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Repair of O^6-ethylguanine in DNA by a chromatin fraction from rat liver: Transfer of the ethyl group to an acceptor protein

(carcinogenesis/mutagenesis/DNA modification)

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ABSTRACT **Incubation of O^6-[^{3}H]ethylguanine-containing DNA with a rat liver chromatin fraction resulted in a decrease in the O^6-ethylguanine content of the DNA. Analysis of the products of this reaction showed that the ethyl group had been transferred from the O^6-ethylguanine to a protein acceptor. When the incubation mixture was separated on a cesium chloride gradient, the radioactivity removed from O^6-ethylguanine appeared in a low-density band. This material has been isolated and subjected to trypsin digestion and high-pressure liquid chromatography analysis; it was sensitive to trypsin and the digest contained new high-pressure liquid chromatography peaks characteristic of oligopeptides. Radioactive peaks from the trypsin digestion have been digested further to the amino acid level and have been shown to contain *S*-[^{3}H]ethylcysteine. Thus, we conclude that the repair activity in rat liver chromatin removes the ethyl group from O^6-ethylguanine and transfers it to a cysteine moiety contained in an acceptor protein.**

The somatic mutation theory of carcinogenesis assumes that the initial step in tumor formation is the production of a mutagenic lesion in DNA. Loveless (1) proposed that O^6-alkylguanine would be such a lesion, and subsequent studies have shown that this modified base causes mispairing in both replicative and transcriptive processes (2–4). Evidence is also accumulating that high levels of O^6-alkylguanine and other *O*-substituted bases correlate with tumor formation in carcinogen-treated animals (5).

Once formed, however, O^6-alkylguanine can be removed from DNA by a repair process. The disappearance of O^6-methylguanine from bacterial DNA was observed by Lawley and Orr several years ago (6). More recently, Cairns and coworkers have described the induction of an activity that removes this lesion from DNA in bacteria adapted to growth in the presence of a methylating agent (7–9). It was originally thought that O^6-alkylguanine might be released by a DNA glycosylase, but Olsson and Lindahl have recently shown that the alkyl group is transferred to a protein in *Escherichia coli* (10).

Disappearance of O^6-alkylguanine from DNA has also been noted in higher organisms, in which the persistence of this lesion seems to correlate with tumor formation (11–13). The rate at which DNA is replicated is important because repair must occur before replication in order for it to be effective (14). Tumor formation may depend on the outcome of what is essentially a race between replication and repair.

The repair of DNA containing O^6-methylguanine has been studied in eukaryotic cells by Pegg and his collaborators, who isolated an active fraction from rat liver homogenates (15). Renard and Verly have purified a chromatin factor from rat liver nuclei that removes O^6-ethylguanine from calf thymus DNA: this chromatin factor has a specific activity that is 50 times higher than the specific activity of proteins from whole liver homogenates (16).

After this discovery, our laboratories began to collaborate in an effort to elucidate the repair mechanism. Our original hypothesis was that O^6-ethylguanine was released in modified form or that the ethyl group was transferred to another site in DNA. Accordingly, we digested DNA that had been partially repaired by the chromatin factor and analyzed for the presence of modified nucleosides by high-pressure liquid chromatography (HPLC). It became apparent that the ethyl group had been transferred from O^6-ethylguanine to an entirely different structure, which we thought might be proteinaceous. These experiments have shown that the ethyl group is indeed transferred to cysteine in an acceptor protein in this mammalian system as well as in *E. coli* (10). The evidence for this conclusion is presented below.

MATERIALS AND METHODS

Materials. Enzymes for the digestion of DNA—venom phosphodiesterase, spleen phosphodiesterase, deoxyribonuclease I, and bacterial alkaline phosphatase—came from Worthington. Trypsin also came from Worthington, while porcine kidney leucine aminopeptidase, type V, was purchased from Sigma, and Pronase, a *Streptomyces griseus* protease, was obtained from Calbiochem.

Standard L amino acid markers and *S*-ethyl-L-cysteine were obtained from Sigma. γ-Ethyl-L-glutamate was purchased from Pfaltz & Bauer (Stamford, CT), and β-ethyl-L-aspartate was synthesized according to the method of Bergmann and Zervas (17). O^6-Ethyldeoxyguanosine (e^{6}dGuo) was synthesized from 6-chlorodeoxyguanosine and sodium ethoxide in a manner analogous to the synthesis of O^6-methyldeoxyguanosine described previously (3). The *o*-phthaldialdehyde used in preparing amino acid derivatives for chromatographic separation was obtained from Sigma. All other materials were from standard sources.

