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Interaction between Chemical Mutagens with a Delayed Effect and Metabolites of Seeds

Communication 2: Changes in mutagenic activity

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Summary. A study was made of chromosome aberrations in *Crepis capillaris* seedlings, induced by the reaction products of chemical mutagens with seed metabolites. Interaction between ethylenimine and seed metabolites of some plants of the family Compositae (*C. capillaris, Taraxacum officinale, Pyrethrum carneum, Helianthus annuus*) has been found to lead to the formation of highly active secondary mutagens whose action remains similar to that of ethylenimine, although the effect of ethylenimine is enhanced dozens of times. The substances responsible for this „enhancement effect" are contained in the fruit coating of the seed. The metabolites of seeds of other plants studied (*Triticum vulgare, Hordeum vulgare, Fagopyrum esculentum*) enhanced the effect of ethylenimine only 1.5–2.0 times. Unlike ethylenimine, the effect of its derivatives (thioTEP and phosphazine) and of ethyl methanesulphonate, HN2 and maleic hydrazide is not enhanced after their interaction with metabolites of compositae plant seeds. Experiments with HN2 revealed an almost complete inactivation of the mutagenic action of NH2 by metabolites of *C. capillaris* seeds. The observed modification of the mutagenic action of ethylenimine and NH2 after successive treatment of seedlings with mutagens and metabolites of seeds points to the preservation of the mutagen in the cell. It is concluded that when chemical mutagens act on the cells, chromosome aberrations are induced not only by the chemical agent itself, but also by its reaction with cell metabolites.

Introduction

It is known that the effect of chemical mutagens can be modified by a variety of chemical substances. For example, the effect of ethyl methanesulphonate (EMS) was enhanced by adding microquantities of copper and zinc salts before or during treatment of *Vicia faba* seeds with the mutagen (Moutschen-Dahmen and Moutschen-Dahmen, 1963). The addition of calcium, magnesium, sodium and iron ions was ineffective. Similar results were obtained when studying the effect of EMS on wheat (Bari, 1963) and *Crepis capillaris* (Grinikh, 1969). The effect of EMS on barley was enhanced by mercury and arsenic ions (Moutschen and Degraeve, 1965 a, b). The mutagenic activity of ethylenimine, another alkylating mutagen, was increased *in vitro*, following its reaction with thymine, thiamine and uracil. Other nitrogenous bases (adenine, guanine, cytosine), some amino acids (histidine, glycine), vitamins (nicotinic acid) and urotropin produced no such effect. In addition to ethylenimine, thioTEP was tested and its action was also intensified by thiamine (Sidorov, Sokolov and Andreyev, 1966). The effect of embichine (HN_2), phosphazine and maleic hydrazide was modified using an extract from *V. faba* seed hull and cysteine (Popa, 1969).

Earlier we found that the mutagenic effect of ethylenimine can be enhanced by its preliminary reaction with metabolites of *C. capillaris* seeds (Protopopova, Shevchenko and Grigoriyeva, 1969). The effect of ethylenimine increased 2.5 times after interaction with thymine and thiamine (Sidorov, Sokolov and Andreyev, 1966), but in our experiments this effect was enhanced dozens of times. The present investigation was designed to study this phenomenon further. An attempt was made to answer the following questions:

1. Does the spectrum of chromosome aberrations change when the ethylenimine effect is enhanced by its interaction with the substances from the seeds?
2. What is the "life span" of the substances isolated from the seeds and of the products of their reaction with ethylenimine?
3. How specific is the action of the substances from *C. capillaris* seeds and can the metabolites of seeds of other plants enhance the effect of ethylenimine?
4. Can the effect of other chemical mutagens be enhanced by seed metabolites?
5. Is the greater effectiveness of ethlyenimine related to *in vitro* formation of highly active secondary mutagens?

