A GENETIC AND CYTOLOGICAL INVESTIGATION OF THE EFFECTS OF ETHYL METHANESULFONATE ON PLANTS

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INTRODUCTION

The induction of mutations by the use of chemicals is still a relatively new field of research. Since 1946, when the first published reports on effective chemical mutagens appeared, many workers have tested a variety of chemical compounds for their ability to produce mutations.

Until very recently no chemical mutagens had been found which equalled or surpassed ionizing radiation in the production of genetic changes. However in 1959 Heslot et al., treated barley with a considerable series of related organic compounds and found one, ethyl methanesulfonate, that induced chlorophyll mutations at a much higher rate than did even the largest tolerated doses of radiation.

The results are extremely important since with high chlorophyll mutation rates the chances of obtaining desirable mutations are presumed to be correspondingly increased. This is only a presumption since there is a possibility that a mutagen might alter certain genes only, or might cause chromosome breaks only in specific (e.g. heterochromatic) regions (McLeish, 1953).

Some chemicals (Ehrenberg, Gustafsson & Lundqvist. 1958) have been found to induce a relatively low proportion of chromosome aberrations and still produce a high mutation rate. From the genetic standpoint, this is of advantage since fewer chromosome aberrations would in turn lead to less disruption of the division mechanism. Mutations produced would be tentatively attributed to true 'point' or gene mutations. It was considered of interest to discover whether or not EMS is a chemical which causes effects of this sort.

Since EMS is the most potent of the plant mutagens described up to the present, a detailed investigation of its effects on plants was undertaken.

LITERATURE REVIEW

The branch of genetics concerned with the study of induced mutations had its beginning when X-rays were found to be mutagenic in Drosophila (Muller, 1927), and in barley (Stadler, 1928). Much work on many species followed, and a wealth of information regarding the mutational effects of X-rays was accumulated. Then, in the 1940's three people independently discovered the mutagenic properties of certain chemicals. Auerbach & Robson (1946) reported mustard gas to be mutagenic in Drosophila. Ethylurethane and potassium chloride proved to be mutagenic in Oenothera (Oehlkers, 1943) and formaldehyde was found to be mutagenic in Drosophila (Rapaport, 1946). Soon after, Hadorn and Niggli (1946) showed that phenol was mutagenic and Vogt (1948) reported that urethane was also effective in Drosophila. In the next ten years investigators tested a large number of varied chemical compounds, many of which induced mutations to a large or small extent.

Some chemicals, although they showed some mutagenic effects, were very inefficient, only doubling the spontaneous rate or equalling effects caused by low doses of radiation: eg. 2:4:6: tri(ethyleneimino) - 1:3:5 triazine (TEM) on Vicia (Arnason & Wakonig, 1959), maleic hydrazide in Vicia (McLeish, 1953) and ethylenediaminetetraacetic acid (EDTA) in barley (Wakonig & Arnason, 1958). Other chemicals were effective in certain species but not in other species; eg. phenol induced mutations in Drosophila (Hadorn & Niggli, 1946) but not in Neurospora crassa (Jensen et al., 1951).

Recently some chemicals have been tested which were more strongly mutagenic than the highest tolerated radiation doses;

e.g. ethyl methanesulfonate in barley (Heslot et al., 1959) and diethyl sulfate in barley (Heslot and Ferrary, 1958; Heiner et al., 1960).

A partial list of some of the chemical mutagens and the more prominent workers associated with them is given below. The chemicals are subdivided here into two groups: non-alkylating and alkylating. The alkylating compounds are those which form an alkyl group which attaches itself to the substrate, chemically changing the nature of the substrate. In the terms of a chemist, Price (1958) explained it this way: If H-X represents the substrate (e.g. chromosome or gene) and R-Y represents the alkylating agent, the alkyl group attaches to the substrate, usually through an oxygen, nitrogen or sulfur atom, to give R-X. The two mechanisms by which this occurs are; 1) First order nucleophilic substitution, which is carried out by the unimolecular agents and requires two steps. 2) Second order nucleophilic substitution, which is carried out by bimolecular compounds and is accomplished in one step, (Price, 1958; Ross, 1958). It has been shown 'in vitro' that alkylating agents react with the nucleic acids, desoxyribonucleic acid (DNA) and ribonucleic acid (RNA), esterfying the phosphate groups. Polyfunctional alkylating agents can react with two amino groups or two phosphate groups thereby causing cross linkage of the DNA molecule (Price, 1958; Ross, 1958; Stacey et al., 1958). Ross (1958) has shown that alkylating agents can cause enzyme inhibition but the high concentrations necessary for this do not appear to occur 'in vivo'.

Stacey et al. (1958) claim that bifunctional reagents have more biological activity than do monofunctional ones. However in plants some monofunctional compounds have been shown to be more effective.

Some known non-alkylating mutagens and the workers associated with them are: Phenols (Hadorn and Niggli, 1946, Jensen et al., 1951), hydrogen peroxide and organic peroxides (Jensen et al., 1951), acridines and purines (Kihlman, 1951), urethane and its derivatives (Oehlkers, 1943, Jensen et al., 1951, and Auerbach, 1951), manganous chloride (Demerec and Hanson, 1951), potassium chloride (Oehlkers, 1943), and oxygen (Moutschen-Dahmen, Moutschen and Ehrenberg, 1959, Gelin, Ehrenberg and Blixts, 1959).

Alkylating agents which have been studied include: mustard gas (Auerbach, 1946 and Klmark and Westergaard, 1953), nitrogen mustards (Auerbach, 1951, and Goldacre et al., 1949), diazomethane (Rapoport, 1948(a), and Jensen et al., 1951), diepoxybutane (Fahmy and Bird, 1952, Fahmy and Fahmy, 1956, Ehrenberg and Gustafsson, 1957, and Kreizinger, 1960), ethylene oxide (Rapoport, 1948(a), Fahmy and Fahmy, 1956, and Ehrenberg and Gustafsson, 1957), ethylene imine and its derivatives (Loveless, 1953, Ehrenberg et al., 1958, and Auerbach, 1958), 2:4:6 tri (ethyleneimino)-1:3:5 triazine (TEM), (Fahmy and Bird, 1952, Fahmy and Fahmy, 1956, 1959 and Arnason and Wakonig, 1959), 2-chloroethyl methanesulfonate (Auerbach, 1958, and Fahmy and Fahmy, 1959), diethyl sulfate (Rapoport, 1946, and Heiner et al., 1960), methyl methanesulfonate (Fahmy and Fahmy, 1959), and ethyl methanesulfonate (Auerbach, 1958, Fahmy and Fahmy, 1959, and Heslot et al., 1959). Formaldehyde (Rapoport, 1946, Auerbach, 1951 and Jensen et al., 1951) is sometimes non-alkylating.

This list is necessarily incomplete as hundreds of chemicals have now been shown to be mutagenic. Prakken (1959) in his review has given a rather complete account of the work which has been done.

Since a vast amount of information had accumulated on the effects

of radiation it was convenient and perhaps desirable to compare chemical with radiation effects. At first, only the similarity of the effects of chemicals to those of radiation were noted (Auerbach, 1947, 1951). Chemicals, like radiation, caused phenotypic mutations and chromosome breakage. Deficiencies, inversions, translocations, fragments and bridges were among the structural changes induced by both chemicals and radiation.

