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Interaction of tRNAs and of Phosphorothioate-Substituted Nucleic Acids with an Organomercurial. Probing the Chemical Environment of Thiolated Residues by Affinity Electrophoresis†

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ABSTRACT: The interactions of 4-thiouridine and 5-[(methylamino)methyl]-2-thiouridine in tRNA and of phosphorothioate esters in nucleic acids with an organomercurial have been investigated. For this purpose, an affinity electrophoretic system has been developed in which the mercury derivative has been covalently immobilized in a standard polyacrylamide gel. The retardation of thiolated macromolecules was found to be sensitive to the chemical environment of the sulfur atom, giving characteristic interaction constants dependent on the nature of the modification and its accessibility to binding. The interaction could, in the case of tRNA, be abolished by conventional specific chemical modification of the thiolated bases, as well as by irradiation with ^{32}P-derived β-emission. Not only has the fractionation of sulfur-modified from unmodified species been attained but a quantitative application of the technique has made it possible to study the binding of mercury and, by competition, that of magnesium in terms of the conformation of tRNA.

Despite the emergence of HPLC systems suitable for the fractionation of macromolecules, electrophoretic methods have, in general, gained preference over chromatographic separations in many areas of molecular biology. However, when specific affinity interactions are considered, the use of electromotive force (electrophoresis) as an alternative to hydrodynamic pressure (chromatography) has, with the exception of immunoelectrophoresis, so far not been widely appreciated. Thus, although affinity electrophoresis has been the subject of a number of reviews (Hořejší, 1981, 1984), its application has been largely restricted to specific enzyme–substrate and glycoprotein–lectin investigations (Hořejší, 1981, and references cited therein).

We have recently demonstrated the utility of boronate-containing polyacrylamide electrophoresis gels as general affinity media for the fractionation of free *cis*-diol-containing nucleic acids (Igloi & Kössel, 1985, 1987). The relatively weak interaction of *cis*-diols with boronate derivatives was found not to be influenced by the chemical environment of the interacting functional group in nucleic acids, whether in terms of a ribose residue or in the case of the Q modification in tRNAs. However, one could expect that a functional group more sensitive to its electronic environment might reflect its altered reactivity by a modulation of its interaction with an appropriate ligand.

The strong interaction of sulfur-containing nucleic acids with organomercurials has been the subject of numerous reports [e.g., Scheit and Faerber (1973), Sunshine and Lippard (1974), and Maguire (1976)], while the use of mercurated column materials for the isolation of sulfur-containing macromolecules is well documented (Melvin et al., 1978; Sun & Allfrey, 1982; Zhang et al., 1984) and the matrices for such batch procedures are commercially available. The wide range of chemical environments available to the sulfur atom provides a range of compounds whose different reactivity toward mercury might be expected to permit high-resolution fractionation by affinity electrophoresis.

In order to test this possibility and to widen the scope of affinity electrophoretic application, the interaction of sulfur-containing nucleic acids, as exemplified by tRNAs containing s^4U[1] or mam^5s^2U and by phosphorothioate-substituted nucleic acids, with an organomercurial polyacrylamide gel during electrophoresis has been investigated.

Experimental Procedures

Synthesis of [(N-Acryloylamino)phenyl]mercuric Chloride (APM). Addition of 8 mL of acetonitrile to 0.35 g of (*p*-aminophenyl)mercuric acetate (Fluka) at 0 °C, followed by 2 mL of 1.2 M $NaHCO_3$, resulted in an almost biphasic suspension in which the solid remained as a white mobile pellet at the base of the reaction vessel. A total of 0.2 mL of acryloyl chloride (Fluka) was now added in 10-μL aliquots over a period of 10 min with vigorous stirring. A voluminous white

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[1] Abbreviations: s^4U, 4-thiouridine; mam^5s^2U, 5-[(methylamino)-methyl]-2-thiouridine; APM, [(*N*-acryloylamino)phenyl]mercuric chloride; APB, [(*N*-acryloylamino)phenyl]boronic acid; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; Temed, *N,N,N',N'*-tetramethylethylenediamine; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

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precipitate was formed, and the reaction was allowed to continue overnight at 4 °C. This resulted in two phases: a clear lower phase and an upper phase containing the white product. The upper phase was removed and centrifuged. The resulting pellet was washed with H_2O and dissolved by warming to 50 °C in 8.5 mL of dioxane and clearing the warm solution by rapid filtration. Crystals formed on standing at room temperature. The solid was washed with H_2O and dried under vacuum. It was stored at 4 °C (yield, 26%; TLC on silica plates using dioxane–EtOAc–triethylamine, 4.5:4.5:1, gave a single ultraviolet-absorbing spot, R_f 0.55).

Preparation of Affinity Gels. Polyacrylamide gels containing APM as a covalently linked component were cast by the addition of an appropriate aliquot of a 1 mg/mL solution of APM in formamide to a solution containing a given amount of acrylamide, bis(acrylamide), and urea in 0.1 M Tris–borate/EDTA, pH 8.3. Polymerization was initiated by the addition of 0.5 mL of 1% ammonium persulfate and 7 μL of Temed (BRL) per 10 mL of gel solution. Horizontal 2% polyacrylamide gels were prepared as described elsewhere (Igloi & Kössel, 1987). Gels were prerun for 0.5 h before application of the samples, and electrophoresis was carried out at approximately 10 V/cm.

Labeling of Samples. For autoradiographic detection, RNA samples were 3′ terminally labeled with [^{32}P]pCp (Amersham; 3000 Ci/mmol) and T4 RNA ligase, as described (Igloi & Kössel, 1987). 5′-Ends were subjected to polynucleotide kinase treatment in the presence of [γ-^{32}P]ATP (Amersham; 5000 Ci/mmol). 3′-Labeling of single-stranded DNA fragments was achieved by using terminal transferase (Kössel et al., 1974). The reaction was carried out in a volume of 50 μL containing 0.1 M potassium cacodylate, 25 mM Tris-HCl, 1 mM $CoCl_2$, and 0.2 mM DTT, pH 7.6, with 50 μCi of [α-^{32}P]GTP (Amersham, 400 Ci/mmol) and 25 units of terminal transferase (Boehringer) at 37 °C for 30 min. Polymeric G tails were reduced to single residues by treatment with 50 μL of 0.6 M KOH at room temperature overnight, and the 3′-terminal phosphate could be removed with alkaline phosphatase. 3′-Labeled material bearing homogeneous 3′-ribose termini was isolated with the boronate affinity electrophoresis system described (Igloi & Kössel, 1987).

