

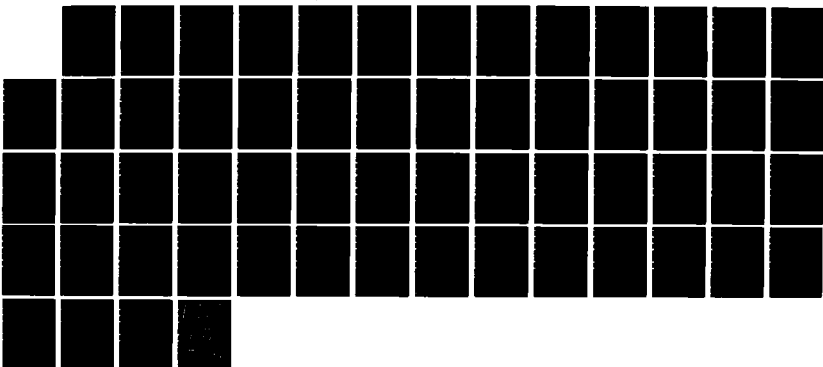
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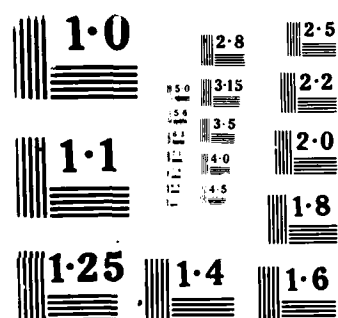
PROTECTION AGAINST THE ACUTE AND DELAYED TOXICITY OF
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Report No. 1

PROTECTION AGAINST THE ACUTE AND DELAYED TOXICITY
OF MUSTARDS AND MUSTARD-LIKE COMPOUNDS

ANNUAL SUMMARY REPORT

DAVID B. LUDLUM, PH.D., M.D.

SEPTEMBER 1983

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

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Albany Medical College
Albany, New York 12208

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) The toxicity of sulfur mustards is evidently related to the damage they inflict on cellular DNA. Previous studies have shown that much of the acute toxicity of bifunctional mustards can be attributed to DNA cross-linking through two guanine moieties in opposite DNA strands. Analysis of the mutagenic effects of these compounds has indicated that delayed toxicity, on the other hand, may be caused by substitution in the 6 position of guanine and perhaps by other minor DNA modifications as well. [Continued]		

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However, previous studies have failed to provide chemical evidence for reaction of the sulfur mustards with the 6 position of guanine. We have reexamined this problem using high pressure liquid chromatography to separate the products of the reaction between chloroethyl ethyl sulfide (CEES) and deoxyguanosine. Using this technology, we have shown conclusively that CEES does react with the 6 position of guanine to produce O⁶-ethylthio-ethyl deoxyguanosine, thereby providing a firm basis for the suggestion that this lesion could be responsible for the delayed toxicities of sulfur mustards.

Current literature suggests that this lesion would be repaired by an enzyme system present in both bacterial and mammalian cells. Enhancement of this repair activity should provide protection against the long-term toxicity, especially the genetic toxicity, of the sulfur mustards.

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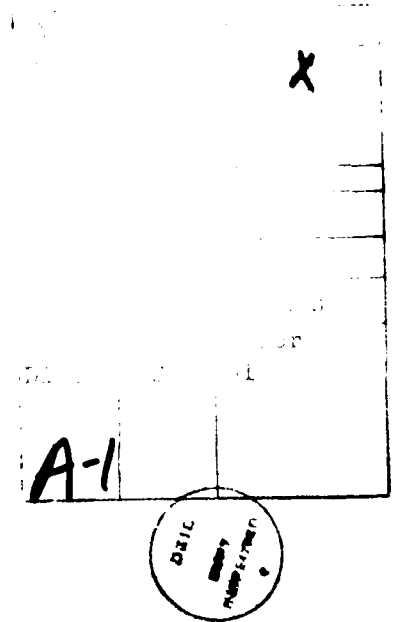
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SUMMARY

The toxicity of sulfur mustards is evidently related to the damage they inflict on cellular DNA. Previous studies have shown that much of the acute toxicity of bifunctional mustards can be attributed to DNA crosslinking through two guanine moieties in opposite DNA strands. Analysis of the mutagenic effects of these compounds has indicated that delayed toxicity, on the other hand, may be caused by substitution in the 6 position of guanine and perhaps by other minor DNA modifications as well.

However, previous studies have failed to provide chemical evidence for reaction of the sulfur mustards with the 6 position of guanine. We have reexamined this problem using high pressure liquid chromatography to separate the products of the reaction between chloroethyl ethyl sulfide (CEES) and deoxyguanosine. Using this technology, we have shown conclusively that CEES does react with the 6 position of guanine to produce O⁶-ethylthioethyl deoxyguanosine, thereby providing a firm basis for the suggestion that this lesion could be responsible for the delayed toxicities of sulfur mustards.

The structure of O⁶-ethylthioethyl deoxyguanosine has been established conclusively by a combination of ultraviolet and mass spectrometry, and by comparison with material synthesized by an unambiguous route. The compound dealkylates in acid pH so that previous attempts to isolate it from DNA presumably failed because it was destroyed at the pH which was used.

Current literature suggests that this lesion would be repaired by an enzyme system present in both bacterial and mammalian cells. Enhancement of this repair activity should provide protection against the long-term toxicity, especially the genetic toxicity, of the sulfur mustards. Thus, we are currently determining whether this repair system operates on O⁶-ethylthioethyl deoxyguanosine, the objective being to provide protection from toxicity associated with this lesion.

This work was presented at the Third Annual Bioscience Review held at Aberdeen Proving Ground in Edgewood, Maryland on June 2-3, 1983. A manuscript describing the formation of O⁶-ethylthioethyl deoxyguanosine has been submitted for publication in Cancer Research.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

TABLE OF CONTENTS

	Page
INTRODUCTION	6
MATERIALS AND METHODS.	7
SYNTHESIS OF O ⁶ -SUBSTITUTED GUANINE NUCLEOSIDES.	7
Table 1: High-Pressure Liquid Chromatography of Nucleoside Derivatives.	9
CHARACTERIZATION OF O ⁶ -ETHYLTHIOETHYL DEOXYGUANOSINE	10
Confirmation of Structure	10
Figure 1: Ultraviolet spectra of O ⁶ -ethylthioethyl deoxyguanosine in acidic, neutral, and basic solutions.	11
Figure 2: Mass spectrum of O ⁶ -ethylthioethyl deoxy- guanosine.	12
Chemical Stability.	13
Table 2: Stability of O ⁶ -Substituted Guanine Nucleosides.	14
Fluorescence of O ⁶ -Ethylthioethyl Deoxyguanosine.	13
Figure 3: Excitation and emission fluorescent spectra of O ⁶ -ethylthioethyl deoxyguanosine.	15
Figure 4: Calibration of the HPLC detector response for O ⁶ -ethylthioethyl deoxyguanosine	16
REACTION OF CHLOROETHYL ETHYL SULFIDE WITH DEOXYGUANOSINE.	17
Figure 5: High pressure liquid chromatography separation of a chloroethyl ethyl sulfide- deoxyguanosine reaction mixture.	18
Figure 6: Ultraviolet spectrum of O ⁶ -ethylthioethyl deoxyguanosine (peak 8) in the deoxy- guanosine-CEES reaction mixture shown in Figure 5	19

Table 3: Products of the Reaction of Chloroethyl Ethyl Sulfide with Deoxyguanosine. Separation by High Pressure Liquid Chromatography	21
REACTION OF CHLOROETHYL ETHYL SULFIDE WITH DNA	20
Figure 7: High pressure liquid chromatographic separation of a digest of a chloroethyl ethyl sulfide-DNA reaction mixture	24
Figure 8: Ultraviolet spectrum of the O ⁶ -ethylthioethyl deoxyguanosine peak identified in Figure 7.	25
PLAN	26
REFERENCES	27

APPENDIX

PREPARATION OF HEMI-SULFUR MUSTARD	28
Figure 9: Gas Chromatography of hydroxyethyl chloroethyl sulfide (hemi-sulfur mustard).	29
Figure 10: Mass spectrum of the hydroxyethyl chloroethyl sulfide (hemi-sulfur mustard) peak from the gas chromatographic run shown in Figure 9.	30
PREPARATION OF O ⁶ -ALKYLGUANOSINE	28
Figure 11: Purification of O ⁶ -ethylthioethyl guanosine by G-10 Sephadex chromatography.	32
Figure 12: Purification of O ⁶ -hydroxyethylthioethyl guanosine by G-10 Sephadex chromatography.	33
Table 4: Ultraviolet Spectra of O ⁶ -Alkylguanine Nucleosides.	34

CHARACTERIZATION OF OTHER MODIFIED NUCLEOSIDES IN CEES- DEOXYGUANOSINE REACTION MIXTURES.	31
Figure 13: Ultraviolet spectrum of Peak 0, Figure 5 . . .	35
Figure 14: Ultraviolet spectrum of Peak 1, Figure 5 . . .	36
Figure 15: Ultraviolet spectrum of Peak 2, Figure 5 . . .	37
Figure 16: Ultraviolet spectrum of Peak 3, Figure 5 . . .	38
Figure 17: Ultraviolet spectrum of Peak 4, Figure 5 . . .	39
Figure 18: Ultraviolet spectrum of Peak 5, Figure 5 . . .	40
Figure 19: Ultraviolet spectrum of Peak 6, Figure 5 . . .	41
Figure 20: Ultraviolet spectrum of Peak 7, Figure 5 . . .	42
Figure 21: Ultraviolet spectrum of Peak 8, Figure 5 . . .	43
Figure 22: Separation of 4 mg chloroethyl ethyl sulfide-deoxyguanosine reaction mixture on a G-10 Sephadex	45
Figure 23: High pressure liquid chromatographic separation of G-10 fraction A.	46
Figure 24: High pressure liquid chromatographic separation of G-10 fraction B.	47
Figure 25: Separation of 8 mg chloroethyl ethyl sulfide-deoxyguanosine reaction mixture on a G-10 column	48
Figure 26: High pressure liquid chromatographic separation of G-10 fraction G'	49
Figure 27: High pressure liquid chromatographic separation of G-10 fraction I'	50
Figure 28: High pressure liquid chromatographic separation of G-10 fraction K'	51
Figure 29: High pressure liquid chromatographic separation of G-10 fraction L'	52
Figure 30: High pressure liquid chromatographic separation of G-10 fraction M'	53
INITIAL DISTRIBUTION LIST.	54

INTRODUCTION

The sulfur mustards are well-known as potential chemical warfare agents. They are acutely toxic to the skin, respiratory tract, eyes, bone marrow, and, in large doses, to other organs as well. Delayed toxicity includes the induction of neoplasms which may occur years later. Substantial evidence has accumulated that many of these toxicities are related to the alkylating activity of sulfur mustards and, specifically, to alkylation of DNA (1).

Investigations of related compounds have led to the accumulation of considerable information about the nature of these DNA modifications (2). It has also been discovered that many cells possess the ability to repair DNA damage, but, until recently, almost nothing has been known about the biochemistry of DNA repair. However, recent studies in several laboratories including our own have led to the isolation of vital components in this repair process. Since repair of sulfur mustard-induced damage to DNA would presumably provide some protection against the toxicity of sulfur mustard, it is important to understand and to be able to facilitate this process.

In studying DNA repair, the nature of sulfur mustard-induced DNA lesions must be defined. Much of the cytotoxicity of sulfur mustards has been attributed to DNA crosslinking, but a wealth of biological data has suggested that alkylation of the 6 position of guanine might be responsible for mutagenesis, teratogenesis, and carcinogenesis (3,4). A major problem with this hypothesis has been the lack of chemical evidence that the sulfur mustards react with the 6 position of guanine (5,6).

We have reexamined this question and have shown conclusively that the sulfur mustards do modify DNA in this manner; information on this point constitutes the main body of this report. Observations concerning other modifications of DNA, as well as some supporting data on the O⁶ alkylation of guanine, are contained in an appendix.

Thus, our first objective, that of showing sulfur mustards alkylate the O⁶ position of guanine, has been achieved. In the course of reaching this objective, we have noted that DNA modification by the sulfur mustards is apparently a far more complex process than previously appreciated.

Our current objectives are focused on developing a quantitative assay for the enzymatic repair of the newly-described O⁶-ethylthioethyl deoxyguanosine lesion in DNA. If, as anticipated, this lesion is repaired by cellular extracts, it will then be possible to isolate and characterize the relevant repair factors.

MATERIALS AND METHODS

Chloroethyl ethyl sulfide was obtained from Dr. Bruno Papirmeister, USAMRICD. Sodium hydride for use in preparing hemi-sulfur mustard came from Alfa Division of the Ventron Corporation. Nucleosides were obtained from P-L Biochemicals. Calf thymus DNA, and the enzymes used for its digestion, were obtained from Worthington. Deoxycoformycin, an adenosine deaminase inhibitor, was supplied by the Division of Cancer Treatment, National Cancer Institute. Ethylhydroxyethyl sulfide and bis-hydroxyethyl sulfide (thioglycol) were obtained from Aldrich Chemical Co., Inc. All other chemicals came from standard sources.

High pressure liquid chromatography was performed on a modular apparatus consisting of a Milton-Roy 5000 psi minipump, a Rheodyne 7125 injector valve, an LDC Fluoromonitor III fluorometric detector, and a Hewlett-Packard 1040A detector system. This detector is interfaced with a Hewlett-Packard 85 computer with disc drive and is able to record spectra of derivative peaks as they are separated. Quantitation of either fluorescence or ultraviolet peaks was achieved with a Perkin-Elmer Sigma 10 data system.

The preparation of hemi-sulfur mustard and of the critical O⁶-substituted guanine nucleosides is described below.

SYNTHESIS OF O⁶-SUBSTITUTED GUANINE NUCLEOSIDES

The O⁶ derivatives of guanine which would be expected from the attack of sulfur mustards on DNA have never previously been reported. Nevertheless, we began this project with the supposition that high pressure liquid chromatography might enable us to detect these derivatives in DNA digests. The use of "marker" nucleosides, i.e., the O⁶-alkylguanine derivatives which would be expected from reaction with sulfur mustard, strengthens this approach since it allows us to focus attention on the region of the chromatograph where these derivatives would be eluted in a DNA digest. Accordingly, we began these studies with the synthesis of appropriate markers.

Attack by sulfur mustard (bis-chloroethyl sulfide) on the 6 position of guanine in DNA could yield two monosubstituted O⁶-alkylguanines, O⁶-chloroethylthioethyl guanine or O⁶-hydroxyethylthioethyl guanine. O⁶-chloroethylthioethyl guanine would be formed by the reaction of one arm of sulfur mustard with the O⁶ position of guanine while O⁶-hydroxyethylthioethyl guanine would be formed either by hydrolysis of the first adduct or by direct attack of hemi-sulfur mustard (chloroethyl hydroxyethyl sulfide) on DNA. Hemi-sulfur mustard would, of course, usually accompany sulfur mustard as a hydrolysis product.

When the difficulty in isolating the O⁶ nucleosides from DNA became apparent, we decided to concentrate on the derivative which would be obtained from the reaction of chloroethyl ethyl sulfide (CEES) with DNA. Chloroethyl ethyl sulfide has been widely used to study the mutagenic effects of sulfur mustard and has the additional advantage that it is more stable than hemi-sulfur mustard.

Work related to hemi-sulfur mustard is, therefore, described in the appendix. We will focus here on the reactions of CEES.

O⁶-substituted guanine nucleosides were synthesized by displacing the chlorine in chloroguanosine (6-chloro-2-amino-purine riboside) or chlorodeoxyguanosine (6-chloro-2-amino-purine deoxyriboside) with the appropriate alkoxide. Thus, O⁶-ethylthioethyl deoxyguanosine was synthesized as follows:

1. Ethyl-S-ethyl-OH + NaH → (ethyl-S-ethyl-O) Na + H₂
2. (Ethyl-S-ethyl-O) Na + 6-chlorodeoxyguanosine → O⁶-(ethyl-S-ethyl)-deoxyguanosine

Since 6-chlorodeoxyguanosine is not commercially available, it was synthesized as follows. Five grams of deoxyguanosine was reacted with an excess of acetic anhydride (15 ml) in pyridine (75 ml) for 3 days at room temperature. The product was filtered, washed with warm pyridine (50 ml) and then with dry ether (50 ml), and finally dried at 65° for 2 days before the next step. This procedure completely acetylates the deoxyribose sugar and protects it from chlorination.

Chlorination of the acetylated deoxyguanosine was accomplished by the method of Gerster et al. (7) with some modifications to avoid decomposition of the glycosidic linkage. All of the glassware was dried *in vacuo* at 75°, and the POCl₃ was freshly distilled immediately before use. Two grams of acetylated deoxyguanosine was added with stirring to a solution of POCl₃ (75 ml) and N,N-diethylaniline (2 ml) at room temperature. The suspension was heated until all of the solid was dissolved and a clear yellowish solution was obtained. (Note that any moisture present at this stage leads to decomposition of the entire deoxynucleoside content.) Excess POCl₃ was removed *in vacuo*, and the resulting syrup was stirred slowly into excess ice until hydrolysis was complete. Then, the solution was extracted three times with 100 ml of dichloromethane. Organic extracts were combined, washed three times with 100 ml of cold 1 N HCl, and finally with cold water until neutral. Then, the dichloromethane solution was dried over anhydrous magnesium sulfate and the solvent was evaporated under vacuum to leave a thin oil. When this oil was extracted with dry ether, amorphous 6-chloro-3',5'-di-O-acetyl deoxyguanosine was obtained.

This material was used without further purification for the synthesis of O⁶-substituted deoxyguanosine. Typically, approximately 5 mg of the acetylated 6-chlorodeoxyguanosine was dissolved in 0.5 ml of dimethyl formamide and mixed with an excess (approximately 100 mg) of ethylhydroxyethyl sulfide. Then, 10 mg of sodium hydride was added to convert ethylhydroxyethyl sulfide to ethylthioethyl alkoxide, and the reaction mixture was incubated at 37° for 18 hr. By this time, the alkoxide had displaced the chlorine in the 6 position of the acetylated deoxyguanosine and the acetate groups had been hydrolyzed from the 3' and 5' positions of the deoxyribose. The reaction mixture was dried *in vacuo* and the product was purified by high pressure liquid chromatography using System A described in the footnote to Table 1 below. A late, intensely fluorescent peak was identified as the expected product, O⁶-ethylthioethyl deoxyguanosine, as described in the next section.

