FACTORS AFFECTING THE PRODUCTION OF CHROMOSOME ABERRATIONS BY CHEMICALS*

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A number of chemical compounds, such as the sulfur and nitrogen mustards (1, 2), diepoxides (3, 4), maleic hydrazide (5, 6) and purine derivatives (7, 8), which differ appreciably in structure and reactivity, are known to be capable of inducing alterations in chromosome structure similar to those resulting from x-radiation. In no single instance is there complete knowledge of the particular chain of events which lead to chromosomal damage, although it is clearly recognized that radiation damage can be related to physical, radiochemical, and chemical events taking place in the treated cells, and that the degree of damage can be amplified or reduced by altering the environmental conditions of the cell. Thus, Sax and Enzmann (9) and Thoday and Read (10) have shown that the temperature and oxygen tension prevailing at the time of exposure determine, at least in part, the frequency of chromosomal aberrations resulting from a given dose of x-rays. The influence of such factors on the effects of radiomimetic chemicals has been but rarely studied, however, even though circumstances would suggest that this would be a profitable area of investigation. This is indicated by the studies carried out on the cytological effects of a purine derivative, 8-ethoxycaffeine (11), in which it appeared that its radiomimetic action is more readily influenced by changes in the environment than is that of the ionizing radiations. The hope is entertained, therefore, that a knowledge of the influence of modifying factors on the effectiveness of chemical agents representative of several groups of radiomimetic compounds would provide a clue as to their mode of action within the cell.

The theoretical and practical importances of such information are apparent, and the studies to be reported were carried out with these in mind. Because the influence of modifying factors on the effectiveness of 8-ethoxycaffeine has been studied in the past (8, 11, 12), comparisons were made in all experiments between this chemical and others of similar radiomimetic action.

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Materials and Methods

The experimental materials used in these experiments were the lateral roots of an English variety "Seville Longpod" of the broad bean, *Vicia faba*. The roots were grown in tap water in the dark at room temperature (23–27°C.). One day before the treatments, the beans were transferred to an incubator and kept at 17°C. Treatment consisted of immersing the lateral roots in the solutions. After treatment the beans were transferred to water and kept in water during the recovery period at a temperature of 17°C.

The radiomimetic chemicals used in this study were maleic hydrazide at a concentration of 2×10^{-4} M, di-(2, 3-epoxypropyl) ether[1] at a concentration of 2×10^{-4} M, and 8-ethoxycaffeine at a concentration of 10^{-2} M. The last was dissolved in distilled water, the other two in a M/150 solution of sodium phosphate, pH 6. The periods of treatment were, as a rule, 2 hours. The anaerobic treatments were carried out as described in a previous paper (12). Before fixation, the roots were treated with a 0.05 per cent colchicine solution for 3 hours and were then fixed in cold alcohol-acetic acid, 3:1, after which they were prepared as Feulgen squashes and made permanent according to the dry ice method (13). Only isolocus breaks (14) and chromatid exchanges were scored.

The following abbreviations will be used: maleic hydrazide = MH; di-(2, 3-epoxypropyl) ether = DEPE; 8-ethoxycaffeine = EOC, and 2, 4-dinitrophenol = DNP.

RESULTS

1. Temperature

The effects of MH and DEPE in *Vicia* have been studied extensively by McLeish (5, 6, 15) and Revell (4). McLeish (6) observed that when the beans were kept at 7°C. during treatment and recovery, the effect of MH was delayed and diminished as compared to beans which were kept at 20°C. No studies previous to those to be reported have dealt with the influence of temperature during treatment with DEPE.

In the present study treatments of the bean roots with MH and treatments with DEPE, both at concentrations of 2×10^{-4} M, with the pH of the treatment solutions adjusted to 6, were executed at temperatures of 3, 12, 17, and 25°C. for 2 hours. As in all the experiments, the beans were kept at 17°C. 24 hours before treatment and during the recovery period.

In Text-fig. 1 the frequencies of isolocus breaks are plotted against the recovery period. In the MH and the DEPE treated roots there is a sharp rise in the frequency of aberrations correlated with each rise in temperature. The inhibition of mitosis induced by MH also increases with increasing temperature, a fact which explains why the appearance of the aberrations is delayed in the 25°C. treatment.

