Incorporation of Phosphorothioate Groups into fd and ϕX174 DNA†

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ABSTRACT: We have synthesized fd and ϕX174 DNA in the presence of 2'-deoxyadenosine 5'-*O*-(1-thiotriphosphate) (dATPαS) and the corresponding phosphorothioate derivatives of dCTP and dTTP using ether-permeabilized *E. coli* cells or crude cell extracts or *E. coli* DNA polymerase I. Reaction rates of enzymes involved in the formation or breakdown of DNA are decreased in the presence of phosphorothioates. The amount of label incorporated with [^{35}S]dATPαS suggests that the dAMP has been completely substituted by 2'-deoxyadenosine 5'-*O*-phosphorothioate (dAMPS). The substituted DNAs have the same sedimentation coefficients, similar buoyant density, infectivity, and thermal stability as the unsubstituted DNAs. The procedure therefore allows specific modification at the 5' position of dA, dC, or dT in the DNA. In view of the recent demonstration of specific binding of Pt^{2+} complexes to the phosphorothioate analogue of poly[r(A-U)] (Strothkamp, K. G., and Lippard, S. J. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 2536), the synthesis of phosphorothioate containing DNA may be of use for DNA sequencing by electron microscopy.

The introduction of functional groups into nucleic acids which may be used for the attachment of electron-dense heavy metal atoms to base-specific positions has been proposed first by Beer and Zobel (1961). Recent improvements in electron microscopy (Whiting and Ottensmeyer, 1972; Wall et al., 1974) seem to provide the technology for visualizing individual heavy atoms and thus meet one requirement for the analysis of DNA sequences by a physical technique. However, the chemistry of modifying the DNA (or RNA) specifically and completely has been less successful until recently. Previous attempts at modification consisted of selective attachment of heavy metal derivatives either directly to specific bases (Beer et al., 1966; Dale et al., 1973; Whiting and Ottensmeyer, 1975) or to chemically altered ones (Gal-Or et al., 1967). Each nucleotide required a different modification procedure. A general method of introducing base-specific modification did not exist.

Based on the observation that ribonucleoside triphosphates carrying phosphorothioate groups in the α position are readily incorporated into poly[r(A-U)] (Matzura and Eckstein, 1968; Eckstein and Gindl, 1970), we wondered whether this could also be achieved with similarly modified deoxynucleotides and DNA. The selective introduction of phosphorothioate groups into DNA adjacent to specific bases via polymerization of modified deoxynucleotides would be a convenient procedure which, moreover, would be methodologically identical for all four bases.

An answer to this question became important as evidence was obtained recently (Strothkamp and Lippard, 1976) that alternating poly[r(A-U)], in which pA is replaced by the corresponding phosphorothioate residue, can selectively bind (i.e., selective for A) certain platinum compounds.

We have studied DNA synthesis using as substrates deoxynucleoside phosphorothioates which are analogous in structure to the previously used modified ribonucleotides (Eckstein and Goody, 1976; Eckstein and Scheit, 1971). They have the general formula shown in Figure 1.

Our results were obtained with the phosphorothioate analogues of dATP, dCTP, and dTTP, respectively, used either separately or in various combinations. We therefore synthesized ϕX-RF[1] either in permeabilized *E. coli* cells or fd-RF in crude extracts of soluble *E. coli* proteins or with *E. coli* DNA polymerase I. In all cases only the newly made DNA carried the phosphorothioate group whereas the template DNA (RF or SS) was unmodified.

The present report describes structural, biochemical, and biological properties of substituted ϕX174- and fd-RF.

Materials and Methods

E. coli Strains and Phages. Wild type phages ϕX174 and fd and the following *E. coli* strains were used: H512 (F$^-$ *end*A2), H514 (F$^-$ *thy end*A2), H570 (F$^-$ *pol*A1 *end*A2 *rns*A100). All strains were obtained from Dr. H. Hoffmann-Berling.

Nucleotides, Enzymes, and Other Materials. Deoxyadenosine, deoxycytidine, and deoxythymidine 5'-*O*-(1-thiotriphosphate) and [^{35}S]dATPαS (specific activity 6.3 mCi/mmol unless stated otherwise) were prepared as published for ATPαS (Eckstein and Goody, 1976). The chemically synthesized mixtures of diastereomers of these compounds were used. [^{3}H]dGTP (specific activity 1.3 Ci/mmol unless stated otherwise) and [^{3}H]dTTP (specific activity 495 mCi/mmol unless stated otherwise) were purchased from Amersham. [α-^{32}P]dTTP (specific activity 10 mCi/mmol) was kindly provided by Dr. H. F. Lauppe. Other nucleotides were commercial products from Boehringer (Mannheim, Germany).

Proteinase K (Boehringer), pancreatic RNase and Pronase (Serva), exonuclease III from *E. coli* (approximately 250 units/mL; from Dr. C. H. Schröder), and *E. coli* DNA poly-

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[1] Abbreviations used: dATPαS, 2'-deoxyadenosine 5'-*O*-(1-thiotriphosphate); dAMPS, 2'-deoxyadenosine 5'-*O*-phosphorothioate; dCTPαS, 2'-deoxycytidine 5'-*O*-(1-thiotriphosphate); dCMPS, 2'-deoxycytidine 5'-*O*-phosphorothioate; dTTPαS, 2'-deoxythymidine 5'-*O*-(1-thiotriphosphate); EGTA, ethylene glycol bis(aminoethylene)-*N,N'*-tetraacetic acid; RF, double-stranded, circular, replicative form of DNA; RFI, double-stranded, circular, covalently closed replicative form of DNA; RFII, double-stranded, circular DNA with discontinuity in at least one strand; SS, single-stranded, circular DNA; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

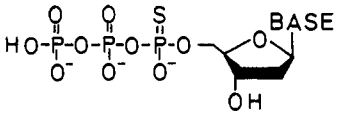

FIGURE 1: Chemical structure of 2′-deoxynucleoside 5′-*O*-(1-thiotriphosphate).

merase I (approximately 10 000 units/mL, specific activity 2500 units/mg of protein, from Dr. K. Geider) were used.

Cesium chloride (Ultra Pure) was from Merck and agarose was from M.C.I. Biomedical, Rockland, Maine.