Table 1. High-pressure liquid chromatography of nucleoside derivatives*

Compound	Retention Time (min)		
	System A	System B	System C
Guanine	6.5	8.9	----
Deoxyguanosine	10.1	10.0	3.6
⁶ O-methyldeoxyguanosine	15.3	16.1	4.6
⁶ O-ethyldeoxyguanosine	16.9	19.4	5.3
⁶ O-ethylthioethyldeoxy- guanosine	24.9	24.8	6.3

* System A: Spherisorb ODS 5 μ m (4.6 x 250 mm) column eluted at a flow rate of 1 ml/min with a 20-min gradient of 3% to 30% acetonitrile in 50 mM KH_2PO_4 , pH 4.5, and continued with 30% acetonitrile in 50 mM KH_2PO_4 , pH 4.5. System B: Spherisorb ODS column as above eluted with a paired ion reagent at a flow rate of 1 ml/min. 20-min gradient of 3% to 30% acetonitrile in 10 mM sodium hexane sulfonate, 10 mM NaCl, pH 3.5, continued with the 30% acetonitrile solution. System C: Adsorbosphere SCX 5 μ m (4.6 x 250 mm) column eluted at a flow rate of 1 ml/min with 15% acetonitrile in 0.2 M $\text{NH}_4\text{H}_2\text{PO}_4$, pH 2.5.

The first step in establishing the structure of this new derivative was, of course, to demonstrate that it was pure. This was done by using two other HPLC systems also described in the footnote to Table 1. These separation procedures, i.e., Systems A, B, and C of Table 1, have great resolving power for derivative nucleosides and clearly demonstrated that we had obtained a pure product. The long retention time exhibited in System A indicates that the new derivative of deoxyguanosine was a rather lipophilic compound.

The O⁶-substituted deoxyguanosine which would be expected as a product of the reaction of hemi-sulfur mustard with deoxyguanosine would have the structure O⁶-hydroxyethylthioethyl deoxyguanosine. This derivative was obtained by exactly the same procedure as described above, but bis-hydroxyethyl sulfide was used in place of ethylhydroxyethyl sulfide in the reaction with sodium hydride.

The O⁶-substituted guanine ribonucleosides which would result from the reaction of CEES or hemi-sulfur mustard with the RNA nucleoside, guanosine, were also synthesized by entirely analogous reactions except that 6-chloroguanosine was used in place of acetylated 6-chlorodeoxyguanosine. 6-Chloroguanosine is available as a commercial product from Waldhof, West Germany. Purification and characterization of these nucleosides is described in the appendix.

CHARACTERIZATION OF O⁶-ETHYLTHIOETHYL DEOXYGUANOSINE

Confirmation of Structure

The structure of this derivative nucleoside was established by a combination of ultraviolet and mass spectrometry, although it could be anticipated that this derivative had the assigned structure from the manner of its synthesis.

Ultraviolet spectrometry revealed a spectrum which is characteristic of an O⁶-substituted guanine nucleoside (Figure 1). The double hump with absorption maxima at 248 nm and at 280 nm is absolutely typical of such compounds and confirms the attachment of the derivative group to the 6 position.

The nature of this substituent was established by mass spectrometry which was kindly performed for us by Mr. Marion Kirk of the Southern Research Institute. Figure 2 shows a mass fragmentation pattern obtained by the electron impact technique revealing a molecular ion peak at m/e 355 which corresponds to the structure, O⁶-ethylthioethyl deoxyguanosine. This spectrum also shows a large peak at m/e 151 corresponding to (guanine + H)⁺. A similar peak appears in the mass spectrum of deoxyguanosine itself and its presence in the O⁶-ethylthioethyl deoxyguanosine spectrum indicates that the O⁶ substituent is labile.

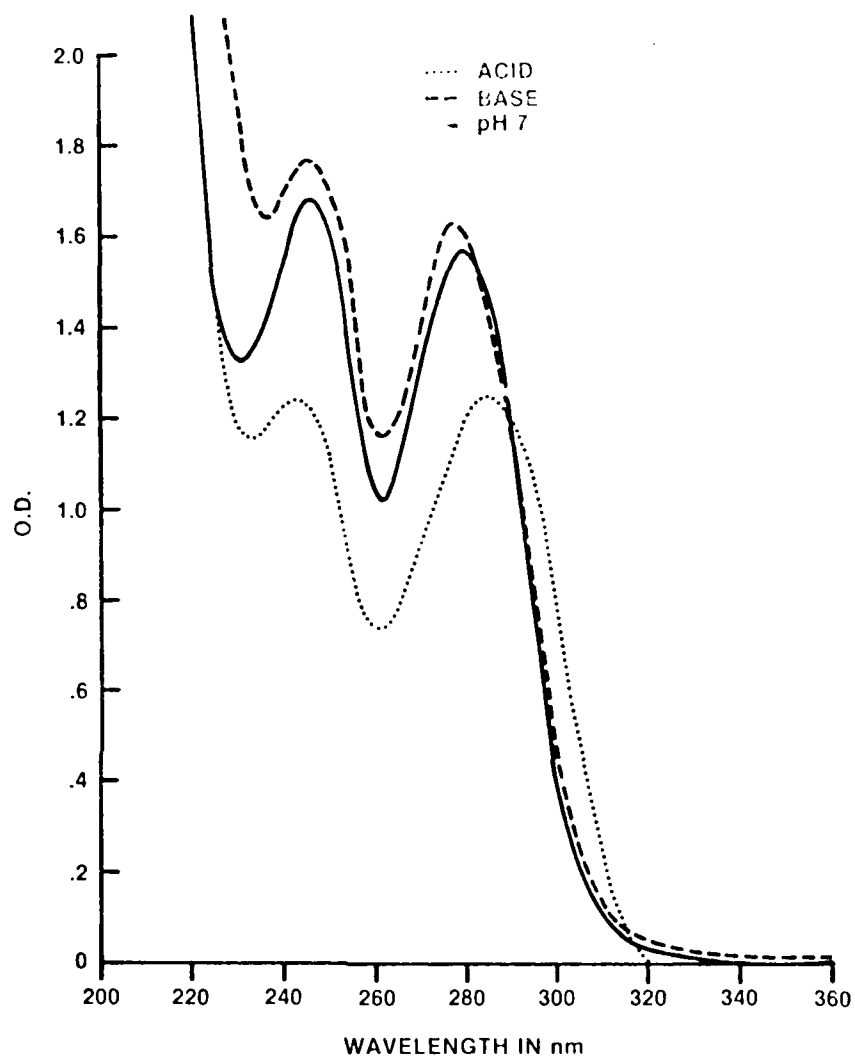


Figure 1: Ultraviolet spectra of 0⁶-ethylthioethyl deoxyguanosine in acidic, neutral, and basic solutions.

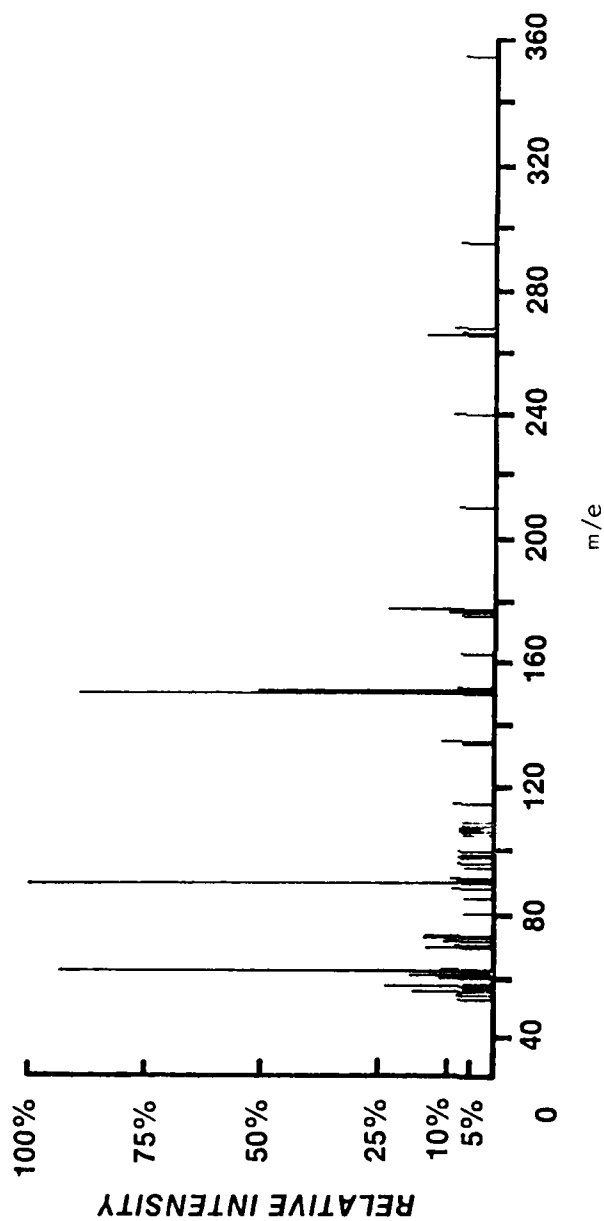


Figure 2: Mass spectrum of 0⁶-ethylthioethyl deoxyguanosine. The spectrum was obtained by the electron impact technique and revealed a molecular ion at m/e = 355.

Mass spectrometry using both positive and negative fast atom bombardment (FAB) confirmed the molecular weight of 355. Again, an intense peak was observed which corresponds to the dealkylated base.

Thus, ultraviolet and mass spectrometry established the structure of the derivative as O^6 -ethylthioethyl deoxyguanosine. Mass spectral evidence of the lability of the O^6 substituent also gave some indication why this derivative had not been detected previously.

Chemical Stability

Although the biological data cited above suggest that an O^6 derivative of guanine would be found in DNA treated with sulfur mustards, such a derivative has never previously been described. One possible explanation for this would be that these derivatives were destroyed in the digestion procedures used to release them from the DNA substrate.

We quickly obtained evidence that the O^6 -thioethyl guanine nucleosides were extremely labile. Not only did the mass spectral data suggest this, but this was demonstrated directly. The first O^6 -thioethyl guanine nucleoside which we synthesized, O^6 -hydroxyethylthioethyl guanosine, decomposed rapidly under mild acid treatment to guanine as shown by HPLC analysis. Since many procedures for releasing modified bases from DNA use acid depurination, this would explain why the derivatives had never been found after a depurination procedure.

The next question was whether such derivatives would be stable under conditions used for the enzymatic digestion of DNA. Table 2 shows the results of studies indicating that O^6 -ethylthioethyl deoxyguanosine is stable for at least 20 hr at 37° and pH 7 or 8. It also remains undecomposed for at least an hour at pH 5.5 and 37° . Table 2, then, establishes two critical points. First, the derivative is stable under physiological conditions and could remain in DNA for a sufficiently long time to effect a permanent change in the genetic information of the cell; second, it is sufficiently stable to be released from DNA by the usual enzymatic digestion.

Fluorescence of O^6 -Ethylthioethyl Deoxyguanosine

O^6 -substituted derivatives of deoxyguanosine have, in general, proved to be intensely fluorescent. This property is of some value in identifying O^6 derivatives of guanine nucleosides and is also a sensitive method of quantitative analysis (8).

Figure 3 shows the excitation and emission spectra which were obtained with purified O^6 -ethylthioethyl deoxyguanosine. These spectra are rather similar to those reported for O^6 -methylguanosine and O^6 -ethylguanosine by Singer (9).

Table 2
Stability of 0⁶-Substituted Guanine Nucleosides

Nucleoside	Conditions	Result*
0 ⁶ -hydroxyethylthioethyl guanosine	0.1 N HCl, 37°, 2 hours	Complete decomposition, mostly dealylation and depurination to guanine
0 ⁶ -ethylthioethyl deoxyguanosine	0.1 N HCl, 100°, 1 hour	Complete decomposition with dealylation, depurination and probable oxidation
0 ⁶ -ethylthioethyl deoxyguanosine	pH 5.5, 37°, 1 hour	Stable
0 ⁶ -ethylthioethyl deoxyguanosine	pH 7, 37°, 20 hours	Stable
0 ⁶ -ethylthioethyl deoxyguanosine	pH 8, 37°, 20 hours	Stable

* As shown by HPLC analysis

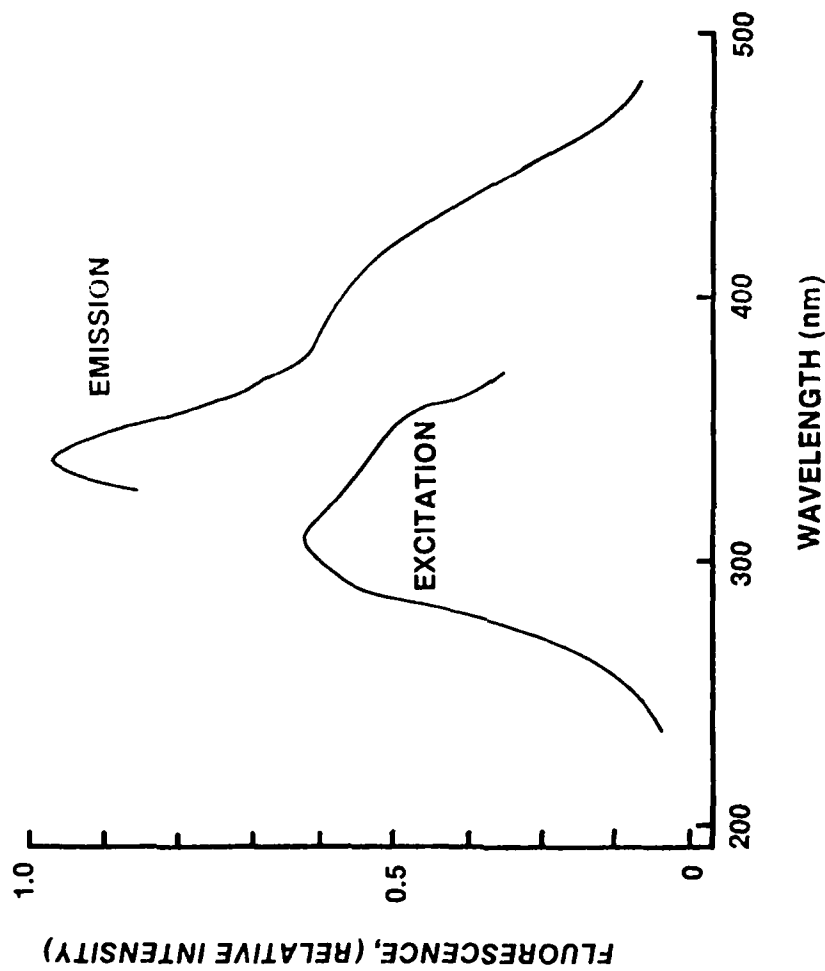


Figure 3: Excitation and emission fluorescent spectra of 0⁶-ethylthioethyl deoxyguanosine. Emission was monitored at 400 nm for the excitation spectrum and the emission spectrum was excited with a wavelength of 310 nm.

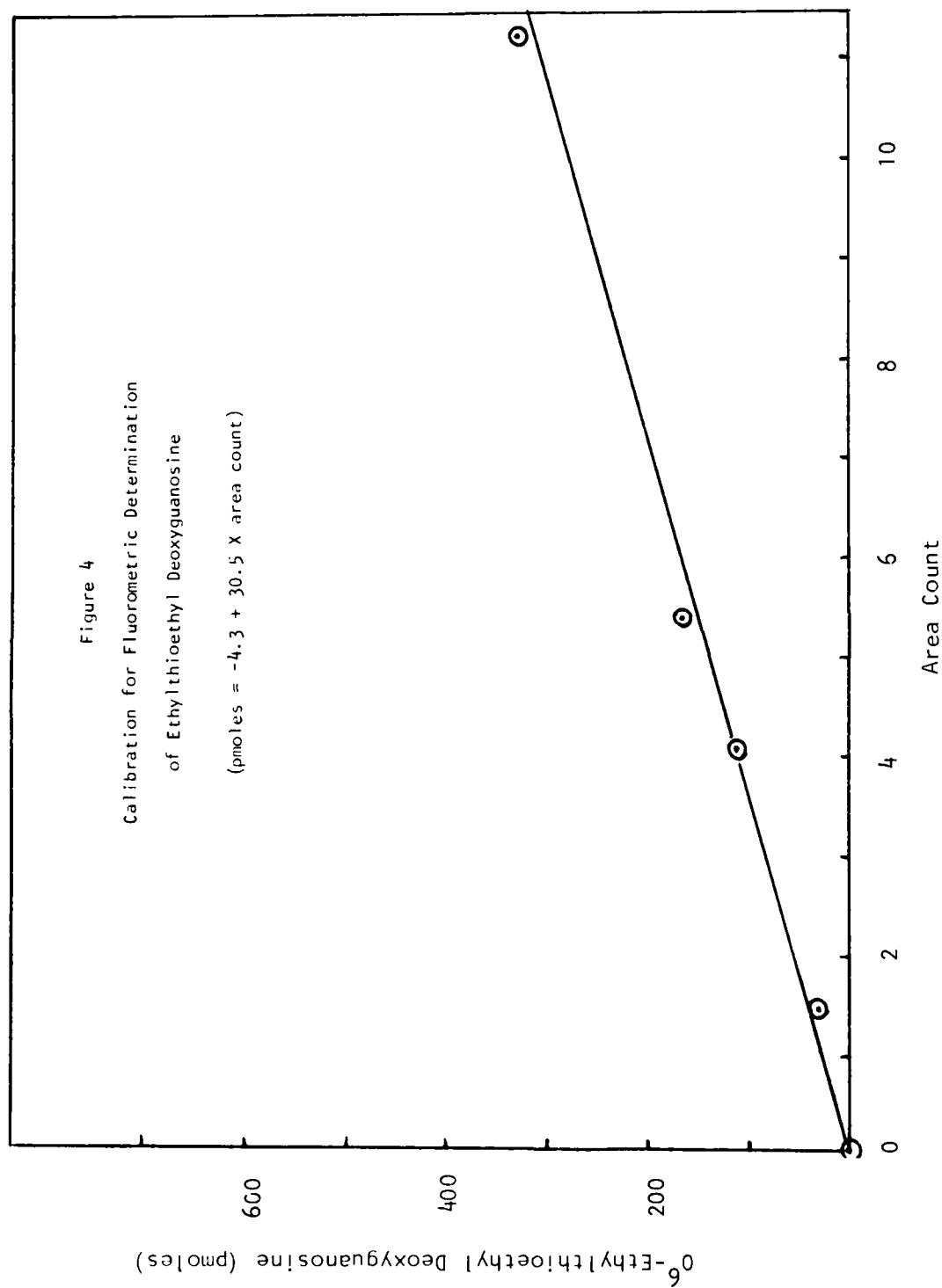


Figure 4: Calibration of the HPLC detector response for 0⁶-ethylthioethyl deoxyguanosine. Fluorescence was excited at 254 nm and monitored between 300 and 400 nm. Fluorescence peak areas were determined by a Perkin Elmer Sigma 10 data system.

