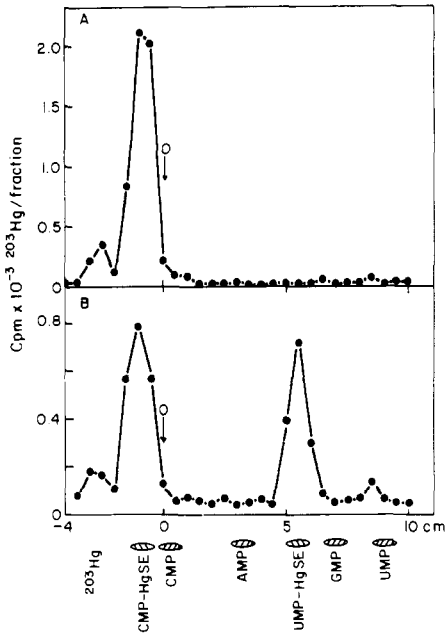


FIGURE 8: Nuclease digestion products of mercurated calf thymus DNA (A) and R17 RNA (B). The polymers were mercurated as previously described (Dale et al., 1975). C.T. DNA contained 21% of the C bases as Hg-C while 28% of the U and C bases were mercurated in the RNA. Enzyme digestions were done in 0.1-ml reactions that contained 0.01 *M* Tris-HCl buffer (pH 7.4), 0.02 *M* NaCl, 0.001 *M* $CaCl_2$, 0.01 *M* mercaptoethanol, 5 μg of micrococcal nuclease, 10 μg of spleen phosphodiesterase and C.T. DNA, 0.22 OD_{260nm} (5.1×10^4 cpm/OD) or R 17 RNA, 0.17 OD_{260nm} (1.3×10^5 cpm/OD). The reactions were incubated at 37° for 30 min. Samples from each reaction were removed and spotted on plastic cellulose-TLE plates with the indicated marker compounds. After electrophoresing for 3 hr at 300 V the plates were dried, and the marker positions located by uv absorption. The sample channels were cut into 0.5-cm strips and counted in a Packard scintillation counter.

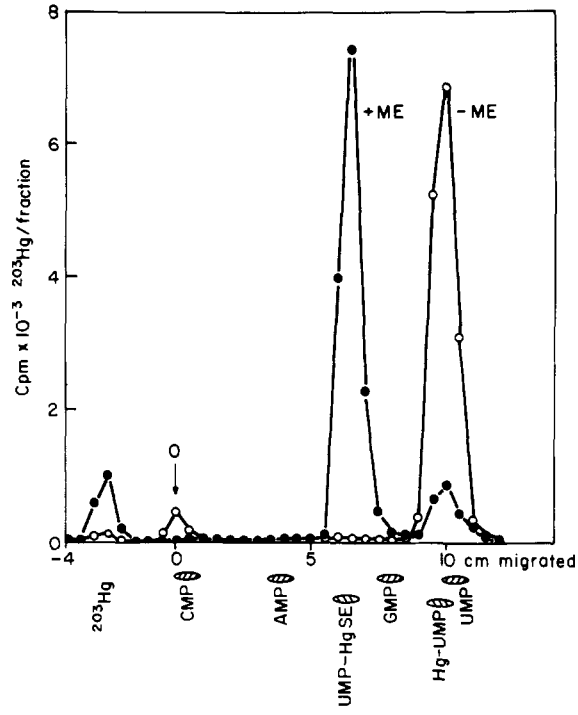


FIGURE 9: Digestion of poly(^{203}Hg-U) by pancreatic RNase in the presence and absence of mercaptoethanol (ME): electrophoretic analysis of the digestion products. The poly(^{203}Hg-U), prepared by direct mercuration of poly(U) (Dale et al., 1975), had a specific activity of 5.9 Ci/mol (9.0×10^5 cpm/OD_{267nm}). 0.1-ml reactions containing 0.2 OD_{267} of poly(Hg-U), 0.02 *M* Tris-HCl buffer (pH 7.0), 0.001 *M* EDTA, 5 μg of pancreatic RNase, and (where required) 0.01 *M* mercaptoethanol were incubated at 37° for 15 min; 10-μl aliquots were removed and electrophoresed as described in Figure 8. When poly(Hg-U) is digested in the absence of ME, over 95% of the ^{203}Hg counts migrate with the mercaptan-free Hg-UMP marker. A small residual, remaining at the origin (○), is often seen in such digests and may reflect mercurinucleotide–protein complexes. Although the mercaptan/Hg ratio in the second enzyme digest was 25:1, electrophoresis of the product yields two Hg-UMP spots, a major one (with) and a minor one without the mercaptoethanol ligand. An increase in the amount of free Hg^{2+} ion is also observed.

