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Biochemistry

The benzene metabolite *p*-benzoquinone forms adducts with DNA bases that are excised by a repair activity from human cells that differs from an ethenoadenine glycosylase

(DNA repair/exocyclic adducts/3,N^4-benzethenocytosine/1,N^6-benzethenoadenine)

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ABSTRACT **Benzene is a ubitiquous human environmental carcinogen. One of the major metabolites is hydroquinone, which is oxidized *in vivo* to give *p*-benzoquinone (*p*-BQ). Both metabolites are toxic to human cells. *p*-BQ reacts with DNA to form benzetheno adducts with deoxycytidine, deoxyadenosine, and deoxyguanosine. In this study we have synthesized the exocyclic compounds 3-hydroxy-3,N^4-benzetheno-2′-deoxycytidine (*p*-BQ-dCyd) and 9-hydroxy-1,N^6-benzetheno-2′-deoxyadenosine (*p*-BQ-dAdo), respectively, by reacting deoxycytidine and deoxyadenosine with *p*-BQ. These were converted to the phosphoamidites, which were then used to prepare site-specific oligonucleotides with either the *p*-BQ-dCyd or *p*-BQ-dAdo adduct (pbqC or pbqA in sequences) at two different defined positions. These oligonucleotides were efficiently nicked 5′ to the adduct by partially purified HeLa cell extracts—the pbqC-containing oligomer more rapidly than the pbqA-containing oligomer. In contrast to the enzyme binding to derivatives produced by the vinyl chloride metabolite chloroacetaldehyde, the oligonucleotides up to 60-mer containing *p*-BQ adducts did not bind measurably to the same enzyme preparation in a gel retardation assay. Furthermore, there was no competition for the binding observed between oligonucleotides containing 1,N^6-etheno A deoxyadenosine (1,N^6-etheno-dAdo; εA in sequences) and these oligomers containing either of the *p*-BQ adducts, even at 120-fold excess. When highly purified fast protein liquid chromatography (FPLC) enzyme fractions were obtained, there appeared to be two closely eluting nicking activities. One of these enzymes bound and cleaved the εA-containing deoxyoligonucleotide. The other enzyme cleaved the pbqA- and pbqC-containing deoxyoligonucleotides. One additional unexpected fact was that bulk *p*-BQ-treated salmon sperm DNA did compete effectively with the εA-containing oligonucleotide for protein binding. This raises the possibility that such DNA contains other, as-yet-uncharacterized adducts that are recognized by the same enzyme that recognizes the etheno adducts. In summary, we describe a previously undescribed human DNA repair activity, possibly a glycosylase, that excises from DNA pbqC and pbqA, exocyclic adducts resulting from reaction of deoxycytidine and deoxyadenosine with the benzene metabolite, *p*-BQ. This glycosylase activity is not identical to the one previously reported from this laboratory as excising the four etheno bases from DNA.**

It has been well established that benzene is carcinogenic to humans, generally in the form of acute leukemia (1), and causes bone marrow toxicity, although other malignancies are also reported. Exposure to benzene affects a large proportion of the population in the world, since it occurs in the environment in automobile exhaust, cigarette smoke, and, most commonly, in industries such as rubber manufacture, refineries, and chemical manufacturing (1).

Although benzene's biological effects have been documented for many years, the responsible metabolite(s) have not been unambiguously identified. Furthermore, although there are many recent studies on metabolism (2–5), research has not yet linked structure and mutation, the accepted first step in carcinogenesis.

One of the biologically important final metabolites of benzene is *p*-benzoquinone (*p*-BQ), which is an oxidation product of hydroquinone (Fig. 1). Both produce the same adducts *in vivo* (6). *In vitro*, the chemically characterized DNA adducts are all exocyclic. They resemble those produced by vinyl chloride *in vivo* (7–9) and chloroacetaldehyde *in vitro* (10, 11), although the *p*-BQ adducts are bulkier.

Reaction of *p*-BQ with deoxycytidine or deoxyadenosine resulted in 3-hydroxy-3,N^4-benzetheno-2′-deoxycytidine (*p*-BQ-dCyd) (12) and 9-hydroxy-1,N^6-benzetheno-2′-deoxyadenosine (*p*-BQ-dAdo) (13) (Fig. 2). Jowa *et al.* in 1990 (14) reported at least two adducts from reaction of *p*-BQ with [^{3}H]deoxyguanosine. One was identified as 7-hydroxy-1,N^2-benzetheno-2′-deoxyguanosine. Reaction with DNA produced the same adducts, but additional products that were detected were not identified (12–15).