A calibration curve for this method of detection is shown in Figure 4. The amount (picoles) of O^6 -ethylthioethyl deoxyguanosine is plotted against the detector response in "area count" as determined by the Sigma 10 data system. The data have been fitted by the least squares method to the equation:

$$\text{pmoles} = -4.3 + 30.5 \times \text{area count}$$

With the HPLC resolution which has been achieved so far, the minimum detectable response is approximately one area count, corresponding to approximately 25 pmoles. A really satisfactory repair assay would require at least an order of magnitude greater sensitivity than this. This will require either modification of the fluorometric analysis or the use of a radiolabelled sulfur mustard. If the chromatographic separation can be run at a pH lower than 4.5, greater sensitivity can probably be achieved in the fluorometric assay, and this approach is currently being investigated. The acid lability of O^6 -ethylthioethyl deoxyguanosine may, however, interfere with this method and it may be necessary to turn to a radiolabel to achieve the necessary sensitivity.

REACTION OF CHLOROETHYL ETHYL SULFIDE WITH DEOXYGUANOSINE

With the chromatographic marker for O^6 -ethylthioethyl deoxyguanosine in hand, it was relatively easy to demonstrate that chloroethyl ethyl sulfide (CEES) forms this compound when it reacts with deoxyguanosine. Deoxyguanosine (35 mg) was dissolved in 35 ml of 50 mM cacodylate buffer, pH 7, at 37° . A total of 125 μ l of chloroethyl ethyl sulfide (approx. 35 μ moles/ml) was added to the reaction vessel with stirring. The pH was maintained at approximately 7 with the addition of small amounts of 1 N NaOH. After one hour, there was no further change in pH, but the incubation was continued for an additional six hours to insure complete hydrolysis of the CEES. The reaction mixture was then filtered and stored frozen at -20° .

As shown in Figure 5, the products of this reaction could be separated by high pressure liquid chromatography on a 5 μ m Spherisorb ODS column eluted with the 25 mM phosphate buffer, pH 4.5-acetonitrile system described in the legend to that figure. It is clear from Figure 5 that the deoxyguanosine was extensively modified; the chromatogram reveals ten significant derivative peaks and several minor products. The major peaks were numbered from 0 through 9, as shown in Figure 5, and partially characterized as described below.

Since pure, well-characterized, O^6 -ethylthioethyl deoxyguanosine had been synthesized, it was easy to identify peak 8 as this long-sought derivative. The chromatographic separation shown in Figure 5 was performed with an HPLC detector capable of providing an ultraviolet spectrum of derivative peaks, and the spectrum of peak 8 was typical of O^6 -ethylthioethyl deoxyguanosine (Figure 6). Further proof that peak 8 was indeed O^6 -ethylthioethyl deoxyguanosine was obtained by collecting this derivative peak, concentrating it, and showing its identity with marker material in the three separate chromatographic systems described in the footnote to Table 1. Thus, we have shown conclusively that

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 Attn (mAU): 10
 Zero: 3.8
 Signal: B: 3.8

Wavelength (nm)
 1. 210, 4
 2. 230, 4
 3. 254, 4
 4. 260, 80
 5. 280, 4
 6. 320, 20
 7. 450, 50
 8. 550, 100

10.40A

Figure 5

High Pressure Liquid Chromatographic Separation
 of Chloroethyl Ethyl Sulfide-Deoxyguanosine Reaction Mixture

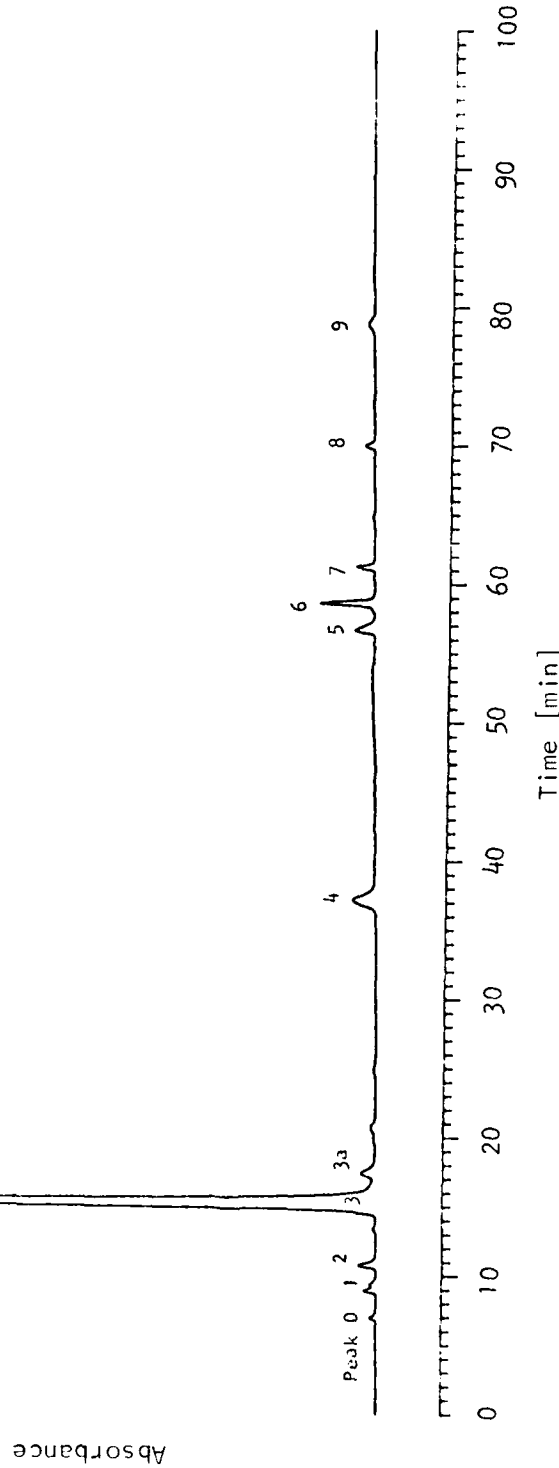


Figure 5: High pressure liquid chromatographic separation of a chloroethyl ethyl sulfide-deoxyguanosine reaction mixture. A sample containing 20 µg of nucleosides was separated on a 5 µm Spherisorb column (4.6 x 250 mm) at a flow rate of 1 ml/min with 3% acetonitrile in 25 mM KH_2PO_4 , pH 4.5, and then with a 3-30% gradient of acetonitrile in the same buffer for 50 min, and finally with 30% acetonitrile in buffer for 25 min. Peak 3 is unmodified deoxyguanosine and peak 8 is O⁶-ethylthioethyl deoxyguanosine.

hp 1040A

RAWDAT

File:

Date:

Spectrum (min): 70.0132

Reference (min): 70.5545

Attn (mAU): 10.1

Absorbance (mAU) (nm): 9.2 (219/ 2)

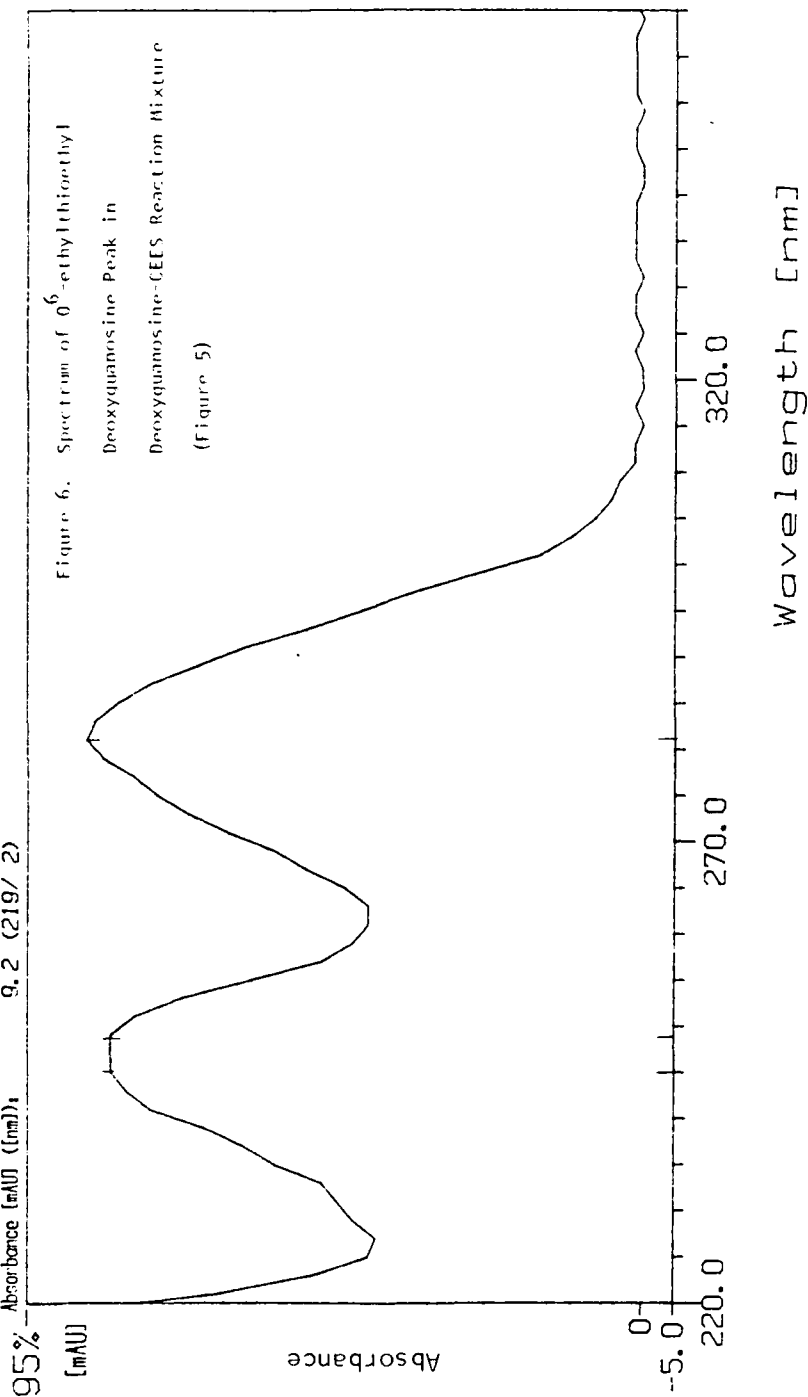


Figure 6: Ultraviolet spectrum of 0⁶-ethylthioethyl deoxyguanosine (peak 8) in the deoxyguanosine-CEES reaction mixture shown in Figure 5.

the sulfur mustards do attack the O⁶ position of deoxyguanosine to produce the predicted derivative nucleoside.

Some attempt was made to identify the other nucleoside derivatives shown in Figure 5 from what has been published in the previous literature about reactions of chloroethyl ethyl sulfide with deoxyguanosine. These tentative assignments are shown in Table 3. Peak 3 was unmodified deoxyguanosine while peak 6 is probably the previously reported major product, N⁷-ethylthioethyl deoxyguanosine (5).

The same article which describes N⁷-ethylthioethyl deoxyguanosine also describes an N² derivative and suggests a separation procedure for these products on G-10 Sephadex columns (5). Since our reaction of CEES with deoxyguanosine had been performed under similar conditions, we also performed a separation of G-10 Sephadex thinking that we could obtain marker material for positive identification of the N⁷ and N² derivatives. However, we found that the peaks isolated on such a column contain several derivatives, and it was not possible to make assignments on that basis with any certainty. These studies are described in an appendix and further work would be necessary to identify the other peaks in Figure 5.

However, we had achieved our objective of showing that CEES modified deoxyguanosine in the O⁶ position, and the next step in assessing its importance was to investigate the reaction of CEES with DNA.

REACTION OF CHLOROETHYL ETHYL SULFIDE WITH DNA

The double helical structure of DNA affects the base positions which are available for alkylation so that quantitative differences are often observed in the amount of alkylation of monomeric nucleosides and DNA. Since the O⁶ position of deoxyguanosine is involved in hydrogen bonding, this factor might decrease the reactivity of the O⁶ position. In general, however, other agents which react with this position of monomeric deoxyguanosine also alkylate the O⁶ position of deoxyguanosine in DNA (10). Having shown that CEES substituted monomeric deoxyguanosine in the 6 position, the next step was to show that this reaction occurred with DNA.

Typically, 3.6 μ l (35 μ mole) of CEES was added per ml to a solution of DNA, 2 mg/ml in 25 mM cacodylate buffer, pH 7.4, and the mixture was incubated at 37°. Small amounts of 1 N NaOH were added to maintain the pH at 7 and after 1.5 hr, the reaction was terminated by precipitating the DNA with four volumes of cold ethanol in the presence of 1 N NaCl. The DNA precipitate was collected by centrifugation in a refrigerated centrifuge at 10,000 g and redissolved in 25 mM cacodylate buffer, pH 7.

The next problem was to digest the DNA to the deoxynucleoside level so that its constituent nucleosides could be separated by high pressure liquid chromatography. We have demonstrated (Table 2) that O⁶-ethylthioethyl deoxyguanosine would be stable over the range of pH's which are normally used in the digestion of DNA. However, it is still not known to what extent modification of the nucleosides interferes with the action of the various

Table 3

Products of the Reaction of Chloroethyl Ethyl Sulfide with Deoxyguanosine.
Separation by High Pressure Liquid Chromatography⁽¹⁾

Peak #	Percent of total derivatives	Absorbance maximum at pH 4.5	Fluorescence	Identity	Basis
0	2.4	243		--	
1	4.3	253	+	--	
2	4.5	252		--	
3	--	253		dGR ⁽²⁾	Comparison with known compound
3a	6.3	253		--	
4	27.2	252			
5	13.0	255	++	N ¹ RG, N ¹ RdGR, N ² RG or N ² RdGR ⁽³⁾	Ultraviolet spectrum and comparison with G-10 fraction G'
6	22.9	255		N ⁷ RdGR ⁽⁴⁾	Ultraviolet spectrum and comparison with G-10 fraction A
7	8.5	254		--	
8	5.6	281, 248	++	O ⁶ RdGR ⁽⁵⁾	Comparison with known compound
9	5.6	253			

- (1) Separation performed on a 5 μ m Spherisorb ODS column (4.6 x 250 mm) eluted at 1 ml/min with 3% acetonitrile in 25 mM KH_2PO_4 , pH 4.5, for 25 min; then with a 40-min 3-30% acetonitrile gradient in the same buffer; and then with 30% acetonitrile in the same buffer for 25 min.
- (2) dGR, deoxyguanosine.
- (3) N^1RG , N^1 -ethylthioethyl guanine; N^1RdGR , N^1 -ethylthioethyl deoxyguanosine; N^2RG , N^2 -ethylthioethyl guanine; N^2RdGR , N^2 -ethylthioethyl deoxyguanosine.
- (4) N^7RdGR , N^7 -ethylthioethyl deoxyguanosine.
- (5) O^6RdGR , O^6 -ethylthioethyl deoxyguanosine.

nucleases which are used in DNA digestion. Assuming that the problems would be similar to those encountered in digesting DNA's modified by other reactive compounds, we adapted a procedure which has been successful for the release of modified nucleosides from DNA modified with the antitumor agent, bis-chloroethylnitrosourea (11).

Following that protocol, CEES-modified DNA (2 mg/ml) was digested in 25 mM cacodylate buffer, pH 7.4, for 24 hr at 37° in the presence of the following amounts of enzyme per ml:

<u>Enzyme</u>	<u>Amount</u>
Deoxyribonuclease I	100 units
Venom phosphodiesterase	2.2 units
Spleen phosphodiesterase	0.5 units
Bacterial alkaline phosphatase	0.8 units

After 24 hr, the solution was filtered and a 0.5 ml sample containing 0.875 mg of digested DNA was analyzed in the same chromatographic system that is described above (legend, Figure 5). The HPLC elution profile for this digest is shown in Figure 7.

Unmodified DNA digested to the deoxynucleoside level and separated in this amount with this chromatographic system shows only three peaks. These contain the unmodified deoxynucleosides: deoxycytidine at 6.4 min, deoxythymidine at 11.4 min, and a mixture of deoxyguanosine and deoxyinosine resulting from the deamination of deoxyadenosine at 15-16 min. Thus, the large number of peaks in Figure 7 is impressive. Significantly, a peak corresponding to O⁶-ethylthioethyl deoxyguanosine was noted at the correct retention time, now 68 minutes since the analytical column had changed characteristics slightly since the time the sample was run in Figure 5. The identity of this peak was confirmed by its characteristic spectrum taken during the chromatographic separation shown in Figure 8. In addition, fluorometric monitoring of the elution profiles showed that this peak had the characteristic fluorescence of O⁶-ethylthioethyl deoxyguanosine.

Thus, we have definitely shown that CEES produces the anticipated modification at the O⁶ position of deoxyguanosine in DNA. In order to establish repair of this modification, however, it will be necessary to develop a reliable quantitative analysis for the amount of O⁶-ethylthioethyl deoxyguanosine contained per mg of DNA. The first question which arises is whether some O⁶-ethylthioethyl deoxyguanosine may remain attached to short runs of undigested DNA. We are currently examining this question by varying the digestion conditions - pH, time, amounts of enzyme added, and the effects of adding exonuclease III (endonuclease VI). The addition of this latter enzyme was suggested by Dr. Papirmeister who noted that CEES might produce apurinic sites in DNA which would be subject to digestion by exonuclease III, but probably not by other nucleases. We have succeeded in reducing the number of unknown peaks in the DNA digest by these procedures and have confirmed the presence of O⁶-ethylthioethyl deoxyguanosine in several different CEES-modified DNA substrates.