yield two spots per mercurated nucleotide (see Figure 9). The use of a large molar excess of mercaptan must also be avoided to circumvent potential demercuration (Figure 10). Excess mercaptan also appears to promote the formation, within the polymer, of covalently cross-linked nucleotides (base–Hg–base), which is especially noticeable after long-term storage. The "split-spot" phenomenon and the potential presence of mercury crosslinks can make the characterization of oligonucleotides containing more than one mercury atom quite difficult. Where sequence analysis is required it is recommended that prior conversion of mercurinucleotides to iodonucleotides be undertaken (Dale et al., 1975; Dale, Livingston and Ward, manuscript in preparation) since such treatment abolishes both of the previous problems. (2) The enzymatic digestion of chemically mercurated calf thymus DNA and R17 RNA (Figure 8) confirms the base specificity of the mercuration reaction; C residues are preferentially modified in DNA while both C and U bases are substituted in RNA.

The interaction between polynucleotide binding proteins and DNA is also unimpaired by mercuration. The gene 5 and gene 32 proteins of bacteriophages fd and T_4 bind efficiently to poly[d(A-HgU)] (R. Anderson and J. Coleman, personal communication) as does the lactose repressor of *E. coli* (T. Richmond and T. Steitz, personal communication). Finally, rearranging the side-chain substituents in mercurated poly[d(A-U)] from base-Hg-SR to base-S-HgR results in a DNA polymer with a T_m that is higher than that of poly[d(A-T)] (Livingston et al., 1975). These various observations strongly suggest that the decreased T_m of the mercurated DNAs described here is not a consequence of a major structural alteration. However, further detailed studies are required for definite proof.

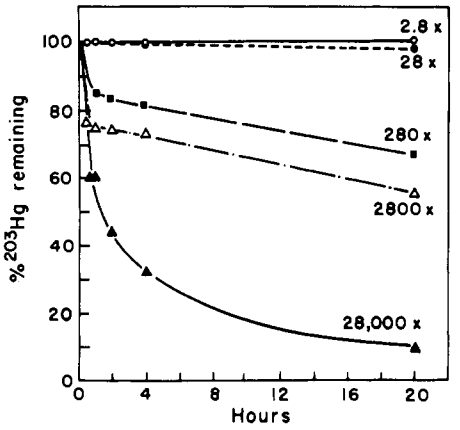


FIGURE 10: The effect of mercaptoethanol concentration on the rate of poly(Hg-U) demercuration. ^{203}Hg-poly(Hg-U) (1.0 OD_{260nm}, 1.04 $\times 10^{-4}$ mol of nucleotide, 2.08×10^5 cpm) was added to thirty 1.0-ml solutions of TNE buffer (0.01 *M* Tris-HCl (pH 7.5)-0.02 *M* NaCl-0.001 *M* EDTA) that contained 0.3, 3.0, 300, or 3000 m*M* 2-mercaptoethanol. Five samples at each mercaptan concentration were used. At the indicated times one sample from each mercaptan concentration was treated with 1.0 ml of quench buffer (0.01 *M* Tris-HCl (pH 7.5)-1.0 *M* NaCl-0.1 *M* EDTA) and dialyzed extensively against TNE. The extent of demercuration was calculated from the ^{203}Hg/OD ratio found after dialysis.

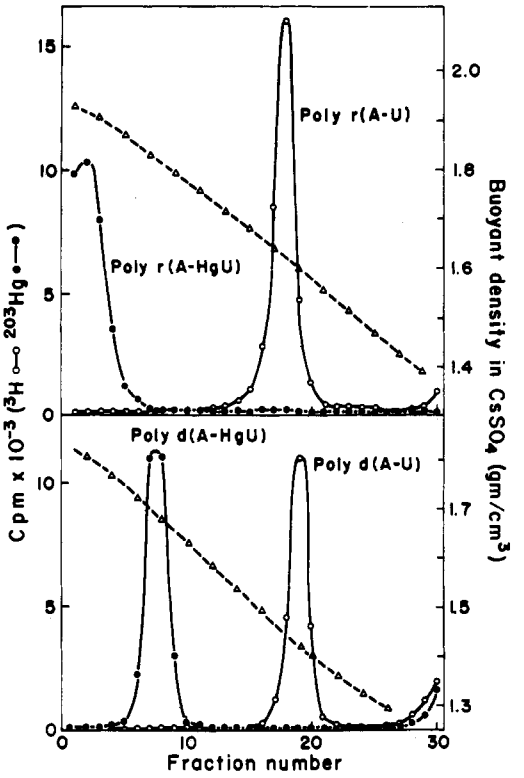


FIGURE 11: Equilibrium centrifugation of [^{3}H]poly[r(A-U)], [^{203}Hg]poly[r(A-HgU)], [^{3}H]poly[d(A-U)], and [^{203}Hg]poly[d(A-HgU)] in $CsSO_4$. Approximately 0.5 OD_{260nm} of each polymer was added separately to 4.5-ml solutions of $CsSO_4$ in 0.01 *M* Tris-HCl buffer (pH 7.5)-0.02 *M* NaCl-0.001 *M* EDTA. The samples, in siliconized cellulose nitrate tubes, were centrifuged for 60 hr at 36,000 rpm in an SW50.1 rotor; 0.15-ml fractions were collected by bottom-puncture, their buoyant density was calculated by refractometry, and 50-μl samples were added to 4 ml of Aquasol and counted in a scintillation counter.