The possible mutagenic effects or the repair of these DNA adducts *in vivo* and *in vitro* has not been elucidated, although it has been shown that benzene metabolites are cytotoxic and leukemogenic in human cells and in benzene-exposed populations (1). Thus, studies on both mutation and repair *in vitro* of modified bases from reaction of DNA with benzene metabolites are warranted.

An analogous carcinogen forming similar exocyclic adducts is vinyl chloride (16, 17). The four known cyclic derivatives have been studied in terms of mutation (18–20) and repair (16, 21, 22) by using site-specifically placed single adducts in defined oligonucleotides. In the present work, we have followed the same approach to study repair of both deoxyadenosine and deoxycytidine adducts resulting from reaction with *p*-BQ; in DNA sequences the notation for these adducts is pbqA and pbqC, respectively. We report that partially purified human cell extracts produce nicks 5′ to the adducts in defined oligonucleotides, but that this repair activity, obtained after

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Abbreviations: FPLC, fast protein liquid chromatography; *p*-BQ, *p*-benzoquinone; *p*-BQ-dCyd, 3-hydroxy-3,N^4-benzetheno-2′-deoxycytidine; *p*-BQ-dAdo, 9-hydroxy-1,N^6-benzetheno-2′-deoxyadenosine; pbqC and pbqA, *p*-BQ-dCyd and *p*-BQ-dAdo in nucleotide pbqA, sequences; εA, 1,N^6-ethenodeoxyadenosine.

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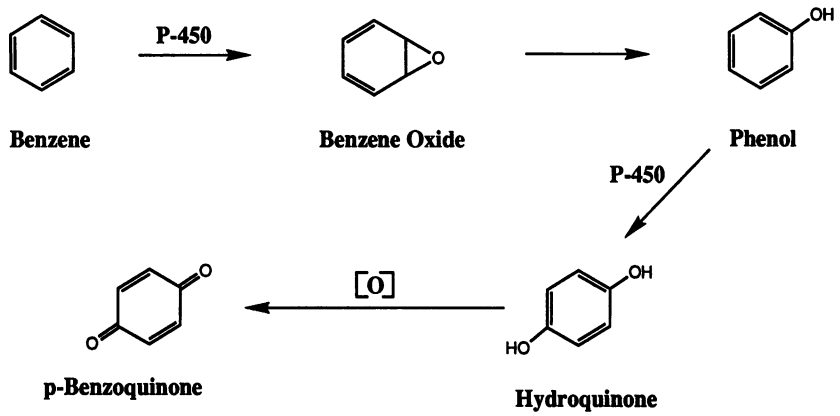


FIG. 1. Partial scheme of metabolic conversion of benzene to yield *p*-BQ.

fast protein liquid chromatography (FPLC) purification, appears to differ from that which excises the etheno derivatives.

MATERIALS AND METHODS

p-BQ was purchased from Aldrich and used without further purification. The synthesis of the adducts formed from 2′-deoxycytidine and 2′-deoxyadenosine and their phosphoramidites will be the subject of a separate paper, as will be the oligonucleotide synthesis and purification (25). All oligonucleotides containing modified bases were HPLC purified to homogeneity. The oligonucleotide containing 1,N^6-ethenodeoxyadenosine (1,N^6-etheno-dAdo; εA in sequences) was a gift from Ashis Basu (University of Connecticut, Storrs). [γ-^{32}P]ATP (specific activity, 6000 Ci/mM; 1 Ci = 37 GBq) was purchased from Amersham. Gel electrophoresis was performed with a Hoefer Poker Face apparatus. All reagents for electrophoresis were of the electrophoresis grade and purchased from Sigma. HeLa cells were purchased from Cell Culture Center, Endotronics Inc., Minneapolis, a National Institutes of Health-sponsored facility, and *Escherichia coli* DNA ligase was from Amersham. FPLC was performed with a Pharmacia/LKB system.