DNA. ^{32}P-labeled DNA (specific activity 0.2 μCi per μmol of P) was freshly prepared from *E. coli* C by the method of Lehman (1960), except that after RNase treatment the DNA was deproteinized with phenol, precipitated in ethanol, redissolved in 0.02 M NaCl and purified over Sephadex 200. DNA from fd phages was extracted with phenol (Schaller, 1969).

Media and Buffers. C medium is a glycerol–salt medium (Hoffmann-Berling et al., 1963) enriched with 0.2% Difco casamino acids. Basic medium contains in mmol/L: KCl, 80; Tris-HCl (pH 7.4), 40; magnesium acetate, 7; EGTA, 2; spermidine·3-HCl, 0.4; and sucrose, 500. KTM is basic medium without EGTA, spermidine, and sucrose.

Nucleotide-Permeable Cells. E. coli cells (H512 and H514) infected by ϕX174 were permeabilized by treatment with ether as previously described (Vosberg and Hoffmann-Berling, 1971). Details of the reaction conditions are specified under Results. Incorporation at 37 °C was measured after removal of 0.1-mL aliquots of the reaction mixture after various times, dilution into 2 mL of ice-cold 0.1 M NaCl and precipitation in 5 mL of 10% Cl_3CCOOH. The precipitates were collected on membrane filters and counted in a Packard Tri-Carb liquid scintillation spectrophotometer.

ϕX-DNA was extracted from the cells as described previously (Hess et al., 1973).

Cell-Free Extracts. Extracts from H570 cells were prepared using a modification of the procedure of Wickner et al. (1972). The modification included treatment of the cells with ether prior to partial lysis of the cells with lysozyme (Dau and Hoffmann-Berling, manuscript in preparation). A typical extract was prepared from a 6-L cell culture grown in C medium and harvested in mid-log phase. Cells were washed once in 25 mL of KTM and resuspended in 25 mL of the same buffer. These steps were carried out at room temperature. Cells were then agitated with an equal volume of ice-cold ether for 45 s. The aqueous phase containing the cells was separated from the ether phase; the cells were washed twice with KTM and suspended in 3 mL of KTM. Lysozyme (0.7 mL) (4 mg/mL freshly prepared in 0.25 M Tris-HCl (pH 8.0)) was added, the cells were kept in ice for 60 min and then centrifuged for 30 min at 40 000 rpm and 4 °C in a Beckman SW50 rotor to remove cell debris and DNA. The supernatant was distributed in 0.3-mL portions and stored at −70 °C after quick freezing in liquid nitrogen. This procedure yields the high protein concentration in the extract (approximately 35 mg/mL) required for the replication in particular of ϕX DNA.

Reaction mixtures for DNA synthesis (between 50 and 500 μL) typically contained 40% of the final volume as cell-free extract, basic medium (without spermidine and sucrose), 2.5 mM ATP, 0.2 mM CTP, GTP, and UTP, respectively, 55 μM of each of the four deoxyribonucleotides including one deoxynucleotide radioactively labeled and single-stranded fd DNA as template in amounts indicated under Results. No addition of priming oligodeoxynucleotides was necessary. Concentrations of deoxynucleoside phosphorothioates replacing the corresponding normal nucleotides are also given in Results. Incubation at 30 °C was stopped by addition of 10 mM EDTA. DNA was extracted after digestion with, first, RNase (50 μg/mL) for 20 min at 37 °C and then with proteinase K (50 μg/mL) for 30 min at 37 °C. DNA was subsequently deproteinized by the phenol/cresol procedure of Kirby et al. (1967), precipitated in ethanol, and redissolved in 10 mM Tris-HCl (pH 7.6), 20 mM NaCl, and 1 mM EDTA.

Synthesis of fd-RFII with E. coli DNA Polymerase I. The reaction conditions were essentially those of Oertel and Schaller (1973). The incubation mixture (between 0.2 and 0.5 mL) contained 0.05 M Tris-HCl (pH 8.1), 0.1 M KCl, 6 mM $MgCl_2$, and 50 μg/mL BSA. Single-stranded fd DNA was added as specified in Results together with oligonucleotide primer (approximately 15 to 20 bases long) prepared from fd-RF by limited digestion with pancreatic DNase (primer oligonucleotides were kindly provided by Th. Meyer). The primer concentration was adjusted so that for every fd DNA molecule about 2 to 3 primer molecules complementary to the template DNA were present. Normal nucleotides were 0.5 mM each; deoxynucleoside phosphorothioates were 1 mM. Radioactivity was introduced either as [^{3}H]dTTP or as [^{35}S]dATPαS.

The amount of DNA polymerase I varied from 2 to 20 units depending on the amount of input single-stranded DNA. Prior to addition of the enzyme, $MgCl_2$, and BSA, the mixture containing template fd DNA and primer was preincubated for 15 min at 45 °C in order to anneal completely the primer oligonucleotides to the fd DNA circles (Heyden, 1970). After 4 h at 20 °C, the reaction was stopped by addition of 10 mM EDTA and the DNA was purified by banding in neutral CsCl in 10 mM Tris-HCl (pH 7.6) and 1 mM EDTA (initial density 1.710 g/cm^3).

Gradient Centrifugation. Neutral or alkaline sucrose gradients were carried out as previously described (Müller-Wecker et al., 1972). Equilibrium banding in neutral CsCl (in 10 mM Tris-HCl (pH 7.6) and 1 mM EDTA) was performed either in a Beckman Ti50 fixed angle rotor or in a Beckman SW65 rotor. Initial densities were adjusted to 1.720 g/cm^3 in the fixed angle rotor and to 1.710 g/cm^3 in the swinging bucket rotor. Centrifugation was carried out for at least 40 h (20 °C) at 40 000 rpm (Ti50) or 35 000 rpm (SW65). In the profiles given below the direction of sedimentation and increase in density is from right to left.

Protoplast Assays. The assay was performed according to Guthrie and Sinsheimer (1963). Samples containing radioactively labeled DNA from neutral sucrose gradients were dialyzed against 0.05 M Tris-HCl (pH 8.1) overnight at 4 °C. Samples from CsCl gradients were diluted 1:5 with 0.05 M Tris-HCl (pH 8.1) and then dialyzed against the same buffer. Infected protoplasts (*E. coli* W6) were plated on *E. coli* C as indicator.