Transcription in the SP6 system of a clone (kindly provided by Dr. D. Dormann of this institute) containing the tRNA (Val)GAC gene from the chloroplast genome of maize (Schwarz et al., 1981) resulted in an approximately 360 nucleotide long RNA. The reaction was carried out as described (Igloi & Kössel, 1987) with ATPγS or GTPγS replacing the corresponding triphosphate. The source of radioactivity was [α-^{32}P]CTP (Amersham; 400 Ci/mmol).

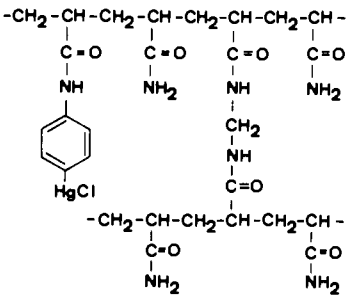
5′-Thiophosphorylation of samples was achieved under the following conditions. The sample, 1–10 μg in 10 μL of H_2O, was transferred to a 0.2-mL Eppendorf tube. The components of the reaction, 2 μL of 10× kinase buffer (200 mM Tris-HCl, pH 8.0, 200 mM $MgCl_2$, and 24 mM 2-mercaptoethanol), 4 μL of 10 mM ATPγS (Boehringer), 2 μL of H_2O, and 2 μL of T4 polynucleotide kinase (20 units; Amersham), were added, and after being mixed and sealed in the tube with Parafilm, the mixture was incubated at 37 °C for 2 days. Phenolization and ethanol precipitation gave a product shown by affinity electrophoretic analysis to contain 50–80% 5′-thiophosphorylated material.

Radiolysis of tRNA. The effect of radiolysis on mam^{5}s^{2}U was studied under the following conditions. A total of 3.5 μCi (0.5 μL) of [^{32}P]pCp was added to 100 ng of tRNA(Glu) (Boehringer) in 10 μL of H_2O, and the resultant mixture incubated at −20 °C for 7 days. A 2-μL aliquot of this and of a nonirradiated control was run on a 5% polyacrylamide–7 M urea gel containing 50 μM APM. The bands were detected by silver staining (Igloi, 1983).

The sensitivity of bulk *Escherichia coli* tRNA to irradiation was tested by carrying out an incubation in the presence or absence of 3.6 μCi of [^{32}P]pCp and 2.5 μg of tRNA (MRE 600; Boehringer) in a volume of 10 μL at −20 °C for 7 days. Samples of 2 μL were analyzed on a 5% polyacrylamide–7 M urea gel containing 25 μM APM. Detection was by silver staining. A 5-μL aliquot, of the reaction products was subjected to aminoacylation (Igloi et al., 1979) using an *E. coli* S100 extract as a source of aminoacyl-tRNA synthetases and a ^{14}C amino acid mixture containing 15 labeled amino acids (New England Nuclear; 55 mCi/matom).

Results and Discussion

Synthesis of [(N-Acryloylamino)phenyl]mercuric Chloride and Its Incorporation into Polyacrylamide Gels. The synthesis of APM in a manner analogous to that used for the synthesis of [(*N*-acryloylamino)phenyl]boronic acid (APB) (Igloi & Kössel, 1987) was hampered by the insolubility of (aminophenyl)mercuric acetate in aqueous solution. Although solvents such as dimethylformamide or dimethyl sulfoxide gave homogeneous solutions, acylation gave multiple products, which were not analyzed further. Acylation in heterogeneous aqueous solution gave essentially a single product. APM was found to be very poorly soluble in water but could be dissolved in formamide to a concentration of 2 mg/mL by stirring for several hours at 37 °C. Similar and greater concentrations of APM could be obtained by dissolving the compound directly in aqueous acrylamide solution. Routinely, polyacrylamide gels containing APM (structure I) were prepared by mixing



Structure I

the desired volume of a 1 mg/mL solution of APM in formamide with the acrylamide monomer solution. APM in formamide was stored at room temperature to avoid slow crystallization at 4 °C. It was assumed that the toxicity of APM was at least comparable to that of unmodified acrylamide and the solution was handled accordingly.

In contrast to APB gels, the much smaller concentration of APM required to produce a retardation (see below) meant that nucleic acids could be visualized by staining with ethidium bromide without the problem of an inner filter effect of the aromatic ring of APM masking the dye fluorescence. Furthermore, despite the presence of a heavy metal, silver staining (Igloi, 1983) was also found to be effective, albeit with a reduced sensitivity. In view of the strength and nature of the affinity interaction, recovery of the retarded material from gels must be carried out in the presence of an elution medium containing 2-mercaptoethanol (see below).

Interaction of APM with Sulfur-Containing Minor Bases of tRNA. Quantification of Mercury Binding. The modified bases of tRNAs constitute the major source of naturally oc-