Although the physical and biochemical properties of mercurated polynucleotides generally resemble those of the corresponding nonmercurated polymers, mercuration dramatically increases polymer buoyant density. Attempts to band poly[d(A-HgU)] or poly[r(A-HgU)] by isopycnic centrifugation in CsCl failed as both polymers pelleted through saturated CsCl (density $\sim$ 1.91 g/cm^3). Satisfactory banding was, however, achieved in gradients of $CsSO_4$ (Figure 11). Complete mercuration of the uracil bases in poly[d(A-U)] and poly[r(A-HgU)] increases the buoyant density from 1.424 to 1.683 and 1.615 to 1.910 g/cm^3, respectively. The density increase is essentially a linear function of the number of pyrimidine bases modified (Figure 12), and increases approximately 0.005-0.009 gm/cm^3 for each percentage of the total bases mercurated.

Noncovalent Hg^{2+}-DNA complexes had previously been shown by Davidson and associates (Nandi et al., 1965, Wang et al., 1965) to enhance the buoyant density separation of DNAs with similar base composition. One would expect to achieve similar results with covalently mercurated DNA or RNA since the chemical mercuration reaction is base specific. The observation that mercurated derivatives of UTP, CTP, dCTP, and dUTP are excellent substrates for a wide range of bacterial, viral, and mammalian polymerases (Dale et al., 1973) also makes it possible to synthesize "heavy" nucleic acids in vitro. Since eukaryotic polymerases in whole cell extracts or in isolated nuclei efficiently utilize these analogs (L. Lowenstein, personal communication), the elevated density of the newly synthesized products should facilitate their separation from the large amount of endogenous nucleic acids. However, chromatography on columns of sulfhydryl-Sepharose (see below) provides a more rapid and efficient fractionation procedure than isopycnic centrifugation. The analysis of mercurated polymers by isopycnic or velocity centrifugation also requires some care as the polymers adsorb to cellulose nitrate and polyallomer tubes much more readily than normal nucleic acids, particularly when handled in submicrogram amounts. Pretreating the tubes with siliconizing agents considerably reduces, but does not totally abolish, the adsorption. In addition, the affinity of the mercury substituents for sulfhydryl groups necessitates the complete removal of all protein in order to prevent the appearance of spurious "light" bands.

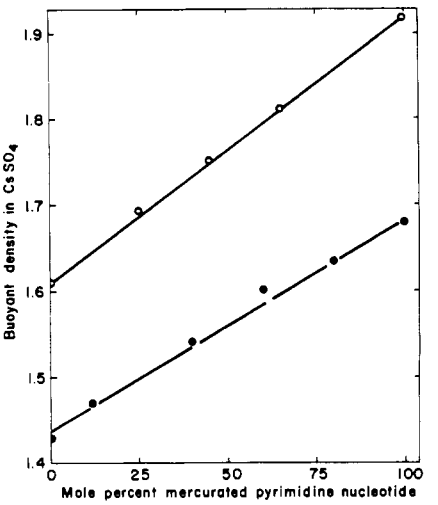


FIGURE 12: The buoyant density of poly[r(A-U)] (○) and poly[d(A-T)] (●) as a function of their 5-mercuriuracil base content. The mercurated polymers were prepared as described under Materials and Methods and the 5-mercuriuracil content calculated from their [^{3}H]A/[^{203}Hg]U ratio. Centrifugation and subsequent analysis was as described in Figure 10.

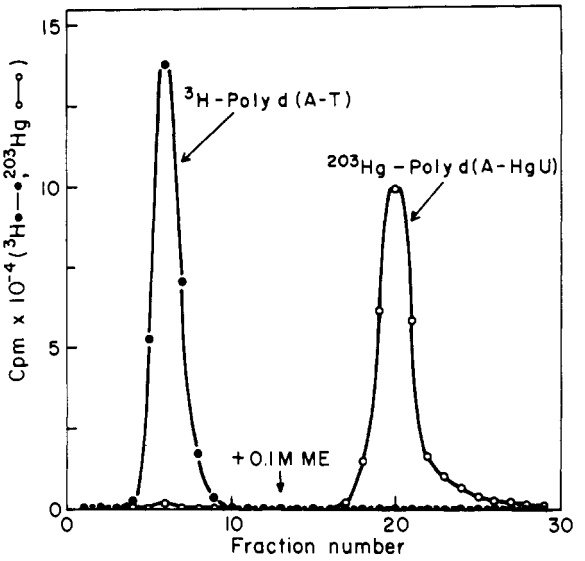
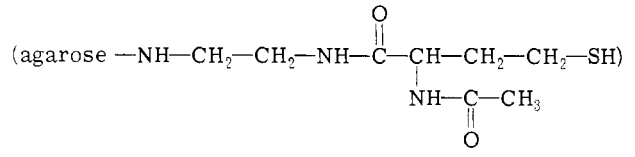