Material and Methods

Interaction between the mutagens and the substances separated from seeds and chromosomal lesions were studied as described in the previous communication (Protopopova, Shevchenko and Grigoryeva, 1971). The metabolites of seeds of the following plants were examined: crepis (*C. capillaris*), dandelion (*Taraxacum officinale*), pyrethrum (*Pyrethrum carneum*), sunflower (*Helianthus annuus*), buckwheat (*Fagopyrum esculentum*) wheat (*Triti-*

cum vulgare) and barley (*Hordeum vulgare*). The seeds of all the plants had been in storage for up to one year. The experiments attempted to modify the effect of the following chemical mutagens: ethylenimine (concentration $2.3 \cdot 10^{-2}$M, treatment period 30 min.), thioTEP ($1.2 \cdot 10^{-3}$M, 30—60 min.), phosphazine ($1 \cdot 10^{-3}$M, 60 min.). EMS ($1 \cdot 10^{-1}$M, 30—45 min.), maleic hydrazide ($5 \cdot 10^{-4}$ M, 60 min.), HN2($1 \cdot 10^{-5}$M, $5 \cdot 10^{-6}$M, $1 \cdot 10^{-6}$M, 60 min.). Solutions of the mutagens were prepared in a phosphate buffer with pH 7.0. The preliminary reaction of the mutagen with seed metabolites was carried out for 30 min.

Results and Discussion

As found earlier, the mutagenic effect of ethylenimine is considerably enhanced following its interaction with substances isolated from *C. capillaris*. It was of interest to see whether the specificity of the ethylenimine effect was retained in this case. It turned out that despite a substantial increase in the number of aberrations, their spectrum did not change (Table 1). As with ethylenimine treatment, mutagenesis was delayed, i.e. all the aberrations were of the chromatid type and appeared a few hours after treatment (Table 2). It should be noted that the location of breakages was also that typically produced by ethylenimine (Shevchenko, 1964): they were frequently seen in the proximal area of the D-chromosome and in the short arms of A- and C-chromosomes. Hence, the effect of the reaction products of ethylenimine with the metabolites of *C. capillaris* seeds proved to be similar to that of pure ethylenimine.

The next important point was to find out the „stability" of the substances isolated from seeds and of the products of their reaction with ethylenimine. It was observed (Protopopova, Shevchenko and Grigoriyeva, 1969) that the enhancement effect attained its maximum when ethylenimine interacted with metabolites isolated from freshly-collected *C. capillaris* seeds, and that it diminished with storage of the

Table 1. *Aberration spectrum after treatment with reaction products of ethylenimine with metabolites of C. capillaris seeds*

Types of aberrations Treatment	Ethylenimine		Metabolites of seeds + ethylenimine	
	Number	%	Number	%
Chromatid deletions	2	2.5 ± 1.7	18	2.2 ± 0.5
Isochromatid deletions with union of ends	20	25.0 ± 4.8	184	22.1 ± 1.4
Isochromatid deletions with ends non-union	0	0	5	0.6 ± 0.3
Chromatid translocations	20	25.0 ± 4.8	207	25.0 ± 1.5
Duplications-deletions	8	10.0 ± 3.4	72	8.7 ± 0.9
Single minutes and rings	28	35.0 ± 5.3	334	40.0 ± 1.7
Aberrations of chromosome type*	2	2.5 ± 1.8	1	0.1 ± 0.1
Total number of aberrations	80	100	832	100
Aberrations per 100 cells		3.9		64.5

* In the control (buffer), seedlings displayed 0,3% of chromatid and 0.3% of chromosome aberrations.