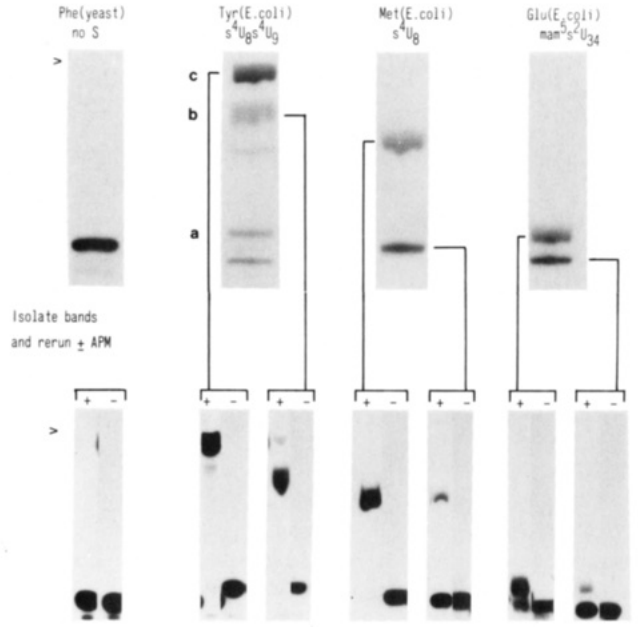


FIGURE 1: Affinity electrophoretic fractionation of thiolated tRNA species. Samples of each of the tRNAs indicated (Boehringer) were 3′ terminally labeled as described under Experimental Procedures. Aliquots were applied to a 5% polyacrylamide gel containing 7 M urea and 25 μM (10 μg/mL) APM. After electrophoresis, bands were detected by autoradiography, eluted by incubation overnight at 37 °C in the presence of 0.3 mL of 0.3 M NH_4Cl/Tris-HCl, pH 7.5/0.1 M 2-mercaptoethanol. After ethanol precipitation, the tRNAs were rerun on a similar gel [5% acrylamide/7 M urea, in the presence (+) or absence (−) of 25 μM APM]. Arrowheads indicate the positions of sample application.

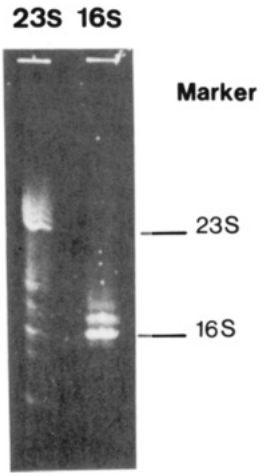


FIGURE 2: Migration of *E. coli* rRNA, labeled in vivo with s^4U (Favre et al., 1986), on an APM gel. A 10-μg aliquot of s^4U-substituted *E. coli* 16S and 23S rRNA was applied to a 2% polyacrylamide gel containing 7 M urea (Igloi & Kössel, 1987) and 0.75 μM (0.3 mg/mL) APM. The positions of migration of commercial 16S and 23S rRNA marker, run alongside the samples, are indicated. RNA was detected by staining with ethidium bromide.

curring sulfur-containing nucleotides. Of the large variety of thiolated nucleotides, s^4U has been detected in many bacterial tRNAs, where it is localized at position 8 of the polynucleotide chain (Sprinzl et al., 1987). The interaction of s^4U as well as of s^4U-containing tRNAs with organic mercurials in solution has been extensively investigated (Scheit & Faerber, 1973; Sunshine & Lippard, 1974; Maguire, 1976). The solution studies had been facilitated by the decrease in the ultraviolet absorbance of s^4U at 330 nm upon complexation with mercury compounds. In order to test the utility of APM for binding s^4U, s^4UMP was titrated with APM and the absorbance at 330 nm was monitored. A typical titration curve analogous to that shown by Scheit and Faerber (1973) demonstrated a 1:1 stoichiometry of complex formation. Unmodified acrylamide had no effect on the absorbance of s^4UMP, while the presence of neither 7 M urea nor 50% formamide affected the APM/s^4UMP interaction. The corresponding Scatchard plot (not shown) yielded a dissociation constant of 0.2 μM compared with 0.55 μM for the binding of (hydroxymercuri)-benzoate to s^2Urd (Scheit & Faerber, 1973). As discussed in the latter work, the exact quantification of such strong binding interactions by this method is difficult and the values obtained probably represent an estimate. In view of this tight binding, similar complex formation between the polyacrylamide-bound APM and s^4U-containing tRNAs could, therefore, be expected with a concomitant retardation of such species.

Although s^4U is the major thiolated nucleoside in tRNA, several other modified bases, mostly derivatives of U, contain sulfur. tRNA(Glu) (*E. coli*), for instance, provides an example of an unmodified U_8 with a $mam^5s^2U_{34}$ in the anticodon loop (Sprinzl et al., 1987). The interaction of s^2U and poly(s^2U) with mercury(II) in solution is well established (Scheit & Faerber, 1973); that of mam^5s^2U in tRNA(Glu) with APM in the affinity system is demonstrated in Figure 1, in comparison with the affinity electrophoretic pattern obtained for tRNA(Met)f (*E. coli*; s^4U_8), tRNA(Tyr) (*E. coli*; $s^4U_8s^4U_9$), and tRNA(Phe) (yeast; no thiolated bases). All resolved components migrated, after elution, to the position of the starting material on a standard, nonaffinity, gel, and no significant reequilibration was observed on reelectrophoresis on the APM gel (Figure 1, lower panels). Any trace cross-contamination of bands after reelectrophoresis may be rationalized by atmospheric oxidation or 2-mercaptoethanol-mediated reduction of thiolated bases during workup. One would, therefore, conclude that the interaction of the tRNAs with the matrix has caused a fractionation of the material, leading to the retardation of those species bearing thiolated nucleotides.

The identity of the retarded bands of Figure 1 presumably corresponds to sulfur-containing tRNA species. Whereas in the case of tRNA(Met)f and tRNA(Glu) single S-substitutions led to single mercury-bound subpopulations, the correlation of bands for tRNA(Tyr) is more tenuous. tRNA(Tyr) is generally accepted as having two adjacent s^4U residues at positions 8 and 9 (RajBhandary et al., 1969), and the pattern obtained was more complex than that observed for tRNA-(Met)f (Figure 1) or for tRNA(Val) (*E. coli*; s^4U_8) (not shown). Several bands were resolved by the gel. As in the case of tRNA(Met)f, a component (a) migrated unretarded. A further major band (b), occasionally appearing as a doublet, was found at a position close to the retarded band of tRNA-(Met)f. A third species (c) was even more strongly retarded. The fastest migrating band consists of impurities in the commercial product, while a further, faint, retarded band is of unknown origin. While one might expect a pair of neighboring s^4U residues to be more tightly bound to the mercury matrix, giving rise to the scarcely migrating band, a logical extension of this interpretation that the less retarded bands correspond to resolvable tRNA species having *either* s^4U_8 *or* s^4U_9 requires the postulation of heterogeneously thiolated species. The behavior of a heterogeneous population of s^4U residues could be examined with rRNA from *E. coli* into which s^4U had been incorporated in vivo (Favre et al., 1986). Figure 2 shows the electrophoretic pattern of 16S (5.6% s^4U) and 23S (2.5% s^4U) rRNA and demonstrates the formation of a "ladder" of RNA species of apparent s^4U heterogeneity. If the behavior of tRNA(Tyr) reflects a situation in vivo, it might explain the

