AN ESTIMATION OF ETHYL METHANESULPHONATE
INDUCED ALKYLATION OF VICIA FABA DNA

by

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1970
STATEMENT BY AUTHOR

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SIGNED: [Signature]

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

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Date: 10 July 1970
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ABSTRACT

DNA extracted from the primary roots of the broad bean *Vicia faba* was reacted *in vitro* to determine the mutagenic specificity of the alkylating agent ethyl methanesulphonate (EMS) for guanine. This study is unique in that it uses DNA from a higher plant which has also been studied cytologically for the effects of EMS on the chromosome complement. In the cytological study, it was inferred that the localization of breaks in the chromosomes resulted from affinity of EMS for guanine rich areas. In the present study, *Vicia faba* DNA was pretreated with the antibiotic actinomycin D (AMD) in an attempt to inhibit alkylation or depurination and to thus facilitate the interpretation of the alkylation reaction.

Buffered solutions of 44 mg and 22 mg DNA were incubated with 0.5 M and 0.25 M EMS respectively for 18 hours at 37°C and the percent of alkylation was determined. An investigation of the alkylation products by ascending paper chromatography indicated that EMS is specific for guanine, and that 7-ethyl guanine was the only detectable product. Therefore, the effect of the *in vitro* alkylation of *Vicia faba* DNA is predominately an ethylation of guanine.
as predicted by current ideas of the molecular mechanism of alkylating agents.

Similar experiments were conducted using DNA pre-treated with 0.004 mM AMD in a buffered solution for 18 hours at 37°C prior to the reaction with EMS. The products were quantified on the basis of their spectrophotometric absorption. Approximately 46% of the total guanine present in the DNA of Vicia faba, reacted with EMS to produce 7-ethyl guanine in all cases, indicating that the AMD pretreatment had no effect. Approximately one-third of the total 7-ethyl guanine produced was found to have been hydrolyzed from the DNA.

The data indicate, on the assumption that AMD did react with the DNA, that the antibiotic neither inhibited alkylation nor prevented depurination.
INTRODUCTION

An *in vivo* cytological study of the mutagenic effect of the alkylating agent, ethyl methanesulphonate (EMS) on the broad bean, *Vicia faba* (Ward, 1966) indicated a definite localization of chromosomal breaks. EMS preferentially mutates certain of the heterochromatic regions of the chromosome in the plant. This apparent specificity suggested that these chromosomal regions attacked are guanine rich, a conclusion based on reports from a number of workers whose research has been reviewed by Lawley (1966).

One group of researchers, Reiner and Zamenhof (1957) found that the nitrogen atom at the N-7 position of guanine was sterically available in the Watson-Crick model (1953) of the DNA double helix. In similar experiments with alkylating agents, Brookes and Lawley (1960) came to the conclusion that in a reaction between DNA and an alkylating agent, the most reactive site was the N-7 atom of the guanine moiety. Further confirmation came from the theoretical calculations of the electron densities in the bases of DNA (Pullman and Pullman, 1959) which suggested that the N-7 position of guanine should be one of the most reactive points in the DNA molecule. This atom
has also been shown to be the site of protonation in guanine by spectral and nuclear magnetic studies (Miles, Howard, and Frazier, 1963). In all alkylation experiments, even at a neutral pH, some hydrolysis of the alkylated product has been reported (Brookes and Lawley, 1961). Bautz and Freese (1960) proposed a mechanism for the depurination of 7-ethylated guanine by hydrolysis at the glycosidic bond. It is this depurination that can result in a transition or transversion (Auerbach, 1967); while an alkylation of the phosphates, which occurs at a low frequency, is responsible for the break in the backbone of the DNA chain. A third possibility is a transition by mispairing of the alkylated guanine to a thymine due to the altered electron configuration of the guanine moiety.

The antibiotic, actinomycin D (AMD), has been shown to be both biologically specific and selective for the guanine moiety (Kersten, 1961). Reich (1964) and Gellert, Smith, Neville, and Felsenfeld (1965) found that in addition to guanine, AMD required a helical structure for binding. According to the model of Hamilton, Fuller, and Reich (1963), the actinomycin chromophore binds directly to the N-3 atom of guanine and its deoxyribose moiety, and is also bound in the minor groove of the helical DNA by seven hydrogen bonds.
Therefore, the unique binding properties of the antibiotic suggested the hypothesis that AMD might provide a useful tool to investigate the mutagenic specificity of EMS by either blocking alkylation of the guanine moiety or preventing depurination of the alkylated product. Since a cytological study has already been completed (Ward, 1966), this investigation is primarily concerned with the in vitro alkylation of Vicia faba DNA.