However as more experiments with chemicals were carried out, striking differences between the effects of the different mutagens became evident. Some chemicals, unlike radiation, show specificity, attacking certain parts of chromosomes more frequently than others (Auerbach, 1951, Gray, 1953, Oehlkers, 1953, Revell, 1953, Fahmy and Fahmy, 1956, Merz et al., 1961). Merz et al., (1961) observed interaction between breaks induced by X-rays and chemically induced breaks. However there was no interaction between breaks induced by two different chemicals. Therefore there maybe some difference in the surface of the chromosomes at the point of breakage when breaks are induced by different chemicals. It may also be that different bonds are attacked.

While radiation effects are produced mainly during treatment, the effects of chemicals are often delayed, causing mosaics in Drosophila (Auerbach, 1951, Fahmy and Fahmy, 1955a and producing chromosome breakage some hours after treatment (Revell, 1953, Gray, 1953). The action of chemicals is often indirect (Revell, 1953, Auerbach, 1951) whereas radiation action is direct or nearly so (Darlington, 1947, Thoday, 1953). Also the effects of chemicals often depend upon their ability to penetrate the cell (Kihlman, 1951, Revell, 1953). Other notable differences include; the occurrence of sensitive stages to

chemicals (Auerbach, 1951, Revell, 1953, Fahmy and Fahmy, 1956, fewer major chromosome rearrangements and an increased number of small deficiencies for the same mutational equivalent dose of X-rays (Auerbach, 1951, Fahmy and Fahmy, 1956, Fahmy and Bird, 1952, Heiner et al., 1960, Ehrenberg et al., 1958), and a different mutational spectrum (Ehrenberg et al., 1958, Heiner et al., 1960).

Early in the study of mutagenic chemicals it was thought that all chemicals must exert their effects by the same mechanism, and various 'common action' theories were proposed. Initially, because many mustard compounds were mutagens, mutagenic action was believed to involve the poisoning of the sulfhydryl groups (-SH) in either the proteins of the chromosomes or of enzymes concerned with chromosome metabolism. This theory fell when two powerful -SH poisons, lewisite and chloropurine, were found to be ineffective. Goldacre, et al., (1949) put forth the hypothesis that mutagenic action was due to cross linkage of protein chains by two active groups in the same molecule. This theory did not completely explain the action since some compounds containing only one active group were found to be mutagenic.

The large number of mutagenic compounds known today include some that are completely unrelated to each other with regard to chemical structure, physico-chemical and pharmacological properties. Mutagens can work at three levels: (1) the self-reproducing information system (2) the translation system which decodes information and transmits it (3) the receptor system (Westergaard, 1957). They may act by direct chemical reactions with the proteins or nucleic acids of the chromosomes, release energy close to the chromosome, inactivate enzymes concerned with chromosome metabolism or interfere with chromosome

reduplication. Effects of chemicals are influenced by differences in penetration ability, degree and speed of detoxication, reactions with cytoplasmic components and other similar factors (Auerbach, 1951). Today it is agreed that there is no single mechanism common to all chemical mutagens.

In 1954, Demerec, working with Escherichia coli demonstrated that the rate of reversion (back mutation) at several nutritional deficiency loci varied markedly under similar treatments with different mutagens. These results were confirmed by Kølmark (1953) and Kølmark and Westergaard (1953) using several chemicals on Neurospora crassa. They found that the adenineless mutants were more susceptible to some chemicals than were the inositolless mutants, but with other chemicals the inositolless mutants were more susceptible. They also found that the reversion rate differed between different adenineless cistrons and even between different mutons within the same cistron. This differential gene response to chemical mutagens has also been studied in Drosophila (Fahmy and Fahmy, 1959).

Some chemicals, therefore, have shown specificity not only for certain parts of the chromosome, but for certain genes (cistrons) and parts of genes (mutons). The gene, then, is a much more complex unit than was originally thought and the geneticist's concept of it is changing. Possibly the specificity of certain chemicals for certain genes, when determined, may ultimately give geneticists greater control over the mutation process than they now have.

Little work has been done on EMS. Heslot et al. (1959) tested EMS along with many other related compounds, and reported that it caused a very high rate of chlorophyll mutations in barley. The only other

publications referring to EMS are those of Fahmy and Fahmy (1959), where EMS was one of the chemicals used to demonstrate the differential gene response to chemicals in Drosophila, and Auerbach (1958), where EMS was among the chemicals compared to X - irradiation for mutagenicity. These reports have stated that EMS is a strong chemical mutagen. However no information is available on the preparation of the compound, and very little on suitable concentrations, treatment techniques, or the mutagenic effects.

MATERIALS AND METHODS

EMS Characteristics

The chemical used in these experiments was Ethyl Methanesulfonate. It was obtained from the Eastman Organic Chemicals division of the Eastman Kodak Company, U. S. A. Ethyl Methanesulfonate is a colorless liquid and is a poison. The molecular formula is $CH_3SO_3C_2H_5$ and its molecular weight is 124.16.

A stock solution, 1/50 by volume of EMS in distilled water, was prepared. Other desired concentrations were obtained by dilution of the stock solution. The pH of the solutions was between 1.6 and 2.2. They were quite stable, lasting under refrigeration for at least two months after preparation.

Experimental plants

The material treated and planted in the field was Hordeum vulgare L., the Montcalm variety. Dry grains which had been selected for uniformity and plumpness were treated. The Thatcher variety of Triticum aestivum L. was also treated with one concentration of the chemical being used. For cytological purposes, root tips of Vicia faba L. were treated.

Germination tests

Germination tests were conducted in order to determine the percentage survival following the various treatments. One hundred seeds were placed in a petri dish between pieces of No. 1 Whatman filter paper. They were treated by being soaked in 15cc. of the chemical for the desired period, in a constant temperature chamber at 20°C. The seeds were then washed in running tap water and transferred to a clean petri dish with two filter papers on the bottom. Fresh tap water was run

into the dish, then was almost completely drained off. It was found that the two pieces of filter paper retained enough moisture for the seeds for at least twenty-four hours. Formerly only one paper was used and the seeds were left in standing water. However mold developed and by the fourth day the seeds were mushy. The number of seeds germinated were counted on the second, fourth, and sixth day. They were discarded after the seventh day.

A seed was counted as germinated as soon as the primary root appeared. In some cases the coleorhiza was pushed through the pericarp but the primary root did not emerge. These seeds were scored as non-germinated.

Treatments and field methods for genetic tests.

Barley lots were treated with the following concentrations of EMS and sown in the field: (1) EMS 1/400 (by volume) for 24 hours at 20°C. (2) EMS 1/200 for 24 hours at 20°C. (3) EMS 1/100 for 6 hours at 20°C. (4) EMS 1/50 for 2 hours at 20°C. (5) EMS 1/100 for 19 hours at 20°C. One treatment of the base pair, Adenine and Thymine alone, 6×10^{-3} M for 4 hours, and in combination with EMS was used. Combinations of EMS Co^{60} radiation were tried, but due to an unavoidable delay in seeding, did not germinate. Hexaploid wheat of the Thatcher variety was treated with EMS 1/100 for 24 hours.

Most treatment lots consisted of 2400 seeds. The seeds were treated in petri dishes for the required length of time, then surface dried (usually for 2 hours) and planted. Control seed lots of Moncalm barley and Thatcher wheat were also seeded.

A V-belt hand-pushed seeder which was set to drop one seed every two inches was used. Rows were six inches apart within treatments.

and one foot apart between treatment lots.