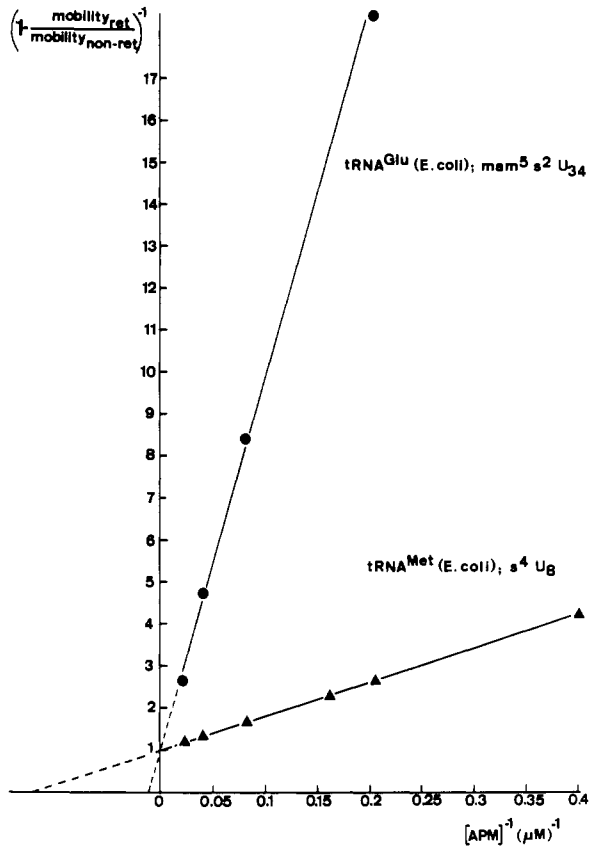


FIGURE 3: Quantitative affinity electrophoresis for the determination of interaction constants between APM and modified nucleotides. Samples of tRNA(Met)f and tRNA(Glu) were run on a 5% polyacrylamide gel containing 7 M urea and varying amounts of APM. The relative retardation of the interacting band was determined in each case, and the results were displayed (Gerbrandy & Doorgeest, 1972) as double-reciprocal plots with the interaction constant being a function of the intercept on the abscissa.

differences in the published tRNA(Tyr) nucleotide sequences: $s^4U_8U_9$ (Goodman et al., 1970) and $s^4U_8s^4U_9$ (RajBhandary et al., 1969).

The APM concentration dependence of the retardation gave rise, in accordance with the theory of affinity electrophoresis (Hořejší, 1981), to linear double-reciprocal plots yielding interaction constants of 8 μM for tRNA(Met)f and 90 μM for tRNA(Glu) (Figure 3). In the case of s^4U_8, no major variation in the binding was detectable between different tRNAs, with tRNA(Val) (*E. coli*) having an interaction constant of 10 μM under the same conditions. These constants may be compared with values determined in solution for the binding of methylmercuric hydroxide to tRNA(Met)f (*E. coli*) of 10 μM (Maguire, 1976), *p*-(chloromercuri)benzoate to tRNA(Val) (*E. coli*) of 11 μM (Sunshine & Lippard, 1974), and *p*-(hydroxymercuri)benzenesulfonate to s^2Urd and poly(s^2U) of 0.5 and 1 μM, respectively (Scheit & Faerber, 1973). The interaction is also demonstrable when large amounts of (aminophenyl)mercuric acetate are present as a noncovalently bound component of the gel (not shown). For a significant retardation of tRNA(Met)f by this free ligand, the concentration of the mercurial must be greater than 100 μM. Apparently, the tRNA–mercury complex is formed and migrates as such through the gel, albeit at a reduced rate compared with the uncomplexed tRNA.

Factors Affecting the Binding of tRNA to APM. Not only does there appear to be a variation in the thiolation of tRNA(Tyr) but a substantial amount of material (approximately 40%), apparently unretarded by the affinity gel, was observed in the case of all three tRNAs. This prompted us to investigate a number of factors involving both the lability of the thiolated bases and the nature of the tRNA–mercury interaction that might be expected to modulate the binding of thiolated tRNAs to the matrix.

Capacity of the Gel. The values of the interaction constants obtained from Figure 3 indicate, as would be expected from a quasi-covalent binding, a much more stable complex formation for all species studied than was observed for the boronate–*cis*-diol system (40 mM). In order to be able to measure such tight binding phenomena by affinity electrophoresis, low concentrations of the APM component must be used. Consequently, such gels are relatively easily overloaded; i.e., free tRNA competes with bound tRNA for the available ligand. The visible result of this competition is a shift of the retarded species to an apparently faster migrating band (Figure 4A).

Chemical Modification. Confirmatory evidence that the retardation observed with the affinity gels was due to the interaction with thiolated components was obtained by a chemical modification reaction described as being relatively specific for such species. Cyanogen bromide, which converts s^4U to uridine via uridine thiocyanate and reacts analogously with mam^5s^2U (Saneyoshi & Nishimura, 1967), was found to give rise to species no longer able to interact with the gel (Figure 4B).

