

A COMPARISON OF IN VIVO AND IN VITRO ALKYLATION  
BY ETHYL METHANESULFONATE IN VICIA FABA

by

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## ABSTRACT

Two experiments were conducted to determine the sites of action of [ethyl-1- $^3\text{H}$ ] ethyl methanesulfonate ( $^3\text{H}$ -EMS) in cells from Vicia faba embryonic axes, and to gain insight into the effects these sites have on the amount of alkylation occurring in the DNA of the cells. Embryonic axes of V. faba seeds were alkylated with  $^3\text{H}$ -EMS and various nuclear fractions were separated and analyzed for tritium. Radioactivity was found in all fractions observed.

Experiments on the alkylation of DNA in its pure form, while associated with isolated chromatin, and in vivo were carried out, and the radioactivity in each DNA was estimated and compared. This experiment indicated that the amount of alkylation of DNA is reduced as the environment of this DNA is made increasingly complex. The DNA alkylated in vitro showed a 3:1 increase in 7-ethyl guanine production over the DNA treated in vivo.

The data indicate that other sites in the nucleus besides the DNA are alkylated and, based upon the reduction in 7-ethyl guanine production from in vivo alkylated DNA, it is suggested that these sites play some role in the chemical activity of EMS.

## INTRODUCTION

Ethyl methanesulfonate (EMS) is well known to induce mutations in plants. A number of workers (Rao and Natarajan 1965, Doll and Sandfaer 1969, Kawai 1969, Zamenhof and Arikawa 1970) have described the effectiveness of this mutagen in regards to the production of chlorophyll mutations and chromosome aberrations in various plants. Because of the effectiveness of this chemical as a mutagen much attention has focused on determining its sites and modes of action on a molecular level. Brookes and Lawley (1961) have established that the primary site of alkylation is at the N-7 position of the guanine nucleotide and that this frequently results in the liberation of 7-ethyl guanine from the DNA complex. According to the prevailing concept of the DNA molecule (Watson and Crick 1953), this site is sterically available (Brookes and Lawley 1961) for alkylation and the reaction follows a general  $S_N2$  nucleophilic substitution (Price et al. 1969). Bautz and Freese (1960) have suggested the hydrolysis of DNA by cleavage of the glycosidic bond as a primary source of damage. Auerbach (1967) pointed out that after the deletion of 7-ethyl guanine, the DNA complex is subject to both transition and transversion. Krieg (1963) suggested a possible mechanism for a GC to AT transition based upon shifts in the hydrogen bonding during replication of the DNA. Cleavage of the phosphate backbone may also be a site of action of EMS resulting in breaks and gaps along the chromosome.



Much evidence is available concerning the in vitro alkylation of DNA by ethyl methanesulfonate, but little is known about the sites of alkylation for this compound in vivo. In the complex environment of the nucleus, it cannot be assumed that alkylation of guanine is the sole mechanism that produces mutation. Nucleoproteins and other macromolecules associated with the chromosome must play an important role in maintaining the integrity of this organelle and any alterations in the structure of these macromolecules may damage this chromosome and possibly enhance mutagenesis. A number of phenomena observed following the treatment of plant tissue with EMS imply the primary damage may be associated with alterations in nucleoprotein structure, and it has been suggested (Auerbach 1967) that many other reactions occur prior to alkylation of DNA.

Several workers have reported a high frequency of gaps and breaks in the heterochromatic regions of chromosomes treated with EMS (Ward 1966, Natarajan and Upadhyaya 1964, Natarajan and Ahnstrom 1969). This implies, assuming the above observations concerning molecular mechanisms of alkylation, that these regions are guanine-rich. There is no evidence to date, however which would suggest a G-C rich region associated with V. faba DNA (Kung, Moscarello and Williams 1971, also see Appendix B). In addition, there seems to be considerable contradictory information regarding the nature of base sequences in heterochromatic regions. Not only do base sequences of heterochromatic regions seem to differ from species to species (Macgregor and Kezer 1971, Eckhardt and Gall 1971) but Ward (1966) has observed that not all heterochromatic regions of V. faba chromosomes were attacked by EMS, implying that these regions

may differ within a single species as well. The nature of this difference could stem directly from differences in base content, or indeed from some other unrelated aspect of the structure of the heterochromatic region. Brown (1966) suggests that heterochromatic regions are characterized by differential coiling of localized sites along the chromosome and it has been postulated that the mechanism of coiling of chromosomes is associated with nucleoproteins (DeLange and Smith 1971). Should this be the case the preference of EMS for heterochromatic regions might be the direct result of a reaction with heterochromatin-associated protein.

Brock (1969) has suggested that the most likely time for attack by EMS is during replication or transcription when the DNA is extended. Mikaelson (1969) has found evidence in barley that the most sensitive stage for EMS-induced chromosome damage is during the  $G_1$  phase of the life cycle which also is a time when the chromosome is extended. Evidence of this nature suggests that chromosomal breaks and gaps may result from alterations of chromosomal protein rather than from cleavage of phosphodiester bonds or from depurination of DNA.

Scalera and Ward (1971) observed that when DNA isolated from V. faba root tips was treated with EMS about 46% of the guanine nucleotides were alkylated. It has since been demonstrated (J. B. Engle, unpublished) that there is a significant protein fraction associated with DNA isolated by the method of these workers. It is possible that the incomplete alkylation is due to this associated protein fraction.

In light of the foregoing, it was felt that an EMS-chromosomal protein interaction was a distinct possibility. The purpose of this research was to investigate the nature of any interaction between EMS

and chromosomal proteins. The study extends the observations on V. faba by utilizing  $^3\text{H}$ -EMS to assay for alkylation in the histone and acidic protein fractions of the chromosomal protein. In addition, a comparison is made between the relative amounts of guanine alkylation following EMS treatment of 1) V. faba DNA in a highly purified form, 2) V. faba DNA isolated as a chromatin complex, and 3) V. faba DNA in vivo.

## METHODS AND MATERIALS

Two experiments are performed in this study and due to their extensive nature they are outlined in Figures 1 and 2 (page 16 and 17). The following parts of this section describe various techniques used while performing these two experiments.

### I. Seed Germination

Seeds of Vicia faba var. "Longpod", were obtained from W. Atlee Burpee Co., Philadelphia. Approximately 100 to 200 seeds were surface sterilized for 30 min in 1:10 (v/v) clorox : water solution to reduce fungal contamination. The beans were rinsed in tap water and placed in a bucket with continually flowing water and constant aeration. The temperature of the water was  $22^{\circ} \pm 2^{\circ} \text{C}$ . After four days the seeds were removed and 1.0 cm of the primary root tip excised. For in vivo experiments the embryonic axis was excised from the seed. This tissue was immediately placed at  $-15^{\circ} \text{C}$ . In most cases the tissue was used within several hours of being excised; however, often the tissue was stored overnight at  $-15^{\circ} \text{C}$ .

### II. Preparation of Native DNA

DNA was extracted from root tissue according to modifications of the technique described by Chen (1971). Chen's technique is based primarily on that described by Marmur (1961). The modifications to Chen's technique were necessary to ensure removal of a yellow color that

is associated with DNA extractions from this plant (Scalera and Ward 1971).

The excised roots were weighed, placed in a Waring blender containing 50 ml of cold saline-EDTA (.5 M NaCl, .1 M ethylenediamine tetraacetate) and homogenized for 1 min at 120 v. The resulting slurry was made 2% with sodium lauryl sulfate (SDS) and shaken for 10 to 15 min at 60°C. Following this an equal volume of a chloroform-phenol mixture (1:1, v/v) was added and the slurry was shaken for 30 min at room temperature (19-22°C).

The chloroform contained 1% n-octyl alcohol to prevent frothing and the phenol was redistilled and saturated with .025 M Tris (tris (hydroxymethyl) amino methane, pH 7.5). The slurry was centrifuged at 10,000 x g for 10 min at 4°C and the upper aqueous phase removed. This aqueous layer was subjected to additional washes with chloroform-phenol as before until the protein interface was reduced to a thin film (Marmur 1961). Three washes were usually required. The DNA was precipitated from the aqueous phase by addition of 1 vol of cold 95% ethanol. The solution was placed at -15°C for 1-2 hr to ensure complete precipitation. The DNA fibers, appearing yellow in color, were spooled and redissolved in fresh .1 X SSC (.015 M NaCl, .0015 M sodium citrate).

The DNA was purified by addition of crystalline bovine pancreas ribonuclease (Calbiochem) at a concentration of 50 µg/ml and incubated for 1 hr at 37°C. Following this, pronase (Calbiochem) was added at a concentration of 1 mg/ml and also allowed to incubate for 1 hr at 37°C. The ribonuclease was allowed to self-digest for 10 min at 80° and the pronase was incubated for 1 hr at 37° prior to use to ensure

deoxyribonuclease free enzymes. The DNA was washed three times in chloroform-phenol as described above to remove the pronase. The DNA was again precipitated with 1 vol of 95% ethanol and placed back in .1 X SSC.