During the summer progress of each treatment was noted. At harvest time, the heads of the M_1 (treated generation) plants were taken, all heads of one plant being tied together.

Part of the M_2 was planted in the greenhouse during the winter. The heads were planted in 'beds' which were approximately four feet by nine feet. They were placed in rows with the heads of successive plants at right angles to each other. When the seedlings were 14 to 18 days old, they were scored for chlorophyll mutations. They were then discarded.

Some of the M_2 seedlings, twenty-five groups of mutants and their siblings, were allowed to grow to maturity. These were harvested and an M_3 grown in the field the following summer to determine segregation ratios in the offspring of heterozygotes.

The rest of the M_2 were grown in the field. These were scored for chlorophyll mutations when they were 14 days old. At about six weeks, pollen mother cells (P.M.C.) were collected. These were taken from plants in segregating M_3 populations in which a segregation ratio higher than 3 green : 1 mutant occurred since aberrant ratios may be associated with chromosomal disturbance.

Scoring mutations

Mutations were scored per M_1 plant and per M_1 spike. In both cases, if two or more spikes of the same plant contained the same mutational type, it was always counted as only one mutation, regardless of the ratios of mutant to green. Heiner et al. (1960) suggested that if neither of the spikes containing the mutation produced progeny showing a 3:1 ratio, the mutations should be counted

separately. However, if one of the spike progenics appeared in a 3:1 ratio, the mutations should be scored as only one. It was found that this method of scoring was impractical, so it was not used. The reasons for this will be discussed later.

Another method of scoring mutations was suggested by Heiner et al. (1960) and Gaul (1960). This involves counting the number of mutations in relation to the total number of M_2 plants (M_2 seedlings). This method was also used.

The chlorophyll mutants were divided into eight phenotypic classes (mod. after Gustafsson, 1940). The classes are:

(1) Albino - No carotenoids or chlorophylls formed.

(2) Xantha - Carotenoids prevail, chlorophylls are absent.

(3) Alboviridis - Different colors at the base and tip of the leaf.

  (a) Alboxantha - Tip white, base yellow.

  (b) Xanthalba - Tip yellowish, base white or faintly colored.

  (c) Viridoalbino - Tip greenish or yellowish green, base white.

  (d) Other combinations.

(4) Chlorina - A heterogeneous group characterized by a yellowish green or light green color occurring already at the seedling stage.

(5) Tigrina - Transverse destruction of pigment. The transverse stripes are usually brown or yellow, narrow and pinched.

(6) Striata - Longitudinal stripes of white or yellow color.

(7) Lutescens - First green appears then color is lost and plant shrivels.

(8) Maculata - Chlorophyll destruction in the form of dots distributed over the leaf.

Cytological techniques

Secondary root tips of the broad bean, Vicia faba, were treated with EMS and examined to determine the extent of chromosome aberrations produced. Vicia was used because of its small chromosome number and its large chromosomes. It was therefore easier to obtain good slides and to see clearly any aberrations. M_3 pollen mother cells of barley were also examined.

Vicia faba root tips were treated for various times; 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 8 hours, and 12 hours with 1/100 EMS. Treatment was carried out at room temperature (24°C). Recovery periods were 24 hours and 48 hours at 20°C.

In order to prepare the beans for treatment, they were first placed in vermiculite where they began to germinate. After 48 hours (2 days) they were removed, the seed coat peeled,and they were replaced in the vermiculite. In 3 or 4 days the beans were again removed and the primary root tip cut off, forcing the development of secondary roots. When the secondary roots were between 1 and 1.5 cm. long the beans were transferred to a water bath which was maintained at 20°C (this varied 2-3 degrees). They were then ready for treatment. After the treatment and recovery period, the root tips were removed and put in freshly prepared fixative (95% ethanol: glacial acetic acid, 3:1).

Hydrolizing and staining

Vicia root tips were hydrolized in 1 N HCl at 60°C for 6-8 minutes. The roots were then placed in the stain for not more than two hours. The Feulgen stain was used, the stain being prepared according to Johansen (1940).

Squash technique

The stained roots were removed from the Feulgen and decolorized in 45% acetic acid for 10-30 seconds. This was done to remove excess stain from the cytoplasm. The roots were then placed on a slide in a drop of 45% acetic acid. A plastic coverslip was placed over them and tapped gently with a wooden match stick or a pencil to separate the cells. The edges were sealed with paraffin and the slides were allowed to stand for two hours. They were then placed in acetone overnight or until the coverslip was dissolved. The slides were transferred through the following solutions; fresh acetone for 5 minutes, xylene:acetone, 1:1, for 5 minutes, and three washes of xylene, each for 5 minutes, and made permanent by mounting in Canada balsam with a glass coverslip. They were then examined.

The dry ice method

This method was used in preparing slides for the examining of P.M.C.'s. Four plants were collected from each desired head. Young inflorescences were fixed in freshly prepared 3:1 fixation (95% ethanol: glacial acetic acid) for 30 minutes to 2 hours. They were then transferred to 95% ethanol, 80% ethanol, and stored in 70% ethanol in the refrigerator. Squashes of the anthers were made in a drop of aceto-carmine placed on an acid clean slide. A not-acid-clean coverslip was placed crosswise on the material. Slight heating

intensified the stain. The slide was then examined and if the right stage was found, it was laid flat on a piece of dry ice until the material was completely frozen. The coverslip was then flicked off and the slide placed in 70% ethanol for 30-60 seconds, then transferred to 80% ethanol, 95% ethanol, and two washes of absolute ethanol, each for 5 minutes. Permanent mounting was done in 'Diaphane'.

RESULTS

Germination Results

The barley seed used for these tests was harvested in 1956. Preliminary runs showed that the seeds germinated better than did other stocks of seed or commercial seed, the percent germination being over 95%.

Germination results were not complete at the time of seeding in 1960, therefore concentrations and treatment lengths similar to those described by Heslot *et al.* (1959) were used for field treatments. Several of these treatments proved to be too strong resulting in such low germination that they were not included in the study.

Table I shows final germination results, obtained by averaging individual runs. The dose at which there is approximately 50% mortality (the LD50) is close to EMS 1/100 for 8 hours at 20°C.

The treated generation (M_1)

Seventy days after seeding (mid-July), the M_1 population was scored for percent germination and average height. A striping effect was noted in most of the M_1 treatments. This was presumed due to a physiological imbalance caused by the chemical treatments. In some treatments nearly 100% striping was observed (Table II). The modifications varied. A ribbing effect was noted; this was a dark green leaf with light green narrow stripes running through the leaf regularily and in alternation. The same effect occurred with a light green leaf having dark green stripes. Regarding the more pronounced striping effect, stripes were white, brass yellow, gold yellow, pale yellow, maroon, and brown.

Table I.

Germination Results
(20°C in Dark)

Conc. EMS (by volume)	Treatment period in hours	Germination in % at 20°C		
		2 days	4 days	6 days
1/50	2	73	89	90
	4	45	66	70
	6	31	65	66
	12	0	1	1
1/100	6	61	73	78
	7	33	64	71
	8	18	36	43*
	12	6	17	18
	24	5	5	5
1/400	2	82	92	92
	12	80	75	75
	24	55	65	70
	48	0	0	0
Control	-	91	96	96

* LD_{50}

Table II.