Of interest with resect to the degradation of these rather labile moieties was a rapid radiolytic destruction of mam^5s^2U in tRNA(Glu) (Figure 4C) and s^4U in tRNA(Met)f (not shown). Incubation of tRNA(Met) or tRNA(Glu) with a source of ^{32}P, as described under Experimental Procedures, resulted in a radiation dose and tRNA concentration dependent disappearance of the retarded bands. Although the sensitivity of these bases to ultraviolet light is a well-documented area of investigation (Hall, 1971; Carrē et al., 1974; Kittler & Löber, 1977; Thomas & Favre, 1980; Thomas et al., 1981), and organic thiols have been applied as radioprotective reagents (Jocelyn, 1972), we are not aware of any previous report describing the destruction of thiolated bases in tRNA by low doses of radiation. In view of the nature of the β-particles emitted by ^{32}P, it is possible (Jelley, 1983) that the damage is caused by the ultraviolet component of the Čerenkov radiation. A more plausible agent in the reaction would appear to be the collection of free radicals generated in aqueous solution by the radioactive decay (Packer, 1974). The product of radiolysis of the thiolated bases is not known, but probably no major structural damage is incurred. Indeed, bulk tRNA (*E. coli*), irradiated under conditions leading to a disappearance of mercury-bound material (Figure 4C), could be aminoacylated with a mixture of 15 ^{14}C amino acids with characteristics indistinguishable from those of the control, nonirradiated sample in terms of kinetics and extent (not shown). Furthermore, the H_2O_2-mediated destruction of s^4U (Watanabe, 1980), which might be considered as a model reaction for a free-radical attack on this residue, leads to dethiolation but no loss of aminoacylation activity.

The possibility that the nonretarded bands in the tRNA samples were the products of an attack by free radicals present in the polymerized gel seems unlikely, since the amount of this material was not influenced by the period of time elapsed between casting and running the gels.

Magnesium Binding and Conformational Considerations. It has been reported that the binding of certain mercury compounds to s^4U in tRNA is inhibited by Mg(II) (Pal et al., 1972; Sunshine & Lippard, 1974). However, denaturing tRNA(Met)f [or tRNA(Glu)] at 56 °C and in 80% form-

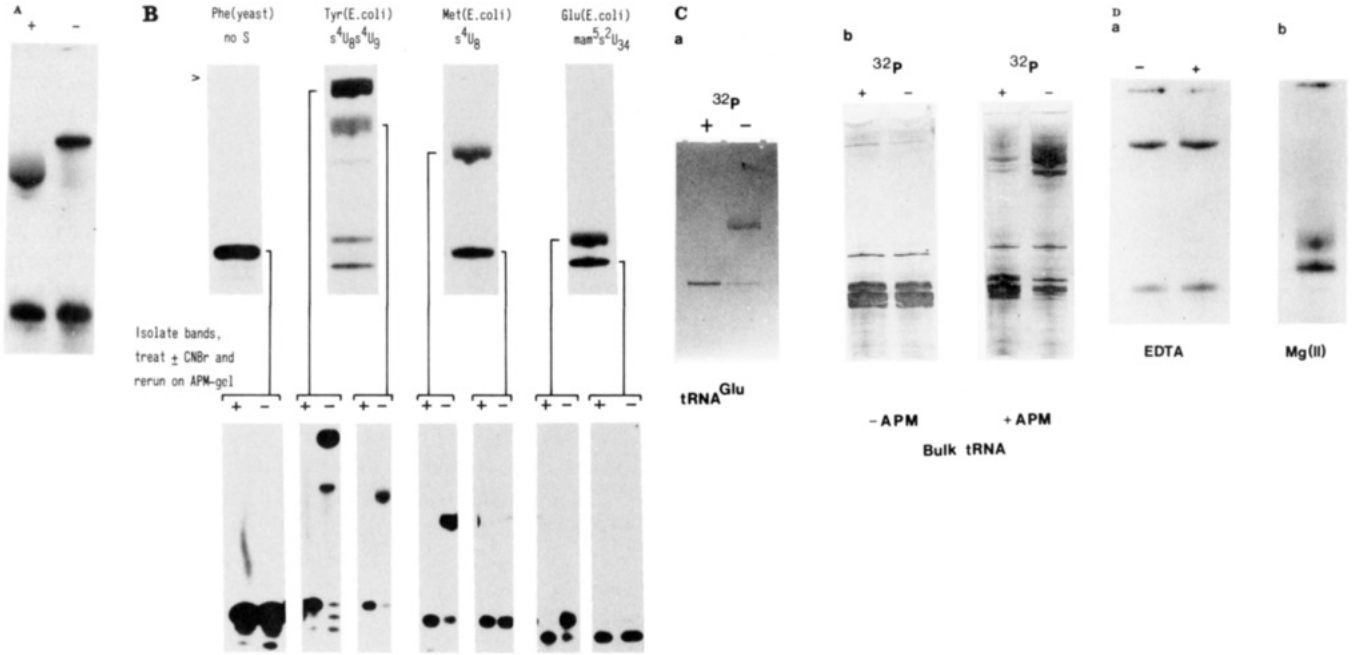


FIGURE 4: Sensitivity of APM/tRNA interaction to chemical and physical factors. (A) The capacity of the gel may be exceeded. 3′ terminally labeled tRNA(Met)f (50 ng) was applied to a 5% polyacrylamide gel containing 15 μM APM either before (−) or after (+) mixing with 10 μg of unlabeled tRNA(Met)f. (B) Cyanogen bromide abolishes the affinity interaction. 3′ terminally labeled tRNAs, as indicated, were fractionated on a 5% polyacrylamide gel containing 7 M urea and 25 μM APM. The autoradiographically detected bands were cut out and eluted in the presence of 2-mercaptoethanol as described in the legend to Figure 1. Modification of each species was at 20 °C for 10 min in 0.1 mL of $NaHCO_3$, pH 8.9, by the addition of 0.25 mg of CNBr in 2 μL of EtOH (Saneyoshi & Nishimura, 1967). Controls were incubated in the absence of CNBr. The samples were rerun on the APM gel described above. (C) Irradiation of tRNAs with ^{32}P-derived β-emission abolishes the affinity interaction. Radiolysis of tRNAs and their analysis were carried out as described under Experimental Procedures. (a) Migration pattern of tRNA(Glu) (mam^5s^2U) on an APM gel after irradiation (+) compared with the untreated sample (−). (b) Migration pattern of *E. coli* bulk tRNA (Boehringer) [>50% s^4U (Sprinzl et al., 1987)] after irradiation (see Experimental Procedures) (+) compared with the untreated sample (−) on a standard gel (−APM) or on an affinity gel (+APM). (D) Mg(II) competes with the binding of APM to tRNA(Met)f. (a) tRNA(Met)f, 3′ terminally labeled, was denatured in the presence (+) or absence (−) of 1 mM EDTA prior to electrophoresis on a 5% polyacrylamide gel containing 7 M urea and 25 μM APM using a 100 mM Tris–borate/1 mM EDTA buffer. (b) tRNA(Met)f was denatured in the presence of 1 mM $MgCl_2$ prior to electrophoresis on a gel as in (a) except that the buffer contained 1 mM $MgCl_2$ instead of EDTA.