Calf thymus DNA was treated with [^{3}H]ethylnitrosourea [3 Ci/mmol; (1 Ci = 3.7×10^{10} becquerels); Institut des Radioéléments, Belgium] and partially depurinated to provide a DNA substrate that contained 16% of the radioactivity as O^6-[^{3}H]ethylguanine (16).

DNA Repair. Rat liver nuclei were purified and the chromatin was isolated as described by Thibodeau and Verly (18). Chromatin proteins were then separated according to the method of Renard and Verly (16); chromatin was treated with heparin-Sepharose and the resulting DNA–protein–heparin-Sepharose complex was extracted with a buffer containing 0.3

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Abbreviations: HPLC, high-pressure liquid chromatography; e^{6}dGuo, O^6-ethyldeoxyguanosine.

M KCl, 10 mM K_2HPO_4, and 10 mM Tris·HCl (pH 8.0). The extract was then dialyzed against a buffer containing 1 mM EDTA, 0.2 mM dithiothreitol, and 20 mM Tris·HCl (pH 8.0); final protein concentration was 250 μg/ml.

This extract was used to repair substrate DNA as follows. One volume of [^{3}H]ethylated DNA (1 mg/ml; 73,000 dpm of [^{3}H]e^{6}dGuo per ml) in 10 mM Tris·HCl/1 mM EDTA (pH 8.0), buffer was incubated with 7 vol of the protein solution for 2 hr at 37°C.

Cesium Chloride Gradient Centrifugation. The following solutions were layered successively in 5-ml tubes: 1.3 ml of 57.5% (ρ = 1.73 g/ml) CsCl in 0.15 M NaCl/15 mM sodium citrate/1 mM EDTA (pH 7.0) buffer; 1.3 ml of 39% (ρ = 1.40 g/ml) CsCl in the same buffer; and, finally, 2.4 ml of the incubation mixture adjusted to 1 M KCl.

These tubes were centrifuged for 24 hr at 10°C and 80,000 × *g* in an SW 65.1 rotor in a Beckman L5-65 centrifuge. The gradients were then collected by injecting Fluorinert FC 48 (Isco) through the bottom of each tube with an Isco gradient fractionator. Absorbance was monitored at 254 nm and 0.2-ml fractions were collected. The radioactivity of these fractions was measured on one gradient in each run. Low-density protein-containing fractions from the other gradients were pooled, dialyzed against 50 mM Tris/phosphate (pH 8.1), and then lyophilized to dryness. High-density fractions that contained DNA were pooled separately, dialyzed against 50 mM ammonium formate, and lyophilized.

DNA Digestion and Analysis. Purified lyophilized DNA from the cesium chloride gradient was dissolved in 0.5 ml of 100 mM Tris/acetate, (pH 4.5), containing 5 mM magnesium acetate, 8 μg of 2′-deoxycoformycin (an adenosine deaminase inhibitor), and 400 units of DNase I. The solution was incubated for 70 min at 37°C, and then the following ingredients were added: 40 μl of 1 M Tris·HCl (pH 8.9), containing 1 M $MgCl_2$; 0.4 unit of spleen phosphodiesterase; 6.4 unit of *E. coli* alkaline phosphatase; and 3.2 unit of snake venom phosphodiesterase. The pH was adjusted to 8.9 and the mixture was incubated for an additional 24 hr at 37°C and lyophilized to dryness.

The nucleosides from each digested DNA were dissolved in 200 μl of water, the pH was adjusted to 4.5 with dilute H_3PO_4, and e^{6}dGuo was added as a marker. The solution was then separated by HPLC on a 5 μm Excalibar octadecylsilane column. The products were eluted isocratically with 0.05 M KH_2PO_4 (pH 4.5), containing 10% (vol/vol) acetonitrile at 1.0 ml/min for 40 min, and then with a linear gradient of 10–50% acetonitrile in 0.05 M KH_2PO_4 at the same flow rate for the next 40 min. The eluate was monitored at 280 nm with a Perkin–Elmer LC-55 variable-wavelength detector and 0.5-ml fractions were collected for scintillation counting. These fractions were dissolved in 10 ml of Hydrofluor (National Diagnostics, Somerville, NJ) and their radioactivities were measured in a Beckman LS-100 scintillation counter. Recovery of radioactivity from the column averaged 100%.