Synthetic Oligonucleotides. Five synthetic oligonucleotides were as follows: oligomers **1** and **2** containing pbqC (5′-CCGCTAGpbqCGGGTACCGAGCTCGAAT-3′ and 5′-CCGCTAGCGGGTApbqCCGAGCTCGAAT-3′), oligomers **3** and **4** containing pbqA (5′-CCGCTpbqAGCGGGTACCGAGCTCGAAT-3′ and 5′-CCGCTAGCGGGTpbqACCGAGCTCGAAT-3′), and oligomer **5** containing εA (5′-CCGCTεAGCGGGTACCGAGCTCGAAT-3′). Each of the above oligomers was annealed to a complementary 25-mer oligonucleotide. The base opposite pbqC was deoxyguanosine (pbqC·G) and opposite pbqA was thymidine (pbqA·T). Oligomer **5** was annealed to one with thymidine opposite εA (εA·T). The 5-mer, 7-mer, 12-mer, and 13-mer used as markers were machine synthesized.

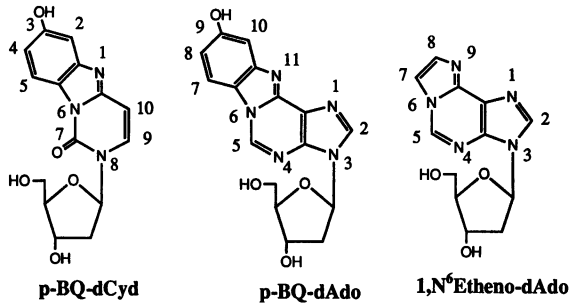
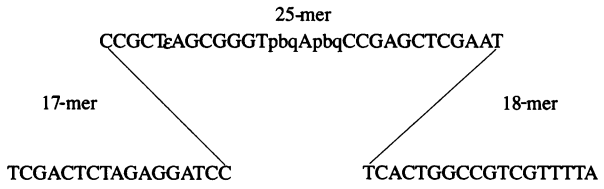


FIG. 2. Chemical structures of p-BQ-dCyd and p-BQ-dAdo compared to that of 1,N^6-etheno-dAdo.

Construction of 60-mer Oligonucleotides. Four 25-mer oligonucleotides were ligated to a 17-mer and an 18-mer at the 5′ and 3′ ends, respectively, to make 60-mer DNAs that are part of M13p19 with an inserted hexamer in the polylinker region. The whole sequence is shown below:



Each ligation contained either pbqC, pbqA, or εA or the unmodified base.

The synthesized and deprotected 17-mer and 18-mer and the complementary 60-mer were 5′-phosphorylated and gel purified. The 25-mers, which are identical to those used in this work, were also 5′-phosphorylated by using unlabeled ATP. For the preparation of 5′-end-labeled DNA, 160 pmol of the 17-mer was end-labeled with [γ-^{32}P]ATP and polynucleotide kinase. Forty picomoles of this labeled 17-mer was mixed in a buffer containing 10 mM Tris·HCl (pH 7.6), 0.6 mM EDTA, and 25 mM NaCl with equal molar concentrations of each of the others, except that a 3-fold excess of the 25-mer oligomers was included. The oligomers were annealed by heating at 95°C for 3 min and then slowly cooled to room temperature. Ligation reactions were carried out at 16°C for 15 hr with *E. coli* DNA ligase. The full-length ligated products were purified through a 12% denaturing gel. The DNA duplexes were obtained by reannealing the single strands under the above conditions.

Preparation of *p*-BQ-Treated DNA. Calf thymus DNA (0.5 mg) was incubated with 0.5 mg of hydroquinone in 550 μl of 20 mM ammonium formate (pH 7.4) containing 1.5 mM $FeCl_3$ at 37°C for 3 days. The reacted DNA was precipitated with 50 ml of 4 M sodium acetate and 1 ml of cold ethanol and centrifuged. The pellet was washed with cold ethanol and dissolved in 400 μl of 0.015 M NaCl/0.0015 M sodium citrate, pH 7.

The relative DNA adduct level was determined as 3.3×10^{-4}—the average value of three determinations with ^{32}P-postlabeling (14, 15). The distributions of the cyclic adducts are as follows: 1.7% cyclic deoxyguanosine adduct, 70.3% of cyclic deoxycytidine adduct, and 25.1% of cyclic deoxyadenosine adduct.