Thermal Denaturation of DNA. The thermal melting of DNA was performed in a Gilford Model 2000 recorder coupled to a Beckman DUR spectrophotometer.

Agarose Gel Electrophoresis. One percent agarose slab gels (Sugden et al., 1975) were used for product analysis of fd-RF synthesized with DNA polymerase I. Agarose was prepared in electrophoresis buffer containing 40 mM Tris–acetate (pH 7.8), 10 mM sodium acetate, 0.5 mM EDTA, and 2 mM $MgCl_2$. In the presence of 2 mM $MgCl_2$, single-stranded fd DNA migrates faster than fd-RFI as compared with magnesium-free buffers, where SS and RFI migrate with almost identical rates. DNA was visualized by illumination from below with short wavelength ultraviolet light (286 nm) after staining of the gels in the dark for at least 30 min in electro-

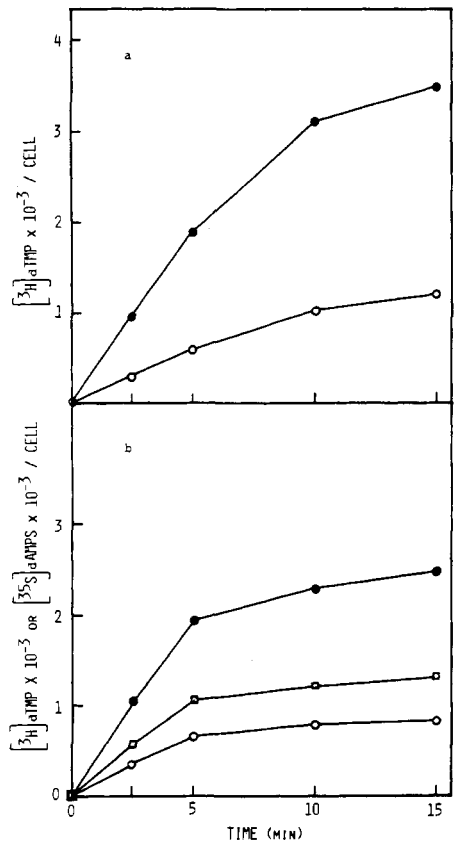

FIGURE 2: Incorporation of dATPαS into φX DNA. The reaction mixture (37 °C) contained in 0.5 mL of basic medium 2.5 × 10^9 φX-infected ether-treated H514 cells in the presence of 1 mM ATP. (a) (●) All four normal deoxynucleoside triphosphates including [^{3}H]dTTP, 20 μM each; (○) dATP replaced by 20 μM dATPαS. (b) Reaction conditions were as in a, except that dTTP was used instead of [^{3}H]dTTP in mixtures containing [^{35}S]dATPαS. (●) All four deoxynucleoside triphosphates, 20 μM each; (○) 22.5 μM [^{35}S]dATPαS and (□) 45 μM [^{35}S]dATPαS instead of 20 μM dATP.

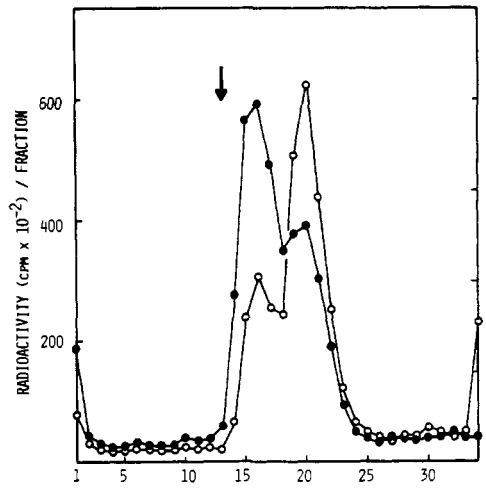

FIGURE 3: Characterization of normal and modified φX-RF from ether-treated cells by velocity sedimentation in neutral sucrose. Phosphorothioate DNA was synthesized for 20 min in the presence of [^{35}S]dATPαS (50 μM), dCTP, dGTP, and dTTP (20 μM each). Normal DNA was synthesized for 20 min with dATP, dCTP, dGTP, and [^{3}H]dTTP (20 μM each). The two DNAs were sedimented in separate tubes of a Beckman SW27 rotor through 5 to 20% neutral sucrose gradient for 12 h at 22 000 rpm, 20 °C. The arrow indicates the position of φX-SS DNA with 24.5 S as determined in a separate run with ^{32}P-labeled φX-SS and ^{3}H-labeled φX-RF.

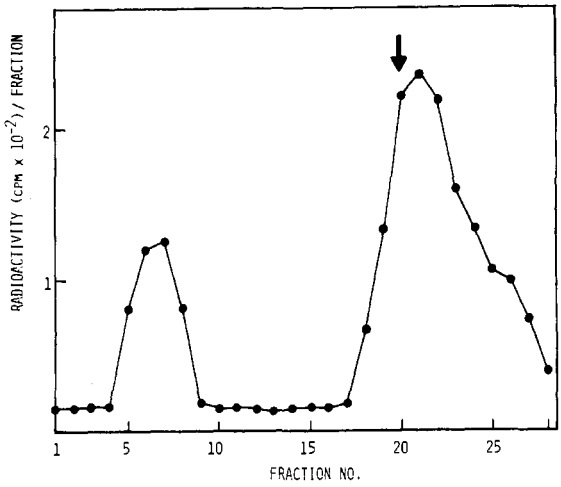

FIGURE 4: Characterization of φX-RF product containing dAMPS by velocity sedimentation in alkaline sucrose. φX-RF was synthesized as described in the legend to Figure 3. Sedimentation was carried out in 5 to 20% sucrose (pH ~12.5) in a SW27 rotor for 12 h at 22 000 rpm (20 °C). The arrow indicates the position of circular native φX-DNA (16 S) as determined in a separate tube of the same rotor.

phoresis buffer containing 1 μg/mL ethidium bromide.

Results

φX DNA Synthesis in Ether-Permeabilized Cells. The incorporation of the four normal deoxynucleotides in φX-infected ether-treated H514 cells was assayed as described in Figure 2. Substitution of dATPαS for dATP caused a reduction of dTMP incorporation by 65% after 15 min. When in a parallel experiment [^{35}S]dATPαS was used to replace dATP, ^{35}S was incorporated into acid-precipitable material suggesting that dATPαS was actually incorporated into DNA (Figure 2b).