Protein Digestion and Identification of *S*-Ethylcysteine. The lyophilized desalted material from the low-density region of the cesium chloride gradient dissolved with difficulty and was not eluted from a reverse-phase HPLC column under conditions that elute oligopeptides. Therefore, this fraction was digested by trypsin. Typically, a lyophilized fraction from a cesium chloride gradient containing 1900 cpm was redissolved in 1 ml of 50 mM Tris/PO_4 (pH 8.1). Then, 125 μl of a 1-mg/ml trypsin solution and 50 μl of a 0.2 M $CaCl_2$ solution were added. After mixing, 100 μl was withdrawn and adjusted to pH 2.5 with H_3PO_4 for HPLC analysis. The remainder of the solution was incubated for 24 hr at 37°C and then adjusted to pH 2.5 for analysis.

These samples were applied to a μBondapak C_{18} reverse-phase column (Waters) and were eluted with a 100-ml linear gradient of 0–50% acetonitrile in 0.01% H_3PO_4 (pH 2.5) at a flow rate of 2 ml/min. Eluate was monitored at 220 nm and 1-ml fractions were collected; 0.2 ml of each fraction was used to determine radioactivity. Fractions containing radioactive material were pooled, neutralized, and lyophilized for subsequent amino acid analysis. Approximately 90% of the radioactivity contained in the material subjected to trypsin digestion was eluted in these peaks.

This material was redissolved in 1 ml of 0.03 M Na_2HPO_4 (pH 7.4) containing 5% (vol/vol) ethanol, and 10 μg (0.75 unit) of Pronase was added (19). This mixture was incubated for 24 hr at 32°C, and then 1 mg (28 units) of aminopeptidase was added and incubation was continued for an additional 24 hr at 37°C (20).

The above treatment resulted in approximately 80% digestion to the amino acid level, so the solution was applied to a Sephadex G-10 column (1.3 × 25 cm) and eluted with water. The eluate was monitored at 220 nm, and the enzymes and oligopeptides in the void volume were separated from the amino acid fractions, which were collected and lyophilized to dryness.

Identification of *S*-ethylcysteine in this amino acid fraction depended on chromatography by three separate modalities and comparison with authentic *S*-ethylcysteine. The digest was separated by: (*i*) chromatography on a μBondapak C_{18} column after conversion to the *o*-phthaldialdehyde derivatives; (*ii*) chromatography on a Partisil SCX column (Whatman); and (*iii*) paper chromatography.

Derivatization with *o*-phthaldialdehyde was performed according to Lindroth and Mopper (21). *o*-Phthaldialdehyde reagent (40 μl) was added to 50 μl of the amino acid digest, which contained approximately 500 cpm. *S*-Ethylcysteine was added as marker, and 4 min were allowed for derivatization to occur at room temperature. The derivatized amino acids were then separated on a μBondapak C_{18} column eluted isocratically at a flow rate of 1 ml/min with 0.05 M KH_2PO_4 (pH 7.2) containing 18% acetonitrile. Fractions (0.5 ml) were collected and their radioactivities were measured while the position of *o*-phthaldialdehyde markers was determined by monitoring for fluorescence with a Laboratory Data Control fluorometer. This was equipped with a 254-nm excitation filter and a 300- to 400-nm emission filter.

Amino acids could also be separated directly with somewhat less resolution on a Partisil SCX (strong cation exchange) column eluted isocratically with 0.2 M $NH_4H_2PO_4$ (pH 2.5) at a flow rate of 0.8 ml/min. Fractions were collected every 0.3 min and their radioactivities were measured. Because *S*-ethylcysteine did not separate widely from two other ethylated amino acids, ethylaspartate and ethylglutamate, this chromatography was performed on a sample of amino acids that had been heated to 100°C for 7 min at pH 7.8. Under these conditions, [^{3}H]ethyl groups attached to ethylaspartate or ethylglutamate would be released as ethanol, whereas *S*-ethylcysteine is stable.