FIGURE 13: Separation of poly[d(A-HgU)] and poly[d(A-T)] by chromatography on sulfhydryl-Sepharose. A mixture of [^{3}H]poly[d(A-T)] (3.2 OD_{260nm}, 2.9 × 10^5 cpm) and [^{203}Hg]poly[d(A-HgU)] (2.2 OD_{260nm}, 3.0 × 10^5 cpm) was prepared in 2.0 ml of 0.05 *M* Tris-HCl buffer (pH 7.5)–0.05 *M* sodium chloride (TN buffer) and the sample applied to a 26 × 1.0 cm (20 ml) column of sulfhydryl-Sepharose 6B (SH content, 0.4 μmol/ml). The column was washed with TN buffer and fifteen 2.0-ml fractions collected at a flow rate of approximately 0.5 ml/min. The eluting buffer was then changed to TN buffer containing 0.1 *M* mercaptoethanol and 15 additional fractions were collected: 0.1-ml samples were added to 4.0 ml of Aquasol and counted in the scintillation counter. The recovery of radioactivity and uv absorption was greater than 95% for both polymers. No [^{3}H]poly[d(A-T)] could be detected in the combined mercaptoethanol eluted fractions.

The facility with which mercurated polynucleotides complex with proteins suggested that chromatography resins containing free sulfhydryl groups might be effectively utilized for polymer fractionation. "Sulfhydryl-agarose" was, therefore, prepared according to the procedure of Cuatrecasas (1970), and its fractionation properties tested. When



a mixture of [^{3}H]poly[d(A-T)] and [^{203}Hg]poly[d(A-HgU)] was applied to the resin, poly[d(A-T)] was quantitatively eluted while over 98% of the poly[d(A-HgU)] was retained on a single passage through the column. Recovery of the mercurated polymer was readily achieved by the addition of a competing mercaptan (0.1 *M* mercaptoethanol) to the eluting buffer (Figure 13). Similar results were obtained with a variety of other enzymatically or chemically mercurated polymers, including poly(Hg-U)·poly(A) complexes, calf thymus, T7, and SV40 DNAs, R17, and ribosomal and transfer RNAs (data not shown). The extent of mercury substitution required to obtain quantitative retention on the resin is quite small. Although an absolute lower limit has not been defined, mercuration of 0.5–1.0% of the total nucleotides is sufficient. Polymers containing a low level of substitution may, however, require two or three passages through the affinity resin for complete adsorption. Single- and double-stranded mercuri polymers appear to be retained with essentially the same efficiency presumably because the sulfhydryl group is at the end of a long "spacer-arm".

Table VI: Selective Fractionation of Hg-tRNA by Sulfhydryl-Agarose Chromatography.[a]

	Cpm Recovered [^{3}H] tRNA (bulk)	[^{203}Hg]-tRNA^{Phe}
Sample applied	1.32 × 10^6	27,300
1st passage through SH-agarose	1.29 × 10^6	5,183
2nd passage through SH-agarose	1.23 × 10^6	823
Eluted in mercaptoethanol wash	167[b]	25,172

[a] Yeast tRNA^{Phe} containing 1.3 atoms/molecule of radioactive mercury was prepared by direct mercuration in 0.5 *M* Tris-acetate buffer (pH 7.0) as previously described (Dale et al., 1975). Bulk *E. coli* tRNA was labeled with tritium in the terminal adenosine moiety by reconstitution of periodate-treated tRNA using [^{3}H] ATP and rat liver tRNA nucleotidyl transferase (Daniel and Littauer, 1963). The [^{3}H] tRNA was kindly prepared by Tom Darling. 0.25 OD_{260nm} (27,300 cpm) of [^{203}Hg] tRNA^{Phe} was mixed with 98 OD_{260nm} (1.32 × 10^6 cpm) of [^{3}H] tRNA in 1.0 ml of 0.02 *M* Tris-HCl buffer (pH 7.5)–0.20 *M* NaCl–0.001 *M* EDTA (TNE buffer) and the solution applied to a 4.5 × 1.2 cm (5 ml) column of sulfhydryl-agarose (SH content, 1.0 μmol/ml). The column was then washed with 15 ml of TNE buffer; 1.0 ml was removed for determination of uv absorption and radioactivity. The remaining sample was applied a second time to sulfhydryl-agarose and washed with an additional 10 ml of TNE buffer. Following the second elution the resin was washed with 10 ml of TNE buffer containing 0.1 *M* mercaptoethanol and 2.0 ml counted in Aquasol. Over 92% of the [^{203}Hg] tRNA was recovered in the TNE–mercaptoethanol wash. [b] This value represents an upper limit as determined by channel-ratio double labeling methods.