Phosphorothioate containing φX DNA labeled either with [^{3}H]dTMP or with [^{35}S]dAMPS was sedimented through a linear neutral sucrose gradient (5–20%). The sedimentation profile for ^{35}S-labeled substituted φX DNA is shown and compared with that of unmodified φX RF (Figure 3). The end products of φX-primed DNA synthesis in ether-treated cells were previously identified as being RFI (21 S) and RFII (16 S) (Dürwald and Hoffmann-Berling, 1971). It is obvious from the profiles in Figure 3 that modified φX-RF obtained from such cells has a sedimentation pattern indistinguishable from that of normal φX-RF. The only difference between modified and unmodified φX DNA was the relative distribution of RFI and RFII. Whereas the ratio of RFI to RFII was 6:4 after synthesis with normal nucleotides, it changed to 3:7 after replacement of dATP by dATPαS.

After sedimentation of phosphorothioate containing φX DNA through alkaline sucrose (5–20%, pH 12.5), two major products were observed (Figure 4). By comparison with unmodified φX DNA in a parallel gradient (not shown), the first peak representing 20% of the radioactive label was found to sediment with approximately 45 S. This is the value reported for covalently closed supercoiled φX-RFI (Hess et al., 1973). The second peak representing 80% of the incorporated label sedimented predominantly with 14 S, the value for linear single-stranded φX DNA. In addition a shoulder of acid-precipitable material trailing the 14S peak was observed. This material was seen only after synthesis with dATPαS but not with dATP. It presumably reflects incomplete polymerization or a deficiency in the joining of discontinuously synthesized DNA strands.

These results show that DNA synthesis in the presence of

TABLE I: Synthesis of fd-RF in Cell-Free Extracts.[a]

Assay composition	[^{3}H]-dGMP incorp (cpm)	Rel incorp (%)
4 dNTP	9594	100
4 dNTP, −DNA	320	3
−dATP, +dATPαS	8551	89
−dCTP, +dCTPαS	8505	89
−dTTP, +dTTPαS	8636	90
−dATP, −dCTP, +dATPαS, +dCTPαS	2068	22
−dATP, −dCTP, −dTTP, +dATPαS, +dCTPαS, +dTTPαS	891	9

[a] The reaction mixture (total volume 50 μL) contained cell-free extract (20 μL), basic medium and 1.2×10^{11} native fd-DNA molecules, [^{3}H]dGTP, dCTP, dATP, and dTTP (66 μM each) in a total volume of 50 μL. Nucleoside phosphorothioates replacing normal nucleotides were 132 μM. After incubation for 30 min at 30 °C, the samples were first digested with 50 μg/mL RNase (30 min at 37 °C) and then with 100 μg/mL proteinase K (30 min at 37 °C) to reduce quenching of tritium label due to the high protein concentration of the extract. The samples were diluted into 2 mL of 0.1 M NaCl, precipitated in 5% Cl_3CCOOH, and collected on membrane filters as described.

one deoxynucleoside phosphorothioate is slower than with unmodified nucleotides, but the end products have the same sedimentation coefficients as normal RFI and RFII. The rate of formation of RFI, however, is considerably decreased. Since most of the newly made strands in RFII (see Figure 4) have full length and since, as will be shown below, the rate of nucleolytic degradation is slower with modified than with unmodified φX DNA, we think that the deficiency in the RFI formation is rather due to a negative effect of the phosphorothioate in the DNA on the ring closure than to preferential nucleolytic breakdown of modified DNA strands.

fd DNA Synthesis in Cell-Free Extracts. Single-stranded circular fd DNA instead of φX DNA was used as a template in experiments with cell-free extracts from H570 cells because due to the low number of protein species involved the former is much more readily replicated than the latter. The results from kinetic experiments and sedimentation analysis of fd DNA products containing one phosphorothioate (dAMPS) were in principal agreement with results obtained for φX DNA from ether-treated cells. The decrease in the rate of nucleotide incorporation was 40%. The formation of fd-RFI was more severely inhibited than that of φX-RFI in ether-treated cells. Not more than 10% of the DNA synthesized in 30 min was converted into RFI as compared with 60% of the control experiment with four normal nucleotides.

In addition we tested whether dATPαS, dCTPαS, and dTTPαS serve equally well as precursors and furthermore we studied the effect of substituting more than one nucleotide on DNA polymerization as measured by the incorporation of [^{3}H]dGMP. Table I shows, first, that no preference exists for any of the three deoxynucleoside phosphorothioates tested as precursors for DNA synthesis as indicated by the constant level of [^{3}H]dGMP incorporation after 30 min at 30 °C. In all three cases approximately 90% of the control value found with four normal nucleotides was obtained. In the second part of this experiment, dATP and dCTP were replaced together by dATPαS and dCTPαS. This time the incorporation of [^{3}H]dGMP was significantly reduced to 22% as compared with the control. An almost complete inhibition was observed after replacing dATP, dCTP, and dTTP together by their corresponding phosphorothioates (see last entry in Table I).

TABLE II: Synthesis of fd-RF with DNA Polymerase I.[a]

fd SS/assay	Nucleotide composition	cpm incorp	fd-RF/assay
(a) 1.72×10^{12}	[^{3}H]dTTP, dATP	8780	1.64×10^{12}
(b) 1.72×10^{12}	[^{3}H]dTTP, dATPαS	9050	1.68×10^{12}
(c) 1.72×10^{12}	dTTP, [^{35}S]dATPαS	26083	1.60×10^{12}

[a] Each reaction mixture contained in a total volume of 200 μL 1.72×10^{12} native fd DNA molecules and two units of enzyme. Nucleotide concentrations were 0.5 mM for each of the normal nucleotides and 1 mM for dATPαS and [^{35}S]dATPαS, respectively. The specific activity was 4 mCi/mmol for [^{3}H]dTTP and 2.8 mCi/mmol for [^{35}S]dATPαS. For other details, see Materials and Methods. The reactions were stopped after 5 h at room temperature by adding 10 mM EDTA. Aliquots of 20 μL were precipitated in 5% Cl_3CCOOH and radioactivity was counted as described.