Percent Germination, Average Height, and % Modification of M_1 Plants Seventy Days After Seeding.

Plant treated	Treatment	% Germination	Average height	% Modification
Barley	EMS 1/400 24 hrs.	80	3' 8"	75
	EMS 1/200 24 hrs.	70	2' 10.5"	98
	EMS 1/100 6 hrs.	70	2' 8.5"	93
	EMS 1/50 2 hrs.	80	3' 2"	75
	EMS 1/100 19 hrs.	4.1	1' 11"	85
	EMS 1/100 19 hrs. & Ad-Thy**	60	1' 1"	92
Wheat	EMS 1/100 29 hrs.	good	1'*	14

*read 40 days after seeding.

**12 hrs. - Adenine-Thymine 6x 10^{-3}M, 19 hrs. EMS 1/100, 2 hrs. Adenine Thymine.

The stripes were broad or narrow; at times these were several stripes in a leaf and these were sometimes different colors (Figures 1 and 2). In a few plants the stripe in the leaf continued down the clasping leaf base.

Germination and growth of the weaker treatment lots were nearly normal; however in the stronger treatments germination was low (as little as 4% in one case). Growth and maturation were also inhibited in these treatments.

In some treatment lots the mature M_1 plants were almost completely sterile. In other treatments partial sterility occurred.

The second generation (M_2)

Fourteen day-old seedlings were scored for chlorophyll mutations, both in the greenhouse and in the field (Figures 3 and 4). When more than one spike in the same plant contained similar mutation types, these were initially counted as one mutational event if the mutant appeared in a 3:1 ratio in one of the spikes, but were scored as two events if neither spike contained a 3:1 ratio, after the method described by Heiner et al. (1959). Difficulty was encountered, however, in establishing a standard deviation from the 3:1 ratio and above which it was considered not to be a 3:1 ratio. The largest tolerated (i.e. insignificant) deviation from the 3:1 ratio increased as the number of seedlings from the spikes decreased. For example, using the Chi^2 method for determining "Goodness of Fit", a ratio of 8:1 was not a significant deviation (but any higher ratio was significant) from the 3:1 ratio, if the number of seedlings from the spike was 36. However, if the number of seedlings from the spike was 18, a ratio as large as 17:1

Figure 1. Striping modifications in leaves of M_1 plants. Normal leaf shown on the extreme right.

Figure 2. Striping modifications in leaves of M_1 plants. Normal leaf shown on the extreme right.

Figure 1.

Figure 2.

Figure 3. View of 'beds' in the greenhouse showing M_2 seedlings ready to be counted.

Figure 4. Close view of M_2 seedlings. Mutants shown are: chlorina, albino and xantha.

Figure 3.

Figure 4.

was not significant. Therefore the problem was at what point a deviation from the ratio was considered large enough to score the mutations as two instead of one. The alternative was to determine the deviation not significant for each spike. Since this was not practical, a ratio arbitrarily chosen would have had to have been used. Because this method could not result in an accurate count, it was abandoned. Mutations, were therefore, counted on a single basis and the rate calculated as the number of mutations per M_1 plant and the number of mutations per M_1 spike.

Gaul (1960) suggested that scoring mutations on the basis of M_2 seedling counts was the most accurate method of determining chlorophyll mutation rates. We therefore also calculated the mutation rate per 1000 M_2 seedlings.

The results in Tables III and IV show that in all treatments the chlorophyll mutation rate is exceptionally high. The treatments involving either lower chemical concentrations and longer treatment time, (EMS 1/400 for 24 hours), or higher chemical concentrations and shorter treatment time (EMS 1/50 for 2 hours), show a higher mutation rate than those of more intermediate chemical concentrations and treatment length. The standard deviation of all mutation rates was calculated.

As part of the M_2 of each of four treatments was grown in the greenhouse and part in the field, it was thought that a comparison of rates might be interesting. From Table V it is seen that in all cases except one the mutation rate was higher in the field. Possible reasons for this will be discussed later.

The chlorophyll mutation spectrum induced by EMS is shown in Table VI.

Table III.

Total mutations rates per M_1 plant and spike.

Plant treated	Treatment	Number examined		Number of mutations	Mutations per plant ± S.D.	Mutations per spike ± S.D.
		Plants	Spikes			
Barley	EMS 1/400 24 hrs.	512	2005	623	1.22±.05	.31±.01
	EMS 1/200 24 hrs.	562	908	361	.64±.03	.40±.02
	EMS 1/100 6 hrs.	510	1118	437	.86±.04	.39±.02
	EMS 1/50 2 hrs.	588	2549	767	1.30±.05	.30±.01
	EMS 1/100 19 hrs.	459	777	149	.32±.03	.19±.02
	EMS 1/100 19 hrs. & A.T.*	34	122	32	.94±.20	.26±.05
	Adenine-thymine-4 hrs. 6 x 10-3M.	750	2815	6	.0080±.0009	.0021±.0008
	Control	4054	11000	11	.0027±.0008	.0010±.0003
Wheat	EMS 1/100 24 hrs.	55	180	0	-	-
	Control	31	120	0	-	-

*Treatment consisted of 12 hrs. Adenine-thymine solution, 6 x 10^{-3}M., 19 hrs. - EMS 1/100, then 2 hrs. Adenine-thymine.

Table IV.

Total mutation rates per 1000 M_2 seedlings.

Treatment	Number of mutant M_2 seedlings.	Total M_2 green seedlings.	Mutation rate per 1000 M_2 seedlings ±S.D.
EMS 1/400 24 hrs.	1431	37037	$37.2 \pm .20$
EMS 1/200 24 hrs.	774	4671	142.1 ± 2.1
EMS 1/100 6 hrs.	792	6779	104.6 ± 1.3
EMS 1/50 2 hrs.	1823	53011*	$33.2 \pm .14$
EMS 1/100 19 hrs.	276	5010	52.2 ± 3.1
EMS 1/100 19 hrs. & Ad. Thy.	93	2583	34.8 ± 3.6

* This figure was estimated; all other figures are from actual counts.

Table V.

Comparison of Mutation Rates in G. H. & Field Scored per M_1 Plant & M_1 Spike

Treatment	Environment of M_2	Number of plants	Number of spikes	Number of mutations	Mutation per M_1 plant	Mutation per M_1 spike
EMS 1/400 24 hrs.	G.H.	236	1033	237	1.0	.23
	Field	276	972	386	1.4	.40
	Total	512	2005	623	1.2	.31
EMS 1/200 24 hrs.	G.H.	463	743	277	.60	.37
	Field	99	165	84	.85	.51
	Total	562	908	361	.64	.40
EMS 1/100 6 hrs.	G.H.	324	767	284	.88	.37
	Field	186	351	153	.82*	.44
	Total	510	1118	437	.86	.39
EMS 1/50 2 hrs.	G.H.	487	2149	615	1.3	.29
	Field	101	400	152	1.5	.38
	Total	588	2549	767	1.3	.30

* In all but one instance mutation rates were higher in the field than in the greenhouse.

Table VI.

Chlorophyll Mutation Spectrum Induced by EMS.