At this point, the DNA solution still appeared straw yellow in color indicating the presence of some impurity. In order to remove this impurity a technique described by Ralph and Bellamy (1964) and Bellamy and Ralph (1968) was used. The DNA was placed in .1 X SSC and a volume each of phosphate buffer (2.5 M  $K_2HPO_4$ , 33%  $H_3PO_4$ , 20:1 v/v) and 2-methoxyethanol were added. The solution was shaken for 10 min at 4°C and centrifuged at 12,000 x g for 5 min. The upper layer was removed and added to an equal volume of .2 M sodium acetate. The DNA was precipitated with 1% CTA bromide (hexadecyltrimethyl ammonium bromide) and placed on ice for 30 min. The solution was again centrifuged at 12,000 x g for 5 min and the pellet washed first in 70% ethanol containing .1 M sodium acetate and then twice in 95% ethanol. The pellet was redissolved in .1 X SSC and dialyzed in a Bio Rad b/HFD 1/20 mini dializer for 4 hr at 4°C.

In a number of experiments treatment with methoxyethanol to remove the yellow color was not practical due to the small volume of solution used. When this was the case, the yellow color was removed by centrifugation in 4.0 M CsCl (cesium chloride) for 18 hr at 35,000 rpm using an SW-65 rotor in the Beckman Model L Ultracentrifuge. The CsCl was biological grade obtained from Schwarz and Mann Laboratories. After centrifugation the aqueous CsCl solution was carefully removed and the pellet resuspended in fresh .1 x SSC and dialyzed as described above.

### III. Characterization of Native DNA

The amount of DNA present in the final solution was determined by ultraviolet absorbance on a Gilford 240 spectrophotometer at 260 nm and by the diphenylamine test as described by Dische (1955). An extinction coefficient of 20 was assumed for a 1 mg/ml concentration of DNA. Highly polymerized calf thymus DNA (Nutritional Biochemical Co.) was used as a standard for the diphenylamine test. Samples were read on a Bausch and Lomb Spectronic 20 spectrophotometer at 600 nm.

Spectral ratios of 280/260 and 230/260 were used as indications of purity and compared to those suggested by Marmur (1961).

The Folin test was used to assess protein contamination (Lowry et al. 1951). Crystalline bovine serum albumin (Nutritional Biochemical Co.) was used as a standard. RNA contamination was determined by the orcinol test. Ribonucleic acid obtained from Nutritional Biochemical Co. was used as a standard. The samples for the Folin test were read on the Bausch and Lomb spectrophotometer at 750 nm and those for the orcinol test at 660 nm.

A chromatographic test for RNA-uracil was also carried out. A sample of the DNA was hydrolyzed in 10% trichloroacetic acid at 100°C for 1 hr. A 60  $\mu$ l quantity of DNA solution with an optical density of .200 at 260 nm was spotted on Whatman #3 paper and the chromatogram was developed in acetonitrile : ammonium acetate (70:30, v/v) as described by Gabriel (1968). Development time was 4 hr at room temperature. The chromatograms were air dried and observed under ultraviolet light to make the various components of the solution containing the DNA visible

and to determine the location of the nucleotides. Uracil obtained from Calbiochem was used as a standard.

#### IV. Preparation of Chromatin

Chromatin was extracted from the root tip tissue as described by Bonner et al. (1968). Seeds were germinated as described in part I of this section and the primary root tips or embryonic axes were excised. All procedures were carried out at 4°C. The tissue was homogenized for 1 min at 120 v in 50 ml of grinding medium containing .05 M Tris, .001 M MgCl<sub>2</sub>, and .25 M sucrose, pH 8.0. The solution was then filtered through cheese cloth and Kimwipe tissue to remove large pieces of cellular material, and centrifuged at 4000 x g for 10 min. The supernatant (yellow in color) was discarded. The pellet was washed once more in grinding medium and collected by centrifugation.

The pellet was washed 5 times by dispersing it in .01 M Tris (pH 8.0) with a Potter homogenizer. After each washing the crude chromatin was pelleted by centrifugation at 10,000 x g for 10 min. The pellet was washed three times in 1 x SSC (.15 M NaCl, .015 M sodium citrate) and each time the pellet was collected by centrifugation at 4000 x g for 10 min.

It was observed that much of the carbohydrate associated with the pellet would sediment faster than the chromatin and this material often stuck to the centrifuge tubes. With care the chromatin could be removed from the carbohydrates by gentle scraping with a glass rod.

An effort was made to use the chromatin pellet immediately after extraction to reduce enzymatic degradation. In some cases however, this



was not possible. In these instances the pellet was stored at  $-15^{\circ}\text{C}$  overnight.

#### V. Separation of DNA from Extracted Chromatin

DNA was extracted from the isolated chromatin as suggested by Beckhor, Bonner, and Dahmus (1969). The chromatin was dispersed in 1 X SSC and made 4.0 M with respect to cesium chloride. The material was centrifuged at 35,000 rpm for 18 - 20 hr in a SW-65 rotor on a Beckman Model L Ultracentrifuge at  $20^{\circ}\text{C}$ . The DNA as well as some RNA was pelleted, collected and redissolved in .1 X SSC. This DNA was purified with ribonuclease and pronase and shaken with chloroform and phenol as described under part II of this section. The DNA was precipitated by addition of 1 vol of 95% ethanol and collected by centrifugation at 10,000 x g for 10 min. The pelleted DNA was dissolved in .1 X SSC and washed with ether to remove any remaining phenol. The DNA was assayed as outlined in part III of this section.

#### VI. Separation of Histone Proteins from Chromatin

The chromatin was dispersed in .4 N  $\text{H}_2\text{SO}_4$  by homogenation in a Potter homogenizer and shaken occasionally for 1 hr at  $4^{\circ}\text{C}$  (Bonner et al. 1968). The material was centrifuged for 10 min at 10,000 x g and the supernatant containing the histone proteins carefully collected. The pellet was saved for extraction of acidic proteins.

A small aliquot of the histone protein was neutralized with 8.0 M NaOH and a Folin test run to determine the amount of protein present (Lowry et al. 1951). In order to separate the histone fractions, approximately 50  $\mu\text{l}$  of the above solution were placed on a 15% acrylamide

gel containing 2.5 M urea. The gels were subjected to electrophoresis at 2 ma/tube for 2 hr in a .01 M acetic acid buffer. The gels were removed and stained overnight in .1% amido black. They were then de-stained in 7% acetic acid for 15-20 min in a Canalco #1801 Quick Gel Destainer. The gel bands were quantified by scanning at 600 nm on a Gilford 240 spectrophotometer equipped with a gel-scanner and a Honeywell and Brown Electronik graph plotter. The gels were stored in 7% acetic acid.

#### VII. Separation of Acidic Proteins from Chromatin

The pellet remaining after extraction of histones was dispersed in .5 ml of .1 N NaOH and allowed to stand with occasional stirring for 1 hr at 4°C. The solution was centrifuged at 10,000 x g for 10 min and the supernatant containing the acidic protein was removed (Bonner et al. 1968).

The amount of protein present was determined by the Folin test as described by Lowry et al. (1951). The solution was neutralized with 2.12 N HCl. Neutralization was necessary since strong acids and bases interfere with the Folin reaction.

#### VIII. Extraction of RNA

Nuclear RNA was separated from chromatin by dispersing the chromatin in 1 X SSC. The chromatin solution was made 4.0 M with respect to CsCl and centrifuged for 18 hr at 35,000 rpm. An SW-65 rotor was used in a Beckman Model L Ultracentrifuge set at 20°C (Beckhor, Kung and Bonner 1969). The resulting pellet containing RNA and DNA was dissolved in .1 X SSC and incubated for 2 hr at 28°C with deoxyribonuclease

(Worthington Biochemical Co.) at a concentration of 20  $\mu\text{g}/\text{ml}$ . The solution was then treated with 1 mg/ml of pronase for 1 hr at 37°C and washed with chloroform and phenol as described in part II of this section. The RNA was precipitated with 2 vol of ethanol, collected by centrifugation at 10,000 x g for 10 min, and dissolved in .1 X SSC. RNA and protein content were determined as previously described.