Nonspecific adsorption of nonmercurated polymers to sulfhydryl-agarose is normally infinitesimally small (also see Materials and Methods). When 8.1 × 10^6 cpm of [^{3}H]poly[d(A-T)] was chromatographed as described in Figure 13, less than 50 cpm of tritium (<0.0006%) was found in the combined 0.1 *M* mercaptoethanol eluate. Similarily, when yeast phenylalanyl-tRNA containing 1.3 Hg atoms per molecule was chromatographed on sulfhydryl-agarose in the presence of a 500-fold excess of nonmercurated bulk *E. coli* tRNA, 96% of the Hg-tRNA^{Phe} but less than 0.01% (the lower limit of detection) of the nonmercurated tRNA was retained (Table VI).

The selective fractionation of mercuri polymers on sulfhydryl-agarose can be done at room temperature under a wide variety of ionic conditions. The method is simple, highly efficient, and applicable to both large and small nucleic acids. The recovery from the resin of bound material is usually greater than 90% although considerable dilution may occur as the sample is competed off the resin by the low molecular weight mercaptan. Dilution effects may be minimized by maintaining a large buffer/resin mercaptan ratio during elution and by allowing the mercaptan–buffer solution to sit in contact with the resin–mercuri polymer complex for 5–10 min prior to elution. The mercury content of polymers eluted in the presence of a vast molar excess of mercaptan will often be reduced after dialysis due to mercaptan-stimulated reductive demercuration (Dale et al., 1975). The extent of demercuration observed has been quite variable (from 0 to 90% loss) and depends upon the nature of the mercurated base (Hg-C is more sensitive to demercuration than Hg-U), the mercaptan/mercury ratio used and the time of exposure. To minimize mercury loss the sample should be dialyzed immediately after elution in a mercaptan-free buffer. Since subsequent product analysis may be facilitated by removal of the mercury substituents (by re-

Table VII: Retention of [^{3}H]SV40 DNA Fragments on Sulfhydryl-Agarose after Hybridization to [^{203}Hg]SV40 CRNA.[a]

RNA used in Hybridization	Distribution of Radioactive Polymers								
	[^{3}H]SV40 DNA (cpm)			[^{14}C]SV40 CRNA (cpm)			[^{203}Hg]SV40 CRNA (cpm of ^{203}Hg)		
	Applied	Eluted	Retained	Applied	Eluted	Retained	Applied	Eluted	Retained
[^{14}C]SV40 CRNA	29,760	31,560	<100	32,100	29,200	<100			
[^{203}Hg]-([^{14}C]SV40 CRNA)[b]	29,760	16,416	11,760				19,350	720	18,050

[a] [^{14}C]SV40 CRNA was prepared in vitro using form I SV40 DNA and *E. coli* RNA polymerase (Westphal, 1970) and mercurated as described under Materials and Methods. Immediately prior to use form II [^{3}H]SV40 DNA, prepared as described under Materials and Methods, was sonicated for 30 sec in an Ultrasonics water bath sonicator, then denatured by boiling for 2 min followed by quick cooling in ice. Hybridization reactions (2.5 ml) were done in 50% (v/v) formamide, 0.3 *M* NaCl, 0.03 *M* sodium citrate, 0.0025 *M* Tris-HCl buffer (pH 7.4), and 0.00025 *M* EDTA. The solutions contained 0.66 μg of fragmented [^{3}H]SV40 DNA and 20 μg of [^{14}C]SV40 CRNA or 20 μg of (^{203}Hg-[^{14}C]-SV40 CRNA) in which 3.2% of the total bases were mercurated. Reactions were incubated at 37° for 20 hr, then applied slowly (over a period of 30–60 min) to 3-ml columns of sulfhydryl-agarose (SH content, 1.0 μmol/ml). Each column was slowly washed with 10 ml of TNE buffer (0.01 *M* Tris-HCl (pH 7.4)–0.1 *M* NaCl–0.001 *M* EDTA) to resolve bound from nonadsorbed polymer; 0.5 ml of each 1.0-ml fraction was added to 4.5 ml of Aquasol for radioactivity determinations. After determining that the column eluates contained only background levels of radioactivity 10 ml of TNE buffer containing 0.1 *M* mercaptoethanol was applied; 1-ml fractions were collected and analyzed as before. The total radioactivity recovered in the "eluted" and "bound" pools are given in the table. [b] The ^{203}Hg-radiolabel was determined using an Intertechnique CG30 automatic γ-counter. The ^{3}H-radiolabel was obtained using a Packard β-scintillation counter by subtracting the combined ^{14}C and ^{203}Hg spillover (<15% of the maximum ^{14}C–^{203}Hg cpm) from the tritium channel counts. The spillover ratios were determined using [^{203}Hg]- and [^{14}C]SV40 in which both isotopes reside in the same RNA sample. The retention of ^{14}C radiolabel on sulfhydryl-agarose was estimated to be greater than 90% (cf. 96% determined for ^{203}Hg).