Noting that some O⁶-modified guanine nucleosides are evidently substrates for adenosine deaminase (12), we have included the potent adenosine deaminase

hp 1040A

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 Zero%: 10
 Signal: B: 3.8

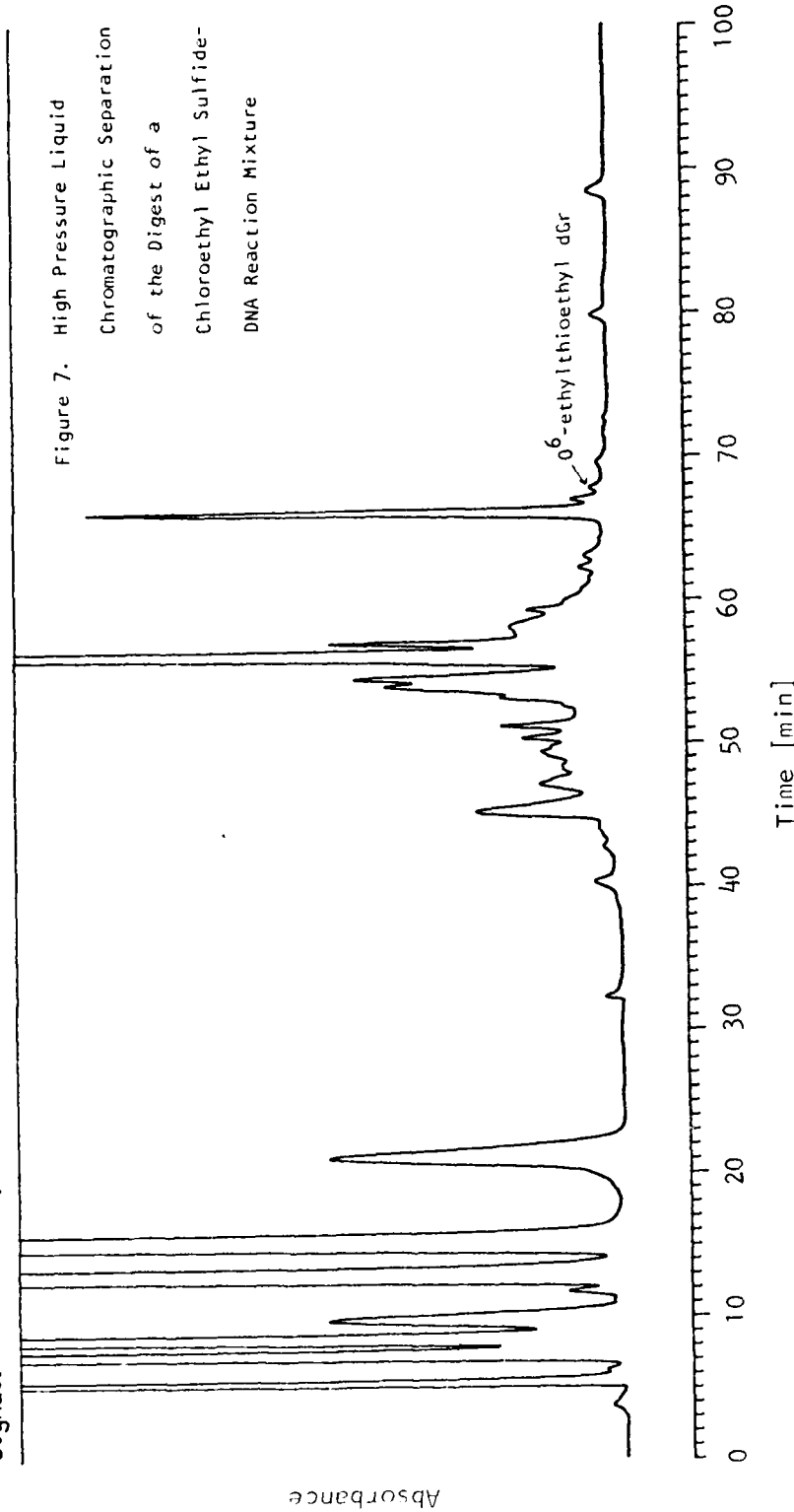


Figure 7: High pressure liquid chromatographic separation of a digest of a chloroethyl ethyl sulfide-DNA reaction mixture. A sample containing 0.5 mg of the digest was separated on a 5 μ m Spherisorb column as described in the legend to Figure 5. A small 06-ethylthioethyl deoxyguanosine peak is observed at 68 min.

hp 1040A

Files RAWDAT
Date 04/20/1983
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Reference (min): 68.7217
Attn (mAU): 9.8
Absorbance (mAU) (nm): 8.9 (219/ 2)

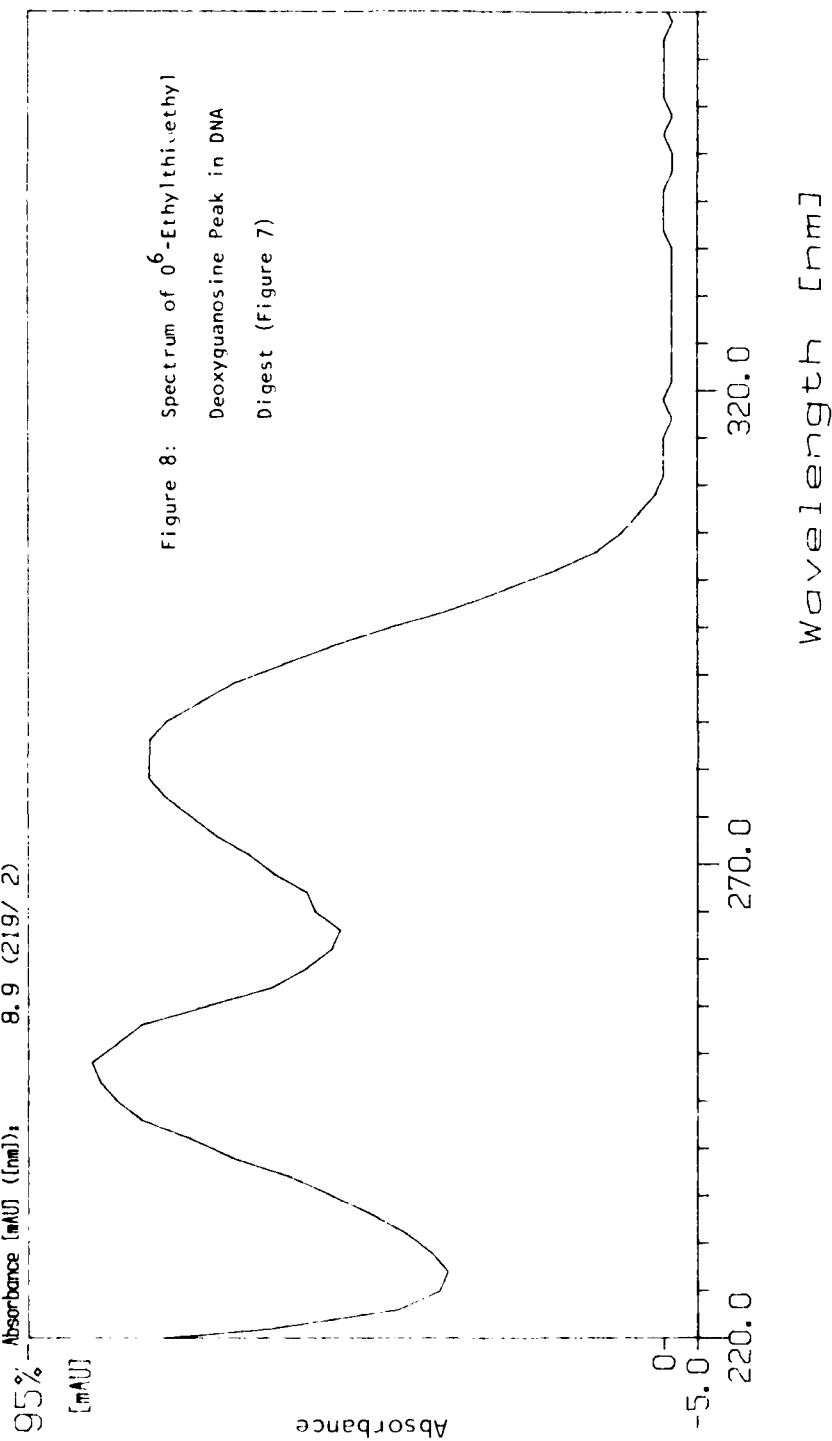


Figure 8: Ultraviolet spectrum of the 0⁶-ethylthioethyl deoxyguanosine peak identified in Figure 7.

inhibitor, deoxycoformycin, to prevent any possible breakdown of the O^6 -ethylthioethyl deoxyguanosine by this enzyme, a frequent contaminant of commercial nucleases. The addition of this inhibitor has not, however, affected recovery of O^6 -ethylthioethyl deoxyguanosine although it would be important in preserving the CEES-modified deoxyadenosines.

PLAN

Following our original objectives, as stated in our contract proposal, the next step in demonstrating repair of O^6 -ethylthioethyl deoxyguanosine in CEES-modified DNA will be to perfect a quantitative assay for the amount of O^6 -ethylthioethyl deoxyguanosine remaining in DNA which has been incubated with rat liver repair proteins. Depending on our success in improving the chromatographic separations of O^6 -ethylthioethyl deoxyguanosine from other peaks in the DNA digest and in improving the sensitivity of the fluorescence assay, we may need to proceed to the use of radiolabelled CEES or other sulfur mustards which might be available in radiolabelled form.

As soon as we have successfully demonstrated the repair of a sulfur mustard-induced lesion in the O^6 position of deoxyguanosine, we will pursue the identification and purification of the repair factors.

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APPENDIX

SYNTHESIS OF HEMI-SULFUR MUSTARD

Chloroethyl ethyl sulfide (CEES) has been studied extensively and some of the best data on the mutagenic effects of sulfur mustard have been obtained with this compound. However, hemi-sulfur mustard is more closely related to sulfur mustard and it can be anticipated that sulfur mustard-modified DNA would contain most of the lesions which hemi-sulfur mustard causes in DNA. Thus, there are some advantages to studying the modification of DNA by hemi-sulfur mustard. Although we are currently studying CEES-modified DNA as explained in the body of this report, we have synthesized hemi-sulfur mustard and obtained, probably for the first time, a satisfactory mass spectral analysis of this compound. Accordingly, this information is included here.

Hemi-sulfur mustard was prepared by the method of Tsou et al. (13). Working in a well-ventilated hood, 75 ml of methanol dried over 3 Å molecular sieves and 10 ml of mercaptoethanol were added to a 200 ml round-bottom flask. Then, a total of 3 grams of sodium hydride was added slowly with stirring and when this had reacted with the mercaptoethanol, 75 ml of 1,2-dichloroethane was added. The reaction mixture was allowed to stand at room temperature overnight and the sodium chloride which was produced in the reaction was allowed to settle to the bottom of the flask.

The supernatant was removed in stages and concentrated on a rotary evaporator. Then, the hemi-sulfur mustard was purified by vacuum distillation. A central cut was taken which distilled at a head temperature of 72° at 0.19 mm pressure.

The purity of this fraction was assessed by gas chromatography. A sample of approximately 5 µg dissolved in 0.1 µl of methanol was injected on an OV 101 column programmed to heat at a rate of 20° per minute from 100° to 280°. Chromatographic peaks were monitored in a Finnigan mass spectrometer interfaced to the gas chromatography apparatus. As shown in Figure 9, the sample contained only one large constituent which had the mass spectrum shown in Figure 10. This spectrum showed the expected molecular ion peak at 140 mass units accompanied by its chlorine isotope at 142 mass units. There was also a small peak which is probably a hydrate of hemi-sulfur mustard at $m/e = 158$ with its chlorine isotope at $m/e = 160$. Thus, we have mass spectral confirmation of the identity of the material prepared by the method of Tsou et al.

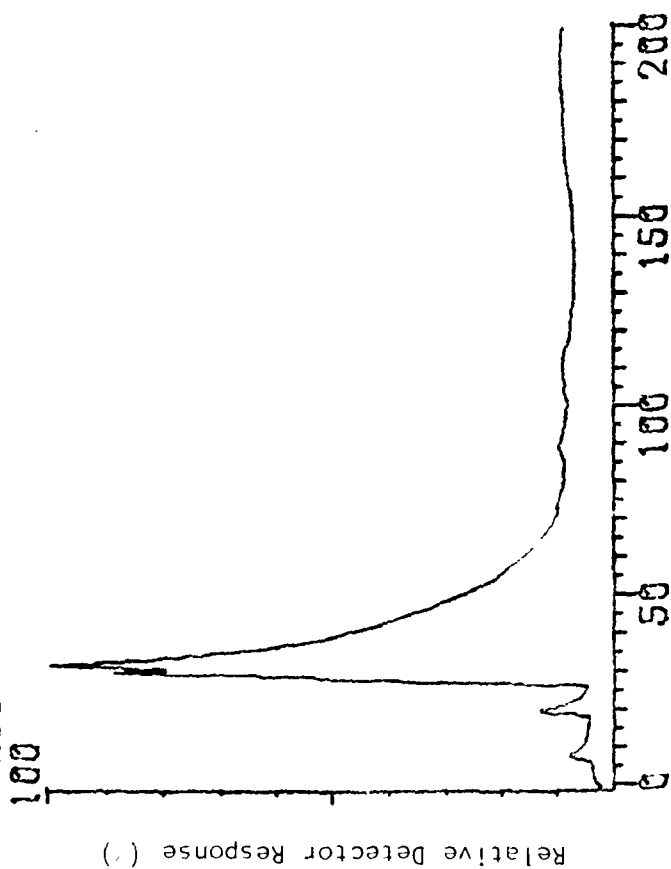
PREPARATION OF O⁶-ALKYLGUANOSINE

Before it was known that the alkyl group in the 6 position was as readily hydrolyzed as the glycosidic bond, it appeared that O⁶-thioethyl guanines could be prepared from the corresponding ribonucleosides. These

HYDROXYETHYL CHLOROETHYL SULFIDE

822370

RGC



Relative Retention Time (200 units = 9 min)

Figure 9: Gas chromatography of hydroxyethyl chloroethyl sulfide (hemi-sulfur mustard). Approximately 5 μ g of hemi-sulfur mustard was injected on an OV 101 column programmed to heat at a rate of 20 $^{\circ}$ /min from 100-280 $^{\circ}$. The GC peak was monitored by mass spectrometry which indicated that it was essentially pure except for a small amount of low molecular weight impurity in the leading edge.

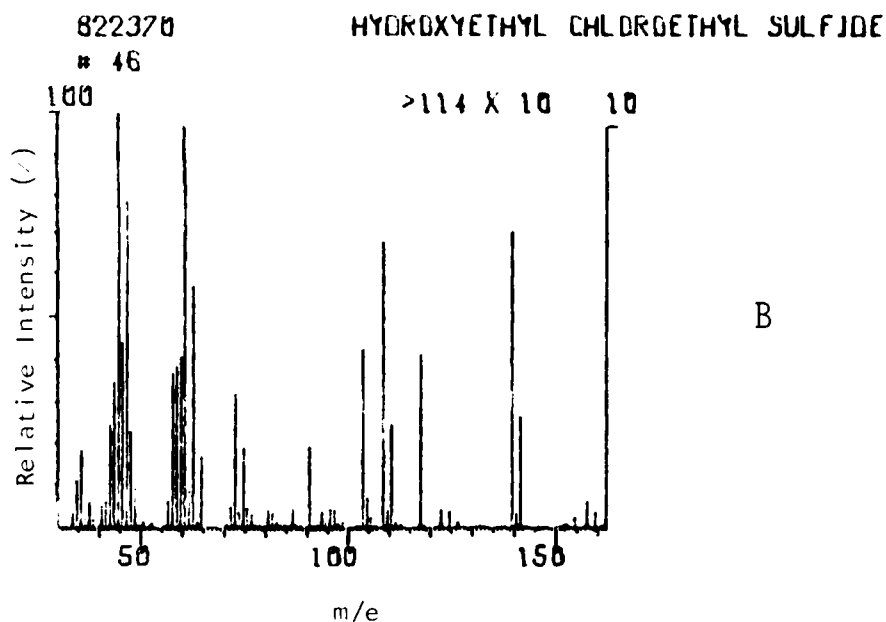
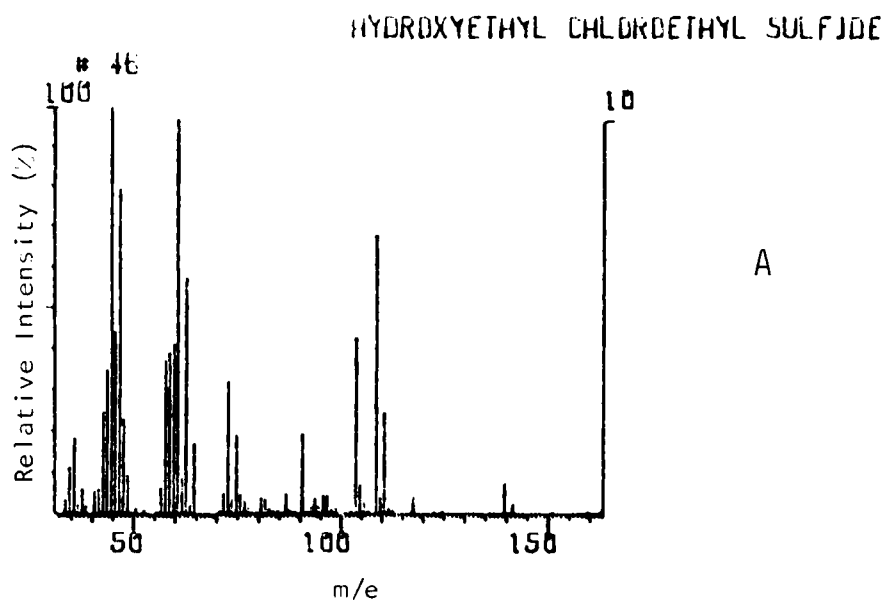


Figure 10: Mass spectrum of the hydroxyethyl chloroethyl sulfide (hemi-sulfur mustard) peak from the gas chromatographic run shown in Figure 9. The spectrum shows a molecular ion peak at $m/e = 140$ corresponding to the correct molecular weight of hemi-sulfur mustard. There is an accompanying peak at $m/e = 142$ attributable to hemi-sulfur mustard containing the chlorine isotope $Cl = 37$. The same spectrum is shown in each panel except that the relative intensity scale is multiplied by 10 for $m/e = 114$ in panel B.

ribonucleosides are easier to prepare than the corresponding deoxyribonucleosides because the starting material for the preparation of the ribosides, 6-chloro-2-amino purine riboside, is commercially available.

In order to prepare O⁶-ethylthioethyl guanosine, 2 ml of ethylhydroxyethyl sulfide was added to 100 mg of sodium hydride in a 50 ml round-bottom flask. After the alkoxide had formed, 50 mg of 6-chloro-2-amino purine riboside was added and the mixture was heated to 50°C with stirring. The solution gradually became homogeneous and after about 1 hr, 4 ml of water was added and the pH was adjusted to 7 with formic acid. This aqueous solution was then extracted with ether to remove the unreacted and very odiferous ethylhydroxyethyl sulfide. The aqueous phase was separated on a 2 x 100 cm G-10 column eluted with 1 mM formic acid. The elution profile for this column is shown in Figure 11.

The O⁶-ethylthioethyl guanosine was found in fractions 80-90 which were pooled and evaporated to dryness. This product was pure by high pressure liquid chromatography in System A of Table 1, and had the characteristic ultraviolet spectrum of an O⁶-substituted guanine nucleoside as shown in Table 4.