Table 2. *Mutagenic activity of metabolites of C. capillaris seeds and products of their reaction with ethylenimine*

Treatment	Time of fixations (hrs.)	No. of metaphases inspected	Metaphases with aberrations		No. of aberrations per 100 cells
			No.	%	
Metabolites of seeds + ethylenimine	9	577	8	1.4 ± 0.4	1.7
	15	570	116	20.4 ± 1.7	23.3
	20	500	200	40.0 ± 2.2	55.0
Ethylenimine (half an hour after preparation)	9	505	4	0.8 ± 0.4	0.8
	15	777	24	3.1 ± 0.6	3.6
	20	536	13	2.4 ± 0.7	2.4
Metabolites of seeds + ethylenimine	9	508	2	0.4 ± 0.3	0.4
	15	677	32	4.7 ± 0.8	4.9
	20	518	43	8.3 ± 1.3	9.8
Ethylenimine (20 hours after preparation)	9	511	2	0.4 ± 0.3	0.5
	15	509	9	1.8 ± 0.6	1.8
	20	728	38	5.3 ± 0.8	5.3
Metabolites of seeds + buffer	9				
	15	1086	6	0.6 ± 0.2	0.6
	20				
Supernatant + ethylenimine	9	553	11	2.0 ± 0.6	2.0
	15	567	161	28.3 ± 1.0	40.3
	20	383	163	42.5 ± 2.5	70.3
Supernatant + buffer	9				
	15	1082	6	0.6 ± 0.2	0.6
	20				

Table 3. *Mutagenic activity of reaction products of ethylenimine with seed metabolites from plants of different families*

Family	Genus and species of the plant	Mutagenic activity
Compositae	1. *Crepis capillaris* 2. *Taraxacum officinale* 3. *Pyrethrum carneum* 4. *Helianthus annuus*	Effect of reaction products is dozens of times greater than that of ethylenimine
Gramineae	1. *Triticum vulgare* 2. *Hordeum vulgare*	Effect of reaction products is 1.5—2 times greater than that of ethylenimine
Polygonaceae	1. *Fagopyrum esculentum*	

seeds. Therefore, the substances responsible for this effect have disappeared from the seeds during or after storage. To find out how long these substances could persist in aqueous solution, ground seeds (collected three months before) were flooded with a phosphate buffer, ethylenimine was added 20 hours later and the resulting mixture was kept for 30 minutes more, after which it was used to treat the seedlings (Treatment 1, Table 2). It was found that even after 20 hours in solution the seed metabolites preserved the ability to enhance the mutagenic action of ethylenimine. It is worth noting that the ethylenimine effect was also enhanced when using the supernatant obtained by centrifuging a ground mass of seeds flooded with a buffer (Table 2). If the ground seeds were mixed at once with ethylenimine, the mixture lost its high activity in 20 hours (Treatment 2, Table 2). Hence the substances responsible for the enhancement effect are sufficiently stable, but their reaction products with ethylenimine break down quite rapidly. (This does not, however, exclude the possibility that because a considerable quantity of different substances were present in the extract, the active products were used up during interaction with them.)

To resolve the question of whether the substances under investigation are specific only for *C. capillaris* seeds or whether they are also contained in seeds of other plants, experiments were performed whose results are summarized in Table 3. Analysis of the action of aqueous extracts from seeds of the plants tested showed that they had no mutagenic effect, except for the barley seed extract which induced 4—8% of aberrations. The ethylenimine effect was considerably enhanced only by metabolites of seeds of plants belonging to the same family, Compositae, as *C. capillaris* (Table 3). An attempt was made to find out which part of the seed contained the substances responsible for the enhancement effect of ethylenimine. Sunflower seeds were separated into the kernel and the fruit coating, and the ethylenimine was mixed with each fraction separately. It was found that the bulk of these substances was contained in the fruit coating of the seed, though a small amount was also found in the kernel. Unlike the Compositae family seeds, the metabolites of *Gramineae* seeds (barley, wheat) and buckwheat enhanced the ethylenimine effect only slightly (by an average of 1.5 times). This means that the substances activating the ethylenimine effect are present in the seeds of all the plants investigated. This could be expected because *in vitro* experiments (Sidorov, Sokolov and Andreyev, 1966) have shown that the ethylenimine effect is enhanced within the same