The aim of my study was to determine the percent of alkylation, and the percent of depurination resulting from the reaction of EMS with extracted native DNA, and with native DNA pretreated with AMD. In a similar experiment, Reich (1964) alkylated calf thymus DNA with labeled mustard gas in the presence of AMD, and reported that the antibiotic did not interfere with the alkylation reaction. Nevertheless, the results of the in vivo study with Vicia faba (Ward, 1966) suggested the possibility of some inhibition of EMS, and therefore warranted further investigation by an in vitro study.
MATERIALS AND METHODS

*Vicia faba* L. (var. Longpod) seeds used in the experiments were obtained from W. Atlee Burpee Company.

**Seed Sterilization and Germination**

To obtain a maximum germination and a minimum contamination, approximately 2,000 seeds for each extraction were sterilized by soaking them for 30 minutes in a Clorox solution, of one part Clorox to 10 parts water, containing approximately one gram of Tide detergent, as a wetting agent, per 500 ml (Ward, 1966). The seeds were rinsed with tap water, and were soaked in slowly running tap water for four days in a plastic bucket. The bucket was provided with a siphon which permitted a change of water every 15-20 minutes. Continuous aeration was supplied from the bottom of the bucket. The temperature of the water was $19^\circ \pm 6^\circ C$. To reduce contamination enough Clorox to make approximately 2% solution was added to the soaking seeds after two days growth.

At the end of a four day growing period, the seedlings had developed primary roots of about 2-3 cm. At this time the entire primary root was cut off and placed on ice. All subsequent procedures were carried out over ice or in a refrigerated centrifuge ($4^\circ C$).
The roots were weighed so that an estimate of the yield of DNA in terms of wet weight could be obtained. The average weight of the roots used for an extraction was approximately 250 g, which yielded 0.54 mg DNA per gram of wet weight.

**DNA Extraction**

The method used for extraction of the total DNA was a modification of both Marmur's method (1961) and that of Lyttleton and Petersen (1964). Approximately 30 g of roots were ground at a time in a cold mortar and pestle using enough freshly made grinding buffer of 1% sodium dodecylsulphate (SDS) in 0.025 M Tris buffer (pH 7.3) to make a thick slurry. A small quantity of acid-washed sand was added to facilitate grinding. Lyttleton and Petersen (1964) found that this solution gives a relative yield of polymerized DNA of approximately 95% of the total DNA in the cell.

To prevent frothing, a drop of octanol was added while grinding when needed. The ground material was then pooled and placed in the refrigerator (4°C) for ½ hour to ensure more efficient lysis of the cell as well as additional deproteination by the detergent SDS.

Following the action of the SDS, an equal volume of redistilled phenol (saturated with 0.025 M Tris) was added to the macerated material. The material was
intermittently shaken for one hour to put the phenol in constant contact with the material, and to thus break up the DNA-protein complex (Kirby, 1957). The slurry was centrifuged in the cold (4°C) for 15 minutes at 17,300 X g. The upper aqueous layer was then drawn off with a large-mouthed pipette. The precipitate was washed with an equal volume of distilled water and then recentrifuged at 17,300 X g for five minutes. The aqueous layer was drawn off and pooled with the previous supernatant (Kirby, 1957).

Approximately one volume of isopropyl alcohol was added slowly into a vortex of the DNA solution of 0.3 M sodium acetate to selectively precipitate the DNA (Marmur, 1961). The mixture was then placed overnight at -15°C. At this stage the granular precipitate was collected by centrifugation for five minutes at 4,340 X g. The alcohol supernatants were discarded, and enough dilute saline citrate (0.1 X SSC = 0.015 M NaCl + 0.0015 M trisodium citrate, pH 7.0) to dissolve the precipitate was added.

**DNA Purification**

Bovine pancreas ribonuclease (grade A obtained from Calbiochem), which had been previously treated at 80°C for 10 minutes to destroy any DNase that was present, was added to the DNA solution at a concentration of 50 µg/ml of solution (Marmur, 1961), and incubated for approximately 15 minutes at 37°C.
After the RNAse treatment, pronase (obtained from Calbiochem) which had been allowed to self-digest for one hour at 37°C to destroy any DNAse, was added at a concentration of 1 mg/ml of DNA solution. The combined mixture of RNAse, pronase, and DNA was incubated for 30 minutes at 37°C in a water bath.

Following the above treatment, two volumes of cold isopropyl alcohol were added to the mixture to selectively precipitate the DNA; and then the mixture was placed at -15°C for one hour. This time, although the DNA appeared thread-like and spoolable, the DNA was spun down at 4,340 X g and the precipitate redissolved in enough dilute SSC as was required. For storage the solution was brought up to standard SSC by adding one-tenth volume of 10 X SSC. One drop of chloroform was added to prevent bacterial contamination.