Specificity of Phosphorothioate Incorporation into fd DNA and Stability of the Incorporated Phosphorothioate Groups. The specificity of incorporation and the stability of phosphorothioate groups in DNA were studied with fd-RF synthesized in vitro with DNA polymerase I. Three separate assays contained (a) normal nucleotides with dTTP being tritiated, (b) dCTP, dGTP, tritiated dTTP, and dATPαS instead of dATP, and (c) same as b but with cold dTTP and [^{35}S]dATPαS. In all cases essentially all of the input DNA was converted from SS to RFII. The extent of the reaction was estimated from agarose slab gels (see Materials and Methods) on which 1% of the input DNA (or 1.72×10^{10} single-stranded fd DNA molecules) initially present in 2 μL of the assay volume (200 μL) would have been detectable by the ethidium staining procedure. No remaining single-stranded DNA could be seen. In addition, banding of a sample of the reaction products (50 μL of the assay volume) in neutral cesium chloride revealed only one symmetrical peak of radioactivity suggesting that no significant fraction of only partially replicated DNA was present.

Under the conditions of this experiment, DNA polymerase I with single-stranded circular DNA as template synthesizes the equivalent of one complementary strand and then stops. The number of complementary strands expected from the reaction was calculated to be 1.72×10^{12} from the known concentration of the fd DNA stock solution used. DNA synthesis in a (with four normal nucleotides including [^{3}H]dTTP) and in b (with cold dATPαS instead of dATP, and [^{3}H]dTTP) was indicated by the incorporation of [^{3}H]dTMP into DNA (Table II). Thus the amount of dTMP incorporated could be directly compared for DNA synthesized in the presence of either dATP or dATPαS. Based on a counting efficiency of 16% for tritium incorporated into DNA and on the fact that dTMP accounts for 0.24 of all nucleotides in the complementary strand of fd-RF, we calculated that in these experiments 1.64×10^{12} complementary strands had been synthesized with dATP and 1.68×10^{12} complementary strands with dATPαS. This corresponds to 95 and 98% of the expected theoretical values, respectively.

In the third experiment (c), with [^{35}S]dATPαS as the only radioactive precursor, the incorporation of the deoxynucleoside phosphorothioate was directly monitored. The counting efficiency for ^{35}S was 83% and the relative occurrence of dAMP in the complementary strand of fd-RF is 0.34. We calculated that the equivalent of 1.60×10^{12} complementary strands or 93% of the expected values had been synthesized.

We tentatively conclude on the basis of these results, first, that the replacement of normal deoxynucleotides by deoxy-

nucleoside phosphorothioates is within the limits of our analysis specific and complete, and second, that no loss of sulfur occurs during or after incorporation of analogue nucleotides into DNA.

Exonucleolytic Breakdown of Normal and Phosphorothioate Containing φX-RFII. Normal and dAMPS-substituted φX-RFII labeled in [^{3}H]dTMP was synthesized in H512 ether-treated cells using nicotinamide mononucleotide as inhibitor of DNA ligase to prevent the formation of RFI (Olivera and Bonhoeffer, 1972) and extracted as described. Nucleolytic breakdown was followed after addition of exonuclease III in excess so that its concentration did not influence the rate of reaction. Both the normal and modified DNAs studied in two parallel assays contained the same amount of newly synthesized DNA corresponding to a radioactivity of 3360 cpm (about 70 pmol of nucleotides). The specific radioactivity of the DNA was not directly determined due to the low concentrations of purified φX DNA. Both normal and phosphorothioate containing φX DNA were obtained from ether-treated cells after extensive in vitro synthesis. In order to compensate for the less efficient incorporation of [^{3}H]dTTP in the presence of an equimolar concentration of dATPαS, the cells were incubated with a fourfold molar excess of dATPαS over the concentration of each of the unmodified deoxynucleotides. Under these conditions, the amount of radioactivity incorporated after 60 min in the presence of dATPαS corresponded to approximately 3800 [^{3}H]dTMP molecules per cell or 90% of the value obtained with dATP. From this result we assumed very similar specific activities for both modified and unmodified φX DNA.

After incubation of φX-RFII with exonuclease III for 30 min at 35 °C, the reaction was not finished (Figure 5). About 43% of the normal RFII but only 17.5% of the modified DNA had been degraded to acid solubility. Thus the ratio of degraded normal DNA to degraded modified DNA was 2.5.

In a further experiment (not shown) the degradation of tritiated normal RFII was compared with that of ^{35}S-labeled modified RFII. The ratio of degraded normal to degraded substituted DNA after 45 min was 2.3, similar to the experiment shown in Figure 5.

Infectivity of Phosphorothioate φX-RF. Normal φX-RF synthesized in ether-treated *E. coli* cells is infectious in a protoplast assay (Geider et al., 1972). We studied the infectivity of phosphorothioate φX-RF isolated from a neutral sucrose gradient and compared it with that of unsubstituted φX-RF. Both normal and dAMPS-substituted φX-DNA was labeled with [^{3}H]dTMP. DNA was synthesized for 45 min with dATPαS present in a 3.5-fold higher concentration than normal nucleotides. From the radioactivity incorporated per cell we calculated a specific activity of approximately 85% for dAMPS-substituted φX-RF as compared with normal φX-RF. The results show (Figure 6) that for both normal and substituted DNA infectivity parallels the radioactivity distribution in the gradients.

In addition, infectivity of fd-RF was tested in Ca^{2+}-treated *E. coli* cells (Taketo, 1972) with normal and phosphorothioate fd-RF containing either dAMPS or dCMPS. These DNAs were synthesized in cell-free extracts, first isolated from neutral sucrose gradients, and then banded in neutral CsCl gradients (initial density 1.710 g/cm^3) in order to remove ribosomal RNA and single-stranded fd DNA which might not have participated in the replication. It was found that phosphorothioate fd-RF was about 50% as infectious as normal fd-RF.

Buoyant Density of Normal and Phosphorothioate fd-RF. Normal fd-RF and phosphorothioate containing fd-RF were synthesized in cell-free extracts from H570 cells in the presence of dATPαS and [^{3}H]dTTP and purified as described in Materials and Methods. Two differently labeled DNAs of known buoyant density were added as density markers to the CsCl solutions containing modified and unmodified fd-RF, respectively: *E. coli* [^{32}P]DNA (ρ = 1.710 g/cm^3) and single-stranded fd DNA, the density of which was taken to be identical with that of the closely related DNA of the phage M13 (ρ = 1.7223 g/cm^3) (Szybalski, 1968).