Table 4. *Interaction between HN2 and metabolites of C. capillaris seeds*

Treatment	Fixation time (hours)	No. of metaphases examined	Metaphases with aberrations		No. of aberrations per 100 cells
			No.	%	
Seed metabolites	9	549	2	0.4 ± 0.3	0.4
+ HN2 (10^{-6}M)	15	325	5	1.5 ± 0.7	1.5
	20	520	4	0.8 ± 0.4	0.8
HN2 (10^{-6}M)	9	471	2	0.4 ± 0.3	0.4
	15	542	17	3.1 ± 0.7	3.1
	20	507	15	3.0 ± 0.7	3.0
Seed metabolites	9	536	1	0.2 ± 0.2	0.2
+ HN2 ($5 \cdot 10^{-6}$M)	15	453	10	2.2 ± 0.7	2.2
	20	480	5	1.0 ± 0.5	1.0
HN2 ($5 \cdot 10^{-6}$M)	9	506	13	2.6 ± 0.7	2.5
	15	361	158	43.9 ± 2.6	64.7
	20	557	303	54.4 ± 2.1	96.0
Seed metabolites	9	533	23	4.3 ± 0.9	4.5
+ HN2 (10^{-5}M)	15	478	5	1.1 ± 0.5	1.1
	20	401	2	0.5 ± 0.4	0.5
HN2 (10^{-5}M)	9	379	197	52.0 ± 2.6	87.4
	15	366	225	61.5 ± 2.5	105.7
	20	177	127	71.7 ± 3.4	138.0

range (2.0—2.5 times) by treatment with thymine and uracil, which are always present in the cell. It appears, however, that the fruit coating of the seeds of the Compositae family contains some special substances which are directly responsible for the multiple enhancement of the ethylenimine effect.

To find out whether the effect of other mutagens is increased by reaction with metabolites of seeds of the Compositae family, some alkylating compounds (two derivatives of ethylenimine: thioTEP and phosphazine and also EMS, HN2) and maleic hydrazide were tested. Unlike ethylenimine, the tested mutagens did not form any active compounds with substances from *C. capillaris* and *P. carneum* seeds. Ethylenimine proved to be the only mutagen from those tested whose effect was enhanced by metabolites of seeds of a group of plants.

An unexpected result was obtained in experiments with HN2. Interaction between HN2 and the substance from *C. capillaris* seeds led to an almost complete inactivation of its mutagenic action (Table 4). This phenomenon was observed at different concentrations of the mutagen. One cannot, however, feel confident that the same substances which intensify the mutagenic action of ethylenimine are responsible for the inactivating effect on NH2. It follows from our experiments that the effect of chemical mutagens can be both strengthened or weakened by preliminary interaction of the mutagen with cellular metabolites which do not possess any mutagenic capacity per se.

In all the above experiments the seedlings were treated with the products of interaction between the mutagen and seed metabolites obtained *in vitro*. Special experiments were carried out to make sure that a more active mutagenic compound is formed as a result of mutagen reaction with seed extract or that, on the contrary, inactivation of the mutagen occurs. We tried to ascertain whether the action of the extract changes the permeability of the cell membranes or chromosome sensitivity to the mutagen. *C. capillaris* seedlings were first treated for 30 minutes with an aqueous extract from pyrethrum seeds, then, after rinsing for 15 minutes they were treated with ethylenimine (Fig. 1-a). In this case the mutagenic effect of ethylenimine did not change. This means that preliminary treatment of the seedlings by the extract increases neither the ability of ethylenimine to penetrate the cell nor chromosome sensitivity to ethylenimine. A different sequence of treatment (first ethylenimine followed, after rinsing, by an aqueous extract) (Fig. 1-c) resulted in an increased ethylenimine effect, though not so great as in the case of treatment with reaction products obtained *in vitro* (Fig. 1-b). This modification of the effect may be accounted for by the preservation of the mutagen in the cell. The ability of the mutagen to persist in the cell is also shown by experiments with HN2 (Fig. 1-e). Although the reaction of HN2 with substances from