**DNA Assay**

A total DNA assay was carried out on the DNA solution using the Dische diphenylamine reaction (Dische, 1955). Standards were made up in 5 mM NaOH using highly polymerized calf thymus DNA (Mirsky - Pollister) obtained from Nutritional Biochemicals Corp. The diphenylamine reagent was freshly prepared by adding 0.5 g diphenylamine to 50 ml glacial acetic acid (99%) and 1.37 ml H₂SO₄
(reagent grade). The reagent was stored in the dark at all times until use.

Two ml of the diphenylamine reagent were added to each 1 ml dilution of DNA of standard solution ranging from 0.4 mg/ml to 0.05 mg/ml. This reagent was also added to 1 ml of the extracted DNA, previously diluted by a factor of one-tenth of the original concentration. All tubes were heated simultaneously for 10 minutes in a boiling water bath.

The transmittance of the solution in each tube was determined by the intensity of the blue color read on a Bausch and Lomb Spectronic 20 spectrophotometer at 600 m\(\mu\). A standard concentration curve was constructed to calculate the approximate amount of DNA-Phosphorus extracted relative to the DNA standards. Once the concentration of the DNA solution was determined, specific portions of it were used in the reaction mixture.

**Alkylation**

Ethyl methanesulphonate (EMS) was obtained from K & K Laboratories, Inc., California. A 0.5 M solution of EMS was prepared fresh for each experiment by adding 0.5 ml EMS stock solution to 9.5 ml of 0.02 M monobasic sodium phosphate buffer (pH 7.2). DNA was prepared by adding two volumes of 95% ethanol to a predetermined amount of DNA solution (depending on the experiment, either 22 or 44 mg
of DNA were used. The mixture was placed at -15°C for one hour to ensure complete precipitation of all DNA. This was followed by centrifugation in the cold (4°C) at 4,340 X g for five minutes. The supernatant was discarded and the precipitate air-dried.

The reaction mixture for preliminary alkylation tests was prepared by dissolving the precipitated DNA in 8 ml of 0.02 M monobasic sodium phosphate buffer (pH 7.2). To this solution 1.2 ml of the 0.5 M EMS solution was added to yield a final concentration of 0.067 M EMS. More than enough EMS was thus present for complete saturation of the DNA guanine, since on a mole/mole basis 0.25 mM EMS would be sufficient for saturation. The mixture was incubated for 18 hours at 37°C ± 1°C in a water bath. The pH of the mixture remained at 7.2 throughout the experiment.

Alkylation of DNA was terminated by the addition of two volumes of 95% ethanol. After overnight storage at -15°C the DNA was centrifuged in the cold at 4,340 X g for five minutes. The precipitate was washed three times by redissolving the DNA in dilute SSC and then re-precipitating the DNA with ethanol as above. All supernatants were pooled and evaporated to dryness over a water bath at approximately 85°C. Evaporation was speeded up by directing a constant stream of cool air over the surface of the solution.
The precipitate was hydrolyzed with 0.2 M $\text{H}_2\text{SO}_4$ for one hour at 100°C to obtain purine bases and pyrimidine nucleotides (Shapiro, 1967). Enough 1 M NaOH was added to bring the solution to neutrality for subsequent paper chromatography analysis. Both the hydrolysate and the supernatant (redissolved in 0.1 N HCl) were used in one dimensional chromatography which will be discussed later.

Pretreatment of DNA with Actinomycin D

Several experiments with EMS were run to determine the minimum amount of DNA required for detection of the alkylated product, 7-ethyl guanine. Once the minimum amount was determined, two experiments were run in which the DNA was pretreated for 18 hours with actinomycin (AMD), obtained from Mann Research Laboratories. The reaction mixture consisted of 22 mg of DNA dissolved in 4 ml of 0.02 M monobasic sodium phosphate buffer (pH 7.2), and 5 mg of AMD (0.004 mM) dissolved in 1.5 ml of distilled water. After incubation for 18 hours at 37°C ± 1°C in the dark, the DNA was precipitated with two volumes of 95% ethanol, and then washed three times with ethanol to remove the unreacted AMD.

At the same time, a control alkylation experiment using an equal amount of DNA was carried out under the same conditions, but with the omission of AMD; that is, 0.5 M EMS (0.6 ml of prepared solution) was mixed with 4 ml of
phosphate buffer (pH 7.2) containing 22 mg of DNA and the mixture was incubated at 37° ± 1°C. As in previous alkylation experiments, two volumes of 95% ethanol were added to terminate the reaction. This was followed by three washings, evaporation of the supernatant, and hydrolysis of the DNA precipitate.