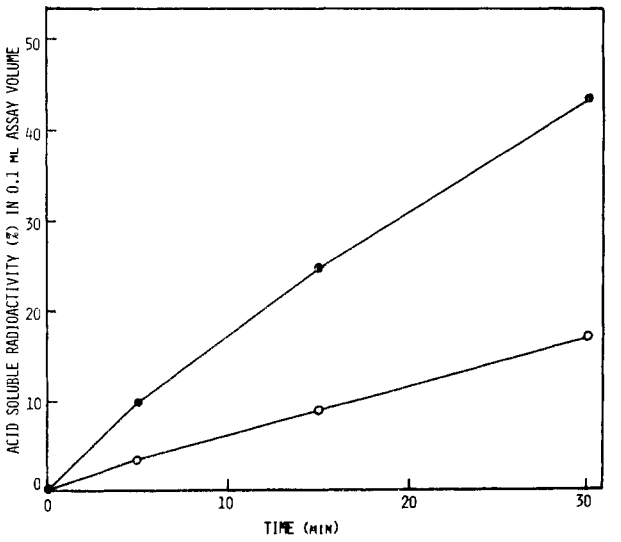

FIGURE 5: Breakdown of normal and phosphorothioate containing φX-RFII by exonuclease III from *E. coli*. φX-RFII was synthesized in H512 ether-treated cells in the presence of nicotinamide mononucleotide (0.1 mM) and [^{3}H]dTTP. The DNA was isolated after extraction from the cells over a neutral sucrose gradient and dialyzed against 66 mM Tris-HCl (pH 8.1) and 1 mM mercaptoethanol. The test volumes for the exonuclease III assay (0.525 mL) contained DNA in 66 mM Tris-HCl (pH 8.1), 0.66 mM $MgCl_2$, 1 mM mercaptoethanol, and 0.25 unit of enzyme. Samples of 0.1 mL were withdrawn from the incubation mixtures immediately after addition of enzyme at 0 °C, after 5, 15, and 30 min, respectively, at 37 °C and mixed with 0.2 mL of buffer (10 mM Tris-HCl (pH 8.0)–5 mM EDTA) containing 1 mg of herring sperm DNA/mL. After addition of 0.2 mL of ice-cold Cl_3CCOOH (1 M), the samples were centrifuged for 10 min at 5000*g* in a Sorvall SS 34 rotor. The supernatants (0.2 mL) were dried on Whatman GF/A filters and counted in a Packard scintillation spectrophotometer. (●) φX-RFII containing dAMP; (○) φX-RFII containing dAMPS.

In a series of four experiments we found an average value of 1.703 ± 0.001 g/cm^3 for the density of normal fd-RF (Figure 7a). fd-RF carrying dAMPS in the complementary strand was found to be identical in density with *E. coli* DNA (Figure 7b). On the basis of our experimental value for the density of normal fd-RF, this result indicates an increase in density of about 0.007 g/cm^3 in the substituted half-synthetic RF relative to the density of normal fd-RF.

Thermal Denaturation of Normal and Phosphorothioate fd-RF. For thermal denaturation experiments, fd-RFII was synthesized in 0.5 mL volume either in the presence of dATP or of dATPαS with 20 units of DNA polymerase I using 4 × 10^{13} fd DNA molecules as templates.

After exhaustive synthesis at room temperature, the DNA was purified by banding in a neutral CsCl density gradient (see Materials and Methods). Fractions containing radioactivity were pooled and dialyzed for 2 × 2 h against 1 L of 0.01 M NaCl and 0.015 M sodium citrate, pH 7.5 to 7.6.

Phosphorothioate fd-RFII showed a T_m of 73 °C which was 1–2 °C below the value we found for normal fd-RFII. In addition, we observed that the width of the melting transition, σ, was slightly broader (1–2 °C) for phosphorothioate fd-RF

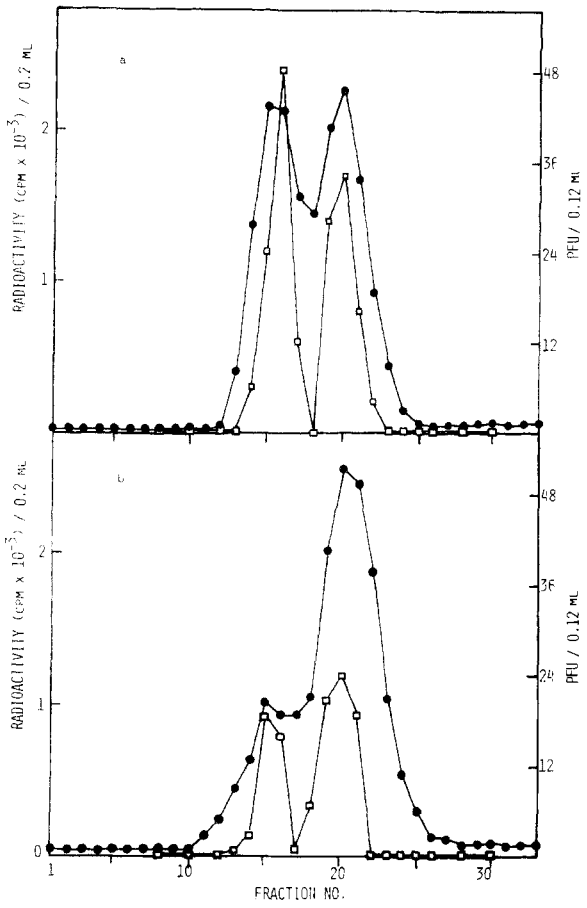

FIGURE 6: Infectivity of normal and phosphorothioate containing ϕX-RF. DNA was synthesized in 5×10^9 H512 ether-treated cells in the presence of [α-^{32}P]dTTP with either dATP, dCTP and dGTP (20 μM each), or 50 μM dATPαS instead of dATP. DNA products were isolated as described (Hess et al., 1973). Separation of RFI and RFII was achieved in a neutral sucrose gradient (5 to 20%, SW27 rotor, 22 000 rpm for 12 h at 20 °C). An aliquot (50 μL) of each fraction was precipitated in Cl_3CCOOH and the radioactivity was counted. The remainder of the fractions was separately dialyzed vs. 50 mM Tris-HCl, pH 8.1, and 0.3 mL of each fraction was incubated with 0.2 mL of W6 protoplasts for 15 min at 37 °C. The plating efficiency of the protoplasts with single-stranded ϕX-DNA was approximately 5×10^{-5}. The titer of infective centers was determined by plating 0.2 mL of each protoplast assay on *E. coli* C. The left-hand ordinate shows the ^{32}P radioactivity in 50 μL of each gradient fraction (●); the right-hand ordinate shows the number of plaques per plate obtained with 0.2 mL of each protoplast assay (□). (a) ϕX-RF containing dAMP; (b) ϕX-RF containing dAMPS.