Paper chromatography was performed on Whatman 3 MM paper in *n*-butyl alcohol/pyridine/water (2:2:1, vol/vol). Solvent was allowed to descend 35 cm, and 1.0-cm strips were cut and their radioactivities were measured.

RESULTS

Following our original premise that [*ethyl*-^{3}H]e^{6}dGuo had been altered to some other nucleoside by the chromatin fraction, we began our investigation by analyzing the radioactivity in incubation mixtures that included both substrate [^{3}H]ethyl-DNA and chromatin proteins. Analysis of nuclease digests failed to reveal any new peaks of radioactivity that had the properties of

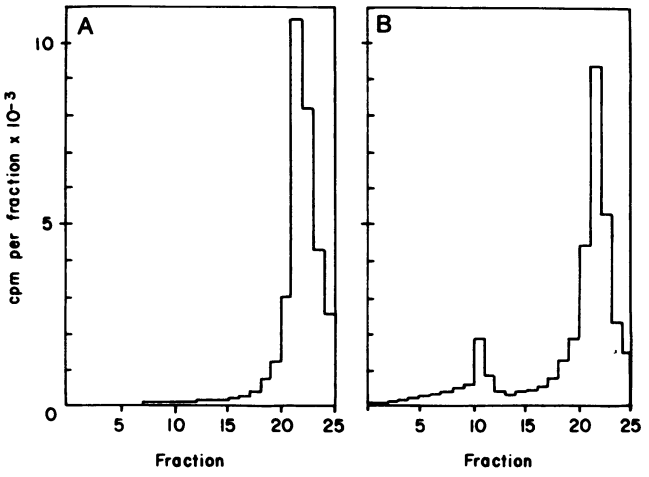

FIG. 1. Separation of [^{3}H]ethyl-DNA/chromatin protein incubation mixture by gradient centrifugation. A sample that had not been incubated (*A*) and a sample that had been incubated for 2 hr at 37°C (*B*) were layered over the top of a 1.40-g/ml layer of cesium chloride, which in turn was layered on top of a 1.73-g/ml layer. After centrifugation, 0.2-ml fractions were collected and their radioactivities were measured; in comparison with the nonincubated control, 10% of the radioactivity was found in the low-density region (left-hand side of figure) after centrifugation.

modified DNA bases or deoxynucleosides. However, a fraction of the radioactivity did behave as if it were associated with protein. Thus, it dissolved with difficulty even after nuclease treatment and was retained almost irreversibly on a μBondapak C_{18} column.

Consequently, incubation mixtures were subjected to cesium chloride centrifugation before further analysis. As shown in Fig. 1, incubation of the [^{3}H]ethyl-DNA substrate with chromatin protein resulted in a marked alteration in the distribution of radioactivity in the gradient. Before incubation, all of the radioactivity was located in the DNA region at a density of 1.73 g/ml; after incubation, 10% of this radioactivity was found at a density of 1.40 g/ml. Indeed, the radioactivity lost from the DNA region corresponded approximately to that which appeared in the low-density band. This was entirely consistent with our hypothesis that the ethyl group was now associated with a protein.

After it had been shown that the distribution of radioactivity on a cesium chloride gradient had changed after incubation, the next step was to characterize the radioactivity associated with each region in the gradient. As described in *Materials and Methods*, the material from the high-density region was collected and digested with a combination of nucleases and alkaline phosphatase. The resulting mixture of deoxynucleosides was separated on a μBondapak C_{18} column as shown in Fig. 2. The profile obtained before incubation with chromatin proteins revealed a variety of peaks including e^6dGuo at a position corresponding to the A_{280} marker. After incubation, this peak was greatly diminshed in size, but all other peaks remained approximately the same. It did not appear as if the radioactivity originally associated with e^6dGuo had become associated with a different nucleoside or base.

Next, we characterized the radioactivity that was found in the low-density region of the cesium chloride gradient after incubation of substrate DNA with chromatin proteins. The sensitivity of this material to trypsin digestion is shown in Fig. 3. An aliquot of this fraction was retained tenaciously on a μBondapak column before trypsin digestion, but the radioactivity appeared in a region typical of an oligopeptide after digestion. There were usually two peaks of radioactivity, although sometimes they merged together. In subsequent experiments designed to identify the amino acid that had accepted the ethyl group, radioactive material from both of these peaks was pooled.