**Paper Chromatography Analysis**

A stock solution of 7-ethyl guanine was obtained from Cyclo Chemicals, California; and was used as a standard both for confirmation of $R_F$ values in the paper chromatography analysis and for quantification of the 7-ethyl guanine produced by alkylation of the DNA. Whatman no. 1 paper was used for ascending chromatography with two different solvents. Solvent I was composed of methanol: water (75:25) and was used for identification of guanine. Solvent II consisted of aq. n-butanol (86 butanol:14 water), with concentrated NH$_3$ solution (sp. gr. 0.88) constituting 5% of the total volume (Wyatt, 1955). Only solvent I was used for quantitative work since Jordan (1960) indicated that solvent II is not suitable for quantification of guanine.

Solvent II was necessary due to multiple spots which occurred when small amounts of unknowns were viewed on the chromatograms of the supernatant. It was believed that nucleotides, the products of incomplete enzymatic
degradation of RNA, were responsible for these unknown spots. There was no explainable reason for their presence as products of alkylation. The problem was alleviated by the use of solvent II since only 7-ethyl guanine was detected in the chromatogram; while the unknowns remained at the origin as expected (Wyatt, 1955). A controlled experiment, with all other conditions the same as those in the alkylation experiments but with the absence of EMS, was conducted to identify the spots. The $R_f$ values were compared to those given by Wyatt (1955).

On all chromatograms, 20 $\lambda$ of 7-ethyl guanine (1 mg/ml in 0.1 N HCl), 20 $\lambda$ of the guanine standard of the same concentration, and aliquots (40 $\lambda$) of the supernatant and the hydrolysate were placed respectively in four small spots 3 cm from the bottom of strips of Whatman no. 1 paper (40 cm X 20 cm). The paper was suspended in a chromatography jar, allowed to equilibrate for one hour, and was then lowered into the solvent without removing the cover of the jar.

The chromatograms were allowed to develop for 13 hours at room temperature. At the end of development, the solvent front was marked and the chromatograms were air-dried. Each chromatogram was then viewed under ultraviolet irradiation (Chromato-vue 260 A, Ultra-Violet Prod. Inc.) and all the absorbing blue spots were circled in
pencil. The $R_P$ values (\textit{= distance moved by the component})
\textit{distance moved by the solvent} were determined for all spots and their identification was
determined by comparison of the $R_P$ values of the standards. Guanine and 7-ethyl guanine were the primary components of concern.

\begin{center}
\textbf{Product Estimation}
\end{center}

Each circle was then cut out and eluted in 2 ml
of 0.1 N HCl (pH 1.2) overnight at room temperature.
Further analysis was performed and confirmation of the
identified spots was obtained by measuring their optical
densities on a Gilford spectrophotometer. Ultra-violet
absorption was measured at 10 m\textmu intervals over a range
of 210 to 290 m\textmu.

The quantities of 7-ethyl guanine and guanine were
estimated by comparing optical densities of the unknowns
with those of the standards. The alkylation product, 7-
ethyl guanine, was found to exist in both supernatant and
hydrolysate; guanine existed only as a free base in the
hydrolysate.

The summation of the moles of 7-ethyl guanine
produced (hydrolysate and supernatant) plus the number of
moles of guanine estimated from the hydrolysate, was used
to determine the percent of recovery of the starting
material. This estimation was based on an expected 18% guanine content (Sober, 1968).
RESULTS AND DISCUSSION

The phenol method (Kirby, 1957) for isolation of DNA is a widely used technique for the extraction of undegraded, and completely biologically active DNA. D. pneumoniae DNA purified by this method (Marmur, 1961) was determined to have all the biological properties of in vivo DNA.

Lyttleton and Petersen (1964) were able to improve Kirby's method and increase the relative yield of DNA. By using a grinding buffer of 1% SDS in 0.25 M Tris buffer (pH 7.3), they were able to obtain a relative yield of 95% of the total DNA in an undegraded and biologically active stage. It was by this method that the relatively high yield of 0.54 mg DNA/g of tissue was obtained in this present study.

One undesirable characteristic of the SDS extractant, also noted by Lyttleton and Petersen, was the production of a brown-colored product. Identical experiments, using DNA from two different extractions, one of which was much browner than the other, produced similar results in terms of alkylation. Initially attempts were made to rid the DNA of this color by using fresh SDS extractant followed by fractionation with a Sepharose 4B column. However,
since the intensity of the brown color had no apparent effect, fractionation was omitted in later experiments. Fresh SDS extraction was carried out even though the product was slightly brown.

The accuracy of the Dische diphenylamine assay of DNA was doubtful at times; to improve its level of confidence it was necessary to run the test three times on each extraction, and take an average of the results. Occasionally as much as 10% deviation was detected among tests.