duction or by conversion to iodo nucleotides) some loss of mercury upon elution should not be critical.

The selective retention on sulfhydryl-agarose and the results of the annealing experiments described earlier suggested that mercurated polymers, especially those with a low level of substitution, should be suitable probes for hybridization. However, before undertaking such studies it was necessary to examine the thermal stability of the polymer-bound mercury. The slight hysteresis in the melting profile of poly[d(A-HgU)] (Figure 3) suggested that some demercuration might be occurring when the polymer is held at elevated temperatures. Poly[d(A-HgU)] was therefore heated under a variety of incubation conditions and the mercury–nucleotide ratio of the resultant polymer examined. The data in Figure 14 show that the mercury–carbon bond in poly[d(A-HgU)] is thermolabile; about 50% of the ^{203}Hg radiolabel is lost after heating for 24 hr at 65°. Qualitatively similar results have been observed with other mercurated polynucleotides. The thermolability of the mercury–carbon bond in polymers seems to be considerably greater when present in polymeric form since no demercuration of Hg-dUMP was observed after heating at 50° for 36 hr. Changes in the electronic character of the pyrimidine base as a consequence of base stacking in the polymer may account for the observed differential lability. The rate of polymer demercuration appears to be solely a function of the temperature. Polymers heated under a variety of ionic and solvent conditions (e.g., solutions containing up to 1.0 *M* NaCl and/or 50% (v/v) formamide) exhibit essentially the same demercuration rate.

Although the mercury–carbon bond is thermolabile, the extent of demercuration that occurs on prolonged incubation at 37° is relatively small (10–15% in 24 hr). Preliminary hybridization experiments were therefore done at 37° in 50% formamide (v/v)–0.3 *M* sodium chloride–0.03 *M* sodium citrate–0.00025 *M* Tris-HCl (pH 7.4)–0.00025 *M* EDTA. The sodium chloride present in the hybridization mixture is of some importance for the chloride ion effectively prevents the covalent mercuration reaction (Dale et al., 1975), thus circumventing a potential problem in the reutilization of any labilized Hg^{2+} ions.

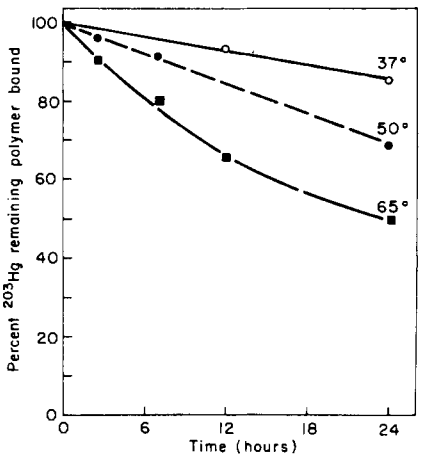


FIGURE 14: Thermolability of the mercury–carbon bond in poly[d(A-HgU)]; 5.0-ml samples of [^{203}Hg]poly[d(A-HgU)] (0.55 OD_{260nm}/ml, 81,000 cpm/ml) in 0.01 *M* Tris-HCl buffer (pH 7.5)–0.05 *M* NaCl (TN buffer) were incubated at 37, 50, and 65°. At the indicated times 1.0-ml samples were removed and dialyzed overnight against 7 l. of TN buffer. The extent of demercuration in the heated samples was calculated from the ^{203}Hg/OD ratio found after dialysis. Samples maintained at 4° and then dialyzed had the same specific activity as the original poly[d(A-HgU)].