Total cellular RNA was isolated according to Hiatt (1962). Approximately 25 root tips were ground in 25 ml of grinding medium (see part IV) and the homogenate made 1% with SDS. An equal vol of 90% phenol containing .1% 8-hydroxyquinoline was added and the solution was shaken for 30 min at 4°C. The solution was centrifuged at 8,000 x g for 10 min and the upper aqueous phase collected. This step was repeated twice and the upper phase was made .1 M with respect to NaCl. Two volumes of 95% ethanol were added to precipitate the RNA. The solution was stored for 4 hr at 4°C to ensure complete precipitation. The precipitate was dissolved in 5 ml of .01 M Tris (pH 7.4) containing .001 M  $\text{MgCl}_2$  and 100  $\mu\text{g}$  of deoxyribonuclease and the mixture was incubated for 2 hr at 28°C. The solution was again made 1% with SDS and ½ vol of phenol was added to extract the deoxyribonuclease. The solution was shaken for 15 min at 4°C and centrifuged at 10,000 x g for 10 min. The RNA was precipitated from the upper aqueous phase as before and the pellet dissolved in 2 ml of .1 M NaCl - .01 M acetate buffer, pH 5.0. This solution was centrifuged at 10,000 x g for 10 min to remove any residual DNA and the supernatant was made 2 M with respect to potassium acetate. Ethanol (95%) was added dropwise until the final concentration was

approximately 20%. The RNA precipitate was collected by centrifugation at 10,000 x g for 10 min and dissolved in potassium acetate buffer (.001 M MgCl<sub>2</sub>, .1 M NaCl, .01 M potassium acetate, pH 5.0).

#### IX. Alkylation of Material with <sup>3</sup>H-Ethyl Methanesulfonate

All alkylations were carried out with a .25 mM concentration of [ethyl-1-<sup>3</sup>H] ethyl methanesulfonate (<sup>3</sup>H-EMS) obtained from New England Nuclear. It was previously estimated (Scalera and Ward 1971) that this concentration would be sufficient to alkylate all guanine nucleotides on a mole to mole basis. This concentration of <sup>3</sup>H-EMS had a specific activity of 17.5 µc/ml which was sufficient for analysis of radioactivity. All alkylations were carried out at 37°C for 9 hr with continuous agitation in a .067 M Sorenson's phosphate buffer, pH 8.0 (Sober 1968).

Alkylation of native DNA for in vitro studies was carried out by dissolving the DNA in 2 ml of buffer and treating it as described above. After the 9 hr incubation time, the DNA was washed three times by precipitation with 1 vol. of cold 95% ethanol. The DNA was spooled and redissolved in .1 X SSC. The remaining aqueous solution was combined and evaporated to dryness on a hot plate at 70°C and the residue redissolved in .1 N HCl.

Chromatin was alkylated by dispersing the pellet in 2 ml of Sorenson's buffer containing .25 mM <sup>3</sup>H-EMS. After incubation, the chromatin and alkylation products were collected by centrifugation at 10,000 x g for 10 min, resuspended in .005 M sodium thiosulfate (Narayanan and Konzak 1969) and allowed to stand for 1 hr at room temperature. The sodium thiosulfate treatment was done to ensure complete

inactivation of any remaining EMS. The chromatin was collected once again and the DNA extracted according to part V of this section.

Embryonic axes were treated with 15 ml of buffer containing .25 mM  $^3\text{H}$ -EMS and following incubation were washed for 1 hr in .005 M sodium thiosulfate at room temperature. The axes were then rinsed in running tap water for 15-20 min to remove all residual radioactivity. The DNA, chromatin, histones, acidic proteins, and RNA were extracted from alkylated tissue as described previously (see also Fig. 1 and 2).

#### X. Hydrolysis of DNA

All DNA was hydrolyzed for chromatographic analysis by incubation in .2 N  $\text{H}_2\text{SO}_4$  for 1 hr at  $100^\circ\text{C}$  (Scalera and Ward 1971). The DNA solutions were neutralized by titration with 1 N NaOH for paper chromatography.

#### XI. Paper Chromatography - Isolation of 7-ethyl Guanine

Ascending chromatograms were run in a solvent containing methanol: water (75:25, v/v). Whatman #1 paper was used and the chromatograms were developed for 12 hr at room temperature (Scalera and Ward 1971).

For the in vitro DNA study, both the pelleted DNA that had been hydrolyzed and the residue from the evaporated supernatant were used for chromatographic analysis. In the case of the chromatin and embryonic axes only the hydrolyzed DNA pellet was subjected to chromatography.

To each spot was added 40  $\mu\text{l}$  of DNA sample plus 20  $\mu\text{l}$  of 7-ethyl guanine (Cyclo Chemical Co.) standard and 10  $\mu\text{l}$  of guanine standard

(Calbiochem). Both standard solutions consisted of 1 mg/ml concentrations dissolved in .1 N HCl.

After development, the chromatograms were observed under ultraviolet light and the spots marked for determination of  $R_f$  values. The 7-ethyl guanine and guanine spots as well as the origins were cut out and assayed for radioactivity.

### XII. Estimation of Radioactivity

All samples of extracted materials were counted on a Packard Tri-Carb Liquid Scintillation spectrometer, set at a 58% gain with window openings at 50/1,000, 50/1,000, 50/1,000. The scintillation fluid contained .017 moles of PPO (2,5-diphenyloxazole) and .45 millimoles of POPOP (1,4-di(2-(5-phenyloxazolyl)benzene) in toluene-triton X-100 (2:1, v/v).

Total DNA, histone and acidic protein, and RNA fractions were spotted on Whatman #3 paper and allowed to dry overnight before being placed in the scintillation fluid (Fig. 1).

The 7-ethyl guanine and guanine fractions from the chromatograms were placed directly into scintillation vials and counted. The various histone fractions were counted after freezing the gels on dry ice, cutting out the bands and allowing them to digest in .5 ml of  $H_2O_2$  (33%) overnight at 60°C. The resulting solution was then added to the scintillation fluid and counted.

In most cases the samples were counted for 1 min; however, in several instances the time was extended to 10 min to minimize counting error (Overman and Clark 1960).

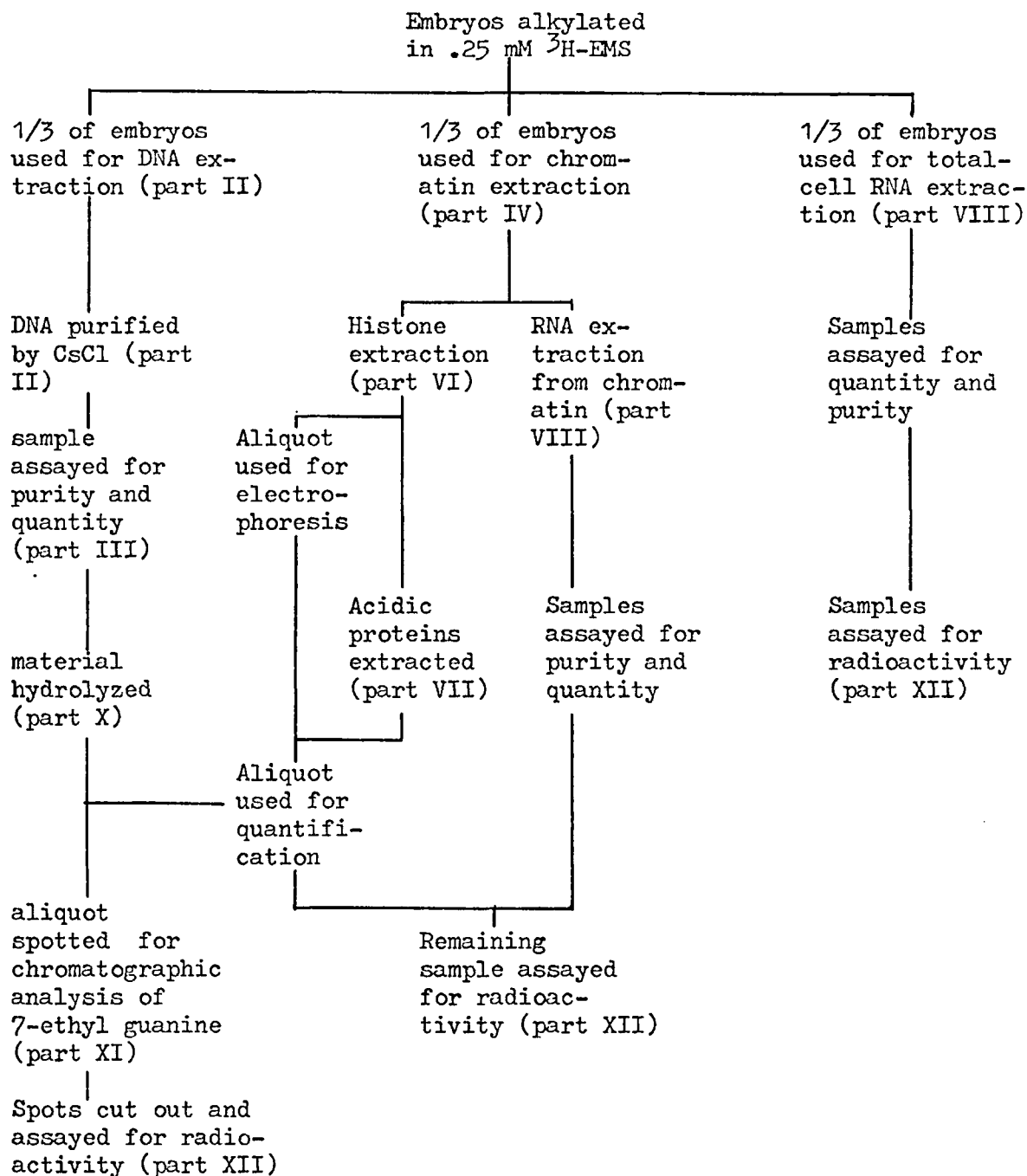


FIGURE 1. Flow sheet for determination of radioactive cell fractions following treatment with  $^3\text{H}$ -EMS.-- Parts refer to sections listed under Methods and Materials.