It will be shown later than an exact estimate of the DNA used was not as critical as it might first appear. The percent of alkylated guanine is a more meaningful presentation of the data, than an approximation in milligrams. Throughout this discussion an attempt will be made to present the data as relative estimates rather than precise values since it is felt that neither the technique nor the equipment used were accurate enough to report precise results.

In the initial experiments, large quantities of DNA (45 mg) were treated with EMS to obtain alkylation products. Examination of the in vitro reaction between DNA and alkylating agents has led to the conclusion that the most reactive site is the N-7 position of guanine moieties, while the N-3 position of adenine is the most reactive minor site of the DNA helix (Reiner and Zamenhof,
Additional evidence from theoretical calculations of electron densities in DNA (Pullman and Pullman, 1959) suggests that the N-7 position of guanine should be one of the most reactive sites in the DNA molecule.

The chromatography of both the supernatant and hydrolysate from the initial alkylation, as well as later reactions with one-half the reaction mixture, yielded six products in solvent I (Tables 1 and 2). Of these six spots, four were readily identified by comparing them to the standards. The R_F values of spots 1 and 4 suggested that these unknowns were the same as the 7-ethyl guanine standard. All the other products had R_F values that were distinctly different from the 7-ethyl guanine standard. Spectrophotometric analysis of spots 1 and 4 confirmed the suggested identity (Table 3).

A problem did arise in the identification of guanine. Spots 3 and 6 (Tables 1 and 2), from the supernatant and the hydrolysate respectively, had R_F values similar to the guanine standard. According to Lawley (1966), there was no reason to expect any bases to be present in the supernatant other than alkylated products. Spectral ratio analysis of the eluted spot suggested that unknown 3 could have been a guanine nucleotide or a purine polynucleotide composed largely of guanine. Unknown 6 was
Table 1. Rp values of control and alkylated DNA products compared with standards (solvent I).

<table>
<thead>
<tr>
<th>DNA Treatment</th>
<th>Rp values of standards</th>
<th>Mean Rp of unknowns</th>
<th>Supernatant</th>
<th>Hydrolysate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>guanine</td>
<td>7-ethyl guanine</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>EMS</td>
<td>.38</td>
<td>.62</td>
<td>.63</td>
<td>.51</td>
</tr>
<tr>
<td>EMS</td>
<td>.35</td>
<td>.57</td>
<td>.57</td>
<td>.44</td>
</tr>
<tr>
<td>EMS</td>
<td>.35</td>
<td>.57</td>
<td>.57</td>
<td>.48</td>
</tr>
<tr>
<td>Untreated</td>
<td>.43</td>
<td>.67</td>
<td>.54</td>
<td>.43</td>
</tr>
</tbody>
</table>

*a45 mg DNA treated with 0.5 M EMS.*
Table 2. $R_F$ values of products from DNA alkylation with and without AMD pretreatment (solvent I).

<table>
<thead>
<tr>
<th>Trial</th>
<th>DNA Treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>$R_F$ values of standards</th>
<th>Mean $R_F$ of unknowns</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>guanine</td>
<td>Supernatant</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7-ethyl guanine</td>
<td>1  2  3</td>
</tr>
<tr>
<td>I</td>
<td>EMS</td>
<td>.41</td>
<td>.72</td>
</tr>
<tr>
<td>I</td>
<td>AMD + EMS</td>
<td>.41</td>
<td>.72</td>
</tr>
<tr>
<td>I</td>
<td>EMS</td>
<td>.42</td>
<td>.63</td>
</tr>
<tr>
<td>I</td>
<td>AMD + EMS</td>
<td>.42</td>
<td>.63</td>
</tr>
<tr>
<td>I</td>
<td>AMD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>.42</td>
<td>.63</td>
</tr>
<tr>
<td>II</td>
<td>EMS</td>
<td>.38</td>
<td>.63</td>
</tr>
<tr>
<td>II</td>
<td>AMD + EMS</td>
<td>.38</td>
<td>.63</td>
</tr>
<tr>
<td>II</td>
<td>AMD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>.38</td>
<td>.63</td>
</tr>
</tbody>
</table>

<sup>a</sup>22 mg DNA treated with 0.25 M EMS, with and without 0.004 mM AMD pretreatment. In the case of AMD pretreatment, DNA was washed with EtOH prior to EMS treatment.

<sup>b</sup>Supernatant from washings of AMD treated DNA.
Table 3. Spectral ratio analysis of critical unknown products from alkylated DNA compared with the standards.