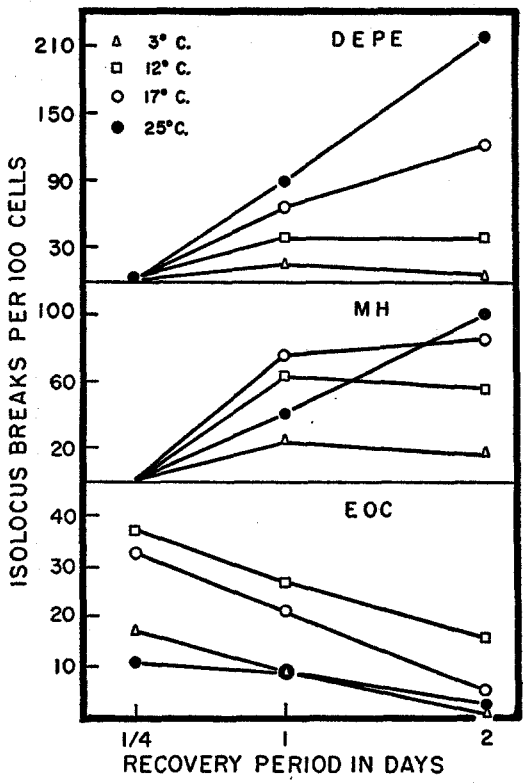
The frequency of aberrations in the root tips treated with EOC (Text-fig. 1), increases with rising temperature from 3° to 12°C. but then begins to decrease, so that at 25°C. the effect is approximately the same magnitude as that at 3°C. In these experiments, the EOC concentration was 10^{-2} M, and the period of treatment 2 hours.

In all three cases the production of chromosome aberrations by chemical

[1] I should like to thank Dr. S. H. Revell for his gift of a sample of the diepoxide.

treatment is affected by temperature, but the influence of temperature on the effect of EOC differs from that of the other two chemicals.

Another difference between the effect of EOC and the effect of MH and DEPE is also apparent from Text-fig. 1, *viz.*, the difference in interphase sensitivity. As has been shown by Revell (4) and McLeish (6), chromosome aberrations are obtained after treatments with DEPE and MH only in those X_1 cells



TEXT-FIG. 1. The influence of treatment temperature on the frequencies of isolocus breaks obtained at various times after treatments with di-(2,3-epoxypropyl)ether (DEPE), maleic hydrazide (MH), and 8-ethoxycaffeine (EOC).

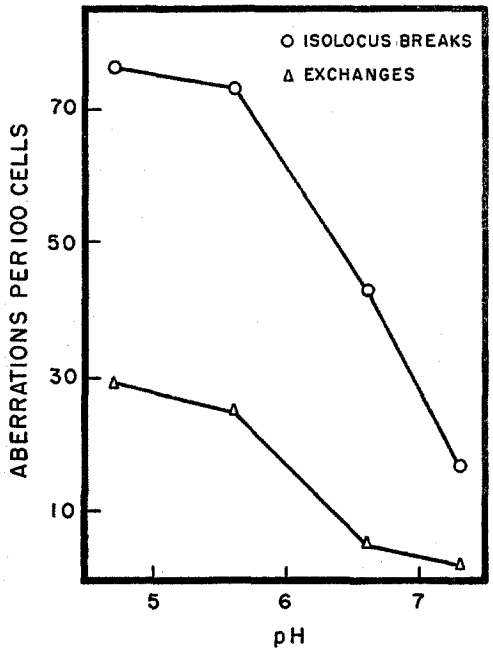
which were in early interphase at the time of treatment. Kihlman (11) has found that EOC, like x-rays, is most effective in late interphase or early prophase. Accordingly, 6 hours after the MH and DEPE treatments (Text-fig. 1) no aberrations were obtained, whereas the highest frequency of aberrations was obtained at this time after treatments with EOC.

2. Hydrogen Ion Concentration

In the experiments with MH and DEPE, the effect of hydrogen ion concentration was tested within a pH range of from 4.7 to 7.3. The pH of the treat-

ment solutions was adjusted with sodium phosphate buffer solution. This buffer was not used in the case of EOC, since the EOC-buffer mixture proved to be very toxic. In the EOC experiments, therefore, the pH of the solutions was adjusted with a few drops of M/10 sodium hydroxide or M/10 hydrochloric acid to include the same pH-range as that in the MH and DEPE experiments.