This material proved to be relatively resistant to further digestion. By combining the action of Pronase and leucine aminopeptidase, however, most of the radioactivity could be shifted to the region typical of amino acids on a Sephadex G-10 column. Additional experiments showing that the ethyl group from e^6dGuo had become associated specifically with *S*-ethylcysteine were performed on material that had gone through the following preliminary purification procedures: cesium chloride gradient centrifugation, trypsin digestion and separation on a μBondapak C_{18} column, Pronase/aminopeptidase digestion, and separation on a Sephadex G-10 column.

Cysteine was identified as the ethyl group acceptor by showing that radioactivity had been transferred to a material having

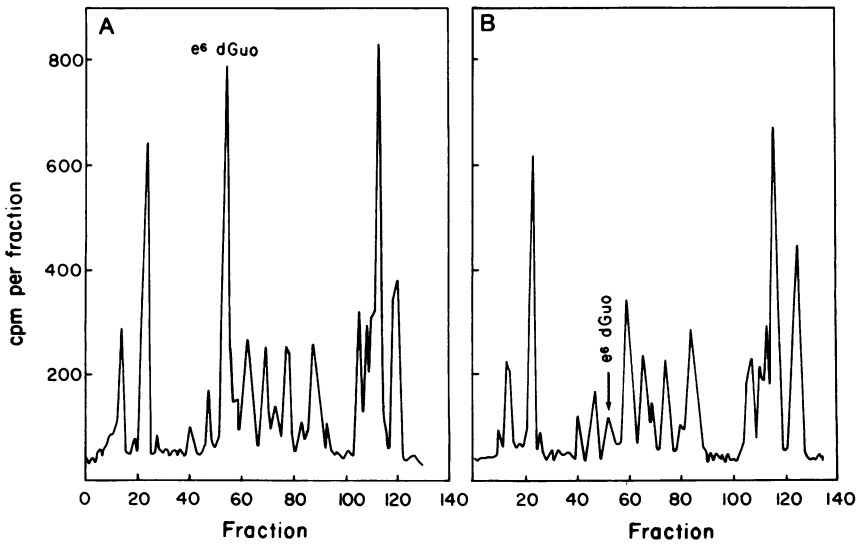

FIG. 2. Analysis of the high-density DNA peak from the cesium chloride gradient. DNA was collected from the high-density region, dialyzed to remove salt, and digested to component nucleosides, which were separated by HPLC. DNA from a control unincubated sample (*A*) shows a large peak of radioactivity corresponding to an e^6dGuo absorbance marker. This peak is much diminished in size after incubation with the chromatin proteins (*B*), but no new peak of radioactivity has appeared in the deoxynucleoside profile.

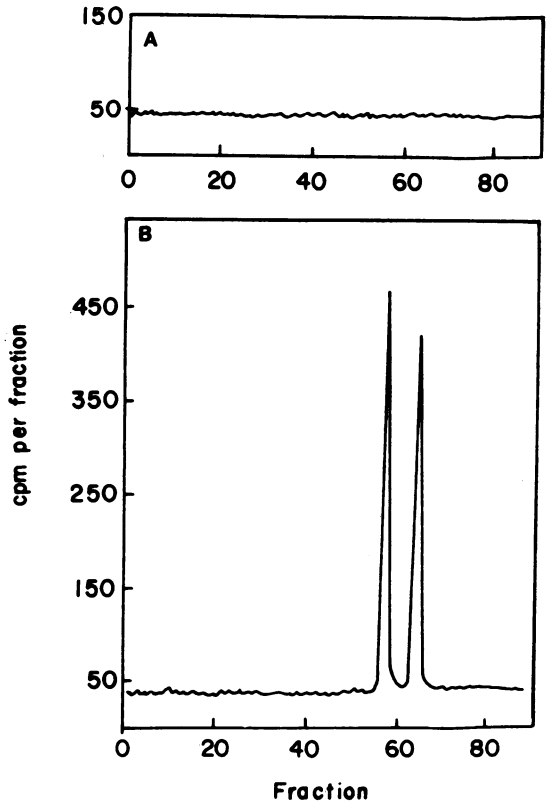

FIG. 3. Separation of material from the low-density band by HPLC. Aliquots from the cesium chloride gradient were applied to a reverse-phase column and eluted with a H_3PO_4/acetonitrile gradient. Before tryptic digestion (*A*), the material was completely retained on the column. After digestion (*B*) two sharp peaks of radioactivity appeared.

the chromatographic properties of *S*-ethylcysteine. Because the ω-carboxyl groups of aspartate and glutamate can also accept alkyl groups (22), authentic markers of ethyl glutamate and ethyl aspartate were also used as chromatographic markers.