<table>
<thead>
<tr>
<th>Substance</th>
<th>pH</th>
<th>Spectral Ratios</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>250</td>
<td>260</td>
</tr>
<tr>
<td>Standards</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>guanine</td>
<td>1.2</td>
<td>1.36</td>
<td>0.84</td>
</tr>
<tr>
<td>7-ethyl</td>
<td>1.2</td>
<td>1.33</td>
<td>0.83</td>
</tr>
<tr>
<td>guanine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknowns&lt;sup&gt;a&lt;/sup&gt; from EMS + DNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.2</td>
<td>1.32</td>
<td>0.82</td>
</tr>
<tr>
<td>4</td>
<td>1.2</td>
<td>1.34</td>
<td>0.83</td>
</tr>
<tr>
<td>6</td>
<td>1.2</td>
<td>1.37</td>
<td>0.85</td>
</tr>
<tr>
<td>Unknowns&lt;sup&gt;a&lt;/sup&gt; from AMD + EMS + DNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.2</td>
<td>1.34</td>
<td>0.82</td>
</tr>
<tr>
<td>4</td>
<td>1.2</td>
<td>1.34</td>
<td>0.83</td>
</tr>
<tr>
<td>6</td>
<td>1.2</td>
<td>1.38</td>
<td>0.84</td>
</tr>
</tbody>
</table>

<sup>a</sup>Unknowns from trial I chromatogram.
identified by spectral ratio analysis (Table 3) as guanine, a product of the mild acid hydrolysis of the DNA. Since tailing is a common problem with guanine in solvents which lack the HCl necessary for increased solubility, some interference may have been responsible for the minor deviations from the expected spectral ratios.

Spectral ratio analysis suggested that unknown 2 could have been an adenine nucleotide or a purine polynucleotide composed largely of adenine. Unknown 5 was confirmed as adenine, the expected product of mild acid hydrolysis, on the basis of $R_F$ values as well as spectral ratio analysis.

It became evident that several questions had to be answered. Were the unknowns 2 and 3 products of alkylation, and if so, what was their identity? If they were not products of the reaction, were these spots artifacts of the preparation, and if so what was their origin?

At this point it was necessary to use a solvent (solvent II) which could distinguish among bases, nucleosides, and nucleotides in the supernatant. A control experiment was run in which 45 mg DNA were incubated for 18 hours under the standard alkylating conditions, but in the absence of EMS. The $R_F$ values of the chromatographed products of the supernatant and the hydrolysate in solvent II (Table 4) were then compared to the $R_F$ values of Wyatt
Table 4. \( R_F \) values of products from DNA alkylation with and without AMD pretreatment compared with those of non-alkylated DNA (solvent II).

<table>
<thead>
<tr>
<th>DNA conc. (mg)</th>
<th>Treatment</th>
<th>( R_F ) values of standards</th>
<th>Mean ( R_F ) of unknowns</th>
<th>Supernatant</th>
<th>Hydrolysate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>guanine 7-ethyl guanine</td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>45</td>
<td>untreated</td>
<td>.16  .42</td>
<td></td>
<td></td>
<td>.32  .15</td>
</tr>
<tr>
<td>45(^a)</td>
<td>EMS</td>
<td>.15  .38</td>
<td>.39</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>22.5(^a)</td>
<td>EMS</td>
<td>.15  .39</td>
<td>.40</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>22.5(^a)</td>
<td>AMD + EMS</td>
<td>.15  .39</td>
<td>.40</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\)Chromatography run on supernatants only.
The absence of spots from the supernatant (Table 4) indicated the absence of free bases or nucleosides. Therefore, unknowns 2 and 3 were judged to be artifacts, since they were present in the preparation, as determined by solvent I (Table 1), even before EMS treatment.

Some idea of the origin of the artifacts has been suggested. Since anything larger than a nucleoside will not migrate in solvent II (Wyatt, 1955), then unknowns 2 and 3 could have been polynucleotides. This would be expected from the treatment of the preparation with ribonuclease, since a 15 minute treatment with RNAse may not have been sufficient time to completely digest the RNA present. It is possible that small quantities of RNA polynucleotides, products of an incomplete enzymatic action of ribonuclease, remained in the DNA solution after the purification process (Schmidt, 1955). These nucleotides then may have co-precipitated with the DNA complex during the isopropanol precipitation. There is no concrete evidence to support this, but at the present time it seems to be the most feasible explanation. Lawley and Brookes (1963) also reported 7-ethyl guanine to be the only alkylated product after treatment of calf thymus DNA with EMS.

Lawley and Brookes (1963) found, in native DNA, that alkylation of the N-3 position of adenine occurs at a low frequency, although this is the second most reactive
site in the molecule. Rare alkylations at this site were detected in their study only through the use of labeled alkylating agents. In this study the small aliquot volume required to obtain optimal product separation on the chromatogram may have further discriminated against the quantitative detection of any 3-ethyl adenine.