amide in the presence of EDTA prior to electrophoresis in an APM gel containing 7 M urea and the standard Tris–borate/EDTA buffer did not lead to a significant change in either the migration or the distribution of the material in the bands (Figure 4D). Presaturation of tRNA (Met)f with Mg(II) and electrophoresis in Tris–borate/Mg(II), on the other hand, caused a large shift of the retarded band (Figure 4D), indicating a competition between Mg(II) and APM for the binding of s^4U and confirming previous observations concerning the organomercurial/s^4U interaction. Mg(II) had no effect on the migration of tRNA(Met) in a standard gel. The strength of the Mg(II)/tRNA interaction could be readily quantified (Hořejší et al., 1977) by using the Mg(II)-induced shift and the previously determined tRNA/APM interaction constant and was found to be 48 μM in 100 mM Tris–borate and 7 M urea.

Although unfolding of tRNA in solution has been reported to lead to a loss of the tight binding of Mg(II) (Stein & Crothers, 1976a), our value obtained in 7 M urea may be compared with the dissociation constant determined in 0.17 M NaCl solution by equilibrium dialysis of 34 μM for a single strong Mg(II) binding site on tRNA(Met)f (Stein & Crothers, 1976b). This comparison should, nevertheless, not be interpreted in a strict sense since the metal ion interaction is known to be sensitive to the ionic environment (Schimmel & Redfield, 1980). While 7 M urea, at room temperature, may be insufficiently stringent to unfold the tRNA structure completely (Reijnders et al., 1973), a further strengthening of the Mg(II) binding was observed by running APM gels in the absence of urea (Table I) and suggests a "loosening" of the Mg(II) binding site in 7 M urea. Loss of the "tight-binding

Table I: Interaction Constants (μM) between tRNAs and Hg(II) or Mg(II)

	native	7 M urea	72% formamide
tRNA(Met)f (s^4U)			
Hg(II) (APM)	8	8	110
Mg(II) ($MgCl_2$)	1	48	36 000
tRNA(Glu) (mam^5s^2U)			
Hg(II) (APM)	20	90	nd[a]
Mg(II) ($MgCl_2$)	80 000	nd[a]	nd[a]

[a] nd, not determined.

conformation" could be induced by casting the gels in 72% formamide, conditions favoring complete denaturation (Reijnders et al., 1973).

The consequences of a partial unfolding of certain regions of the tRNA on the binding of mercury were also investigated, since the competitive effect of Mg(II) and previous studies by both Eu(III) fluorescence energy transfer (Wolfson & Kearns, 1974) and NMR (Schimmel & Redfield, 1980) have suggested a localization of a strong metal ion binding site to be near the s^4U_8 position. The binding of s^4U_8 in tRNA(Met)f to the affinity gel is not influenced by a change in the urea concentration from 0 to 7 M (Table I), although the unfolding of this region, generally considered to be less susceptible to chemical modification (Kim, 1978), might be expected to expose the sulfur to a more intimate binding to the matrix. The fact that there is no change in the interaction constant supports the conclusion concerning the lack of complete unfolding derived from the binding of Mg(II). It is also apparent that the covalent nature of the binding of APM is less sensitive

Table II: Interaction between Phosphorothioate-Substituted Nucleic Acids and Hg(II)[a]

	ssDNA	dsDNA	RNA
5′-phosphorothioate monoester			
monophosphorothioate	strong (0.8)	strong	strong (0.3)
triphosphoro-γ-thioate	nd	nd	strong (<0.1)
3′-phosphorothioate monoester			
monophosphorothioate	nd	nd	strong[b]
phosphorothioate diester	no detectable interaction		

[a] Interaction constants (μM), where measured, are given in parentheses. All gels were run in the presence of 7 M urea. nd, not determined. [b] Detected as the product of an RNase T_1 digest of RNA transcribed in the presence of ATPαS.

to conformational variations than is the case for Mg(II), where a precise arrangement of the coordination site seems to be critical. Indeed, the migration of the retarded s^4U band in a 72% formamide APM system yields an interaction constant for tRNA(Met)/APM of only an order of magnitude weaker at 110 μM than in either the native or the 7 M urea gel.

Comparison of the migration of tRNA(Glu) in 7 M urea gels with that in gels containing no urea (native) shows a tighter binding of the native molecule (Table I). This effect is not unexpected in view of the exposed nature of mam^5s^2U in the anticodon loop of tRNA(Glu) (Yokoyama et al., 1985) leading to a more favorable interaction in the native three-dimensional structure than that with partial unfolding of this region. As would be expected from the preceding discussion, the binding of Mg(II) to the anticodon region of tRNA(Glu), as determined by the affinity gel system, was weaker than the detection limit.

Quantitative affinity electrophoresis leads us, therefore, to the conclusion that mercury binds to the site normally occupied by a strongly associated Mg(II) and that this site involves the favorable positioning of s^4U_8 within the three-dimensional structure. Thus, for both modifications in question, denaturation, which at first sight might be expected to bring the residues into a more exposed situation, results, in fact, in a weaker binding: in the case of $mam^5s^2U_{34}$ by disturbing the accessibility of a rigidly held exposed group (Yokoyama et al., 1985) and in the case of s^4U_8 by destroying the architecture of a metal ion binding site (Schimmel & Redfield, 1980).