Excellent separation of the amino acids was obtained on a μBondapak C_{18} column after derivatization with *o*-phthaldialdehyde (20). When this procedure was applied to the Pronase/aminopeptidase digest, most of the radioactivity was found in a region corresponding to the *o*-phthaldialdehyde derivative of *S*-ethylcysteine as shown in Fig. 4. Underivatized amino acids appeared in the front in this system, and the radioactivity found in the early fractions is probably associated with some underivatized ethylcysteine. None of the radioactivity, however, appeared at the positions corresponding to ethyl aspartate or ethyl glutamate.

The underivatized amino acids were also separated directly on a cation-exchange column. Because the separation from ethyl aspartate and ethyl glutamate was minimal, advantage was taken of the ease with which these two esters can be hydrolyzed to improve resolution. Heat treatment in a mildly basic solution completely hydrolyzes ethyl aspartate and ethyl glutamate, whereas *S*-ethylcysteine remains stable. Taking advantage of this fact, the chromatography shown in Fig. 5 was performed on material that had been heat treated. Again, most of the radioactivity appeared at a position corresponding to *S*-ethylcysteine.

Finally, an aliquot of the heat-treated Pronase/aminopeptidase digest was chromatographed on Whatman 3 MM paper as described in *Materials and Methods*. As shown in Fig. 6, radioactivity was again found associated with the *S*-ethylcysteine region. Thus, we conclude that the ethyl group originally found in e^6dGuo has been transferred to a cysteine acceptor.

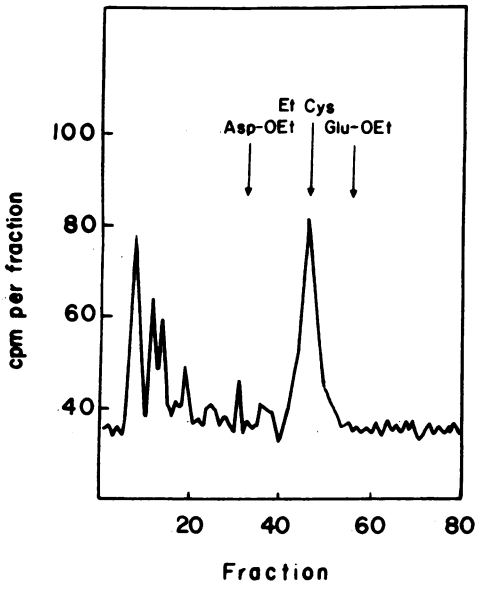

FIG. 4. HPLC separation of the *o*-phthaldialdehyde-derivatized amino acids from the fragment produced by trypsin digestion shown in Fig. 3*B*. Fractions containing radioactivity were pooled and digested to the amino acid level, derivatized with *o*-phthaldialdehyde, and separated on a reverse-phase column. Most of the radioactivity appeared in a position corresponding to the fluorescence from an *o*-phthaldialdehyde *S*-ethylcysteine marker.

DISCUSSION

In the years since Loveless suggested that alkylation of guanine in the 6 position was a mutagenic and procarcinogenic lesion, evidence has gradually accumulated that supports this hypothesis. This evidence, as well as information on the repair process, has been reviewed recently (5).