Fractionation of the Binding Activity from HeLa Cells. The cell-free extracts (fraction I) were ammonium sulfate-

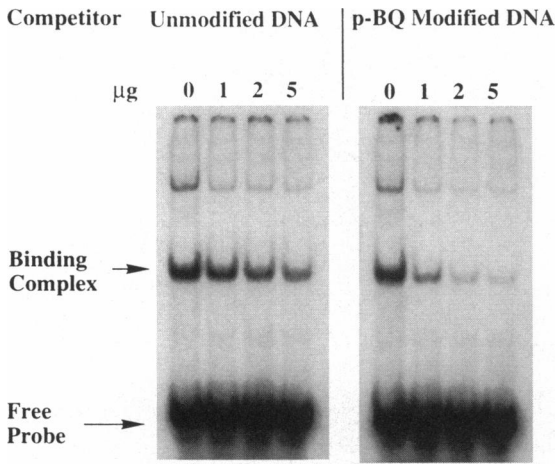


FIG. 3. Autoradiogram showing the effect of p-BQ-modified DNA or control DNA on binding of an εA-containing oligonucleotide. (*Left*) Competition with unmodified salmon sperm DNA. (*Right*) Competition with p-BQ-modified DNA. DNA (0–5 μg as shown at the top of lanes) was added to the reaction mixture containing oligomer and HeLa cell extract. Arrows indicate the position of the binding complex and the free probe. The gel assay procedure follows that of Rydberg *et al.* (21).

precipitated (fraction II), desalted, and passed through a Whatman P11 phosphocellulose column as described by Rydberg *et al.* (22) (fraction III). The fractions showing binding to the εA·T probe were pooled and sequentially twice fractionated on a double-stranded DNA-cellulose column eluted with a KCl gradient of 0–100% containing per liter 25 ml of 1 M Tris·HCl (pH 7.8), 0.3 ml of 2-mercaptoethanol, 2 ml of 0.5 M EDTA, 1 ml each of 1 M benzamidine and 0.5 M phenylmethanesulfonyl fluoride (PMSF), and 100 ml of glycerol. The peak fractions of binding activity toward the εA·T probe were pooled from the first column (fraction IV) and applied to the second column. This protein (fraction V) was used in binding studies.

FPLC Purification of Partially Purified Nicking Activity from HeLa Cells. Prior to FPLC purification, HeLa cell extract was taken from the P11 phosphocellulose fractions containing the highest activity for εA·T binding (fraction III). These were pooled, concentrated, and desalted by using Amicon concentrators (Centricon-10; molecular weight cutoff of 10,000). The fractions were then collected, diluted with buffer A [25 mM Hepes adjusted with KOH to pH 7.0/0.5 mM EDTA/0.125 mM phenylmethanesulfonyl fluoride/3 mM 2-mercaptoethanol/10% (vol/vol) glycerol], and directly loaded onto a 1-ml Pharmacia FPLC Resource S column equilibrated in buffer A. The column was run at 1 ml/min with a linear gradient from 0 to 0.5 M KCl in buffer A. All fractions (2 ml) were stored at −70°C. All fractions were assayed for nicking of oligonucleotides containing εA or pbqC or pbqA. The peak of activity for εA was at 18–20% KCl (fractions 11 and 12). The peak of activity for pbqC and pbqA was at 6–14% KCl (fractions 5–9). Binding to the enzyme with the εA probe was found only in fractions 11 and 12.

RESULTS

Evidence of *p*-BQ-Treated DNA Binding to a Glycosylase from HeLa Cells. The *p*-BQ-modified DNA was used as a competitor for binding by a 5′-^{32}P-labeled 25-mer with εA at position 6, annealed to a complementary 25-mer with T opposite (21). The binding assay has been described by Rydberg *et al.* (21). Unmodified DNA (Fig. 3 *Left*) does not significantly decrease the ^{32}P binding band of the oligonucleotide to the protein when up to 5 μg of unmodified DNA was used. In contrast, the *p*-BQ-modified DNA competes effectively for binding under the same conditions (Fig. 3 *Right*).

Enzymatic Assay. The procedure for measuring binding followed that of Rydberg *et al.* (21). Quantitation of nicking (21) was in the presence of purified T4 endonuclease V at 0.1 μg/ml to cleave abasic sites resulting from enzymatic removal of a base.

Cleavage of pbqC-Containing Oligonucleotides. The two oligomers containing pbqC differed in the position of the modified base (see *Materials and Methods*). Both oligomers **1** and **2** were cleaved by the partially purified HeLa cell extract (fraction II) containing endonuclease V more effectively than was the εA-containing oligomer **5** (Fig. 4). Oligomer **1** was cut 5′ to the adduct, leaving a 7-mer, whereas oligomer **2** was cleaved to form a 13-mer (Fig. 4 *Center* and *Right*). The oligomers with the adduct in the middle (**2** and **4**) were cleaved less efficiently than those with the adduct closer to the 5′ end. A time course of the rates of cleavage of oligomers **1**, **2**, and **3** is shown in Fig. 5. Note that this figure presents the data as percent nicking.