The utility of mercuripolynucleotides as hybridization probes was tested by examining the ability of mercurated SV40 RNA to retain [^{3}H]SV40 DNA on columns of sulfhydryl-agarose. SV40 DNA was transcribed in vitro using SV40 form I DNA and *E. coli* RNA polymerase, conditions which give asymmetric transcription (Westphal, 1970; Khoury and Martin, 1972). Mercuration of the complementary SV40 RNA in vitro (see Materials and Methods) yielded an RNA in which 3.2% of the total bases were modified. The SV40 CRNA, with and without mercury substitution, was hybridized for 20 hr with fragmented [^{3}H]SV40 DNA (under the conditions described above) and then chromatographed on sulfhydryl-agarose. As shown in Table VII, 40% of the [^{3}H]SV40 DNA was retained on a single passage through the column when the SV40 CRNA was mercurated while essentially none was retained in the ab-

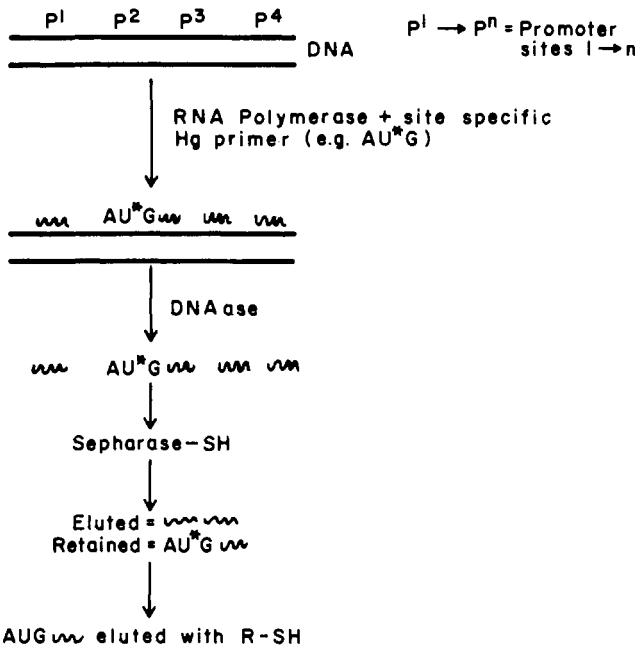


FIGURE 15: A method for the isolation of primer specific transcription products based on the use of mercurated primers and sulfhydryl-agarose chromatography.

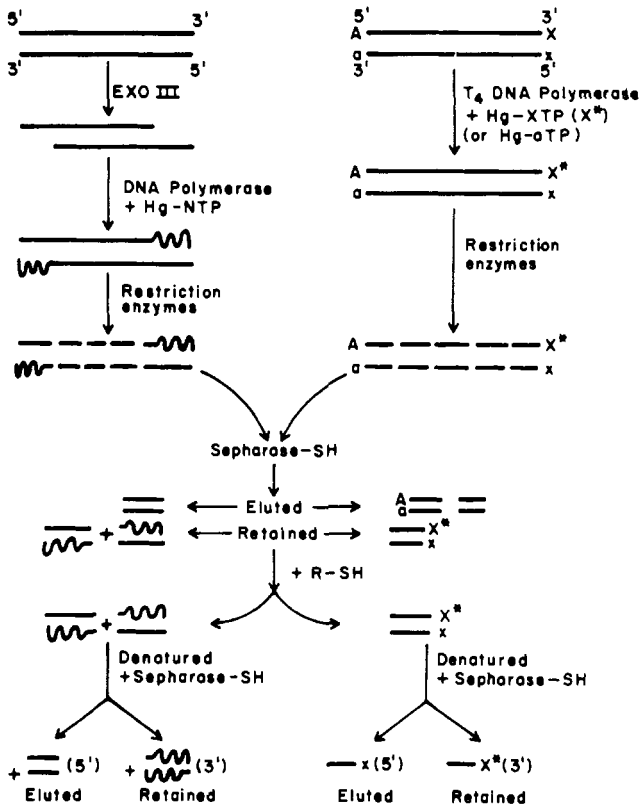


FIGURE 16: A method for the selective isolation of thermal fragments from duplex DNA.

sence of mercuration. In similar hybridization experiments (Westphal, 1970; Khoury and Martin, 1972) it was shown that approximately 50% of the input DNA is hybridizable to in vitro made SV40 CRNA. While the value given in Table VII is lower than previously reported, no attempt was made here to optimize hybridization yields. In addition, since only 96% of the [^{203}Hg]SV40 CRNA was retained in the single column passage, the level of complementary DNA could be as high as 44%. Although a detailed analysis of the effect of mercury substitution on hybridization kinetics, etc., is required, it is apparent that polynucleotides containing a low level of mercury substitution can be utilized effectively in hybridization studies and that, in conjunction with sulfhydryl-agarose chromatography, they can achieve a rapid fractionation of hybrid product. Since retention on the affinity resin is mediated only through the interaction of probe-associated mercury atoms and resin-bound sulfhydryl groups, the method is highly selective. Unlike fractionation methods that rely on discriminating between single- and double-stranded polymers, for example, hydroxylapatite, reassociated DNA is not retained with the hybrid. The possibility of using this and related techniques (see Discussion) for the isolation of unique polynucleotide sequences of viral and eukaryotic origin is currently being investigated.