Phenotypic Categories	EMS 1/400 24 hrs.		EMS 1/200 24 hrs.		EMS 1/100 6hrs.		EMS 1/50 2 hrs.		EMS 1/100 19 hrs.		EMS 1/100 19 hrs. AT	
	No. of mut.	% of total mut.	No. of mut.	% of tot. mut.	No. of mut.	% of tot. mut.	No. of mut.	% of tot. mut.	No. of mut.	% of tot. mut.	No. of mut.	% of total mut.
Albino	176	28.3	101	28.2	119	27.2	224	29.2	44	29.5	9	28.1
Chlorina	195	31.3	154	42.5	149	34.1	239	31.2	23	15.4	7	21.9
Xantha	38	6.1	24	6.6	25	5.8	57	7.4	13	8.7	3	9.4
Striata	46	7.4	21	5.8	23	5.3	39	5.1	11	7.4	4	12.5
Lutescens	78	12.5	26	7.2	53	12.1	87	11.3	27	18.1	5	15.6
Alboviridis	78	12.5	31	8.6	53	12.1	98	12.8	30	20.1	4	12.5
Tigrina	10	1.6	3	.8	15	3.4	15	2.0	1	.1	0	-
Maculata	2	0.3	1	.3	0	-	8	1.0	0	-	0	-
Total	623		361		437		767		149		32	

Approximately 28% of the mutants are albino. This is a much lower percentage than is induced by irradiation. A larger proportion of the mutants is chlorina; approximately 30% of the total mutants. The more rare types of mutants appear more frequently than with irradiation. The spectra are quite similar for all treatments.

Table VII deals with the comparative frequencies of the different categories of mutational types in the greenhouse and in the field. It can be noted that lutescens is consistently more frequent (more than twice as frequent) in the field than in the greenhouse; alboviridis is consistently less frequent (half as frequent) in the field than in the greenhouse. The other types seem to occur fairly similarly in both environments.

During the harvest of the mature M_2, a large sample from each treatment (from 1/6 to the complete treatment) was examined closely; spike mutants were scored and collected. The modifications included: curly stem, wavy sub-spike internode and curly awns, long wavy base internode of the rachis, hooded awn, thick straw, extra glume, double awn, ' rounded and crowded spike', and semi-sterility.

The 'rounded and crowded' spike was the most striking mutation. The rachis was shortened, the spikelets crowded, and the spike had a nearly spherical appearance. Two M_2 plants out of 15 showed this phenotype. The plants were from a single M_1 spike.

The 'rounded and crowded' spike is shown in Figure 5; some other modifications are shown in Figure 6.

An estimate of the percent semi-sterility induced was made by scoring five groups of 100 plants taken from different parts of the field in each treatment, the total count for each treatment being 500 (Table VIII).

Table VII.

Comparison of Frequencies of Mutant Types in the Greenhouse and in the Field.

	EMS 1/400 for 24 hrs.			EMS 1/200 for 24 hrs.			EMS 1/100 for 6 hrs.			EMS 1/50 for 2 hrs.		
	% of mut. in G.H.	% of mut. in field.	% of mut. in Field and G.H. combined.	% of mut. in G.H.	in field	combined	% of mut. in G.H.	in field.	combined	% of mut. in G.H.	in field	combined.
Albino	36	23	28	29	25	28	26	30	27	30	25	29
Chlorina	24	35	31	40	50	43	38	28	34	31	32	31
Lutescens	6	16	12	4	17	7	5	26	12	9	23	11
Xantha	5	7	6	8	4	7	7	4	6	8	6	8
Striata	7	7	7	8	-	6	7	3	5	5	4	5
Alboviridis	17	9	12	10	4	9	15	7	12	14	6.5	13
Tigrina	1	1.7	1.6	1	-	.8	4	2	3	2	2	2
Maculata	-	.5	.3	-	1	.3	-	-	-	.8	2	.1

Note: Lutescens always occurs more frequently in the field. Alboviridis always occurs less frequently in the field.

Figure 5. Side and front views of the 'rounded and crowded' spike compared to the normal spike.

Figure 6. Some striking modifications occurring in the M_2 after treatment with EMS. Left to right: malformed stem with spike turned upside-down, curly stem and wavy awns, accordian rachis, first internode of the rachis long and curly.

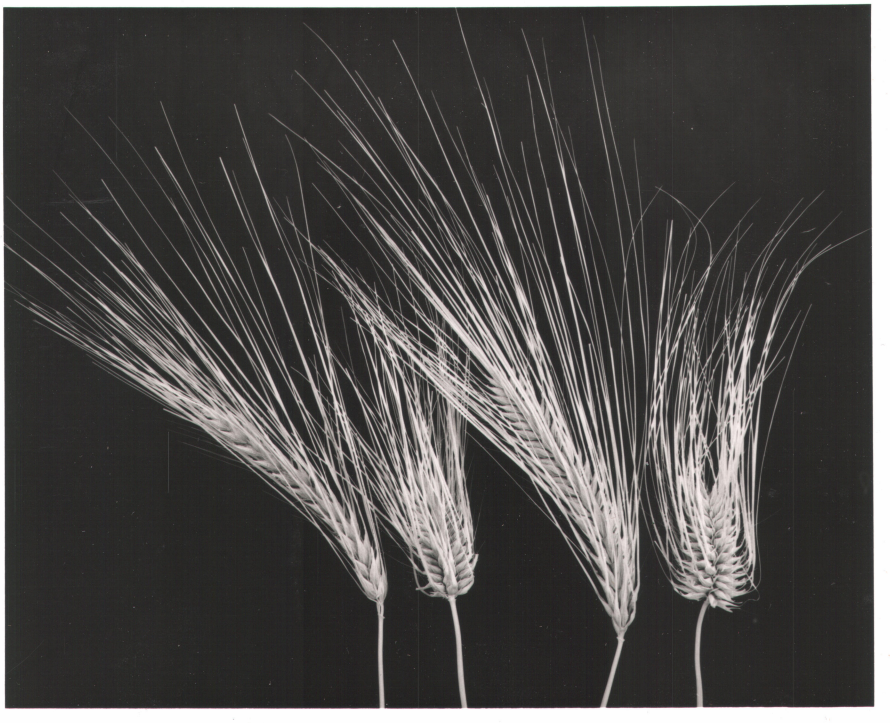

Figure 5.

Figure 6.

Table VIII.

Semi-Sterility Frequencies in the M_2.

Treatment	No. of normal plants	Semi-sterile	Sterile	Total	% Semi-sterile & sterile
1/400 EMS for 24 hrs.	66	32	2	100	
	63	34	3	100	
	71	25	4	100	
	67	26	7	100	
	67	31	2	100	
	334	148	18	500	33%
1/200 EMS for 24 hrs.	20	61	19	100	
	46	43	11	100	
	18	58	24	100	
	21	64	15	100	
	105	226	69	400*	74%
1/100 EMS for 6 hrs.	59	31	10	100	
	54	32	14	100	
	48	38	14	100	
	32	53	15	100	
	39	42	19	100	
	232	196	72	500	54%
1/50 EMS for 2 hrs.	56	33	11	100	
	48	42	10	100	
	55	39	6	100	
	44	53	3	100	
	52	37	11	100	
	259	204	41	500	49%
Control	98	2	-	100	
	98	2	-	100	
	196	4		200	2.0%

* Only a count of 400 was made as this was a small population.

Although the figures for each hundred within the total 500 show considerable variation, they are sufficient to indicate that at least 33% semi-sterility was present in all treatments, and that in the second treatment, EMS 1/200 for 24 hours, it is definitely higher than in the other treatments. Since this was an exceptionally dry summer, drought damage may have caused these figures to be somewhat higher than they would have been if more favorable weather conditions had prevailed.