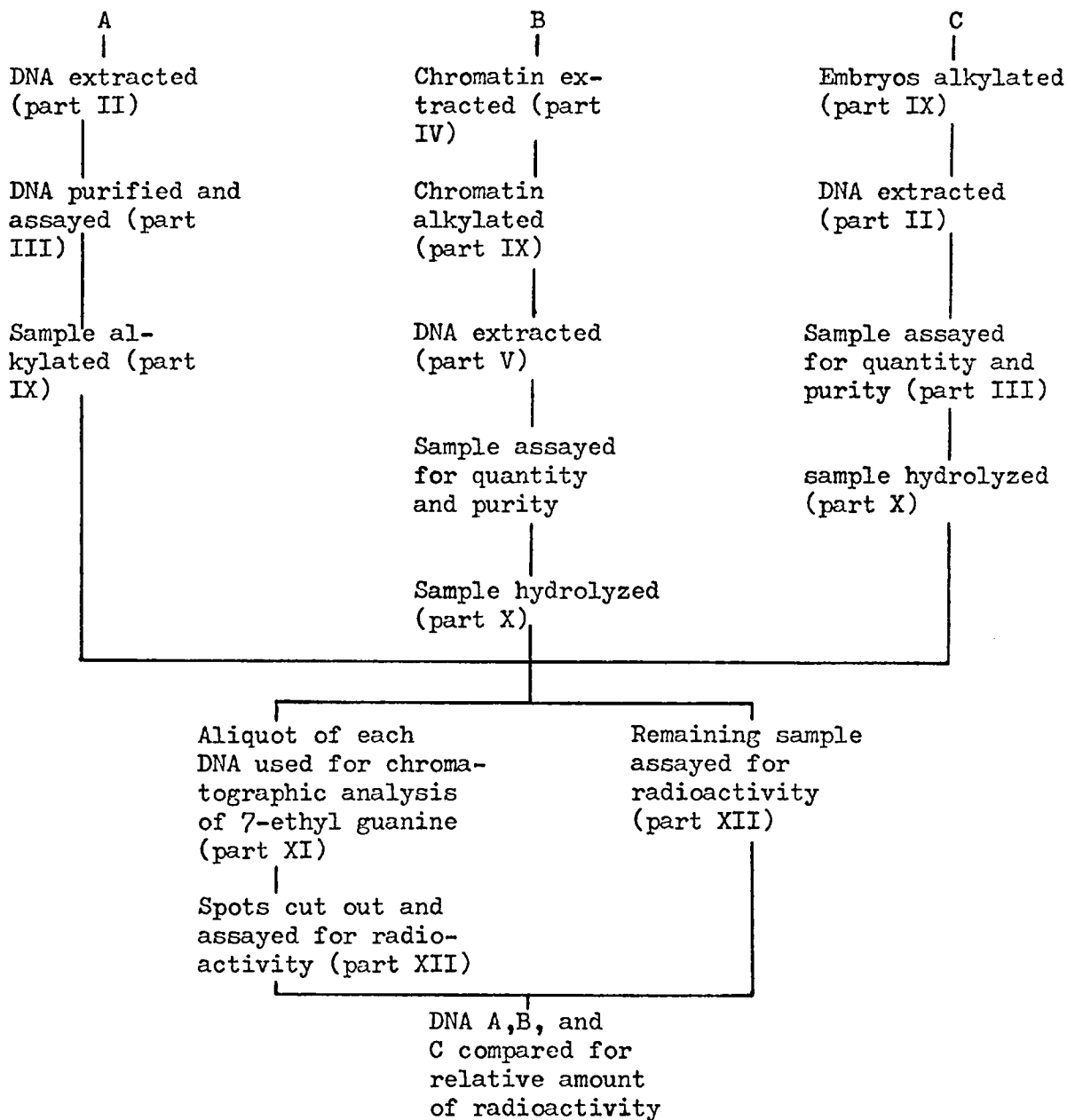


FIGURE 2. Flow chart for estimation of the amount of EMS-alkylated guanine in (A) pure DNA, (B) DNA isolated from alkylated chromatin, and (C) DNA alkylated in vivo. -- Parts refer to sections listed under Methods and Materials.



## RESULTS AND DISCUSSION

In order to determine the sites of action of EMS in a living system and the proportional amount of alkylation in the various nuclear fractions of this system the experiment was performed as outlined in Fig. 1 and the results are summarized in Table I. It can be seen by comparing the radioactivity of the total 7-ethyl guanine fraction to that of the DNA fraction that most of the activity observed in the DNA is due to guanine alkylation as expected (Lawley 1966). The discrepancy in the number of counts observed between these two fractions can be accounted for by the fact that there are other reactive sites in the DNA molecule besides the 7-N position of guanine (Lawley 1966). These other sites should contribute 28% of the activity observed in the total DNA fraction on a mole/mole basis. When the DNA fraction is reduced by 28% to take these additional reactive sites into account the activity in the DNA sample is not different from that of the 7-ethyl guanine fraction indicating that what is being measured in the total DNA is in fact, 7-ethyl guanine (Table IV). Slight contamination of the DNA fraction could contribute to some of the difference. However, analysis for radioactivity in the spot taken from the origin of the 7-ethyl guanine chromatogram (Appendix C, Table C-1) and spectral ratios of the DNA (Table II) suggest this interference is minimal.

Incorporation of  $^3\text{H}$ -EMS is observed in both the histone and acidic protein fraction (Table I). It can be seen from Table I that the amount of radioactivity observed in the histone fraction is considerably

TABLE I. Radioactivity observed in various fractions of *V. faba* cells after *in vivo* alkylation with  $^3\text{H}$ -EMS.--- The experiment was performed twice and the counts/10 min were adjusted for weight so that a comparison between fractions could be made. The error in counting ( $\sigma_{x_{10}}$ ) is listed as the standard deviation of the mean assuming a Poisson distribution (Overman and Clark 1960).

Sample	Weight of material used (mg)	Counts/10 min	Expected counts/.05 mg of material	Average counts/fraction adjusted to .05 mg of sample (a)	$\sigma_{x_{10}}$	Ratio* (a/342)
DNA I	.019	509	1339	1667	40.82	4.9
DNA II	.008	319	1994			
Histone I	.040	285	356	342	18.46	1.0
Histone II	.029	195	327			
.1 N NaOH						
Acidic protein I	.299	5520	920	935	30.57	2.7
Acidic protein II	.225	4280	950			
.3 N KOH**						
fraction I	.296	5340	900	955	30.90	2.8
fraction II	.232	4690	1010			
7-ethyl guanine I	.0016	45	1424	1318	36.30	3.9
7-ethyl guanine II	.0006	16	1212			
RNA I	.300	2317	386	394	19.90	1.2
RNA II	.300	2417	403			

\* The ratio is calculated based upon a proportion of the number of adjusted counts in each fraction (a) divided by the adjusted counts in the histone fraction (342).

\*\* Acidic protein fraction re-extracted with .3 N KOH (see text for discussion).

TABLE II. Spectral ratios of  $^3\text{H}$ -EMS treated DNA samples. -- Alkylation was performed after extraction and purification, while complexed with the chromatin pellet, and with excised embryonic axes for the in vitro, chromatin and in vivo DNA samples respectively. The values are compared to .515 and .450 for 280/260 and 230/260 respectively (Marmur 1961).

Ratio	<u>in vitro</u>		Chromatin		<u>in vivo</u>	
	I	II	I	II	I	II
280/260	.572	.565	.696	.639	.506	.515
230/260	.534	.493	.500	.498	.432	.485
250/260	.904	.904	.317	.315	.864	.897

less than that found in the DNA, as expected. This is probably explained by the number of reactive sites in the histone fraction as compared to those found in the DNA, although little information is available concerning the sites available for alkylation in histones.