Similar data for these oligomers were obtained when protein concentration was increased for 1 hr (Fig. 6). Oligomer **1** was maximally cleaved (≈70%) with 0.5 μg of protein, whereas oligomer **2** required 2.5 μg for ≈60% cutting. Surprisingly, the εA-containing oligomer was only cleaved to a maximum of ≈25% even with 5 μg of protein.

Cleavage of pbqA-Containing Oligonucleotides. The same protocol as used for the pbqC-containing oligomers was followed to measure the cleavage of pbqA-containing oligomers. Considerably more protein was required to visualize the cleaved ^{32}P-labeled oligomers. In Fig. 7 a similar scheme to that in Fig. 4 is shown, except that the protein concentration range is 3–30 μg rather than 0.1–5 μg, the range used for cleaving the pbqC-containing oligomer. Under these condi-

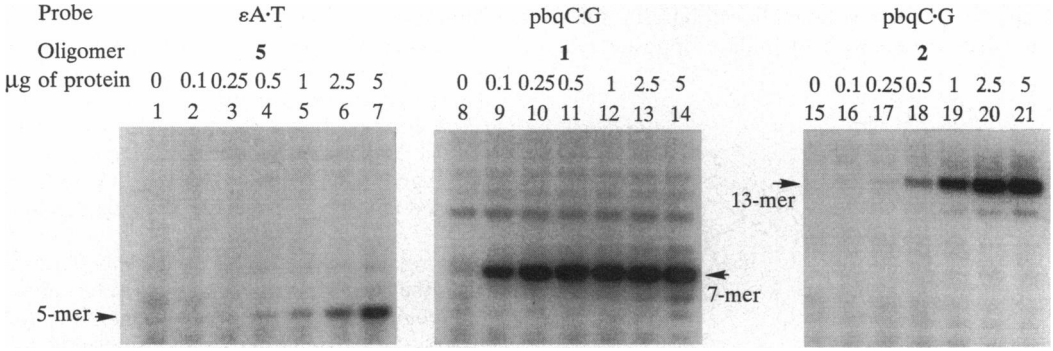


FIG. 4. Autoradiogram of gel electrophoresis of 5′-^{32}P-labeled 25-mer double-stranded oligonucleotides (16 fmol) after incubation for 1 hr with 0.1–5 μg of HeLa cell protein (fraction II). Oligomer **5** has εA at position 6 from the 5′ end so that a 5-mer is the product. Similarly oligomer **1** has pbqC at position 8, yielding a 7-mer; and oligomer **2** has the same adduct at position 14, yielding a 13-mer. The arrows show the position of the markers used.

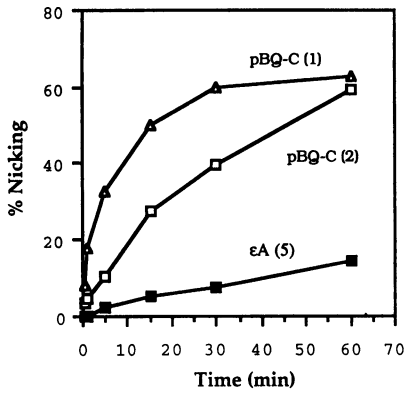


FIG. 5. Rate of nicking of the 25-mer double-stranded oligonucleotides containing εA (oligomer **5**) (■), pbqC (oligomer **2**) (□), (adduct position 14 from the 5′ end), and pbqC (oligomer **1**) (△) (adduct position 8 from the 5′ end). The protein concentration was 3 μg of HeLa cell-free extract (fraction II). The time course was 30 s to 1 hr. The gel (not shown) was scanned, and the data were plotted.