The Third generation (M_3)

An M_3 was grown from twenty-five groups of M_2 mutant siblings. These included siblings of chlorina, albino, xantha, striata, viridoalba, xanthalba, and lutescens. It was found that these segregated with fairly low ratios, from 1:1 to 12:1. These ratios are within the standard deviation for a 3:1 ratio obtained from the numbers occurring. No exceptionally high ratios, such as 30:1 occurred. Also, approximately 2/3 of the siblings of the mutants segregated for the mutation. One chlorina sibling out of four showed segregation. Five out of six siblings of another chlorina showed segregation and albino siblings segregated for the mutation in 2 out of 3 plants. Combining the group of siblings, 34 plants out of 59 showed segregation of mutant to green. The expected values for a 2/3 ratio would be 39/59. The experimental values do not differ significantly from the expected. A 2/3 segregation would be expected if a single gene was involved. Offspring of some chlorina mutants were grown and these bred true.

The indication is, therefore, that these mutants are due to single genic changes and are not caused by small deficiencies. This

is supported by cytological observations.

The siblings of some mutants segregated for other phenotypes as well as for the phenotype expressed in the M_2. Table IX shows the M_3 segregation ratios of the siblings of mutant M_2.

Cytological results

Vicia faba root tips were treated with 1/100 EMS for various lengths of time, allowed to recover for 24 hours or 48 hours, squashed, stained and counted. Al least 100 metaphase and anaphase cells from each treatment time were examined. The results, indicated in Table X, show that a very small percentage of the cells was abnormal due to breakage. Counting first division pollen mother cells of siblings of mutant M_2 plants also indicated a very small percentage of abnormal cells.

These results can only be taken as an indication of what might be. Much more detailed studies are needed to determine the extent of chromosome breakage or the lack of it.

Table IX.

M_3 segregation ratios of siblings of mutant M_2.

Siblings of mutant type	M_2 progeny	Mutant:Green	Other phenotypes occurring	Ratio of mutant:green
Chlorina	1a	26:51	albino	1:2
	1c	11:12	xantha	1:1.1
	2c	62:125	-	1:1.2
	4a	1:12	-	1:12
	4c	26:78	-	1:3.1
	10a	14:48	xanthalba	1:3.4
Albino	2a	8:51	xantha	1:6.4
	5a	4.23	-	1:5.8
	6b	26:72	-	1:2.8
Xantha	5c	12:34	chlorina	1:2.6
	6a	6:29	chlorina	1:4.8
Striata	4b	15:53	lutescens	1:3.5
Viridoalba	3b	39:47	-	1:1.2
	5b	1:2	-	1:2
	7a	27:37	chlorina	1:1.3
	8a	3:9	striata & albino	1:3
Xanthalba	3d	12:33	lutescens	1:2.6
Chlorina & Albino	7b	2:1	-	2:1
	9a	9:39	chlorina-alba	1:4.3
Lutescens	10b	1:8	chlorina	1:8

Table X.

Percent abnormal metaphases and anaphases in *Vicia* root tips treated with EMS 1/100.

Treatment time	Recovery time 24 hrs. Cells counted	abnormal	%aberrant	Recovery time 48 hrs. Cells counted	abnormal	%aberrant
15 min.	250	3	1.2	111	0	0
30 min.	129	0	0	202	1	.90
45 min.	147	0	0	174	0	0
1 hr.	82	0	0	202	0	0
2 hrs,	138	1	.72	161	1	.62
4 hrs.	163	1	.61	124	1	.81
8 hrs.	183	1	.55	131	1	.76
12 hrs.	329	4	1.2	167	1	.60
Control				105	0	0

DISCUSSION

The germination results as shown in Table I indicate that with quite long treatment times the various concentrations of EMS caused high mortality rates. The treatments slowed germination, with the result that some roots emerged after the fourth day. At any given day the control roots were longer than the treated ones.

The treatments used for the field study all, except one, showed good germination, well above the LD_{50} (Table II). The exception was EMS 1/100 for 19 hours. Although this treatment proved to have drastic effects, it was retained in order to compare the effects of EMS alone and the effects of EMS 1/100 for 19 hours in combination with Adenine and Thymine.

In the M_1 a high percentage of striping occurred in all treated barley populations, however only 14% modification occurred in the wheat. Because Thatcher wheat is hexaploid it is believed that genetic disturbances and partial physiological imbalance can be tolerated better than in the diploid barley.

Gaul (1960) claims that scoring mutations per 100 M_1 plants is the least accurate method of estimating the mutation rate. It is greatly affected by variations in the amount of tillering and usually leads to an underestimation of the rate because it does not recognize multiple mutation events which may occur in different spikes of the same plant.

Since most mutations are recessive and therefore become apparent only in the second generation the count should, ideally, be based on the proportion of mutated M_1 inflorescence-sectors. Or, putting it another way, it is only the mutation frequency of the initial cells

in the embryo which will ultimately form the generative tissue of the inflorescences in which we are interested. If the 'sector' of generative tissue derived from the embryonic cells were recognizable in the adult plant, it would be on this sector that the mutation frequency would be based. Generally, one mutated sector results in the production of only one mutated spike. Therefore, scoring mutations per 100 M_1, spikes is the best method. Gaul (1960) concludes that only a few cells in the embryo form the generative tissue of the spike. Each such cell may give rise to a 'spike-sector'. Increased strength of treatment may more often result in the survival of one or a few cells of the embryo stem meristem which these form all the generative tissue of the spike. Therefore the sector size would be increased. Partial sterility in the M_1 would also lead to the failure of some mutations to be expressed.

Scoring mutations per M_2 seedlings is, according to Gaul, the most "suitable" method as it is not subject to errors due to serility, small progeny size, variations in tillering, or in possible different size of mutated sectors. However, although this method gives the proportion of M_2 seedlings mutated and not mutated, it cannot give an estimate of the actual number of mutational events which have occurred. Moreover, when counting our results according to this method it was found that the seedling count tended to underestimate the mutation rate as determined on the spike basis, rather than overestimate it as Gaul suggested. Heslot and Ferrary (1958) point out that as sterility increased, the number of seedlings per spike decreases and therefore the probability of the appearance of mutants is also decreased. It is also evident that counting every M_2 seedling

is impractical, requiring an enormous amount of tissue since up to 50 seedlings may develop from one spike. With large M_2 populations it would be almost impossible.

We therefore agree with Heslot and Ferrary (1958) that scoring mutations per 100 M_1 spikes is the most desirable method at the present time. This method combines a high degree of accuracy with practicality.

EMS induces a high rate of chlorophyll mutations in barley. This is indicated clearly in Tables III and IV. The results obtained here are entirely comparable to those obtained by Heslot et al. (1959). They expressed their mutation rates as the rate per 100 M_1 spikes. For the treatment EMS 1/400 for 24 hours Heslot obtained 31% mutated spikes. We also obtained this figure, stated as .31 mutations per spike. Heslot used EMS 1/250 for 24 hours and obtained 43% mutated spikes. Using EMS 1/200 for 24 hours we obtained .40 mutations per spike.