Because some activity was observed in the total histone fraction an attempt was made to assay for radioactivity in each of the separate histone fractions. When the alkylated histone sample was subjected to electrophoresis 4 peaks were observed (Fig. 3) corresponding to those fractions described by Johns (1967). These histone fractions have been grouped into three classes: the lysine-rich f1 fraction, the slightly lysine-rich f2b and f2a2 fractions, and the arginine-rich f2a1 and f3 fractions (DeLange and Smith 1971). Upon comparison of the alkylated and non-alkylated samples (Fig. 3) one sees a reduction in the height of all peaks in the treated material. This is probably due to differences in the amount of histone sample applied for electrophoresis. In order to facilitate layering of the histone samples on the gels, a few drops of 1 M sucrose were added to increase the density of the solution. As a result the concentration of the histone solution was diluted. This dilution, as well as pipetting error could account for the differences in height of the peaks observed between the two samples. When the area under each peak within one trace is determined and related to the other fractions of the same trace on a percent basis, one observes a reduction in the f2a1, f2a2 and f1 peaks in the treated material (Fig. 3 and Table III). In a recent review by DeLange and Smith (1971) it is pointed out that Smart and Bonner (in press) have obtained evidence that the f1 fraction is more exposed to the aqueous environment of the nucleus than are

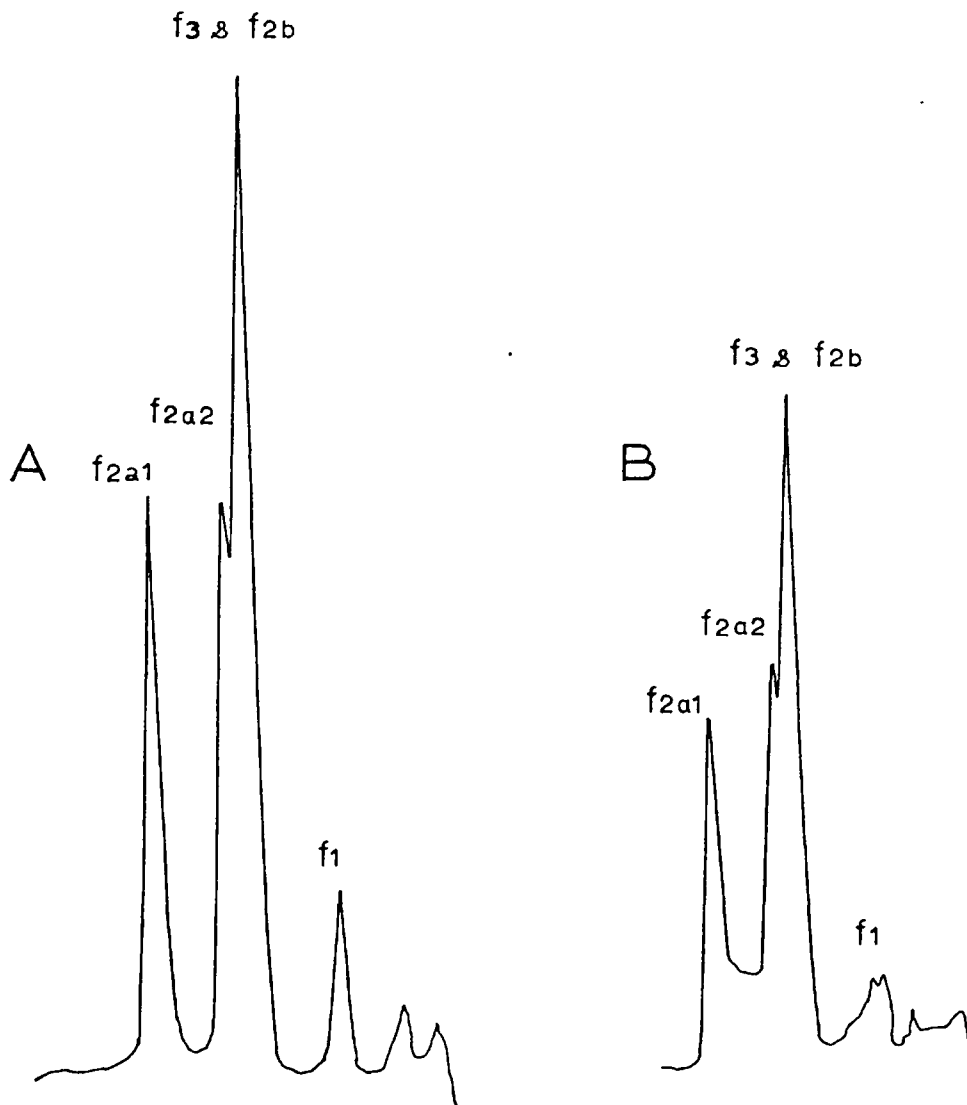


FIGURE 3. Densitometer tracings of acrylamide gels containing treated and untreated histones.--A is a trace of untreated histones and B is a trace of histones extracted from chromatin treated with  $^3\text{H}$ -EMS.

TABLE III. Distribution of alkylation-induced radioactivity in various fractions of the histone proteins extracted from *V. faba* primary root tips.--The weights of the histone fractions were determined by calculating the area under the curves from the ultraviolet absorption scans of acrylamide gels containing the histone fractions (see Fig. 3).

Fraction	% by wt of non-alkylated histones	Weight of alkylated histones	% by wt of alkylated histones	Counts/10 min	Expected counts/10 units of wt	Observed counts/10 units of wt	Ratio*
f2a1	23.1	7.817	20.8	358	145	458	1.2
f2a2	28.5	8.026	21.3	121	152	151	.4
f3 and f2b	42.4	19.610	52.1	198	363	101	.3
f1	6.1	2.200	5.8	----**	----	----	----

\* Ratios correspond to those calculated for the total samples (Table I) and were determined by dividing the number of counts observed in the separate histone fractions by the counts observed in the total histone fraction (i.e., 342).

\*\* No counts were observed in this fraction due to the small amount of material available.

the other fractions of histones. If this is true, the f1 fraction might be more susceptible to damage by alkylation than the other histone fractions, thus explaining the reduction in this peak. However, since some reduction in the relative weight of the f2a1 and f2a2 fractions is also observed after alkylation, some alteration in structure of these fractions is also implied.

In addition to the above observation, an interesting relationship between the fractions appears when they are assayed for radioactivity (Table III). If one assumes that each fraction has the same number of reactive sites on a weight/weight basis, then an expected number of counts can be obtained based on a proportion of the total counts observed. When this is done, the peak containing the f3 and f2b fractions should have the largest number of counts (Table III). This, however, is not observed. The f2a1 peak, showing the smallest weight of the three fractions exhibits the highest activity and the f3 and f2b fractions show the lowest. Possible sites of alkylation are the sulfhydryl groups of the cysteinyl residue (Loprieno 1964, Narayanan and Konzak 1969) and the primary, secondary or tertiary amines (Morrison and Boyd 1966). Since it has been found that the histone fractions contain few cysteinyl residues (DeLange and Smith 1971) it is felt that these sites do not contribute significantly to the  $^3\text{H}$ -EMS induced radioactivity observed. It is an established fact however, that the f2a1 fraction is very rich in arginine and contains more amino groups than any other fraction. This may well be the reactive site explaining the large amount of radioactivity in this peak. Because of the reduction in size of the f1 peak, the band was too light in color to cut out for radioactive analysis.

For this reason nothing could be determined about the amount of activity in this fraction.

When the total number of counts obtained from the three fractions is compared to the counts observed in the total histone sample alone (Table IV), there is no difference at the 5% confidence level as determined by the F test. Based on this observation, the total histone fraction is apparently pure. One must remember, however, that some discrepancy in the weight of the two samples may exist and that there is a lack of information about activity in the f1 fraction. Together these two points may explain the apparent difference between the two values.

It is important to point out the possible relationships between the alkylation activity observed in the histone proteins and the proposed role of histones in the cell. It was pointed out earlier that the apparent breaks and gaps observed in the heterochromatic regions of V. faba chromosomes following EMS treatment could be due to localized uncoiling of the DNA (Ward 1966) and also that there is some specificity of breaks even within the heterochromatic regions. Furthermore, other workers infer that the lysine rich histones are associated with regions of DNA rich in adenine and thymine and that arginine-rich histones are associated with regions rich in guanine and cytosine (Combard and Vendrely 1970). If one assumes, based on the localization of chromosome breaks in heterochromatic regions, that these regions are guanine-cytosine rich, then it would follow that the f3 and f2a1 fractions would show the most radioactivity of the various fractions. The greatest activity is observed in the f2a1 fraction, supporting the suggestion.