Once 7-ethyl guanine was determined to be the only detectable alkylation product of DNA, an attempt was made to quantify this product and to determine the percent of alkylation (Table 5). It was felt that the percent of alkylation based on recovery of the products best explains the results. The data in Table 5 is based on 90% or higher recovery of the product from this technique. Any one of a number of reasons could be responsible for the slight deviations within the results. The quantity of the product was determined by comparing the absorption of the unknown to the absorption of a known quantity of the standard. To minimize the absorption error, the standards were run on the chromatograms under the same conditions as the unknowns; and thus interference in the absorption of any of the eluted material, due to the solvent, was constant. A human error, believed to be the major cause of deviation among experiments, was that produced in pipetting the initial DNA solution as well as in spotting the supernatant and hydrolysate on the chromatography paper.
Table 5. Quantitative values of recovered alkylation products.

<table>
<thead>
<tr>
<th>DNA conc. (mg)</th>
<th>Treatment</th>
<th>7-ethyl guanine</th>
<th>guanine</th>
<th>Total f(c+e)</th>
<th>% alkylation c/f</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>a (mg)</td>
<td>b (mg)</td>
<td>c(a+b) (mg)</td>
<td>c (mM)</td>
</tr>
<tr>
<td>45</td>
<td>EMS</td>
<td>0.95</td>
<td>3.19</td>
<td>4.14</td>
<td>23.0</td>
</tr>
<tr>
<td>45</td>
<td>EMS</td>
<td>0.91</td>
<td>3.09</td>
<td>4.00</td>
<td>22.0</td>
</tr>
<tr>
<td>45</td>
<td>untreated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Trial I

| 22.5          | EMS       | 0.53   | 1.63  | 2.16        | 12.0           | 2.08    | 13.9   | 25.9 | 46.5 |
| 22.5          | AMD + EMS | 0.50   | 1.56  | 2.06        | 11.5           | 1.99    | 13.3   | 24.8 | 46.3 |

Trial II

| 22.5          | EMS       | 0.56   | 1.59  | 2.15        | 12.0           | 2.21    | 14.7   | 26.7 | 45.0 |
| 22.5          | AMD + EMS | 0.51   | 1.56  | 2.07        | 11.6           | 2.05    | 13.7   | 25.3 | 46.0 |

^a Recovered from supernatant.  
^b Recovered from hydrolysate.
The percent guanine alkylation was determined on the basis of the number of moles of 7-ethyl guanine recovered, relative to the total moles of the guanine moiety present (Table 5). The data indicates that 45.8 ± 0.8% of the guanine present in the DNA reacts with EMS to produce 7-ethyl guanine. From a review of the literature, it appears that this experiment was unique with respect to the relatively high concentration of EMS used, as well as the species of DNA treated.

Of the total alkylated product determined in Table 5, 32.1 ± 2.8% of the 7-ethyl guanine was found present in the pooled supernatant resulting from three ethanol washings. Thus, about one-third of the 7-ethyl guanine produced, hydrolyzed from the DNA. The apurinic acid thus formed is produced by an instability of the guanine nucleotide due to the alteration of the electronic configuration. In agreement with this observation, Brookes and Lawley (1961) reported that the 7-alkylguanines were readily released from the alkylated DNA even at a neutral pH.

The remaining two-thirds of the 7-ethyl guanines appeared to remain associated with the DNA helix and could only be removed by mild acid hydrolysis. If this concentration is not lethal, it would be responsible for irregular base pairing due to a tautomeric shift. This
would produce mutations by preferential pairing of the alkylated product with thymine rather than cytosine during replication.

The DNA treated with EMS and that treated with AMD and EMS, yielded an alkylated product in both the supernatant and the hydrolysate indicating that alkylation occurred in both cases. Therefore, at this concentration (0.004 mM), AMD had little, if any, effect in blocking alkylation. Spectral ratio analyses were run on the products and the unknowns were quantified by relative absorption. The quantity of the alkylated product, and the percent of alkylation in each trial was similar to that of the control. Therefore, this data also indicated that pre-treatment with AMD had no effect in blocking alkylation. These results were in agreement with the work of Reich (1964) who reported that alkylation of calf thymus DNA with radioactive mustard gas (which also attacks N-7 of guanine) was uninhibited in the presence of AMD.

In slightly different experiments, Reich (1964) using mustard gas, and Troll (1964) using β-propiolactone, alkylated DNA for a short period of time followed by the addition of AMD to determine if the guanine was still complexed with the AMD. Both reported that the amount of AMD complexed with alkylated DNA was the same as that complexed with non-alkylated DNA. This suggested that there
was no competition between the alkylating agent and AMD for the DNA.