Treatment with Adenine and Thymine alone approximately doubled the spontaneous mutation rate. This rate however is extremely low compared to the mutation rate induced by EMS treatment. EMS (1/100 for 19 hrs.)alone induced approximately .20 mutations per spike, or 20% spike mutations. However, EMS treatment in combinations with Adenine and Thymine resulted in an increase in the mutation rate to 26% spike mutations (Table III). This combination treatment had been done with a proposed theory in mind that providing the cells with an excess amount of Adenine and Thymine, one of the two base pairs occurring in the DNA molecule, during treatment, may reduce the mutation rate caused by EMS, as the raw material needed for repairing DNA breaks would be easily available. Adenine and Thymine treatment

alone increased the mutation rate slightly, which indicates that the Adenine-Thymine solution penetrated the seeds and that it was active within the cells. If there is as little chromosome breakage induced by EMS, as our results indicate, then the Adenine and Thymine were not needed for repairs of gross chromosome breaks. Rather, the base pair as well as the treatment chemical were chemically active in causing the genetic changes produced. The exact nature of this activity is not known.

The frequency of mutations and of certain mutant types varied noticably depending on whether the plants were grown in the greenhouse or in the field. The mutation frequencies were generally higher in the field. A partial explanation may be that the environment of the greenhouse, weak light and even temperature, has a 'covering' effect resulting in less distinct mutants, modifying their characteristics or even rendering them completely undetectable (Holm, 1954). On the other hand, the strong light and sometimes great fluctuations in temperature which occur in the field tend to accentuate the mutant type, different temperatures being responsible for slight color variations in any one mutant type. As it has been known for some time that light and temperature greatly influence chlorophyll formation in plants, we are inclined to accept this type of explanation.

The greater frequency of 'lutescens' in the field could be due to the inability of some mutant types, such as a weak chlorina, to survive under extreme conditions. The young mutant, unable to survive, may begin withering at the tip and continue to wither, thus being indistinguishable from a true 'lutescens'. There is also

the possibility of error in classification. For example, a xantha and a pale chlorina often appear similar.

Our results concerning the mutation spectrum induced by EMS also agree with those of Heslot. According to our results 28% of the total mutants were albino. He found 27.6% of the total mutants to be albino. However he did not further categorize his mutants. Resulting from the fact that the proportion of albino mutants is low following chemical treatment (compared to those induced by irradiation treatments) the number of so called 'rare' types of mutations increased. This trend seems to be more extreme for EMS than for other chemicals. However, Heiner (1960) using a closely related chemical, diethyl sulfate, obtained comparable results.

Where mutant siblings segregated in the M_3 for phenotypes not expressed in the M_2, two explanations are possible. Since the M_2 was grown in the greenhouse and the M_3 grown in the field, the 'covering' effect of the greenhouse environment may have rendered some of the phenotypes indistinguishable, while the 'accentuating' environment of the field could have 'brought out' mutant types already present. Another possible explanation is that the other mutations were present in the heterozygous condition in the M_2, and that homozygotes first appeared in the M_3.

Semi-sterility (between 20% and 95% sterility) was present in fairly high frequencies both in the M_1 and M_2. Often semi-sterility is a result of chromosome breakage, causing chromosome aberrations and abnormal meiosis and mitosis, leading to the production of non-functioning pollen. Chromosome breakage does not, however, seem to be the reason for the semi-sterility incurred here. Two facts point

away from this explanation. Firstly, the segregating ratios of the mutant siblings in the third generation were all without exception in close agreement with the 3:1 ratio. The occurrence of the Mendelian ratio in the M_3 strongly suggests that a genetic factor is involved. Extremely high ratios, which usually indicate chromosome abnormality, were absent. Also, cytological examination of a limited number of pollen mother cells indicates that no more aberrations than normally occur in untreated barley were present.

This leads to a consideration of the mode of action of EMS. It seems reasonable to suggest that the mutagenic action of EMS is mainly that of producing changes at the molecular level; that is, by modifying the structure of the DNA molecule, possibly by esterification of the phosphate groups. A very minor amount of chromosome breakage could also be involved. This explanation is supported by the fact that treated Vicia root tips, examined for chromosome aberrations showed no significant increase in chromosome breakage over that seen in untreated roots. It is realized that much more comprehensive cytological studies are needed to determine the extent of chromosome breakage and the fate of centric and acentric fragments after breakage.

It seems to me that the next step is to undertake a detailed biochemical study of the action of EMS. This interesting problem, if solved, would reveal new information which would considerably help the geneticist in his attempt to understand and partially control the mutation mechanism.

SUMMARY

The mutagenic properties of Ethyl Methanesulfonate (EMS) were investigated. Montcalm barley seeds were treated with different concentrations of EMS and were grown in the field. One treatment of Adenine and Thymine alone, and in combination with EMS was included. One treatment of Thatcher wheat with EMS was also included.

The M_1 germinated quite well in the treatments used for this study. Stronger treatments resulted in poorer germination and slower growth. A striping effect on the leaves, presumed to be due to physiological imbalance caused by the chemical treatments, occurred frequently.

EMS induced high rates of seedling chlorophyll mutations in the second generation. Maximum rates observed were 1.30 mutations per M_1 plant and .40 mutations per M_1 spike. Adenine and Thymine alone doubled the spontaneous rate but the mutation rate induced was still extremely low compared to EMS induction. The combination treatment of EMS and Adenine and Thymine resulted in an increase in the mutation rate.

The mutation spectrum induced by EMS contained a relatively low percentage of albino mutants and therefore a relatively high percentage of the mutation types that are relatively rare after irradiation treatments.

It was noted that observed mutation rates differed depending on whether the M_2 was planted in the greenhouse or in the field; the rate being higher if the plants were in the field. The frequencies of some mutant phenotypes also varied depending on the environment.

Three methods of determining the mutation rates were used and

compared. These were; number of mutations per M_1 plant, number of mutations per M_1 spike, and number of mutations per 1000 M_2 seedlings. It was concluded that the second method, number of mutations per M_1 spike, was the most accurate and practical.

Several spike mutations were noted in the mature M_2. The most striking mutation was the 'rounded and crowded' spike.

A high percentage of the M_1 and M_2 plants was partially sterile. There is some reason to believe that most of this sterility is genetic and not due to gross chromosome abnormalities.

A small M_3 population from siblings of mutant M_2 segregated for the mutant phenotypes in good agreement with expected values on a 3:1 ratio basis. This, plus the fact that after EMS treatments few abberations were found in first division M_2 pollen mother cells, or in Vicia root tips, tend to indicate that the main mutagenic action of EMS is not through chromosome breakage, but by chemical changes in the chromosomes at the molecular level.

BIBLIOGRAPHY

Arnason, T. J. and Wakonig, R. 1959. The mutagenic effects of 2:4:6 Tri (Ethyleneimino) - 1:3:5 Triazine (TEM) on plants. Can. J. of Gen. and Cytol. 1:15-20.

Auerbach, C., Robson, J. M. 1946. Chemical production of mutations. Nature 157:302.

Auerbach, C., Robson, J. M., and Carr, J. G. 1947. The chemical production of mutations. Science 105:(2723), 243-247.

Auerbach, C. 1951. Problems in chemical mutagenesis. Symp. on Quant. Biol. 16:199-213.

Auerbach, C. 1957. Genetical effects of radiation and chemicals. Experientia. 13:217-224.

Auerbach, C. 1958. Mutagenic effects of alkylating agents. Ann. of the N. Y. Acad. Sci. 68:(3)731-736.

Darlington, C. D. and Koller, P. C. 1947. The chemical breakage of chromosomes. Heredity 1:187-221.