TABLE IV. F test for purity of samples.--(Fryer 1966).  $\lambda_1$  represents counts observed in total DNA or histone samples as extracted from root tips.  $\lambda_2$  represents the 7-ethyl guanine fraction of DNA + 28% of  $\lambda_1$  to account for alkylation in the other sites of the DNA (see text for discussion). For the histone samples,  $\lambda_2$  represents the sum of counts observed in the f2a1, f2a2, f2b, and f3 fractions. Confidence intervals are taken at a .05 probability level with 6 degrees of freedom. A Poisson distribution is assumed (Overman and Clark 1960). The confidence interval is  $.234 \leq \frac{\lambda_1}{\lambda_2} \leq 4.28$  where  $\frac{\lambda_1}{\lambda_2}$  is the ratio of the two samples to be compared.

Sample	Counts/10 min in total sample $\lambda_1$	Counts/10 min in sample fractions $\lambda_2$	$\frac{\lambda_1}{\lambda_2}$
<u>in vitro</u> DNA	47186	49309	1.044
<u>in vivo</u> DNA	1728	1667	1.069
chromatin DNA	49	---*	---
histones	342	677	1.663

\*No counts were observed in this fraction due to small yield.

made previously that the amino groups may be the reactive sites in the histone fractions. A question arises however, when the amount of activity in the f3 fraction is observed. According to the above argument this fraction should also contain a large amount of activity. That this is not observed might be explained by the fact that the f2b fraction bands in the same place as the f3. Since the f2b fraction is a slightly lysine-rich fraction containing fewer arginine residues it should demonstrate less radioactivity. It is possible that the combined nature of these two fractions obscures the radioactivity that should be observed in each separately. Although the large amount of activity in the f2a1 fraction further implies that the heterochromatic regions are G-C rich, there is no hard evidence to date indicating that such G-C rich regions exist in V. faba DNA (Fig. B-2). At best the information available to date is highly contradictory and inferences regarding histone-DNA interaction should be interpreted with care. Smith, DeLange and Bonner (1970) suggest that histones may play a role in the supercoiling of DNA observed in metaphase chromosomes. Alkylation of the histone proteins may effect this coiling in some way. If this is the case the uncoiled section of DNA would be more susceptible to alkylation and mutation, however, it is beyond the scope of this paper to suggest any model which would explain the nucleoprotein-DNA interaction.

Little is known about the function of residual non-histone proteins (the acidic proteins) associated with chromatin. There is evidence that these proteins are bound to the DNA (Bonner et al. 1968) and therefore they play some role in the structure of the chromosome. When the acidic protein fraction was assayed for radioactivity following

alkylation with  $^3\text{H}$ -EMS in the current study, a relatively large amount of activity was detected (Table I). There is some indication that the value of 935 counts/.05 mg on the table under the acidic protein fraction does not represent the total fraction and that this fraction may not be pure. As described in the methods section, these proteins were separated from the chromatin pellet by precipitation with .1 N NaOH. When this pellet is further incubated with .3 N KOH for isolation of chromatin associated RNA (Fleck and Munro 1962), high activity is observed again (Table I). This, in addition to indicating the presence of RNA, suggests that the first precipitation did not extract all the acidic proteins. These inferences were verified by the results of the orcinol and Folin tests performed on the two samples. In order to estimate the amount of acidic protein extracted by the second precipitation, chromatin associated RNA was isolated by centrifugation in cesium chloride (see part VIII, Methods and Materials section). The yield of RNA obtained, however, was too small to assay for radioactivity, for the counts observed were close to background. Therefore, it was decided to extract all of the RNA from the cell and estimate the alkylation activity from this. When this was done, a value of 394 counts/.05 mg (Table I) was obtained for the RNA fraction. Upon subtracting this figure from the total of the two acidic protein precipitations, a minimum estimate of activity in the acidic protein fraction is obtained which is 1536 counts/.05 mg. One must remember, however, that the value of 394 counts obtained for RNA is for total cellular RNA. This is certainly higher than that which would be found in nuclear RNA alone if this material could be assayed. Although some activity is expected in the RNA fraction due to

the presence of guanine, alkylation here is not thought to be of great significance to the action of EMS in the cell, since there is a rapid turn over of RNA in the nucleus (Darnell 1968). Thus if this RNA is damaged by alkylation, it will not be permanent. In addition, there is no evidence that RNA plays an important role in maintaining the structural integrity of the chromosome.

It can be concluded from this in vivo experiment that DNA, and the guanine moiety in particular, is not the only reactive site in the nuclei of V. faba roots. However, based upon Table I, the DNA fraction does show the greatest response to  $^3\text{H}$ -EMS when compared to the other fractions observed.

Because alkylation was found to occur in the protein fractions of the chromatin, it was decided to determine whether or not this protein might act so as to protect the DNA from alkylation. Figure 2 outlines the experiment performed to investigate this hypothesis. The experiment is based upon the assumption that purified DNA will be alkylated to a greater extent than DNA associated with the complex environment of isolated chromatin or in the in vivo state.

Earlier work (Scalera and Ward 1971) on the alkylation of V. faba DNA used DNA extracted by a modification of techniques described by Marmur (1961) and Lyttleton and Petersen (1964). It has since been demonstrated (J.B. Engle, unpublished) that there is a significant amount of impurity associated with the DNA when this technique is used. It was imperative that purity of the DNA used in the current study be known, for any contamination containing residual label would obscure the results. Therefore, the DNA was characterized before use (Appendix B).

When the extraction and purification technique described in this study is used, a large reduction in impurity over that occurring in the extracts of Scalera and Ward's is observed (Appendix B, Table B-I). It should be pointed out that small amounts of impurity still exist in this DNA and that further purification could be employed but at the expense of a large reduction in yield.

There are a number of sources of contamination of the DNA in the current study. After alkylation with  $^3\text{H}$ -EMS the pure DNA is washed with ethanol 3 times to remove any residual, labeled material. This is not completely effective and it is possible that some contamination remains. Also the supernatant of the alkylated DNA contains 7-ethyl guanine residues liberated during the alkylation reaction. This supernatant is evaporated, the 7-ethyl guanine is deposited as a residue and is then suspended in HCl. It is possible that label may remain on the glass or become associated with the salts or other impurities in the supernatant, also providing a source of contamination.

When DNA is separated from chromatin by CsCl centrifugation, some contamination remains, possible due to pronase. To remove this contaminant the DNA is washed with chloroform-phenol solution (see part II, Methods and Materials) and precipitated with ethanol. This apparently fails to completely purify the DNA as can be seen by the spectral ratios of Table II. If an attempt is made to further purify the DNA by CsCl centrifugation, the yield is reduced to the point where no radioactivity can be detected in the sample. In fact, the yield before additional purification in CsCl is so small as to prevent observation of activity in the 7-ethyl guanine fraction after chromatography.

Even with these limitations, one can still obtain some information concerning the proportional amount of incorporated  $^3\text{H}$ -EMS in DNA treated in its pure form (in vitro), DNA alkylated while associated with chromatin, and DNA alkylated in vivo. The relative activity observed in the three DNAs appear in Fig. 4 (a more detailed summary of results from this experiment can be found in Appendix A, Table A-I). DNA treated in vitro has about 3 times as much activity as DNA treated in vivo, and the DNA treated as chromatin shows about as much activity as the in vivo-treated DNA. The data suggest that some blockage of DNA alkylation occurs with chromatin as well as with the in vivo DNA. These results are expected because one would expect interference from cellular material.

The accuracy of these data can be obtained by comparing radioactivity of 7-ethyl guanine samples isolated from each DNA treatment. When this is done and the activity in the 7-ethyl guanine fraction compared to the radioactivity in the total DNA on a weight/weight basis, one finds that the samples are not different by the F test (Table IV). Note that no information could be obtained from the chromatin treated DNA due to small yield. A value for alkylation in chromatin treated DNA is listed (Fig. 4) even though it could not be checked by the F test. It is felt that even though this value may not be accurate it is representative of the amount of activity expected in DNA treated while associated with isolated chromatin.