O⁶-hydroxyethylthioethyl guanosine was prepared in an entirely analogous way using 2 ml of bis-hydroxyethyl sulfide in the reaction with sodium hydride. Again, after hydroxyethylthioethyl alkoxide had reacted with 6-chloro-2-amino purine riboside to produce the O⁶-substituted guanosine, the reaction mixture was applied to a G-10 column. Separation of this reaction mixture is shown in Figure 12. O⁶-hydroxyethylthioethyl guanosine, being somewhat less lipophilic than O⁶-ethylthioethyl guanosine, eluted with 1 mM formic acid earlier than did O⁶-ethylthioethyl guanosine and was found in fractions 32-43. These fractions were pooled, evaporated to dryness, and again, the product was found to be essentially pure by high pressure liquid chromatography. It had an ultraviolet spectrum typical of an O⁶-substituted guanine nucleoside (Table 4).

When it was discovered that the O⁶-alkyl bond was as acid labile as the glycoside bond in these compounds, they were not characterized further. However, they are available if it becomes important to characterize the modifications of RNA produced by the sulfur mustards.

CHARACTERIZATION OF OTHER MODIFIED NUCLEOSIDES IN CEES-DEOXYGUANOSINE REACTION MIXTURES

As described in the main body of this report, there were 10 significant peaks in the chromatographic separation of a chloroethyl ethyl sulfide-deoxyguanosine reaction mixture. An attempt was made to obtain ultraviolet spectra on each of these during the separation procedure, and the results are shown in Figures 13-21. Except for the absolutely characteristic spectrum of O⁶-substituted deoxyguanosines, however, the other products all have rather similar spectra at pH 4.5. The absorbance maxima for each of

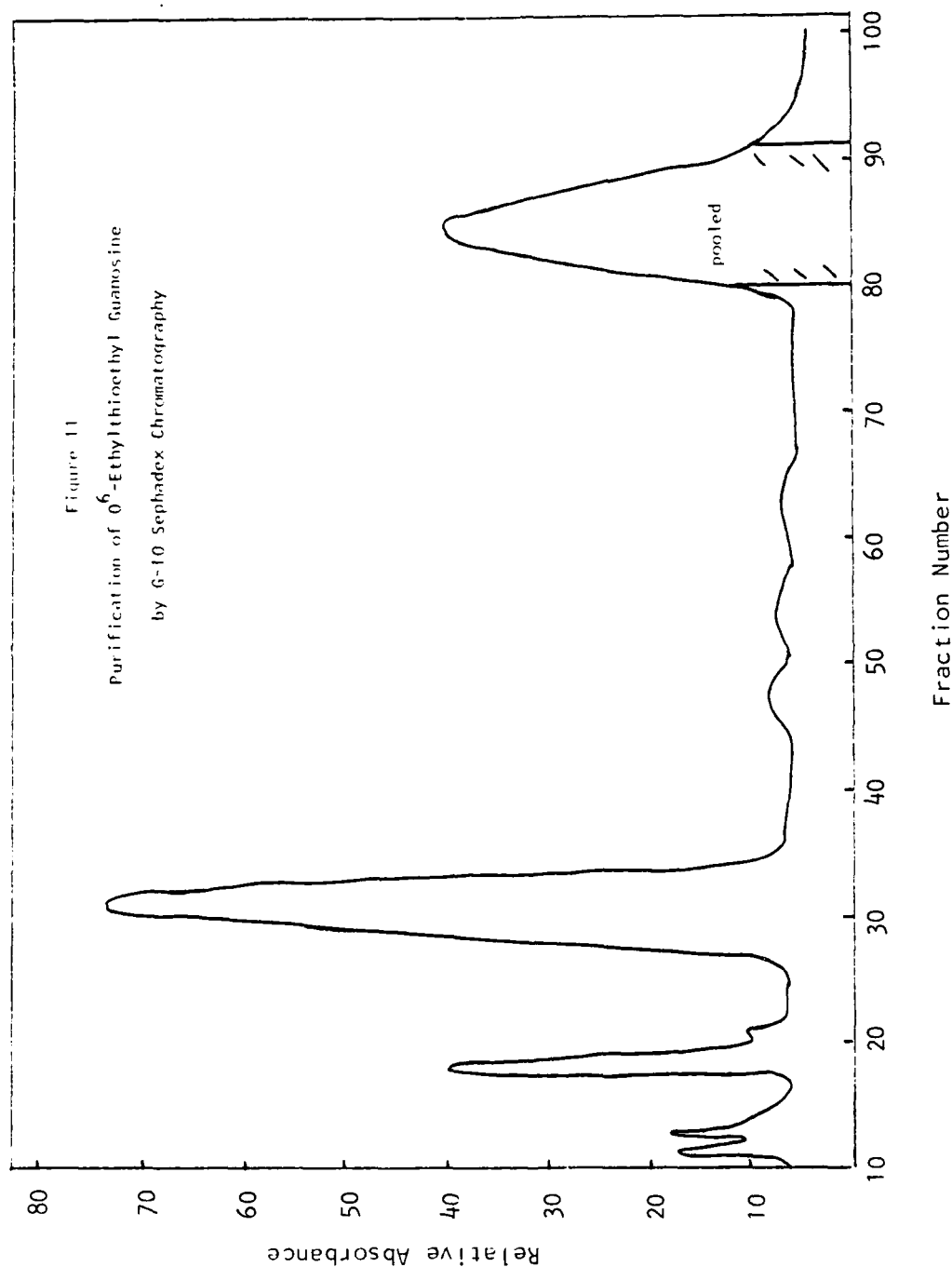


Figure 11: Purification of 0⁶-ethylthioethyl guanosine by G-10 Sephadex chromatography. Products of the reaction between 50 mg of 6-chloro-2-amino purine riboside and an excess of ethylthioethyl alkoxide were separated on a 2 x 100 cm G-10 column eluted with water at 1 ml/min. Fractions were collected at 20-min intervals and the product, 0⁶-ethylthioethyl guanosine, was found in fractions 80-91.

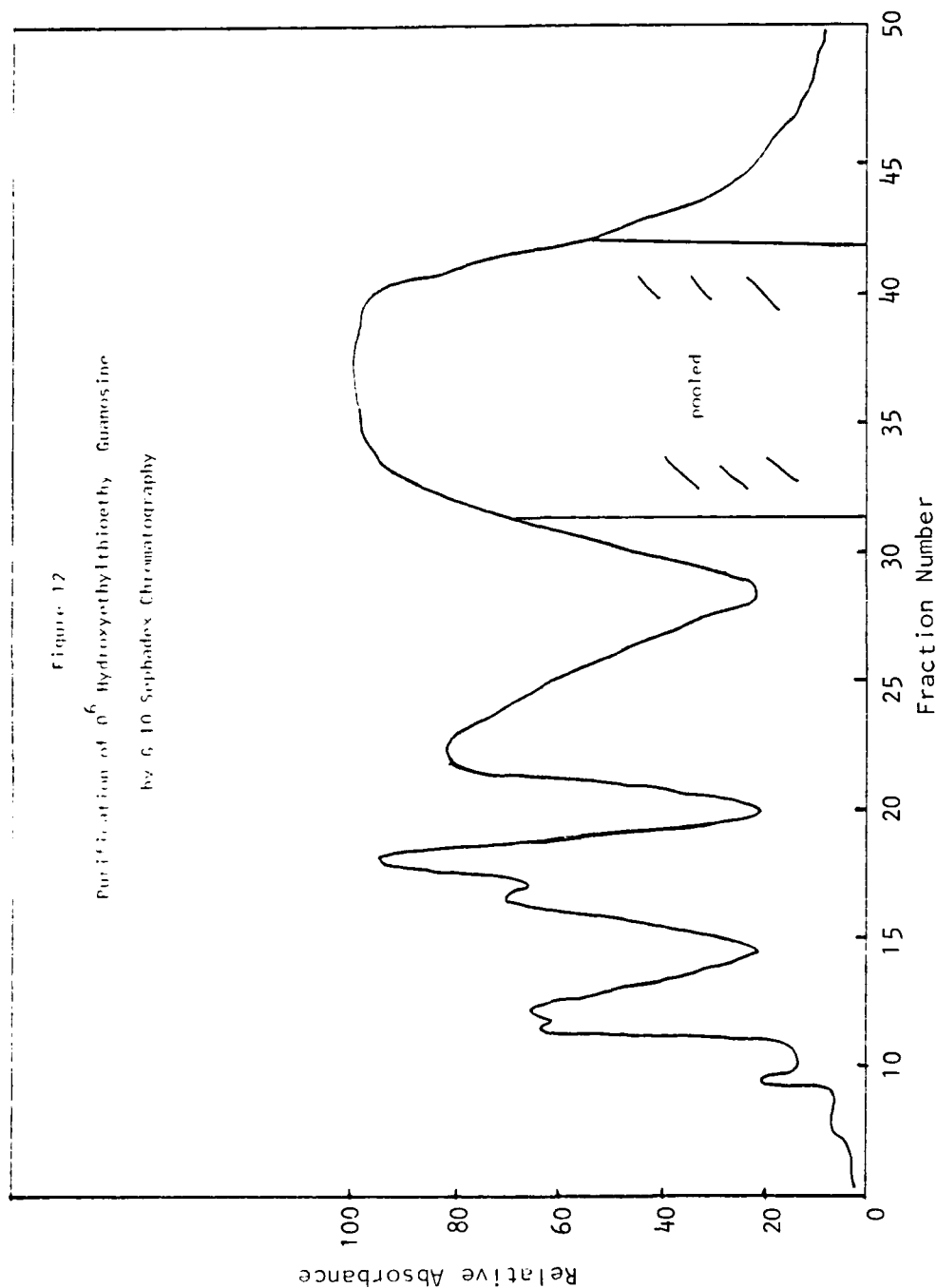


Figure 12: Purification of 0^6 -hydroxyethylthioethyl guanosine by G-10 Sephadex chromatography. Products of the reaction between 50 mg of 6-chloro-2-amino purine riboside and an excess of hydroxyethylthioethyl alkoxide were separated on a 2 x 100 cm G-10 column eluted with water at 2 ml/min. Fractions were collected at 20-min intervals and the product, 0^6 -hydroxyethylthioethyl guanosine, was found in fractions 32-42.

Table 4
Ultraviolet Spectra of O⁶-Alkylguanine Nucleosides

Compound	Acid		pH 7		Base	
	max	min	max	min	max	min
O ⁶ -Ethylthioethyl deoxyguanosine	284 243	261	279 246	262	277 245	262
O ⁶ -hydroxyethylthio- ethyl deoxyguanosine	288 243	261	281 247	262	281 246	261
O ⁶ -ethylthioethyl guanosine	282 246	262	280 248	262	275 249	261
O ⁶ -hydroxyethylthio- ethyl guanosine	---	---	280 247	262	---	---

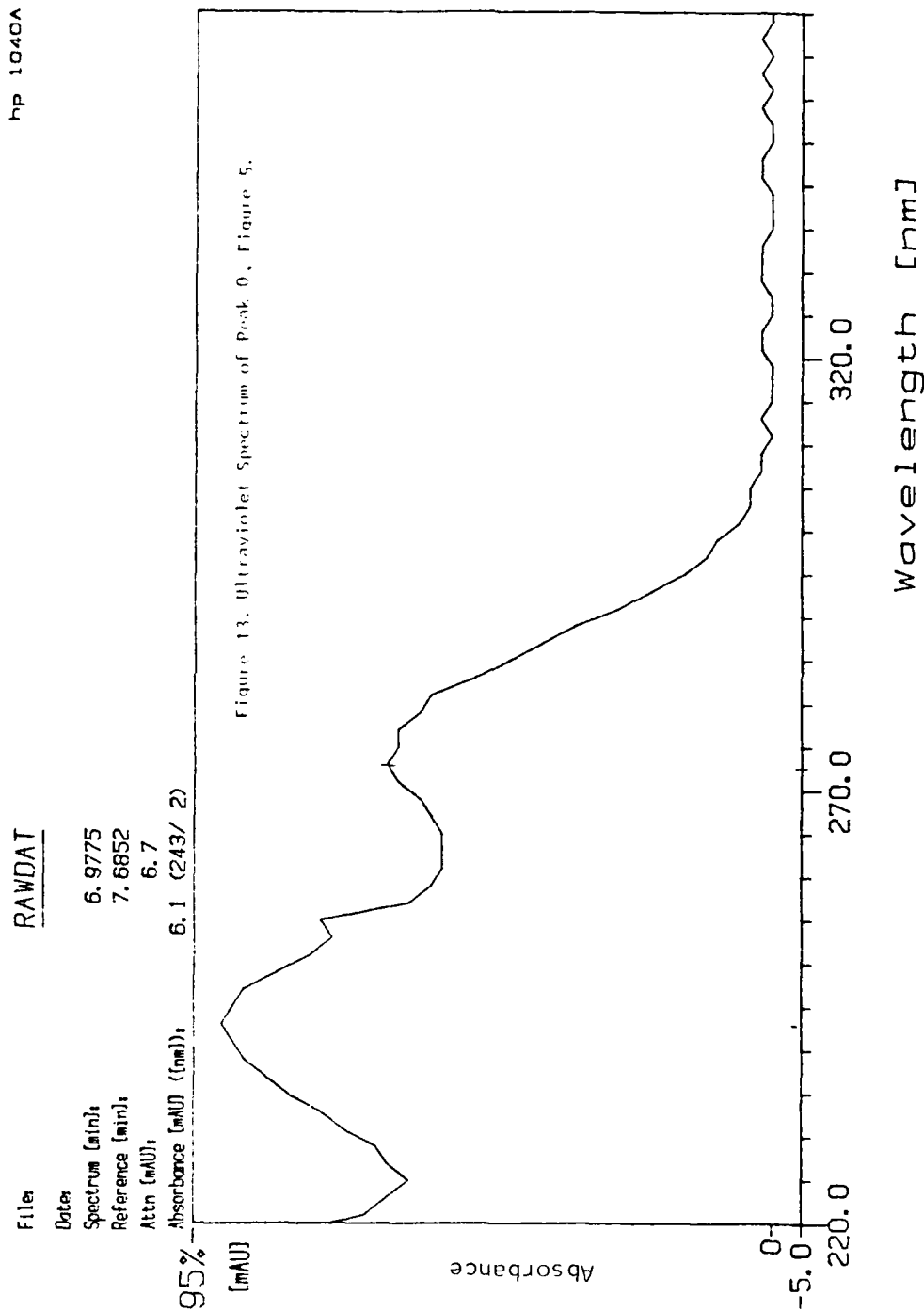


Figure 13: Ultraviolet spectrum of Peak 0, Figure 5.

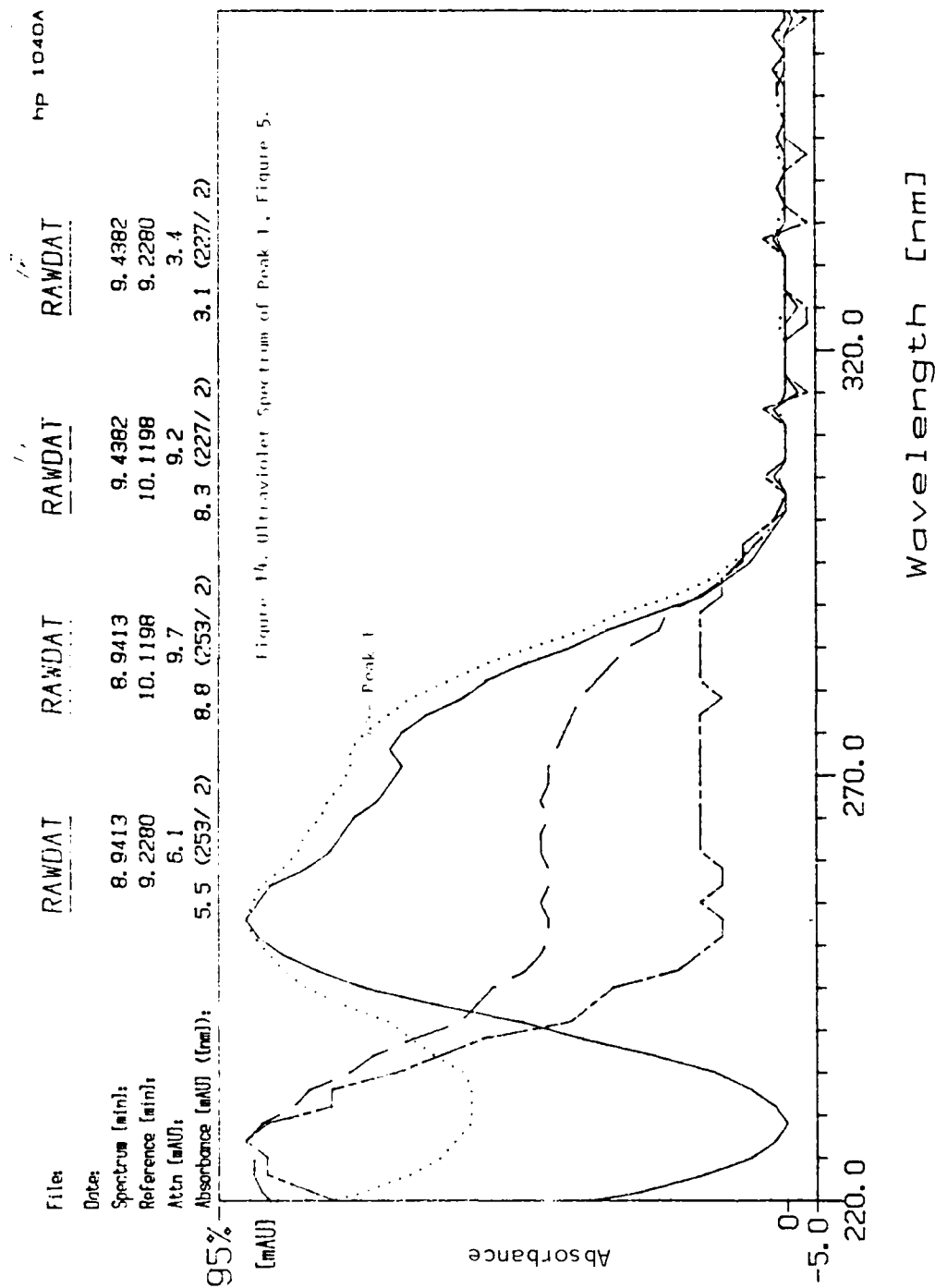


Figure 14: Ultraviolet spectrum of Peak 1, Figure 5.