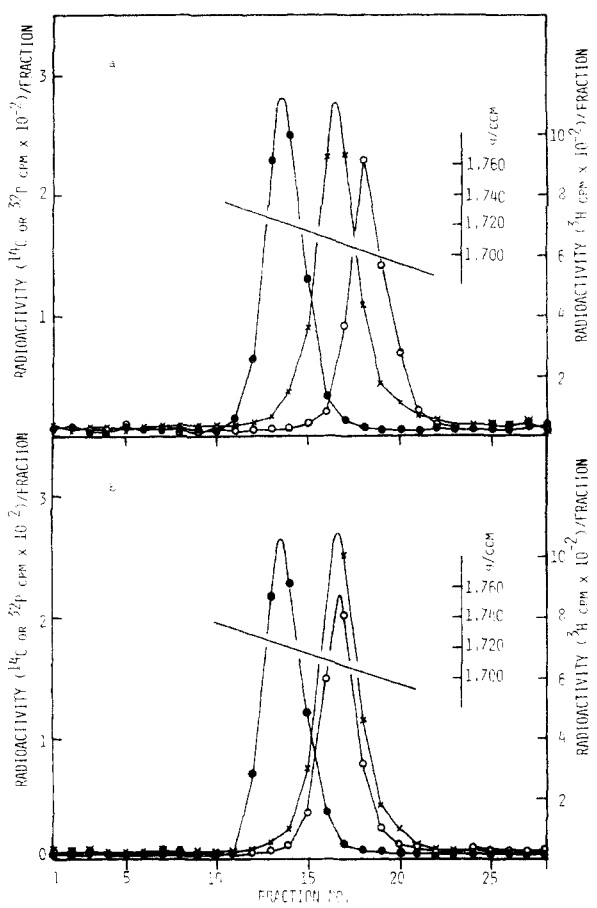

FIGURE 7: Buoyant density of normal and phosphorothioate-containing fd-RF in neutral CsCl. fd-RF was synthesized in cell-free extracts either with 66 μM dATP or with 132 μM dATPαS in the presence of [^{3}H]dTTP. [^{14}C]fd-SS and [^{32}P]DNA from *E. coli* were added as density markers to the CsCl solution (initial density ρ = 1.710 g/cm^3). Centrifugation was for 40 h at 35 000 rpm (20 °C) in a Beckman SW65 rotor. Gradients were fractionated by dripping and radioactivity was counted as described. The radioactivity profiles were corrected for overlapping isotope counting. (●) [^{14}C]fd-SS; (X) [^{32}P]DNA from *E. coli*; (○) [^{3}H]fd-RF. (a) Normal fd-RF; (b) fd-RF containing dAMPS.

than for normal DNA. The effect was small but reproducible. The hyperchromicity was the same for both DNA preparations.

Discussion

Deoxynucleoside phosphorothioates substituting for normal nucleotides during DNA synthesis are readily incorporated into DNA by permeabilized *E. coli* cells or cell-free extracts from such cells or DNA polymerase I. DNA synthesis was tested using ϕX174 or fd DNA as templates. We found that the reaction rates of enzymes involved either in the formation of DNA (polymerization and ligation) or in the nucleolytic breakdown were decreased if one of the normal nucleotides dATP, dCTP, or dTTP was replaced by the corresponding phosphorothioate (dGTPαS was not used). The rate of DNA polymerization with equimolar substitution of one nucleotide (dATP) was depressed to different degrees depending on the system used. DNA polymerase I was the least affected (only 20% decrease with calf thymus DNA as template). Permeabilized cells showed a significant decrease by 65% with ϕX DNA as template. Cell-free extracts appear to be of intermediate efficiency in utilizing phosphorothioates as indicated by a 40% decrease in the rate of fd DNA synthesis at 30 °C (not shown).

The ring closure of ϕX-RF in permeabilized cells and that of fd-RF in cell-free extracts was severely inhibited. Recent evidence showed that after extensive incubation of fd-RFII with T4 ligase in vitro the same amounts of RFI of both normal and modified DNA were formed.

In contrast to the effects of phosphorothioates on enzyme activities, the modified ϕX or fd DNA actually was not significantly altered in its properties as compared with the respective unmodified DNA. The newly made strands of RFII are of essentially full length and RFI and RFII (of ϕX174 or fd) have identical sedimentation rates with or without phosphorothioate groups.

The infectivity of ϕX-RF containing dAMPS was checked with DNA synthesized in permeabilized cells. We conclude that the relative infectivity of phosphorothioate ϕX-RF is between 60 and 80% of that of normal ϕX-RF. These values are in qualitative agreement with those found with fd-RF in Ca^{2+}-treated *E. coli* cells. It should be mentioned, however, that infectivity tests with both normal and modified fd or ϕX DNA had a large spread of error (up to 60%). Therefore, data

presented in Figure 6 have to be considered semiquantitative. Furthermore we cannot exclude from the present results the possibility that the infectivity of modified DNA is due to a mechanism which removes the possibly inhibiting phosphorothioate strand and thus preserves or restores the infectivity of the remaining unmodified strand. This uncertainty about the mechanism of the residual infectivity is not relevant to the main purpose of the thiomodification, i.e., to introduce groups into DNA serving as base specific attachment sites for electron-dense heavy metal compounds.