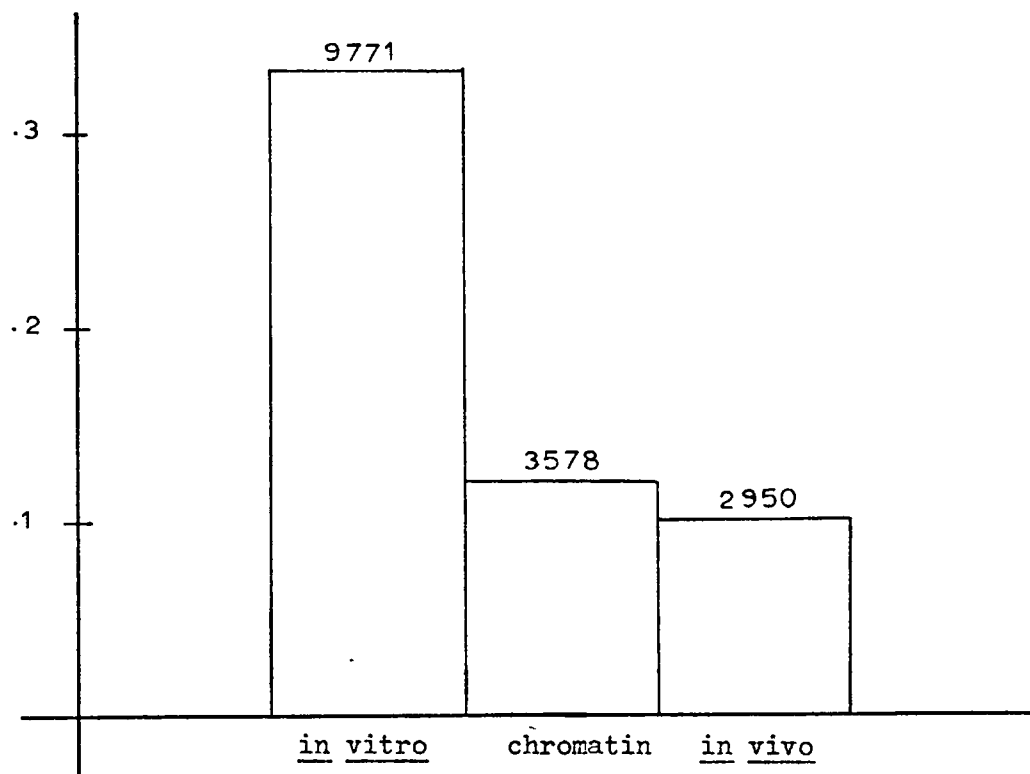


FIGURE 4. Comparison of activity of  $^3\text{H}$ -EMS on DNA alkylated in vitro, while associated with chromatin, and in vivo.-- The values at the top of each column are the counts observed in the total DNA sample - 28% (see text for discussion). It is assumed that these values closely represent the expected counts in the 7-ethyl guanine fraction of the cell. The height of each bar is based on a ratio between the sample in question and the in vivo sample (refer to Appendix A, Table A-I).

## CONCLUSION AND SUMMARY

The data presented in this paper suggest that many sites within the nucleus of V. faba root tip cells are subject to alkylation by <sup>3</sup>H-ethyl methanesulfonate. The majority of the activity is associated with the DNA and the most reactive site in the DNA is the N-7 position of guanine as indicated in other studies (Rao and Natarajan 1965, Doll and Sandfaer 1969, Kawai 1969, Zamenhof and Arikawa 1970). However, in addition, the nucleoproteins are affected by this mutagen. This observation may help to explain the breaks and gaps observed in root tip chromosomes following EMS treatment, although all that can be said concerning this study is that both the histone and acidic protein associated with the DNA are involved in reactions with EMS. Cellular RNA is also alkylated to a lesser degree and it is assumed that guanine nucleotides are responsible. Damage resulting from alkylation of this material is considered minor with respect to mutagenic activity of the chemical because of the rapid turn-over of RNA in the cell (Darnell 1968).

When the DNA is alkylated in its native cellular environment a 3:1 reduction in the quantity of 7-ethyl guanine is observed over that observed in the DNA treated in its pure state. This is presumably due to competition between reaction sites present in other fractions of the chromatin. When the DNA is alkylated as isolated chromatin, the amount of <sup>3</sup>H-EMS activity in the DNA is increased over that observed from the in vivo treated DNA. This suggests that some of the EMS reacts with or is absorbed or metabolized by other cell components before it reaches



the nucleus. DNA alkylated in vitro shows the largest activity, presumably due to the lack of any other competing sites.

In order to fully explain the mode of action of EMS in the living cell, much more work is required concerning the role of the nucleoproteins in chromosome structure. It appears however, that the activity of this chemical is not limited to the DNA and that the nucleoproteins do react to some degree with this mutagen.

APPENDIX A

RADIOACTIVITY IN ALKYLATED DNA

TABLE A-I. Radioactivity observed in DNA alkylated with  $^3\text{H}$ -EMS in vitro, while associated with chromatin and in vivo.--The observed counts are adjusted to .1 mg of DNA on a weight/weight basis for comparison. The column listed as counts - 28% shows the amount of activity in the total DNA sample adjusted for any possible activity in other sites of the DNA besides the 7-N position of guanine. This column should reflect the activity due to alkylation of the 7-N position of guanine alone. The ratio is expressed as the average adjusted count for each fraction (a) divided by the average counts in the in vivo DNA (2950).

DNA sample	Weight of DNA	Counts/10 min	$\sigma_{\bar{x}_10}$ *	Adjusted count for .1 mg of DNA	Adjusted count - 28%	Average count (a)	Ratio (a/2950)
<u>in vivo</u>							
I	.019	509	22.5	2679	1929	2950	1.0
II	.008	319	17.8	3987	2871		
<u>in vitro</u>							
I	.360	40229	245.3	11175	8046	9771	3.3
II	.290	46309	257.5	15966	11496		
chromatin							
I	.001	49	7.0	4900	3578	3578	1.2

\* This value represents the standard deviation of the mean and is an indication of error in counting (Overman and Clark 1960).

## APPENDIX B

### CHARACTERIZATION OF VICIA FABAE DEOXYRIBONUCLEIC ACID

DNA was extracted from V. faba primary roots according to the technique described in part II of the methods and materials section of this paper. Characterization of this DNA in terms of relative yield, protein and RNA content, temperature profile, G-C content, and density was performed. The purity of the DNA was determined as described in part III of the methods section.  $T_m$  values were determined according to Mandel and Marmur (1968) on a Gilford U.V. spectrophotometer. The DNA was sheared as described by Goldberg (1971) and was denatured and renatured for analysis of satellite DNA as described by Vodkin and Katterman (1971). The buoyant density of the DNA was determined by isopycnic centrifugation (Schildkraut, Marmur and Doty 1962).

Chen's technique was modified by incorporation of 2-methoxyethanol treatment to remove the yellow color which occurs with V. faba DNA. Regardless of the number of ribonuclease and pronase purifications and chloroform and phenol washings, the DNA retained the color. Treatment with 2-methoxyethanol as described by Ralph and Bellamy (1964) removed this color, but considerable loss of original DNA also resulted (Table B-I). The yield values of Table B-I may be questionable if the yellow color interferes with DNA quantification. The methoxyethanol treatment was necessary since the color did interfere with the Folin and orcinol tests. The color-producing material absorbs at 280 nm which tends

TABLE B-I. Purity indices and yield of ribonuclease- and pronase-treated V. faba DNA after additional purification techniques. -- Yield is expressed as mg DNA/g wet tissue and contamination is expressed as percent of DNA + protein and percent DNA + RNA respectively.

Additional purification	Spectral Ratios			Yield (mg/g)	Contamination (%)	
	280/260	230/260	250/260		protein	RNA
methoxyethanol	.531	.444	.930	.10	4.8	- 1.0
cesium chloride	.511	.456	.880	.05	- 1.0	- 1.0
none	.582	.489	.774	.57	>15.0	>20.0

TABLE B-II. Buoyant densities of DNA in cesium chloride.

DNA	Density
<u>V. faba</u>	
native	1.693
sheared	1.691
2 hr renatured	1.716
6 hr renatured	1.707
<u>M. lysodeikticus</u>	1.731

to inflate the diagnostic 280/260 spectral ratio. Treatment with methoxyethanol may be omitted provided the DNA can be pelleted through 4.0 M CsCl (Table B-I).

Marmur (1961) suggests typical ratios for absorption of the DNA at 280/260 and 230/260 nm. These values are .515 and .450 respectively for relatively pure DNA. The values in Table B-I which most closely fit these ratios are those for the DNA purified with CsCl. The greater the divergence from the Marmur ratios, the more contaminated the DNA. A large reduction in yield occurs after treatment with CsCl or 2-methoxyethanol (Table B-I).

Protein and RNA contamination are also described in Table B-I. Values in this table represent averages of 3 experiments. Although the amount of protein contamination obtained by 2-methoxyethanol treatment was higher than that for the DNA treated with CsCl, the former is similar to values observed by others (Lyttleton and Petersen 1964). If care is exercised during this extraction, protein contamination can be reduced to almost 1.0%. The values observed for protein content in DNA purified with CsCl correspond with those observed by other workers (Beckhor, Kung and Bonner 1969). DNA not treated with CsCl or 2-methoxyethanol appeared to have considerable protein and RNA contamination but these high values may have been due to interference from the substance producing the yellow coloration.

Results of the orcinol test suggested the presence of small amounts of RNA; however, when an aliquot of the DNA was hydrolyzed and subjected to chromatography no uracil was detected.