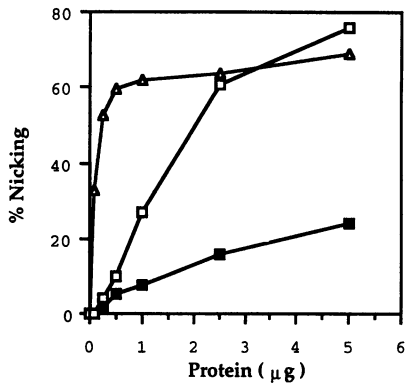


FIG. 6. Data from Fig. 4 plotted as a function of the amount of protein used. The symbols are the same as in Fig. 5. The data were obtained from densitometry of the radioactive bands.

tions, the εA-containing oligomer (Fig. 7 *Left*) is maximally cut with 15 μg of protein. The cleavage leaves a 5-mer as described by Rydberg *et al.* (21). The two oligomers containing pbqA are cut (Fig. 7 *Center* and *Right*), but much less so than those with εA or pbqC (Fig. 4). The pbqA-containing oligomers **3** and **4** showed that the cleavage was also 5′ to the adduct, thus leaving a 5-mer (oligomer **3**) or a 12-mer (oligomer **4**).

Binding Assay of pbqC- and pbqA-Containing Oligomers with Cell-Free HeLa Extracts. All of the oligomers listed as **1–5** were tested for binding to HeLa cell-free extracts (fraction II) by the previously described method (21). Only oligomer **5** (εA·T) led to the gel bond shift indicative of protein–nucleic acid binding. Variations of the method were attempted. The time of incubation prior to electrophoresis was decreased to as little as 30 sec, KCl was used instead of spermidine, and the temperature was decreased from 20°C to 4°C. None of these changes led to perceptible binding except with the εA-containing oligomer as control.

Since it seemed possible that the relatively small size of the oligonucleotides (25-mers) containing the bulky *p*-BQ adducts might interfere with the necessary annealing to form a double strand, 60-mers containing pbqC, pbqA, or εA were constructed with the modified base in the center position (*Materials and Methods*). These longer oligonucleotides would be presumed to form a more stable complex than the 25-mers. Again, no binding band was observed except with the 60-mer containing εA. However, all of the oligomers were cleaved 5′ to the adduct (data not shown). These results, together with the results with the 25-mers, indicated that poor annealing was probably not the major cause of the lack of binding. Unfortunately, the technique used for a binding assay cannot measure fast association–disassociation of the enzyme and substrate. It does appear that an enzyme–substrate complex is formed, since specific cleavage occurs only if the glycosylase is present.

DISCUSSION

Environmental agents leading to carcinoma in humans are inescapable. They include airborne chemicals as well as those generated by industry and normal human metabolism. Examples are aflatoxin, acrolein, *N*-nitroso compounds, many α,β-unsaturated carbonyl compounds, vinyl chloride, aromatic amines, etc. (1). Few of these have been well studied in terms of the repair of adducts formed by exposure of DNA. If DNA containing potentially mutagenic adducts is not repaired, the adduct can block replication (lethality) or miscode (mutation).

We have reported that the four identified vinyl chloride adducts are excised from oligonucleotides and DNA containing these exocyclic etheno bases (16). The glycosylase responsible is found in human cells and organs (21). The observations reported in this paper extend the range of substrates for human repair activity to DNA modified by a benzene metabolite, *p*-BQ. The two such derivatives in this study are also exocyclic compounds, *p*-BQ-dAdo and *p*-BQ-dCyd (Fig. 2). They resemble structurally, in some respects, 1,N^6-etheno-dAdo (Fig. 2) and 3,N^4-etheno-dCyd (εC in sequences) (not shown). However, there are major differences in recognition of the *p*-BQ adducts by the partially purified human glycosylase or the FPLC-purified enzyme.

The most obvious is that the isolation of a binding complex of oligonucleotides containing site specifically placed *p*-BQ-dAdo (pbqA) and *p*-BQ-dCyd (pbqC) with the protein cannot be demonstrated under the same conditions where such binding is found for εA- or εC-containing oligonucleotides (16, 21). Modification of conditions for binding were tried unsuccess-