It is interesting that organisms as far apart on the evolutionary

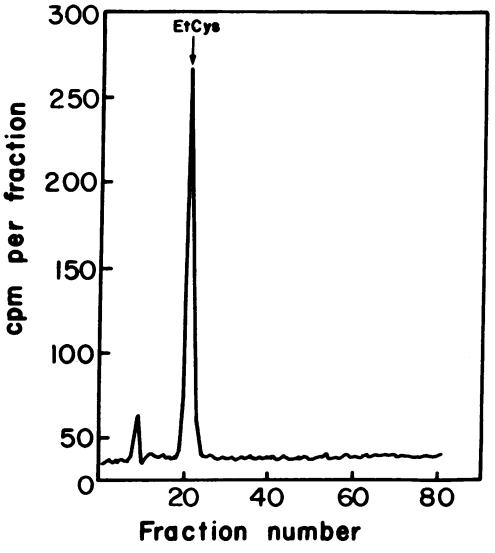

FIG. 5. HPLC separation of heat-treated amino acids obtained from digestion of the radioactive peak in Fig. 3*B*. An aliquot of the amino acid digest was heated to hydrolyze any ethyl aspartate or ethyl glutamate that might be present and then was applied to a strong cation exchange resin, which was eluted with 0.2 M $NH_4H_2PO_4$ (pH 2.5). Again, radioactivity appeared coincident with an absorbance marker of *S*-ethylcysteine.

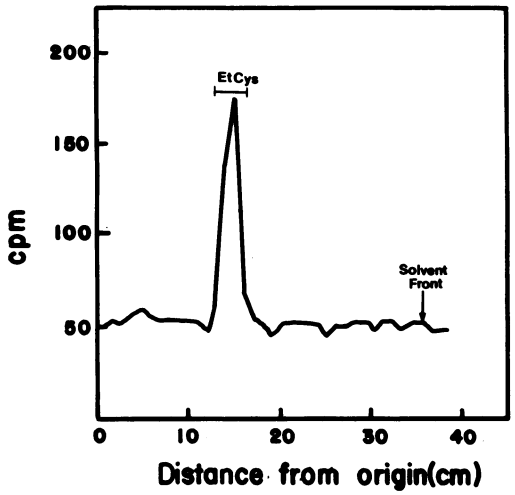

FIG. 6. Paper chromatogram of heat-treated amino acids obtained from digestion of the radioactive peak in Fig. 3*B*.

scale as bacteria and mammals both have mechanisms for removing this lesion from their DNA. In bacteria, this may prevent too high a mutation rate, whereas in mammals repair may serve as a defense mechanism against carcinogens, many of which are known to occur in nature.

Quite obviously, a detailed knowledge of this repair process in mammals might lead to methods of increasing the protection it affords. Even if the process cannot be controlled, its capacity might be evaluated and this knowledge used to assess the threshold level of exposure that could be tolerated.

The kinetics of repair by the rat liver chromatin fraction have been described (16, 23). The objective of this investigation was to determine how, biochemically, the O^6-ethylguanine was repaired.

DNA alkylated with labeled ethylnitrosourea was incubated with proteins from rat liver chromatin. Isopycnic separation of DNA and proteins followed by nuclease digestion of the DNA showed that the ethyl group from e^6dGuo had been transferred to a protein fraction (Figs. 1 and 2). Digestion of the protein fraction with proteases released radiolabeled *S*-ethylcysteine (Figs. 3–6), suggesting that the ethyl group from e^6dGuo had been transferred to an acceptor protein.

Because of the difficulty in digesting the acceptor protein completely, we cannot rule out the possible presence of another acceptor amino acid. However, most of the ethyl group removed from e^6dGuo has been transferred to cysteine.

This is a point of similarity between repair of O^6-alkyl-guanine in the bacterial and mammalian systems. Olsson and Lindahl (10) have shown that the methyl group is transferred from O^6-methyldeoxyguanosine to a protein acceptor in bacteria, whereas we have shown that the ethyl group is transferred from e^6dGuo to a protein in rat liver. However, we do not yet know how similar the bacterial acceptor is to the mammalian one, or whether the same acceptor is involved for both methyl and ethyl groups in either system.

Although it is clear that there are similarities between the bacterial and mammalian systems, there appear to be some differences as well. Activity in the mammalian system is evidently constitutive, whereas it must be induced in the bacterial system. Rates are higher in the bacterial system, but the extent of reaction appears to be limited by the stoichiometric consumption of some factor that is necessary for repair (9). In the mammalian system, the rate of repair does not appear to be limited in this way, but rather to be slowed down by product inhibition (23). The work of Olsson and Lindahl suggests that the same protein acts as a transferase and an acceptor of the methyl group in *E. coli* (10). The kinetic data obtained with the chromatin proteins indicates that the situation might be different in mammals. Further purification of all of the components of these systems will be necessary for a more detailed comparison.

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