Analysis of the temperature profile of the DNA revealed a  $2\sigma$  value of 3.5, which may be taken as a measure of the heterogeneity of the DNA (Doty, Marmur and Sueoka 1959). In addition, purified DNA of high molecular weight exhibits an increase in hyperchromicity of approximately 40% (Mandel and Marmur 1968). The increase in hyperchromicity observed in V. faba DNA is 36%, which implies a small degree of impurity as indicated by Table B-I. A similar value is observed by Kung and Williams (1969). It is inferred from the 36% figure, that the DNA is of high molecular weight and has not been extensively sheared by the extraction technique. A  $T_m$  of  $69.4^\circ\text{C}$  (Fig. B-1) corresponds to a G-C content of 37%. When this value of  $69.4^\circ\text{C}$  is corrected for concentration of saline citrate solution, the  $T_m$  is  $86.0^\circ\text{C}$  (Mandel and Marmur 1968).

The bimodal curve in Fig. B-1a and the apparent drop at the lower end of the probability graph in Fig. B-1b were initially taken to indicate the presence of two species of DNA (Knittel et al. 1968). Additional tests were made by isopycnic centrifugation to check for a possible satellite component. Densitometer tracings resulting from this centrifugation are presented in Fig. B-2. An obvious increase in the density of the native DNA results when the DNA is allowed to denature in 1 N NaOH (pH 12.5) and renature for 2 hr (Diagram III, Fig. B-2). However, when subjected to shearing the DNA bands at about the same density as the native DNA. If a satellite were associated with this DNA, it would appear as a shoulder on the peaks in diagrams II and III of Fig. B-2. To be assured that the DNA was undergoing renaturation, the sample was checked after 6 hr of renaturation (Diagram IV, Fig. B-2). Table B-II presents the density values obtained and it can be seen that

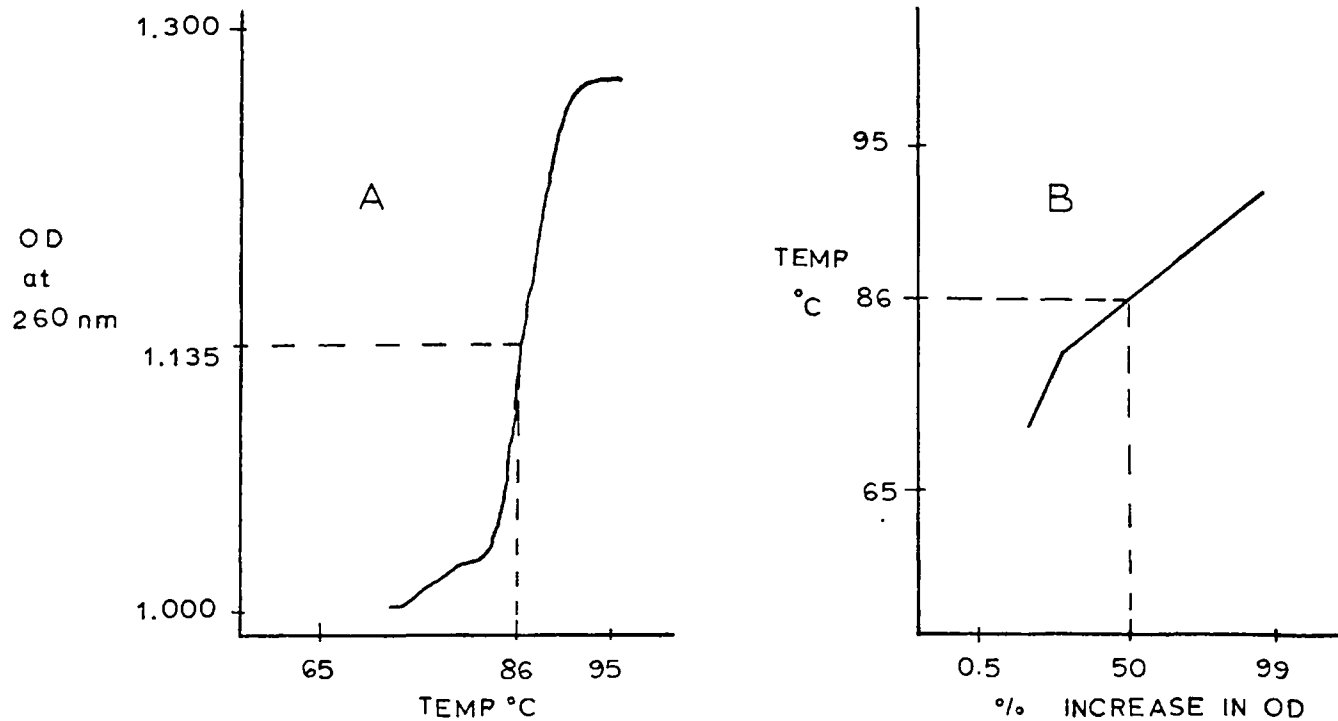


FIGURE B-1. Temperature profile and probability graph of *V. faba* root tip DNA. -- An increase in hyperchromicity of 36% is obtained from curve A. The  $T_m$  value indicated on the graph is corrected for salt concentration (see text). Note the drop at the lower end of the probability graph (B) indicating the lack of homogeneity of the DNA. Since the drop is at the bottom, it would imply the presence of an A-T rich satellite; however, see text for discussion.



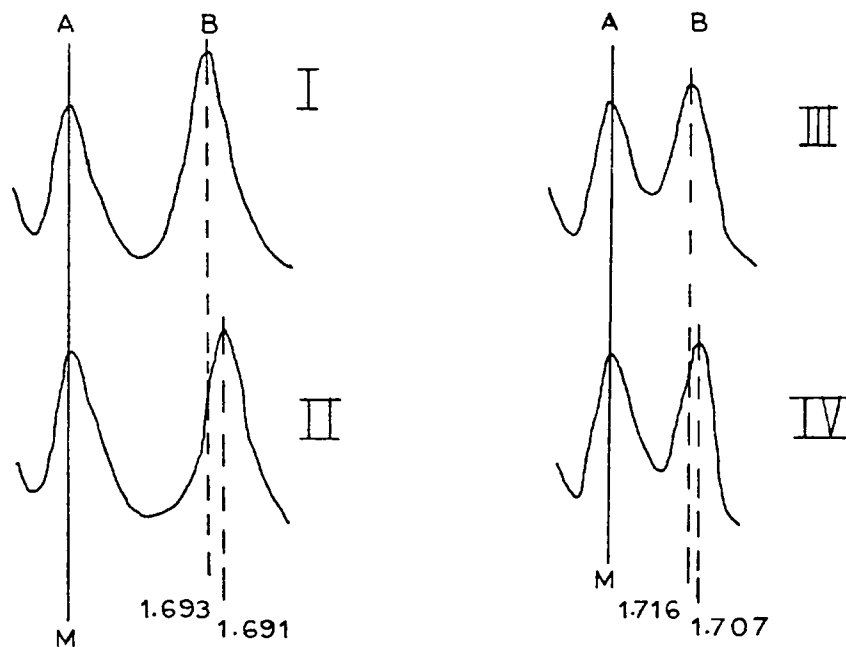


FIGURE B-2. Densitometer tracing of *V. faba* root tip DNA prepared by isopycnic centrifugation in CsCl.--Diagram I represents native DNA; II, sheared DNA; III, DNA that has been denatured and renatured for 2 hr; and IV, DNA that has been denatured and renatured for 6 hr. Peak A represents marker DNA, (*M. lysodeikticus*) in all diagrams. The tracings do not support the presence of a satellite associated with this DNA.

the value of the denatured sample is receding back to that of the native DNA, thus verifying renaturation. The increase in density from 1.693 to 1.716 reflects the transition from double to single stranded configuration during denaturation (Table B-II). The bimodal curve of Fig. B-Ia could be due to slight impurities in the DNA.

It is concluded that this technique provides for the extraction of a high molecular weight DNA with minimal amounts of associated impurities. There is at present no evidence for the existence of a satellite component of DNA from V. faba primary roots.

APPENDIX C

INDICATION OF PURITY IN DNA FRACTIONS

TABLE C-I. Amount of radioactivity observed in the guanine, 7-ethyl guanine and origin spots resulting from chromatography of  $^3\text{H}$ -EMS alkylated and hydrolyzed DNA. -- The amount of activity observed is an indication of contamination of the DNA. Values represent averages of two experiments.

Source of DNA	Spot		
	guanine	origin	7-ethyl guanine
treatment <u>in vitro</u>	156	1192	802
treatment <u>in vivo</u>	background*	background*	1318
treatment of chromatin	background**	background**	background**

\*For the in vivo treatment, the sample was measurable. The fact that no counts were observed is due to the lack of contamination.

\*\*The fact that no counts were observed here is due to the very low yield of DNA obtained.

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