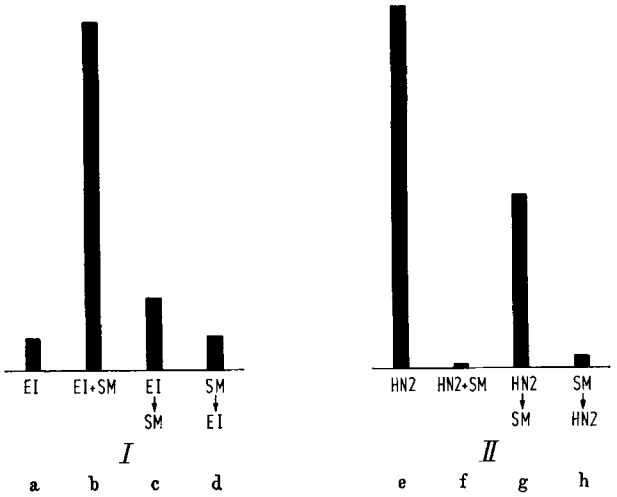

Fig. 1. Dependence of the magnitude of the mutagenic effect in case of successive action on seedlings by mutagen and metabolites of *Pyrethrum carneum* (I) and *Crepis capillaris* (II) seeds. — (*I*) The effect is expressed as percentage of the effect in case of the ethylenimine + seed metabolites treatment taken as 100%. — (*II*) The effect is expressed as percentage of the effect of the HN2 treatment taken as 100%

seeds *in vitro* (Fig. 1-f) led to nearly complete disappearance, treatment of the seedlings first with HN2 and then with an extract (Fig. 1-g) resulted in the effect being only halved (the sum of three fixations). As in the experiment with ethylenimine, it is evident that the modification concerned that part of the mutagen which did not have enough time to react in the cell, or which formed new compounds capable of reacting with seed metabolites which gained access into the cells later.

It should be noted that when the sequence of treatment was reversed (first the extract and then the mutagen) (Fig. 1-h), the effect of ethylenimine did not change, but that of HN2 was almost completely eliminated. The lack of increase of the ethylenimine effect may be explained by the fact that, unlike HN2, ethylenimine reacts faster with the cell substances than with the exogenous seed metabolites.

Our results suggest that interaction between ethylenimine and the metabolites of the seeds of some plants of the Compositae family leads to the formation of some secondary highly active mutagens whose mechanism of action on the chromosomes seems to be similar to that of ethylenimine. Such interaction occurs both *in vitro* (preliminary reaction between ethylenimine and seed extract) and *in vivo* (treatment of seedlings first with ethylenimine and then with extract). The modification of the mutagenic action of ethylenimine observed *in vivo* testifies to the preservation of the mutagen in the cell. The same conclusion was reached by earlier researchers using ethylenimine (Sidorov, Sokolov and Andreyev, 1965; Andreyev, Sidorov and Sokolov, 1966). It may be concluded that when ethylenimine is used, the lesion is not solely the result of direct action of ethy-

lenimine on the chromosomes; an important part is also played by the reaction products of ethylenimine with cell metabolites.

It is also important that, in contrast with ethylenimine, no active secondary mutagens were formed in experiments with its derivatives (thioTEP and phosphazine). This may be due to the peculiarities of their structure: both substances are N-substituted derivatives of ethylenimine. Possibly, the reaction leading to the formation of a secondary active mutagenic compound occurs precisely along the NH bond. The absence of an enhancement effect on the action of other mutagens (EMS, HN2, maleic hydrazide) also points to the specificity of the above reaction. Moreover, the effect of ethylenimine considerably enhanced was only when metabolites of seeds of some plants of the family Compositae were used, and it is clear from experiments with sunflowers that the substances from the fruit coating of the seed exerted a much stronger effect than did those from the kernel.

It would be of great interest to elucidate the nature of the substances enhancing the mutagenic action of ethylenimine and the reactions leading to the formation of highly active secondary mutagens. The data obtained here suggest ways for further study of these points.

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