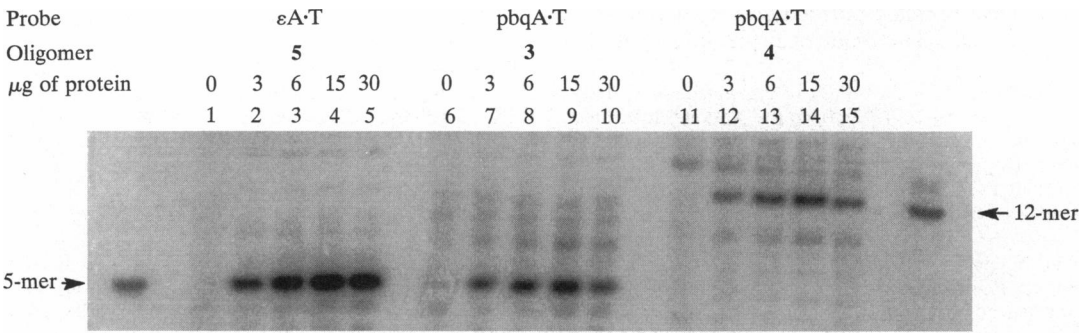


FIG. 7. Autoradiogram of nicking of 25-mer double-stranded oligonucleotides (16 fmol) as a function of protein concentration. (*Left*) Probe εA·T (oligomer **5**) yields a 5-mer. (*Center*) Probe pbqA·T (oligomer **3**) yields a 5-mer. (*Right*) Probe pbqA·T (oligomer **4**) yields a 12-mer. Note that the protein concentration ranges from 3 to 30 μg, whereas in Figs. 4 and 6 the range is 0.1–5 μg.

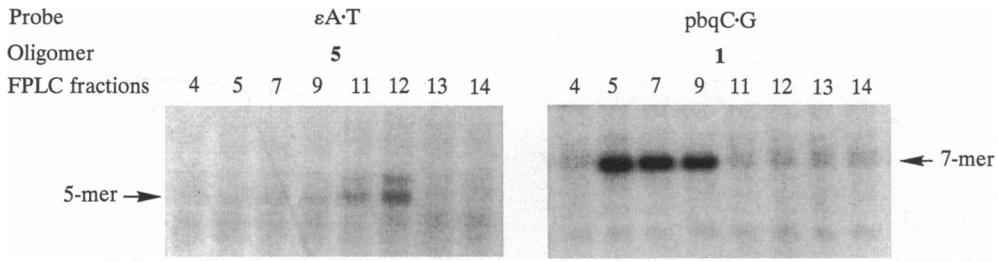


FIG. 8. Autoradiogram of nicking of εA·T (oligomer **5**) (*Left*) and pbqC·G (oligomer **1**) (*Right*), both 25-mer double-stranded oligonucleotides, when using purified fractions from the FPLC column (see *Materials and Methods*). Individual fractions were tested for their activity against both oligomers. The peak of activity for εA·T was at 18–20% KCl and for pbqC·G was 6–14% KCl. The only protein binding was coincident with the nicking of εA·T (data not shown).

fully. In addition no binding was detected with 60-base-long oligonucleotides containing these adducts. Thus, it appears that the size of the DNA is not a critical factor.

However, unexpectedly, there was efficient nicking of four different oligonucleotides containing pbqA or pbqC. In the case of pbqC, the nicking was several times faster than that of excision of εA and required 1/50th as much enzyme (Figs. 5 and 6). When pbqA-containing oligomers were used, they were also nicked, but at a slower rate than the excision of εA. It appears that enzyme recognition depends on factors such as structural details or conformation of the adduct but not of the oligonucleotide *per se*, since the same 25-mer sequence was used for all adducts.

The most tantalizing aspect of repair of the closely related adducts from metabolites of vinyl chloride and benzene is that there is evidence from our protein purification using FPLC that there are two nicking activities (Fig. 8). Nicking of the εA-containing oligomer is in fractions 11 and 12 (Fig. 8 *Left*), whereas nicking of the pbqC-containing oligomer is in fractions 5–9 (Fig. 8 *Right*). If the human 3-methyladenine-releasing DNA glycosylase (EC 3.2.2.21) is responsible for excision of the etheno compounds, there must be another enzyme excising the benzene adducts. Although these results indicate the presence of two distinct repair activities, it is reasonable to consider that these results reflect the presence of isozymes in mammalian cells. This would be in accord with recent studies that identify differential specificities of recombinant alkylpurine DNA glycosylases (23) as well as production of two transcripts from the corresponding human gene (24). Similarly, isozymes containing differing primary and tertiary structures may display dissimilar kinetic parameters with respect to their individual DNA-binding properties.

Of the two major pathways of DNA repair in human cells, nucleotide excision–repair and base excision–repair, only base excision–repair involves nicking directly 5′ to the adduct, after removal of the base by a glycosylase. Although we do not present direct evidence for a released base or an abasic site, the nicking directly 5′ to the adduct is a strong indirect evidence for a glycosylase activity. We therefore tentatively propose that the enzyme(s) we have partially purified and studied in this paper is a DNA glycosylase. This requires further investigation.

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