Interaction of APM with Phosphorothioate-Substituted Nucleic Acids. In contrast to the thiolated bases present naturally in tRNA, the phosphorothioate nucleoside triphosphates are an example of a P–S link that may be incorporated into the backbone of nucleic acids by chemical or enzymatic methods (Eckstein, 1983). These nucleic acid analogues have been widely used in mechanistic studies or as substrates for other sulfur-specific interactions (Eckstein, 1985). The binding of Cd(II) to the parent phosphorothioate triphosphate is the basis for stereochemical analysis of optical isomers resulting from the O to S substitution. Furthermore, mercury-bearing affinity columns have been used to isolate 5′-S–PPP terminating primary transcripts (Zhang et al., 1984). It was, therefore, anticipated that although no systematic study of the binding of Hg(II) to phosphorothioates has been reported, APM affinity electrophoresis would also permit resolution of sulfur-substituted species with migration characteristics dependent on the chemical environment of the sulfur atom.

The interaction of APM gels with a number of DNA and RNA model compounds, substituted at various sites and in different chemical environments, was investigated. As indicated in Table II and shown in Figure 5, the binding of 5′-terminal phosphorothioate monoesters is significantly stronger than that of s^4U. This interaction is further enhanced when

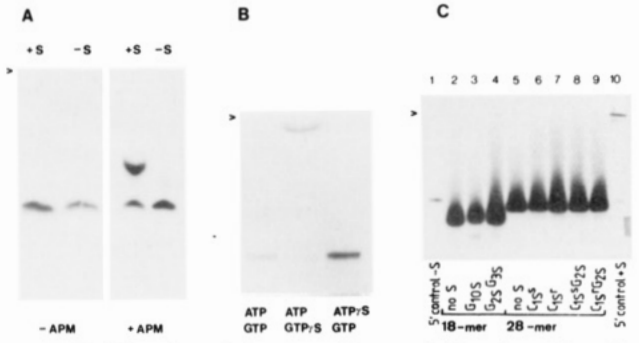


FIGURE 5: Migration of phosphorothioate-substituted nucleic acids in the APM affinity system. (A) 5′-Terminal monophosphorothioate monoester. A sample of a single-stranded deoxyoligonucleotide 33-mer (5′-ATTGAACTATCCATGTGGCTGAGAGCCCTCACA), synthesized on a Model 380A DNA synthesizer from Applied Biosystems, was 3′ terminally labeled with [α-^{32}P]GTP and 5′-thiophosphorylated with ATPγS (+S) or ATP (−S) as described under Experimental Procedures and run on a 5% polyacrylamide gel containing 7 M urea (−APM) or, additonally, 0.25 μM (0.1 μg/mL) APM (+APM). (B) 5′-Terminal triphosphoro-γ-thioate monoester. Transcription in vitro in the SP6 system in the presence of ATP/GTP, ATP/GTPγS, or GTP/ATPγS, as indicated, was performed as described under Experimental Procedures. After isolation of the products on a standard polyacrylamide gel, the samples were applied to a 5% polyacrylamide gel containing 7 M urea and 1.25 μM (0.5 μg/mL) APM. (C) Phosphorothioate diester. The following deoxyoligonucleotides were tested for interaction with APM after 5′-terminal labeling with [γ-^{32}P]ATP, where s designates the position of the phosphorothioate internucleotide link. The enantiomers, where relevant, are indicated in brackets. Lane 2, 5′-CGGCCAGTTGATTCGTAA; lane 3, 5′-CGGCCAGTTGsATTCGTAA; lane 4, 5′-CGsGsCCAGTTGATTCGTAA (18-mers kindly supplied by Prof. F. Eckstein, Göttingen); lane 5, 5′-CGCCAGGGTTTTCCCAGTCACGACGTTG; lane 6, 5′-Cs(S_P) GCCAGGGTTTTCCCAGTCACGACGTTG; lane 7, same as lane 6 but R_P isomer; lane 8, 5′-Cs(S_P)-GsCCAGGGTTTTCCCAGTCACGACGTTG; lane 9, same as lane 8 but R_P isomer (28-mers generously provided by Prof. H.-J. Fritz, Munich). Lanes 1 and 10 were used for negative and positive controls, respectively, by applying the samples (−S) and (+S) from panel A to the gel. The matrix consisted of 5% polyacrylamide, 7 M urea, and 250 μM (0.1 mg/mL) APM.

an RNA transcript is formed in the presence of NTPγS, leading to a 5′-terminal triphosphoromonothioate (S–PPP–). In the case of transcripts obtained in the SP6 system in vitro, it could be shown, in agreement with the DNA sequence, that the transcript initiated with GTP (Figure 5); the S of ATPγS was not incorporated. Thus, not only is it feasible to isolate primary transcripts by this method but information concerning the initiation of transcription may be more conveniently obtained than by the corresponding affinity chromatography.

For preparative purposes, in view of the very low interaction constant, one must bear in mind that those APM gels that permit entry of the thiolated sample into the gel contain very low concentrations of APM (<1 μM) and that they may easily become overloaded (see above). The strong association between the gel and the phosphorothioate monoesters also raises the question of recovery of such species from the gel. The results in Table III indicate that standard salt-mediated elution by diffusion from gel pieces leads to low recovery yields. However, inclusion of 0.1 M 2-mercaptoethanol in the elution buffer restores the efficiency.