Demerec, M. 1954. Genetic action of mutagens. Caryologia Suppl. 6:201-217.

Demerec, M., and Hanson, J. 1951. Mutagenic action of manganous Chloride. Symp. on Quant. Biol. 16:215-228.

Ehrenberg, L., Gustafsson, Å., and Lundqvist, U. 1958. The mutagenic effects of ionizing radiations and reactive ethylene derivatives in barley. Hereditas 45:(2-3)351-368.

Ehrenberg, L., and Gustafsson, Å. 1957. On the mutagenic action of ethylene oxide the diepoxybutane in barley. Hereditas.43:595-602.

Fahmy, O. G. and Bird, M. J. 1952. Chromosome breaks among recessive lethals induced by chemical mutagens in Drosophila melanogaster. Supp. Heredity 6:149-159.

Fahmy, O. G., and Fahmy, M. J. 1955a. Cytogenetic analysis of the actions of carcinogens and tumor inhibitors in Drosophila melanogaster. III. Chromosome structural changes induced by 2:4:6 Tri (Ethyleneimino) 1:3:5 Triazine, J. of Genetics 53:181-199.

Fahmy, O. G., and Fahmy, M. J. 1955b. Cytogenetic analysis of the actions of carcinogens and tumor inhibitors in Drosophila melanogaster. IV. The cell stage during spermatogenisis and the induction of inter genetic mutations by 2:4:6 Tri (Ethyleneimino) 1:3:5 Triazine. J. of Genetics 53:563-584.

Fahmy, O. G., and Fahmy, M. J. 1956. Differential genetic response to the alkylating mutagens and X-radiation. J. of Genetics 53:146-164.

Fahmy, O. G. and Fahmy, M. J. 1959. Differential gene response to mutagens in Drosophila melanogaster. Genetics 44:(6)2,1149-1171.

Gaul, H. 1960. Critical analysis of the methods for determining the mutation frequency after seed treatment with mutagens. Genetica Agraria 12:(3-4):297-318.

Gelin, O., Ehrenberg, L, and Blixt, S. 1959. Quantitative studies of induced mutations in peas. II. Mutagenic effect of oxygen. Agri Hortique Genetica 17(3-4)265-273.

Goldacre, R. J., Loveless, A. and Ross, W. C. 1949. Mode of production of chromosome abnormalities by the nitrogen mustard. Nature, London, 163, 667-669.

Gray, L. H. 1953. Characteristics of chromosome breakage by different agents. Suppl. Heredity 6:311-314.

Gustafsson, Å. 1940. The mutation system of the chlorophyll apparatus. Lunds Univ. Arsskr. N. F. Aud 2:36, 1-40.

Hadorn, E. and Niggli, H. 1946. Mutations in Drosophila after chemical treatment of gonads 'in vitro'. Nature, Lond. 157:162-163.

Heiner, R. E., Konzak, G. F., Nilan, R. A. and Legault, R. R. 1960. Diverse ratios of mutations to chromosome aberrations in barley treated with diethyl sulfate and gamma rays. Proc. of Nat. Acad. Sci. Wash. 46(9)1215-1221.

Heslot, H., and Ferrary, R. 1958. Action génétique comparée des radiations et de quelques mutagènes sur l'Orge. Annales des l'institut National Agronomique 43-45:135-151.

Heslot, H., Ferrary, R., Levy, R. and Monard, C. 1959. Recherches sur les substances mutagènes (halogeno-2 éthyl) amines, dérivés oxygénés du sulfure de bis- (chloro-2 éthyl) esters sulfoniques et sulfuriques. Comptes Rendus 248:729-732.

Holm, G. 1954. Chlorophyll mutations in barley. Acta Agricultural Scandinavica 4:457-471.

Jensen, K. A., Kirk, I., Kølmark, G. and Westergaard, M. 1951. Chemically induced mutations in Neurospora. Symp. of Quant. Biol. 16: 245-261.

Johansen, D. A. 1940. Plant Microtechnique. McGraw-Hill Book Co. Ltd. New York.

Kihlman, B. 1951. The permeability of the nuclear envelope and the mode of action of purine derivatives on chromosomes. Symbol. Botan. Upalienses 11:1-37.

Kølmark, G. and Westergaard, M. 1953. Further studies on chemically induced reversions at the adenine locus of Neurospora. Hereditas. 39:209-224.

Kølmark, G. 1953. Differential response to mutagens as studied by the Neurospora reverse mutation test. Hereditas 39:270-276.

Kreizinger, J. D. 1960. Diepoxybutane as a chemical mutagen in Zea mays. Genetics 45:143-154.

Levan, A. 1951. Chemically induced chromosome reactions in Allium cepa and Vicia faba. Symp. on Quant. Biol. 16:233-243.

Loveless, A. 1953. Chemical and biochemical problems arising from the study of chromosome breakage by alkylating agents and heterocyclic compounds. Suppl. Heredity 6:293-298.

McLeish, J. 1953. The action of maleic hydrazide in Vicia. Suppl. Heredity 6:125-147.

Merz, T., Swanson, C. P. and Cohn, N. S. 1961. Interaction of chromatid breaks produced by X-rays and radiomimetic compounds. Science 133:703-705.

Moutschen-Dahmen, M., Moutschen, J. and Ehrenberg, L. 1959. Chromosome disturbances and mutation produced in plant seeds by oxygen and high pressure. Hereditas 45(2-3):230-243.

Muller, H. J. 1927. Artificial transmutation of the gene. Science 66:84-87.

Oehlkers, F. 1953. Chromosome breaks influenced by chemicals. Suppl. Heredity 6:95-105.

Prakken, P. 1959. Induced mutations. Euphytica 8:(3)270-322.

Price, C. 1958. Fundamental mechanisms of alkylation. Ann. of N. Y. Acad. Sci. 68(3):663-668.

Rapoport, J. A. 1946. Carbonyl compounds and the chemical mechanism of mutation. C. R. (Doklady) Acad. Sci. URSS 54:65-67. (Russian with English summary).

Rapoport, J. A. 1948(a). The action of ethylene oxide, glycine and glycolonides on gene mutation. C. R. (Doklady) Acad. Sci. URSS 60:469-472.

Revell, S. H. 1953. Chromosome breakage by radiomimetic substances in Vicia. Suppl. Heredity 6:107-124.

Ross, W. C. J. 1958. In vitro reactions of biological alkylating agents. Ann. of the N. Y. Acad. Sci. 68:(3)669-681.

Stacey, K. A., Cobb, M., Cousens, S. and Alexander, P. 1958. The reactions of the radiomimetic alkylating agents with macromolecules in vitro. Ann. of N. Y. Acad. Sci. 68:(3)682-701.

Stadler, L. J. 1928. Mutations in barley induced by X-rays and radium. Science 68:186-187.

Thoday, J. M. 1953. Sister-union isolocus breaks in irradiated Vicia faba: the target theory and physiological variation. Suppl. Heredity 6:299-310.

Vogt, M. 1948. Mutationsauslösung bei Drosophila durch Athylurethan. Experientia 4:68-69.

Wakonig, T. and Arnason, T. J. 1958. Some effects of the chelating agent EDTA on plant chromosomes. Proc. of Gen. Soc. of Canada. 3:(2)37-40.

Westergaard, M. 1957. Chemical mutagenesis in relation to the concept of the gene. Experientia 13:224-234.