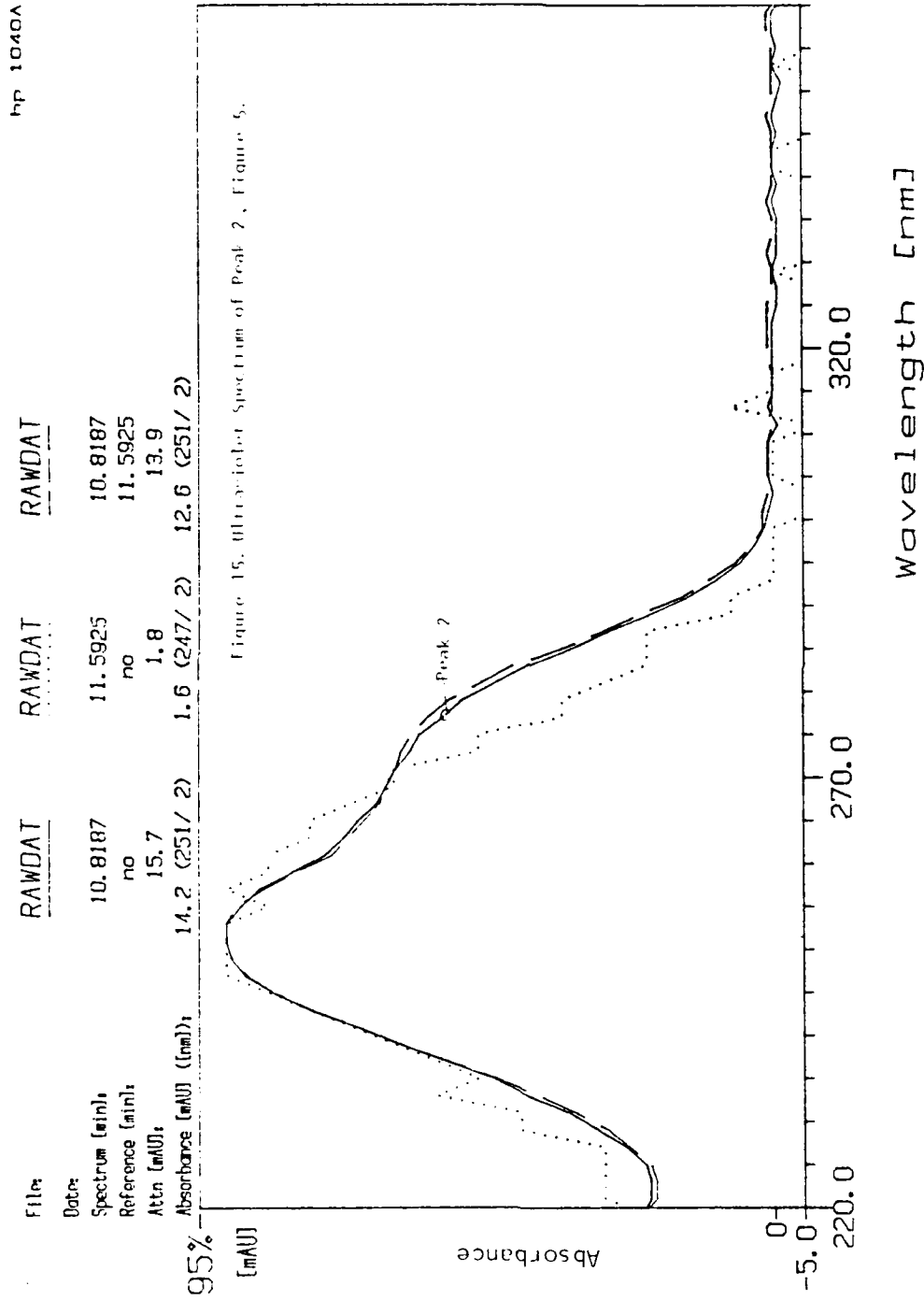


Figure 15: Ultraviolet spectrum of Peak 2, Figure 5.

HP 1040A

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Date:		
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Reference (min):	18.7622	18.7622
Attn (a.u.):	967.1	5.3
Absorbance (mAU) (nm):	875.0 (251 / 2)	4.8 (253 / 2)

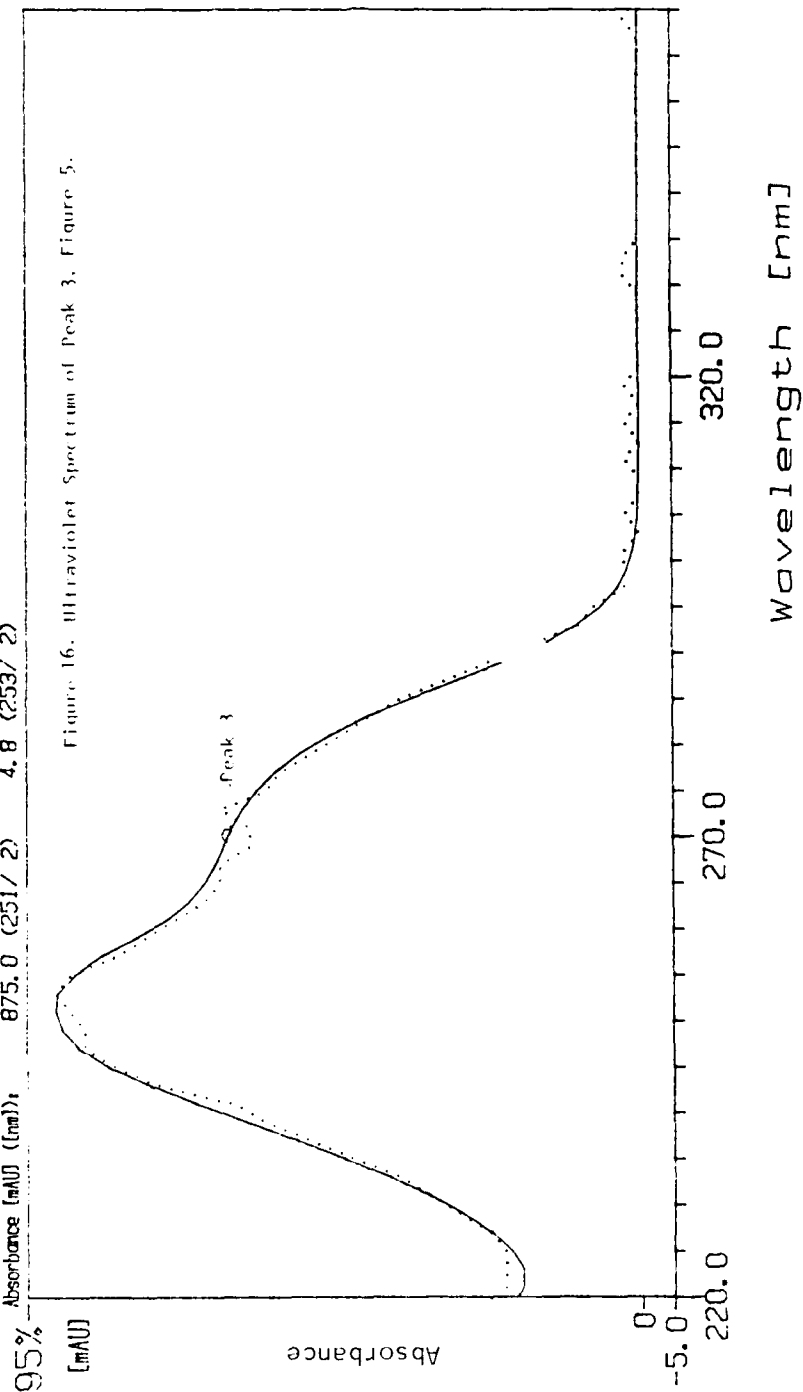


Figure 16: Ultraviolet spectrum of Peak 3, Figure 5.

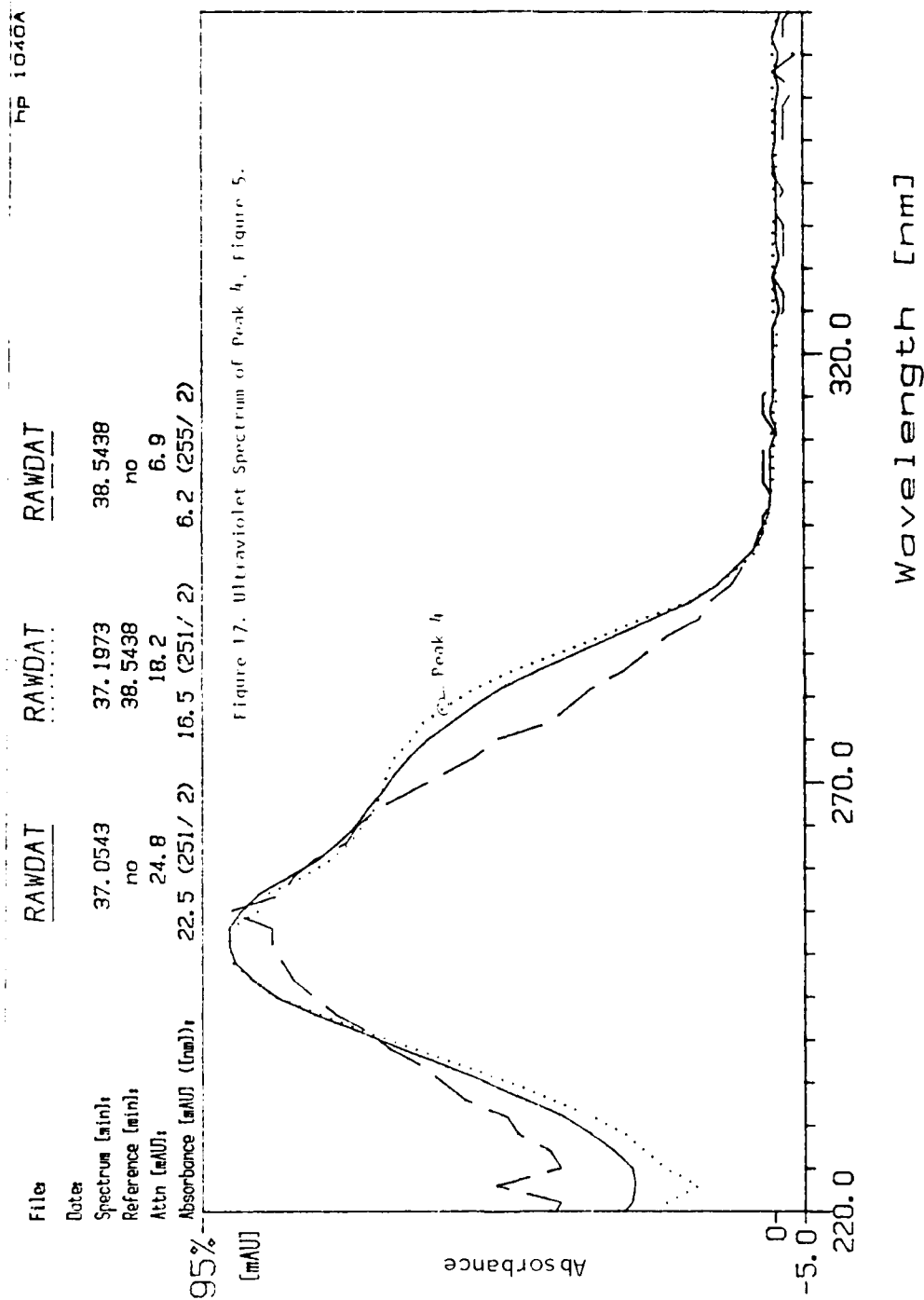


Figure 17: Ultraviolet spectrum of Peak 4, Figure 5.

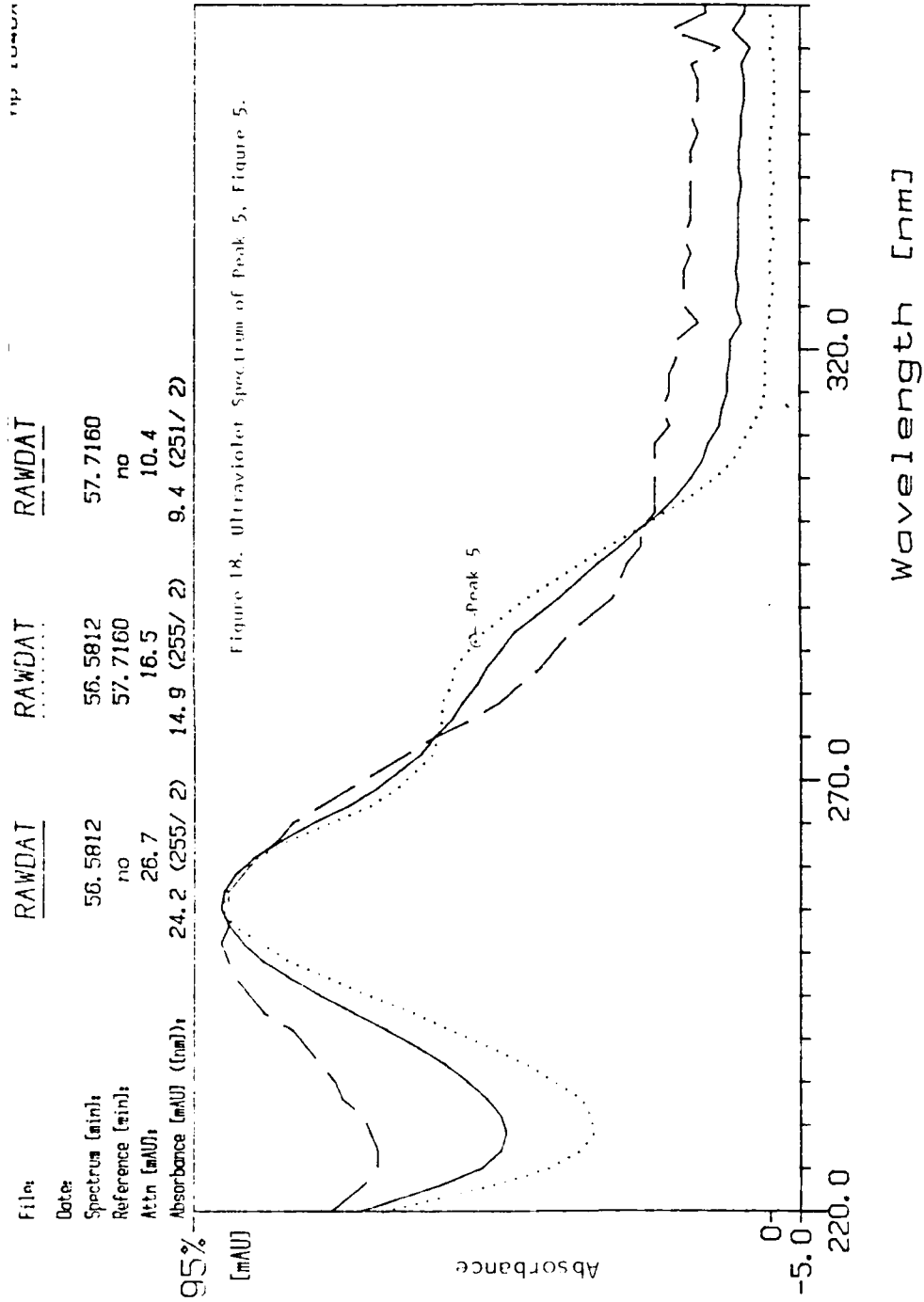


Figure 18: Ultraviolet spectrum of Peak 5, Figure 5.

HP 1040A

3.1.3

Dates

Spectrum [min]:

Reference [min]:

Act 1 [MAU]:

Absorbance [mAU] ([nm]):

RAWDAT

58. 5953

no

56.8

51.4 (255/ 2)

RAWDAT.....

58. 5953

59. 6263

46.0

41.6 (255/ 2)

RAWDAT

59. 6263

no

12.3

11.1 (219/ 2)

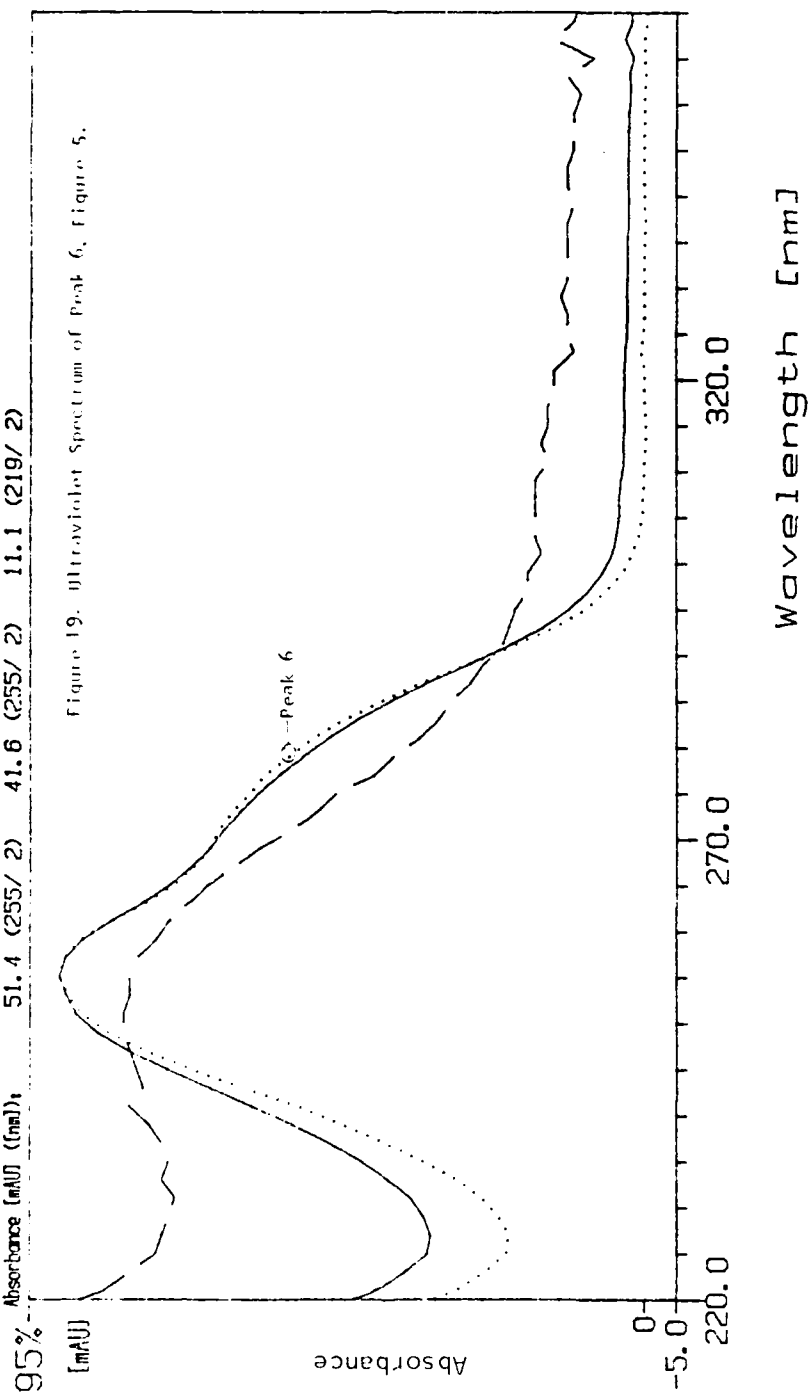


Figure 19: Ultraviolet spectrum of Peak 6, Figure 5.