The model of Hamilton et al. (1963) suggested that AMD would not effect alkylation of the N-7 guanine. According to this model, AMD binds directly to the N-3 guanine, and its deoxyribose moiety, and also binds to the minor groove of the DNA helix by hydrogen bonds. The N-7 guanine position is located in the major groove of the DNA double helix, and, therefore, is not affected by AMD.

In this experiment, it is assumed that the DNA-AMD complex was formed since the procedure used was similar to Reich (1964) and that of Gellert et al. (1965). The procedure used in this paper differed from those above in that the reaction was carried out at a slightly higher temperature. Previous studies by Reich (1964) and Gellert et al. (1965) were carried out at room temperature, but it is believed that the slightly higher temperature (37°C) used in this work did not change the mode of reaction.

Goldberg, Rabinowitz, and Reich (1962) found that the amount of AMD bound parallels the guanine content, but is not directly proportional to it. Cavalieri and Nemchin (1964) reported that at low AMD to DNA ratios, as used in this study, the bond strength of the DNA-AMD complex is high due to the availability of AMD binding sites on the DNA molecule. As the concentration of AMD is increased
relative to a constant amount of DNA, the complex approaches a 1:1 relationship between the AMD molecule and the guanine present in the DNA, and the strength of the complex is thereby reduced.

AMD is toxic to an organism because of its inhibiting effect on RNA synthesis when bound to the DNA helix (Reich, Franklin, Shatkin, and Tatum, 1961). The DNA-AMD complex thus formed has been found to be relatively stable, as Kirk (1960) demonstrated by showing the resistance of the complex to disassociate by dialysis. Nevertheless, the results suggest that the N-3 guanine and hydrogen bonds are not strong enough to prevent hydrolysis and therefore depurination of the alkylated guanine. This hypothesis is supported on the basis that no guanine-AMD complex was detected by chromatography in either the supernatant or the hydrolysate. When the electron configuration of guanine is changed due to the addition of the ethyl group, it becomes sterically unstable in the DNA helix causing hydrolysis (Brookes and Lawley, 1961; Bautz and Freese, 1960). It has thus been concluded from the results of the chromatograms that hydrolysis by mild acid or alkylating agents takes place despite the AMD complex.

It was hoped that the DNA-AMD complex would prevent alkylation or at least stabilize the alkylated guanine molecules in the DNA double helix. The results cited in
this paper support the studies of Reich (1964) and Troll (1964), and indicate that AMD does not inhibit the reaction of EMS with DNA guanine. Therefore, AMD cannot be used as an agent to compete with EMS for DNA guanine.

This study also determined the amount of alkylation, as well as the percent of 7-ethyl guanine hydrolyzed during depurination from *Vicia faba* DNA by a specific concentration of EMS. It is hoped that these results will be an aid to those pursuing quantitative studies with EMS.
A qualitative and quantitative investigation has been conducted to determine the biochemical effects produced by the in vitro alkylation of *Vicia faba* DNA with EMS. The study has attempted to further define the molecular mechanism of the interaction of EMS with this DNA. In addition, an assay was made to determine the effect of the antibiotic AMD on the reaction system.

A reaction mixture containing 44 mg DNA/0.5 M EMS in 0.02 M monobasic sodium phosphate buffer (pH 7.2) was incubated for 18 hours at 37°C and the percent of alkylation was determined. Subsequent experiments using one-half the reaction mixture, both with and without pretreatment with 0.004 mM AMD, were conducted.

Ascending paper chromatography was carried out on the supernatants and hydrolysates using two different solvents. Solvent I was used to identify and quantify products of alkylation, while solvent II was used to confirm the results obtained with solvent I as well as to identify artifacts of the preparation.

Eluted unknowns were further identified by spectral ratios and were quantified by their relative absorbance as compared to the standards. The only detectable alkylated
product was 7-ethyl guanine, thus confirming the specificity of EMS for guanine. Approximately 46% of the DNA guanine present was alkylated. One-third of this alkylated product was hydrolyzed from the DNA and existed as a free base in the supernatant. It was suggested that this 33% hydrolysis would be responsible for transversions and transitions in an *in vivo* system, while the remaining 67% would be responsible for mispairing of the 7-ethyl guanine with thymine instead of cytosine.

The DNA was pretreated with AMD according to a procedure conducted by other workers. It was assumed but not confirmed, that a DNA-AMD complex was formed. On this basis, a comparison of the results between DNA treated and that untreated with AMD prior to alkylation indicated that the antibiotic had no effect on alkylation or on depurination. This conclusion supports the findings of other workers who have conducted similar experiments with AMD and different alkylating agents.

Alkylation may be an important genetic tool in the future, and it is hoped that the results of this study will be of value to those wishing to pursue the genetic engineering of chromosomes through the use of this tool.