In direct contrast to the interactions of APM with phosphorothioate monoesters it is notable that the association between the APM gel and a sulfur substitution is abolished for phosphorothioate diesters (Figure 5). No retardation was observed for oligodeoxyribonucleotides containing single or double sulfur substitutions at various positions in the chain, even at APM concentrations of 0.25 mM or greater. At sufficiently high concentrations of the mercurial (6 mM), the

Table III: Effect of 2-Mercaptoethanol on Recovery of a 3′ Terminally Labeled, 5′-Thiophosphorylated Deoxyoligonucleotide 33-mer from an Affinity Gel Containing 1.2 μM APM

buffer[a]	band	eluted material (%)
0.3 M NH_4Cl/20 mM Tris, pH 7.5	lower (−S)	88
	upper (+S)	31
0.3 M NH_4Cl/20 mM Tris, pH 7.5, + 0.1 M 2-mercaptoethanol	lower	95
	upper	94

[a] At 37 °C, overnight.

direct interaction with the bases becomes observable (not shown), as would be expected from solution studies (Gruenwedel & Davidson, 1966).

This result is in apparent conflict with the observation of Sun and Allfrey (1982), who isolated thiolated DNA on a mercurated affinity column after labeling cells in vivo with [^{35}S]thiophosphate. While the latter experiment demonstrated that ^{35}S becomes incorporated from NTPαS, the authors' interpretation assumed a potential interaction of all phosphorothioate residues with the matrix. Assuming that the binding properties of the mercurated column material are comparable to those of the APM gel system, the 30% recovery of ^{35}S-labeled material as column-bound species reflects, in the light of our observations, probably a fractionation of 5′ terminally labeled phosphorothioate monoesters (bound) from only internally labeled phosphorothioate diesters (not bound).

The dependence of the binding of APM on the environment of the sulfur atom may be rationalized by an extrapolation of the complexes envisaged between Cd(II) and ATPβS (Pecoraro et al., 1984). These structures have been depicted as octahedral coordination complexes. Hg(II), on the other hand, while able to form octahedral structures (Cotton & Wilkinson, 1974), has commonly also been found in square-planar complexes (Aylett, 1975; Saenger, 1984), although it has been ascribed a linear bidentate structure in its interaction with s^{4}U (Sunshine & Lippard, 1976). The results we observe may be rationalized in terms of a square-planar structure as shown in (Figure 6). An octahedral coordination is, however, not ruled out. A successive loss of stabilization is apparent on going from the triphosphoro-γ-thioate monoester to the phosphorothioate diester. Whereas the former may interact in a bidentate fashion directly through P-bound S and O atoms, the latter is deprived of the P–O–Hg interaction, and the strength of the H bond to the esterified O may be further weakened by electronic neighboring group effects. In particular, the requirement of a nucleophilic S atom for formation of the practically covalent bond with Hg(II) is much less well met by the sulfur in the diesterified phosphorothioates (pK_a <2) than by the more nucleophilic monoester ($pK_a \approx 5.5$) (Peacock & Nickless, 1969). Steric factors, due to the presence of an additional, bulky, nucleotide R_2, may also play a role in preventing an effective coordination. The intermediate case of the monophosphorothioate monoester is then a consequence of a relief from steric and electronic destabilization, together with the possibility of forming a strong H bond, as indicated.

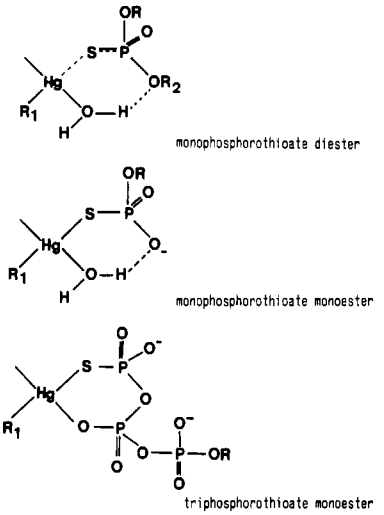


FIGURE 6: Putative structure of Hg(II) complexes of phosphorothioate esters, as indicated. R_1 represents, in this case, the gel matrix.

CONCLUSIONS

We have previously reported the use of affinity electrophoresis for the fractionation of those nucleic acids bearing a free ribose residue (Igloi & Kössel, 1985, 1987). In this paper it has been demonstrated that the method can be extended to another class of nucleic acids by an appropriate selection of the immobilized ligand. Once again, it could be shown that a single substitution within a polynucleotide chain, be it one s^{4}U in 76 bases of tRNA or one phosphorothioate in 360 phosphates of an RNA transcript, is sufficient to achieve a binding interaction.

Not only does the interaction of the organomercurial with sulfur modifications permit a fractionation of these molecules on an analytical and preparative scale but the sensitivity of the binding to the chemical environment of the participating groups, as reflected in the characteristic migration properties (interaction constants), may be of potential value in the identification of structures under investigation.

A number of factors have been shown to modulate the binding of sulfur-containing tRNA species to the gel. These include chemical modification and radiolytic destruction of s^{4}U and mam^{5}s^{2}U, as well as metal ion competition. These approaches have, nevertheless, not been able to establish whether the appearance of nonretarded tRNA bands reflects a heterogeneous situation in vivo or whether it is merely an indication of a preparative artifact (e.g., light-induced cross-linking of s^4U_8 with C_{13}) (Carrê et al., 1974). Clarification of this requires further investigation for which a method as described here may be well suited.

The sensitivity of the modified bases to radiolytic damage has practical consequences for the sequencing of radiolabeled tRNAs where the presence of these residues, and possibly of others, may easily be masked by their radiolytic degradation during workup. Furthermore, one may speculate whether *E. coli* and its component tRNAs may provide a system for radiation monitoring in vivo.

The binding of Hg(II) to phosphorothioate monoesters is much stronger than to the heterocyclic derivatives studied here. Whereas the structure of the phosphorothioate–Hg complex cannot be determined by the method described in this paper, any proposed complex must accommodate the dramatic loss of binding on going from a monoester to a diester. A practical application of this phenomenon in the fingerprinting of phosphorothioate-containing transcripts is currently under investigation.

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stituted rRNA. E. Schiefermayr and S. Krien gave enthusiastic technical assistance.

Registry No. s^4U, 13957-31-8; s^4U monophosphate, 4145-46-4; APM, 72136-45-9; mam^5s^2U, 32860-54-1; Mg, 7439-95-4; (*p*-aminophenyl)mercuric acetate, 6283-24-5; acryloyl chloride, 814-68-6.

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