Substitution of dAMP by dAMPS in the complementary strand of fd-RF results in the replacement of phosphate by phosphorothioate in approximately one-third of the nucleotides in this strand (dAMP accounts for 34% of all nucleotides in the complementary strand). It is reasonable to expect that this substitution is accompanied by an increase in molecular weight of about 0.8% due to the higher atomic weight of sulfur replacing oxygen in the phosphate. This calculation is based on a molecular weight of 1.92×10^6 for native fd DNA (Berkowitz and Day, 1974). It is of interest whether this increase in mass is paralleled by an increase in buoyant density. We observed a buoyant density in neutral CsCl of phosphorothioate fd-RF (containing dAMPS) which is approximately 0.007 g/cm^3 or about 0.4% higher than that of normal fd-RF (Figure 7). This increase in density is qualitatively consistent with the increase in average nucleotide mass due to the introduction of sulfur. Because various other physical parameters like hydration and ionic radius also go to determine the buoyant density, the change in density cannot be explained in terms of nucleotide mass alone.

Thermal denaturation experiments with fd-RFII indicated a small decrease in the thermal stability of the phosphorothioate DNA. The T_m was 1-2 °C below the T_m of normal fd-RF and the transition width for the melting of the DNA was slightly broadened. These effects were small and a complete assessment of their magnitude requires further studies.

Phosphorothioate groups in DNA are stable under the conditions of polymerization. In addition we tested the stability of these groups against hydrolysis with loss of sulfur under a variety of other conditions with fd-RF containing [^{35}S]dAMPS. No loss of acid-precipitable radioactivity was observed on treating the DNA at pH 12.5 for up to 12 h at 37 °C as well as in 3 M KCl or NaCl under neutral conditions and boiling for 10 min also at neutral pH. Approximately 25% loss of acid-precipitable radioactivity was found on treating the DNA for 12 h at pH 2.3 at 37 °C. These results show that at least at neutral and alkaline pH the phosphorothioate group is stable.

Moreover we investigated whether nicks were preferentially introduced into DNA strands containing phosphorothioates by acid or alkaline treatment. Analysis of the length of polydeoxynucleotides in modified and unmodified fd-RFII on alkaline sucrose gradients showed that phosphorothioate containing DNA retained its original length of a complete linear fd DNA molecule (14 S) after a 3-h incubation at pH 12.5 and 37 °C. After a 3-h incubation at pH 2.3, less than 30% of the DNA was found in the position of complete linear strands. A considerable portion (50%) sedimented close to the top of the gradient and some 20% were found between the top fraction and the 14S material. The same sedimentation profile was obtained with unmodified fd-RFII after acid treatment. We conclude from these results that phosphorothioate fd-RF is as stable against alkaline treatment as normal DNA. The fragmentation of modified fd-RFII under acid conditions was not different from that observed with normal fd-RFII and is therefore considered to be a consequence of partial depurination and subsequent breakage of phosphodiester bonds rather than a consequence of the presence of phosphorothioate groups in the DNA.

The most important question arising from these experiments is that of sequence fidelity in phosphorothioate containing DNA. Two lines of evidence allow the conclusion that the degree of fidelity is probably very high. First, we calculated the number of nucleotides incorporated into fd-RF with DNA polymerase I under conditions where a defined number of native DNA circles was completely converted into RF. A comparison of the amount of [^{3}H]dTTP incorporated in the presence of dATP and dATPαS, respectively, gave almost identical values for normal fd-RF and phosphorothioate fd-RF (Table II). The difference of 3% is within the accuracy of the test system. In addition, we measured the number of dAMPS incorporations into DNA using [^{35}S]dATPαS. Based on the number of single-stranded template molecules used (1.72×10^{12}), we calculated that the number of ^{35}S-labeled nucleotides incorporated was some 93% of the number expected for dAMP, a close agreement.

The second line of evidence comes from preliminary experiments obtained with various restriction enzymes (Vosberg and Eckstein, unpublished results). From the data available, it is apparent that a modification in a nucleotide which does not occur in a sequence recognized by any of the restriction endonucleases tested (*hae*III, *hpa*II, and *hin*dII) has little if any effect on the rate and the products of endonucleolytic action. Substitution of a nucleotide at the cleavage site, however, significantly decreases the rate of cleavage and results in intermediate fragments which are only gradually converted into the normally observed end products of restriction. These results were obtained by polyacrylamide and agarose gel electrophoresis. From the fact that the modification at the cleavage site predominantly affects the rate of nucleolytic action, but not the size of the end products, we tentatively conclude that mismatching due to the erroneous incorporation of nucleotides in the presence of deoxynucleoside phosphorothioates is at least not a frequent event. Whether it occurs at all, and if so, how frequently, requires further studies.

DNA or RNA molecules modified by incorporating chemically altered nucleosides or nucleotides are used for different purposes. 5-Iodo-5′-amino-2′,5′-dideoxyuridine, for instance, appears to be a highly selective antiviral agent (Chen et al., 1976). 5′-Amino-5′-deoxythymidine 5′-triphosphate can be incorporated into DNA in vitro resulting in a high acid lability of DNA regions containing phosphoroamidate (P–N) bonds (Letsinger et al., 1976). The introduction of a heavy metal derivative into DNA (or RNA) has been achieved using 5-mercuripyrimidine nucleoside 5′-triphosphates. These compounds (HgUTP and HgCTP) were found to be substrates for a variety of nucleic acid polymerases (Dale et al., 1973) and have been successfully applied for a selective fractionation of polynucleotides (Dale and Ward, 1975). This method is restricted to pyrimidine derivatives and therefore does not lend itself for possible sequencing by electron microscopy.

The deoxynucleoside phosphorothioates we describe here can largely be commended for three principal reasons. First, in contrast to any modification used so far, identically modified nucleoside phosphorothioates are available for all four deoxynucleotides. Second, the modification does not affect the bases and therefore probably does not interfere with the base-pairing mechanism in double-stranded DNA. Third, from the results presented we tentatively suggest that the sulfur has only indirect and little, if any, influence on the intramolecular forces stabilizing the DNA duplex. The location of the sulfur on the periphery of the DNA helix should provide an easily accessible

target for the attachment of heavy metal atoms to the DNA as a first step in the development of a physical method to sequence DNA.

Acknowledgment

We wish to thank Drs. H. Schaller for supplying ^{14}C-labeled native fd DNA and R. Herrmann for performing infectivity tests with Ca^{2+}-treated *E. coli* cells and Miss I. Erdmann for technical assistance.

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