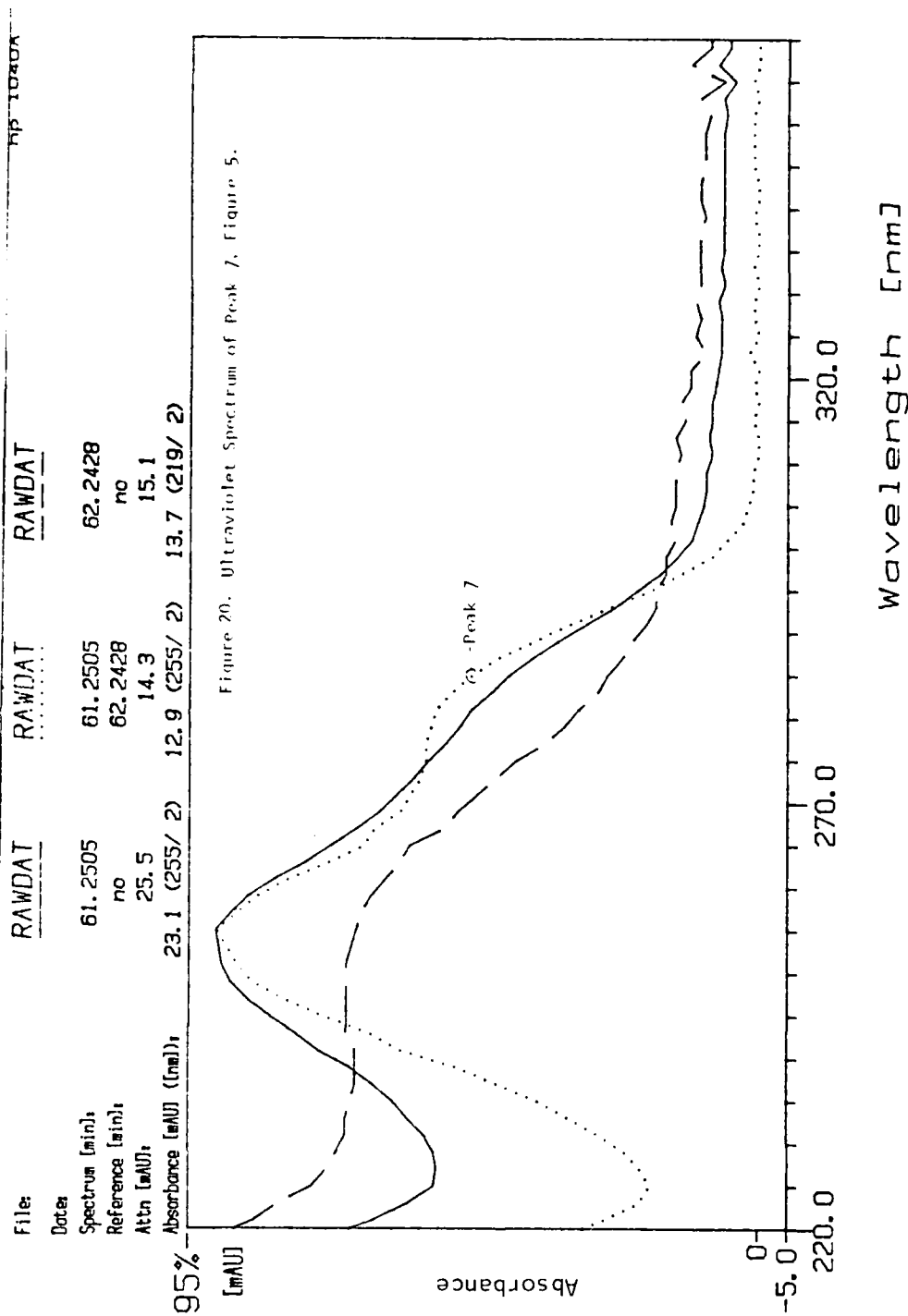


Figure 20: Ultraviolet spectrum of Peak 7, Figure 5.

hp 1040A

RAWDAT

File:

Date:

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Reference [min]: 70.5545

Attn [mAU]: 10.1

Absorbance [mAU] (1ml): 9.2 (219/ 2)

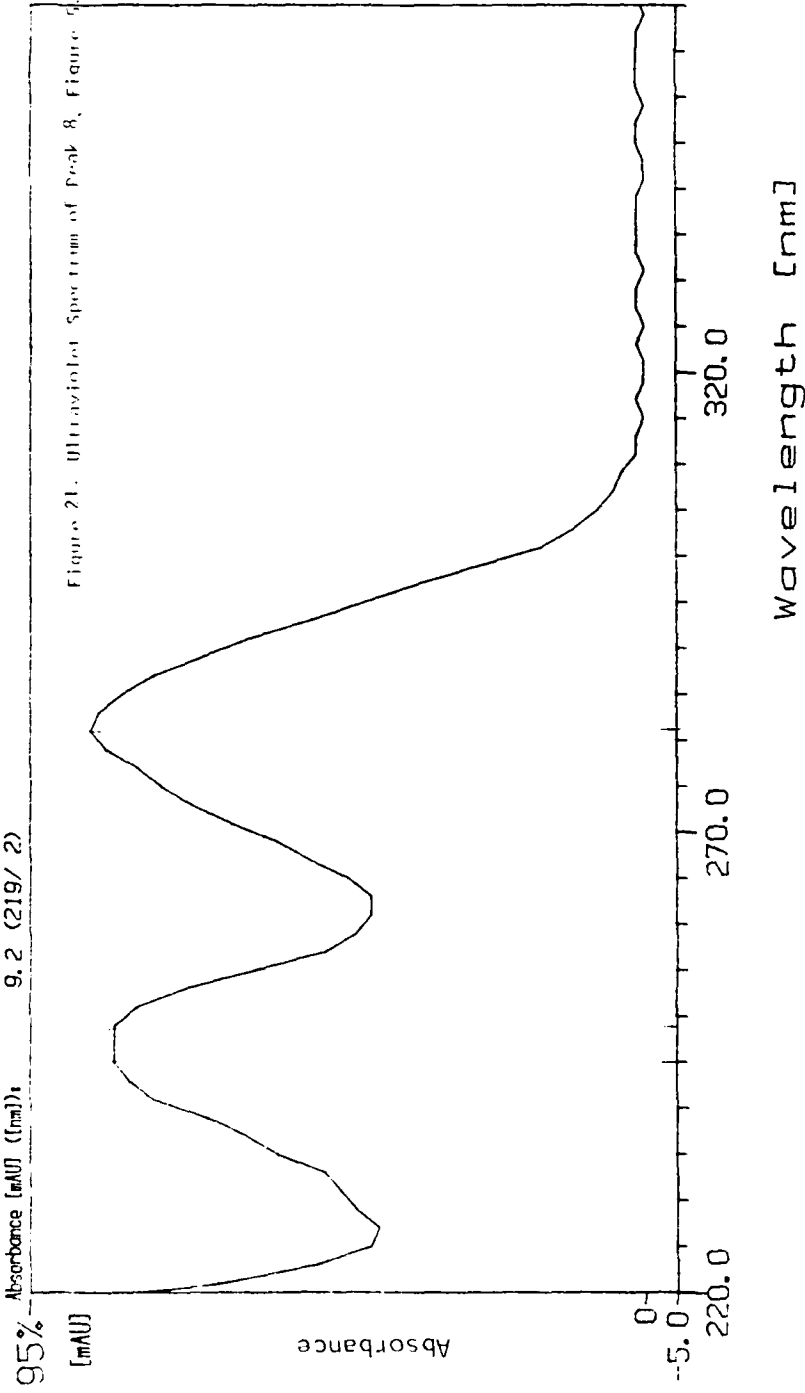


Figure 21: Ultraviolet spectrum of Peak 8, Figure 5.

these spectra were included in Table 2 but further studies would be necessary to identify these peaks with certainty.

Since the literature contains a description of the separation of a CEES-deoxyguanosine reaction mixture on G-10 Sephadex and describes the major derivative found in each of these peaks, we decided to repeat these experiments and use the information obtained to assign structures to some of the unknowns observed in our HPLC separation (Figure 5). A portion of CEES-deoxyguanosine reaction mixture prepared in the manner similar to that described in the literature (5) was applied to a G-10 column (1.5 x 40 cm) and eluted with 50 mM ammonium formate buffer at a flow rate of 0.9 ml/min. As shown in Figure 22, two partially resolved peaks appeared before the major deoxyguanosine peak. Fractions were pooled as indicated in Figure 22 and subjected to high pressure liquid chromatography as shown in Figures 23 and 24. The literature would suggest that the material in "B" would be primarily N'-ethylthioethyl deoxyguanosine and that "A" would consist of its decomposition products. Based on this information, the HPLC peak with a retention time of 58 minutes may be N'-ethylthioethyl deoxyguanosine while some of the other peaks in the 55- to 65-minute region may be products of its decomposition.

In order to characterize the smaller peaks which appeared after deoxyguanosine on G-10 Sephadex chromatography, a larger sample of CEES-deoxyguanosine reaction mixture was applied to the same column and eluted under similar conditions, but with a higher detector sensitivity. The results of this experiment are shown in Figure 25. It is apparent that, just as reported in the literature, three additional ultraviolet absorbing peaks appear after deoxyguanosine. These fractions were pooled as shown in Figure 25. Judging from the literature, pool "F" might have been N'-ethylthioethyl deoxyguanosine, "I" might have been N'-ethylthioethyl guanine, and "L", N'-ethylthioethyl guanine. When these various fractions were characterized by high pressure liquid chromatography, however, they were found to be somewhat impure, and we could not be certain of their identity. Some of these products do, however, appear in the same region as our O⁶ derivatives and may be of use in further refining our chromatographic separations.

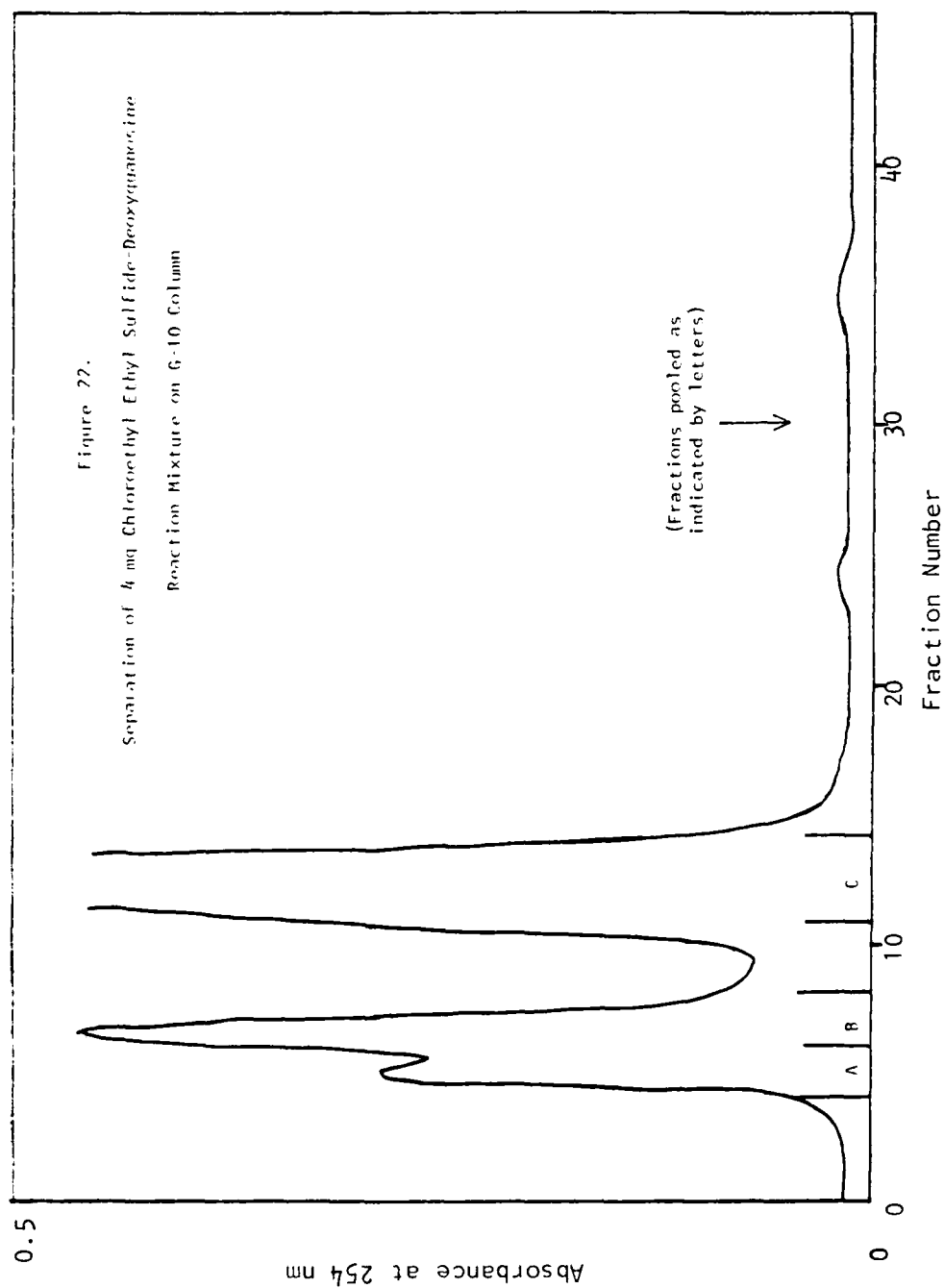


Figure 22: Separation of 4 mg chloroethyl ethyl sulfide-deoxyguanosine reaction mixture on a G-10 column. A sample containing 4 mg of nucleosides was applied to a G-10 column (1.5 x 40 cm) and eluted with 50 mM ammonium formate, pH 6.5, at a flow rate of 0.9 ml/min.

hp 1040A

File: RAWDAT
 Date: 05/17/1983
 Inj. Time: 13:28
 Attn (mAU): 1500.0 (895.1)
 Zero: 10
 Signal: B: 3.8

Wavelength (nm)
 1. 210. 4
 2. 230. 4
 3. 254. 4
 4. 280. 80
 5. 280. 4
 6. 320. 20
 7. 450. 50
 8. 550. 100

Figure 23: High Pressure Liquid Chromatographic Separation of G-10 Fraction A.

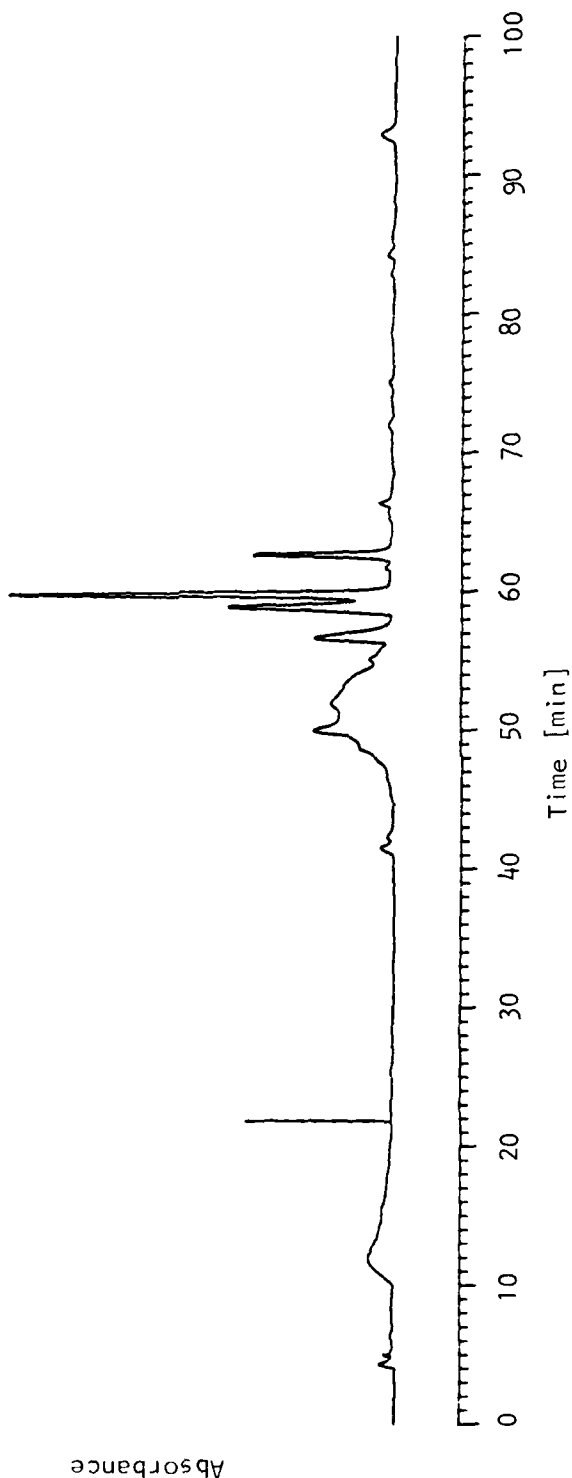


Figure 23: High pressure liquid chromatographic separation of G-10 fraction A.
 HPLC performed as described in the legend to Figure 5.

hp 1040A

Novelength (nm)
1. 210. 4
2. 230. 4
3. 254. 4
4. 280. 80
5. 280. 4
6. 320. 20
7. 430. 50
8. 550. 100

File: RAWDAT
Date: 05/18/1983
Inj. Time: 10:44
Attn (mAU): 1500.0 (775.8)
ZeroZ: 10
Signal: B: 3.8

Figure 24. High Pressure Liquid Chromatographic
Separation of G-10 Fraction B.