Discussion

The introduction of mercury atoms and their associated ligands into a polynucleotide might, on first thought, be expected to alter grossly the normal polymer structure. However, the molecular models in Figure 6 show no steric grounds for structural perturbation in duplex DNA (or RNA) even when there is one mercury substituent per base pair. The mercurithioester side chains, by virtue of their attachment to the C-5 carbon of the pyrimidine bases, project into the major groove. As long as the mercury ligands are not excessively large (mercaptans as bulky as 1-thioglucose can be easily accommodated) there are no steric interactions either between the individual mercurithiol substituents or between the substituents and the phosphodiester backbone. Although other parameters of structure, for example, the extent and sites of hydration, may be modified, mercurated polynucleotides still function as templates for nucleic acid polymerases, they retain full sensitivity to nuclease digestion, and they denature and reanneal efficiently. The activity of the polymerases and nucleases examined, therefore, does not appear to depend on interaction with specific recognition sites located within the major groove since filling the groove with bulky mercurithiol substituents does not significantly impair their activity.

Although the biochemical properties of mercurated nucleotides and polynucleotides closely resemble those of their nonmercurated counterparts, the mercury atoms should provide a set of unique "handles" which can be used in the discrimination and fractionation of discrete polynucleotide sequences. The preliminary data given in this report clearly demonstrate that mercurated polynucleotides can be retained selectively on columns of sulfhydryl-agarose. Since mercury substituents can be introduced into DNA and RNA under mild conditions by both enzymatic and direct chemical means, and since low levels of mercuration do not appear to significantly alter hybridization and reannealing kinetics, the method could prove to be a highly versatile one for polynucleotide fractionation, even though the thermal lability of the mercury–carbon bond may limit its utility in hybridization reactions done at elevated temperatures. Since retention on the sulfhydryl-agarose resin results from the in situ formation of mercurithioester bonds between the mercurated polymer and resin-bound sulfhydryl groups, the fractionation method should also be highly selective. Indeed, while polynucleotides containing as few as one mercury atom per 200 nucleotides can be fractionated rapidly in

near quantitative yields, the background adsorption of nonmercurated polymer has been found to be as low as 0.0006% of that applied to the affinity resin. In addition, since the mercury–mercaptan interaction is relatively insensitive to the presence of formamide or variations in ionic strength, the adsorption of a mercurated polymer probe (and its associated complementary sequences) can be achieved under a wide variety of experimental conditions. However, to minimize nonspecific adsorption, solutions applied to the resin should contain at least 0.05 *M* salt.

Sulfhydryl-agarose can in effect be used as a general affinity resin for the isolation of either DNA–DNA, DNA–RNA, or RNA–RNA hybrids since polymer retention depends only on the prior mercuration of an appropriate hybridization probe. In this regard the method offers certain advantages over other procedures in which the hybridization probe is first immobilized by cross-linkage to cellulose or phosphocellulose (Shih and Martin, 1973, 1974). Preparation of the probe–resin complexes takes several days and a separate resin must be prepared for each hybridization probe. Sulfhydryl-agarose should be compatible for use in the continuous hybridization system described by Shih and Martin and also offer the additional advantage that the hybrid can be isolated intact for further analysis by eluting the affinity resin with buffer containing a competing mercaptan.

The selective retention of mercurated nucleotide derivatives on sulfhydryl-agarose also can be exploited to achieve the fractionation of polynucleotide fragments which cannot be readily obtained by hybridization techniques. For example, the use of mercurated oligonucleotide primers should facilitate the isolation of specific transcription (or replication) products (Maizels, 1973; Minkley and Pribnow, 1973; Sanger et al., 1973; Padmanabhan et al., 1974) even though transcription may be initiated from a number of sites (see Figure 15). In addition, since mercurated nucleotide 5′-triphosphates are excellent polymerase substrates (Dale et al., 1973), reconstitution of exonuclease-digested duplex DNA with mercurinucleotides should provide a method for the selective isolation of terminal DNA sequences. Appropriate molecules for reconstruction can be prepared with *E. coli* exonuclease III (Richardson et al., 1964) or the 3′ → 5′ exonuclease of T_4 DNA polymerase (Goulian et al., 1968; Cozzarelli et al., 1969). Englund and collaborators (Englund, 1972; Weigel et al., 1973) have recently described a reaction, catalyzed by T_4 DNA polymerase, which can be used to incorporate a single nucleotide at the 3′-terminus of duplex DNA. Selective terminal labeling can be obtained if the terminal nucleotide of the r and l strands are different (Price et al., 1973). Preliminary experiments with exonuclease III and T_4 DNA polymerase digested T_7 DNA (Ward and Dale, unpublished results) suggest that the isolation schemes outlined in Figure 16 can be used for the fractionation of such terminal sequences. The application of these and related techniques to the isolation of unique fragments of viral DNA and RNA is currently under investigation.

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