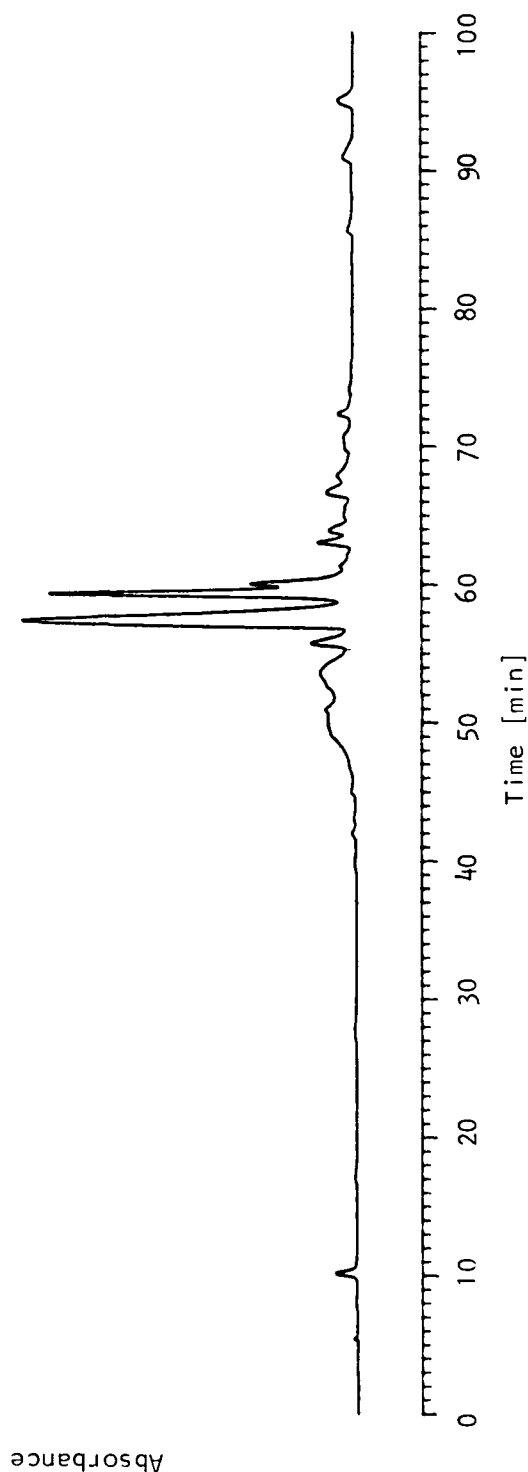


Figure 24: High pressure liquid chromatographic separation of G-10 fraction B.
HPLC separation as described in the legend to Figure 5.

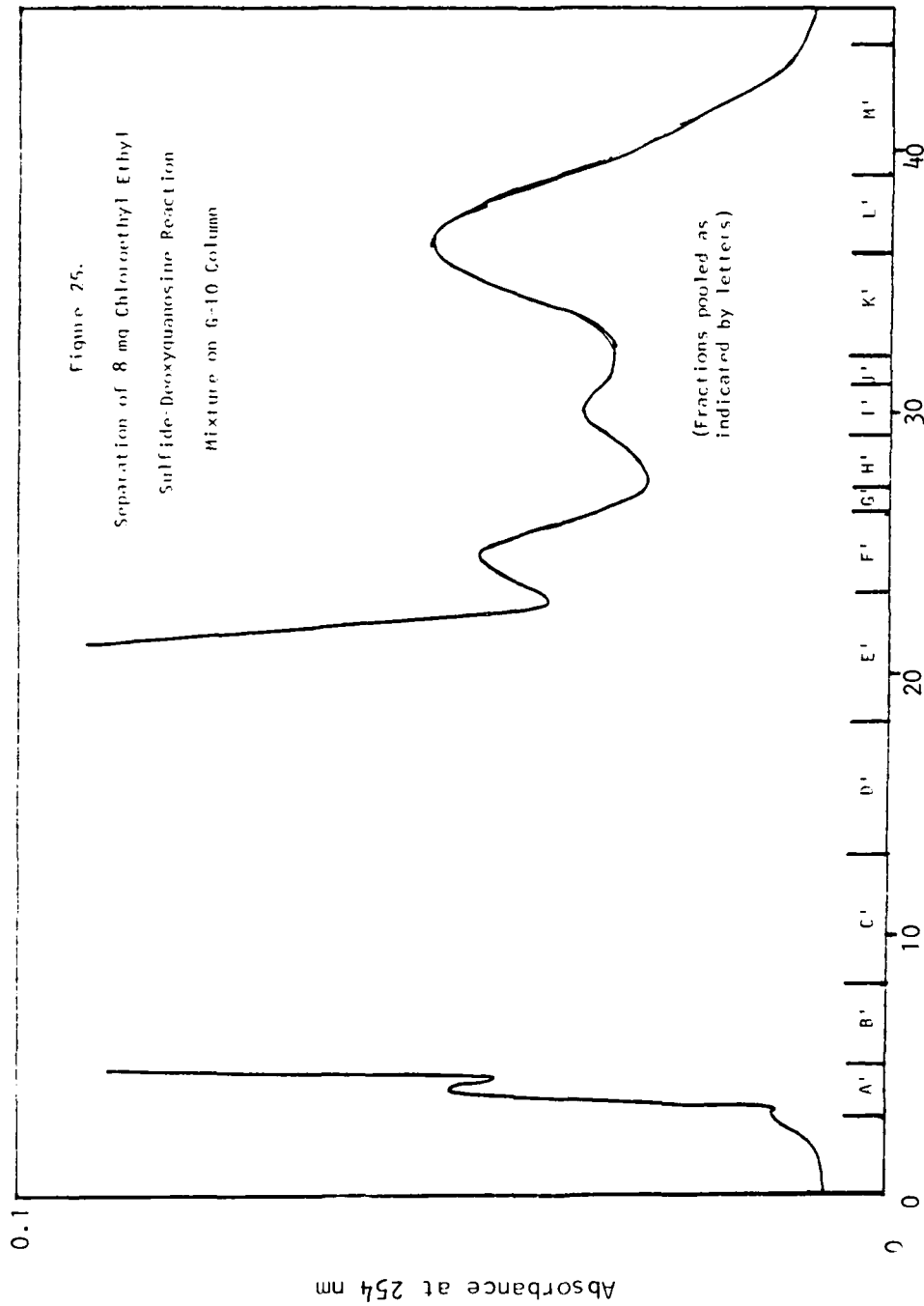


Figure 25: Separation of 8 mg chloroethyl ethyl sulfide-deoxyguanosine reaction mixture on a G-10 column. A sample containing 8 mg of nucleosides was applied to the same G-10 described in Figure 22 and eluted under the same conditions. The greater sample size and a higher detector sensitivity allowed later peaks to be resolved.

hp 1040

File: RAWDAT
Date: 05/26/1983
Inj. Time: 14:05
Attn (mAU): 500.0 (50.1)
Zero%: 10
Signal: B: 3.8

Wavelength (nm)
1. 210. 4
2. 230. 4
3. 254. 4
4. 280. 80
5. 280. 4
6. 320. 20
7. 430. 50
8. 520. 100

Figure 26. High Pressure Liquid Chromatographic Separation of G-10 Fraction G'.

Absorbance

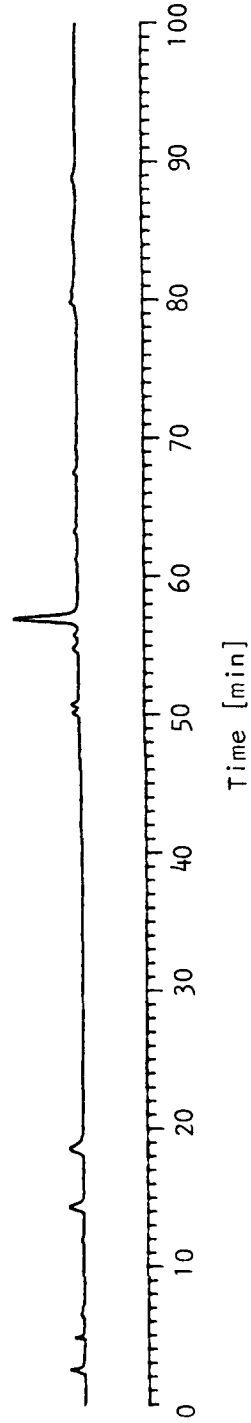


Figure 26: High pressure liquid chromatographic separation of G-10 fraction G'.
HPLC separation was performed as described in the legend to Figure 5.

hp 1040A

Wavelength (nm)
1. 210, 4
2. 230, 4
3. 254, 4
4. 280, 80
5. 280, 4
6. 320, 20
7. 450, 50
8. 520, 100

File: RAWDAT
Date: 05/26/1993
Inj. Time: 10.16
Attn (mAU): 500.0 (94.5)
Zero: 10
Signal: B: 3.8

Figure 27. High Pressure Liquid Chromatographic Separation of G-10 Fraction I'.

Absorbance

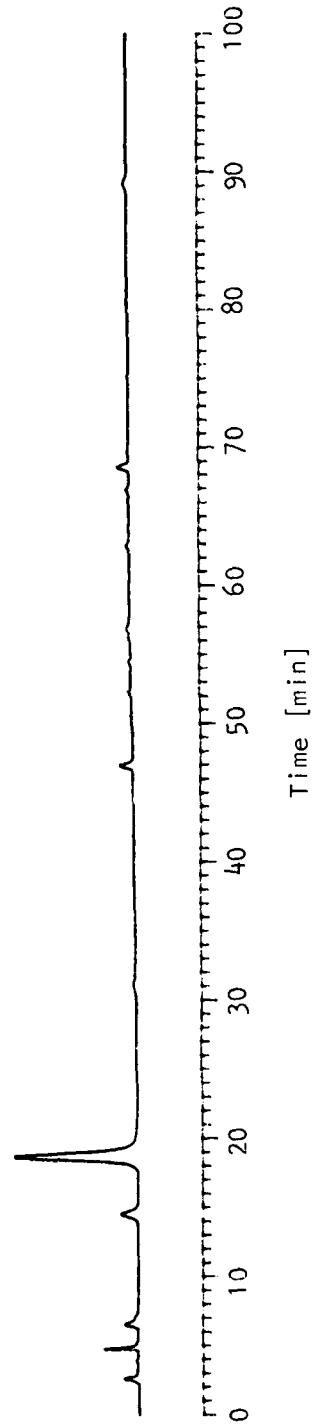


Figure 27: High pressure liquid chromatographic separation of G-10 fraction I'.
HPLC separation was performed as described in the legend to Figure 5.

hp 104

File: RAWDAT
 Date: 05/25/1983
 Inj. Time: 14:09
 Attn (mAU): 500.0 (143.3)
 Zero%: 10
 Signal: B: 3.8

Wavelength (nm)
 1. 210. 4
 2. 230. 4
 3. 254. 4
 4. 280. 80
 5. 280. 4
 6. 320. 20
 7. 450. 50
 8. 550. 100

Figure 28. High Pressure Liquid Chromatographic
 Separation of G-10 Fraction K'

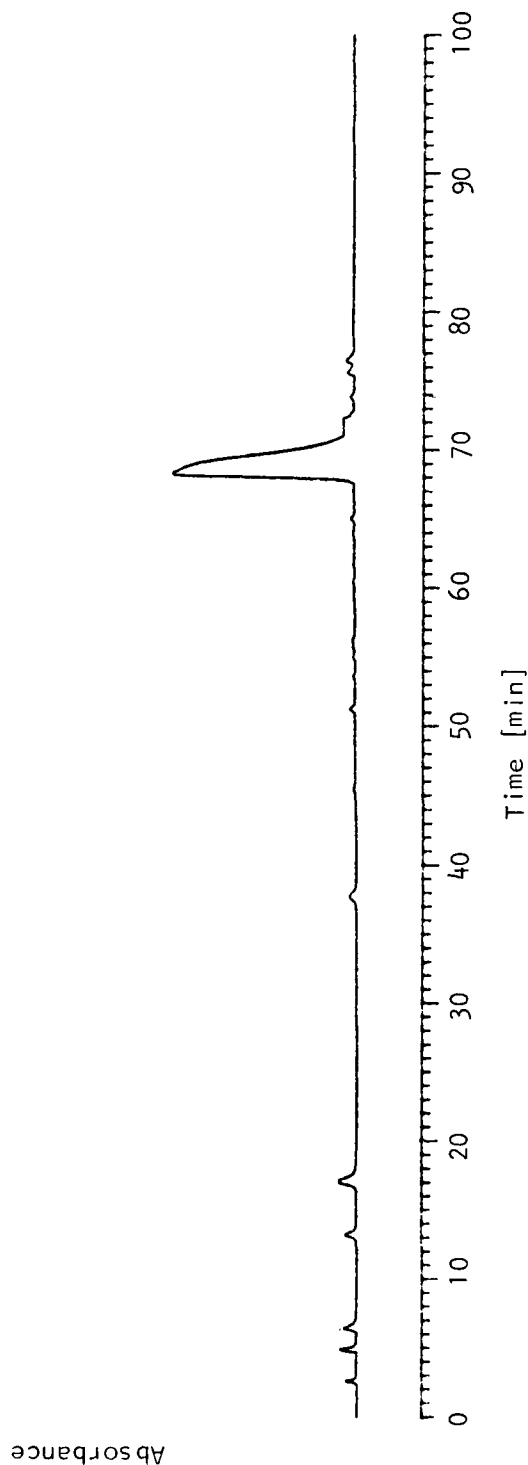


Figure 28: High pressure liquid chromatographic separation of G-10 fraction K'.
 HPLC separation was performed as described in the legend to Figure 5.

File: RAWDAT
 Date: 01:20
 Inj. Time: 500.0 (40.3)
 Attn [mAU]: 10
 Zero: B: 3.8
 Signal:

hp 1040A

Wavelength (nm)
 1. 210. 4
 2. 230. 4
 3. 254. 4
 4. 280. 80
 5. 280. 4
 6. 320. 20
 7. 450. 50
 8. 550. 100

Figure 29: High Pressure Liquid Chromatographic
 Separation of G-10 Fraction L'

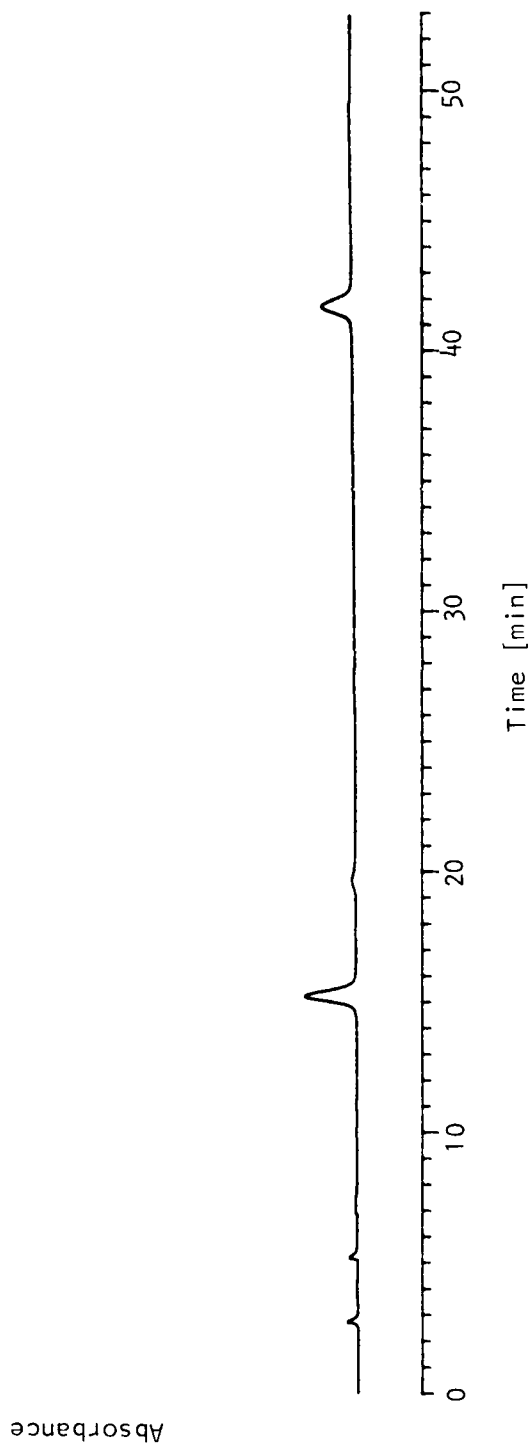


Figure 29: High pressure liquid chromatographic separation of G-10 fraction L'.
 HPLC separation was performed as described in the legend to Figure 5.

hp 1040A

File: RAWDAT
 Date: 05/25/1983
 Inj. Time: 10:19
 Attn [mAU]: 500.0 (255.6)
 Zero%: 10
 Signal: B: 3, 8

Wavelength [nm]
 1. 210. 4
 2. 230. 4
 3. 254. 4
 4. 280. 80
 5. 280. 4
 6. 320. 20
 7. 450. 50
 8. 550. 100

Figure 30. High Pressure Liquid Chromatographic
 Separation of G-10 Fraction M'.

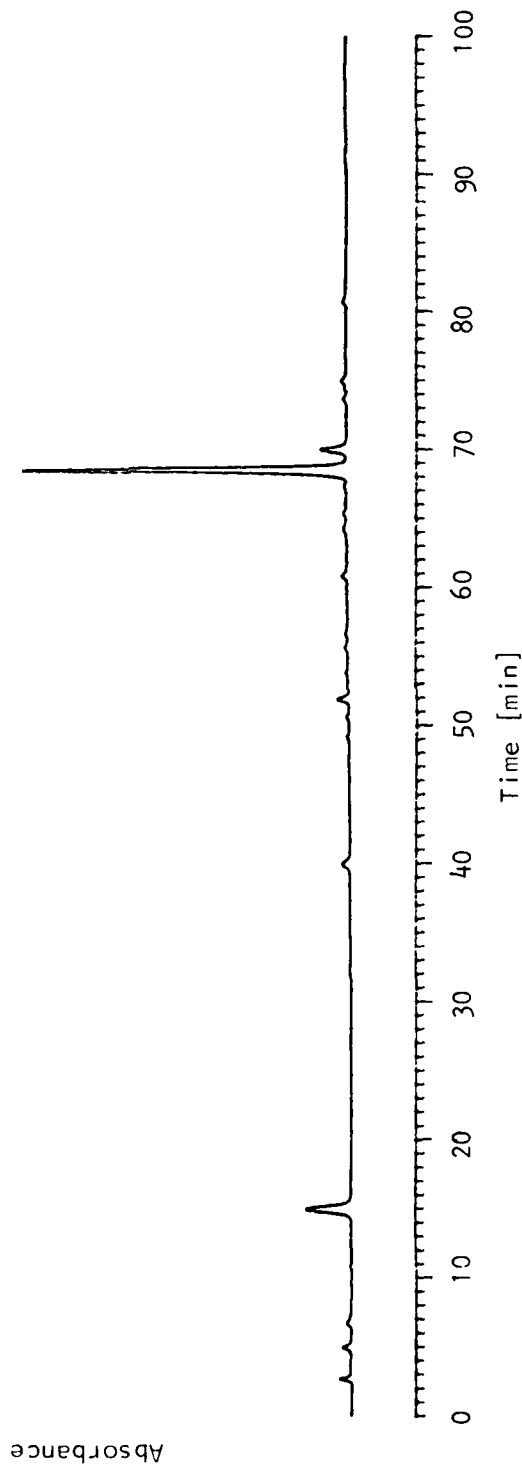


Figure 30: High pressure liquid chromatographic separation of G-10 fraction M'.
 HPLC separation was performed as described in the legend to Figure 5.

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