Variations in the pH of the treatment solutions within the pH-range mentioned did not appreciably change the frequencies of chromosome aberrations resulting from either the EOC or the DEPE treatments. In contrast, the



TEXT-FIG. 2. The influence of pH of the treatment solution on the effect of 2 hour treatments with 2×10^{-4}M maleic hydrazide at 12°C.

effect of MH was much reduced when the pH was increased from 4.7 to 7.3. As is shown in Text-fig. 2, MH produces only one-fourth as many isolocus breaks at pH 7.3 as at pH 4.7, and only one-seventh as many exchanges. Also, at the higher pH values, the frequency of cell division was less inhibited by the MH treatment. The data on which the graph is based were obtained from roots fixed 24 hours after treatment. Similar values were obtained when roots were fixed after a recovery period of 48 hours.

3. Oxygen Tension

From a previous study (12) on the effect of oxygen tension on the production of chromosome aberrations by EOC, the following facts have been

established. The effect of EOC is almost completely inhibited when (1) oxygen is excluded from the solution during the treatment, (2) respiration is inhibited by azide or cyanide, and (3) phosphorylation is uncoupled from respiration by DNP. From these facts it was concluded that respiration energy in the form of energy-rich phosphate is involved in the production of chromosome aberrations by EOC, and that oxygen is necessary for the EOC effect because it greatly enhances the formation of energy-rich phosphate.

In connection with these experiments, it was also found that the effect of nitrogen mustard, di-(2-chloroethyl)methylamine hydrochloride, was independent of the oxygen tension during treatment. Like nitrogen mustard, DEPE is an alkylating agent (16) and the cytological effects produced by it in *Vicia* are remarkably similar to those produced by nitrogen mustard. Thus, for instance, interphase sensitivity to breakage by nitrogen mustard and DEPE seems to be identical as is the distribution of breaks (3, 4). It may be mentioned that whereas breaks produced by DEPE and nitrogen mustard are mainly concentrated in heterochromatic knobs in the short chromosomes (2, 3), those produced by MH or EOC have a quite different localization. The MH produced breaks tend to be localized close to the centromere in a heterochromatic segment of the nucleolar arm of the long chromosome (5, 6). After EOC treatments of cells at early or middle interphase most breaks are found in the nucleolar constriction (12).

Since nitrogen mustard and DEPE are so similar in respect to their chemical properties and cytological effects, an oxygen effect in the case of DEPE was not to be expected. As appears from Table I, the frequencies of isolocus breaks and exchanges obtained after DEPE treatments in nitrogen are not different from those obtained after similar treatments in air. In the case of MH, on the other hand, the effect is significantly reduced when the treatments are performed in the absence of oxygen (Table I). In this respect, MH is similar to EOC and not to DEPE or nitrogen mustard. The reduction in frequency of aberrations, although great, is not as complete as in the case of EOC, however.

Table II shows that the effect of MH, like that of EOC, is also reduced when, during the treatment, respiration is inhibited by azide or phosphorylation uncoupled by DNP. Therefore, the explanation of the oxygen effect in the case of MH is probably the same as that of EOC.

Tables III and IV show the effects of pre- and posttreatments with DNP on the production of isolocus breaks and exchanges by EOC, MH, and DEPE. Pretreatments with DNP for two hours (Table III) reduced the frequencies of aberrations produced by EOC and MH and, in fact, the reduction was as great as that obtained when DNP is added to the treatment solutions after ½ hour pretreatment with the uncoupling agent. Posttreatments with DNP (Table IV) do not change the magnitude of the effect produced by EOC. In the case of MH, the initial reduction found at 24 hours is very probably

TABLE I

The Influence of Oxygen on the Effects of 10^{-2} M 8-Ethoxycaffeine (EOC), 2×10^{-4} M Maleic Hydrazide (MH), and 2×10^{-4} M Di-(2,3-epoxypropyl)Ether (DEPE)

Mutagen	Period of treatment	Treatment temperature	pH of treatment solution	Treatment atmosphere	Recovery period	No. of metaphases analyzed	Abnormal metaphases	Aberrations per 100 cells	
								Isolocus breaks	Exchanges
	hrs.	°C.			*hrs.*		*per cent*		
EOC	1¾	20	6	Air	5½	200	64.5	14.5	94.5
"	1¾	20	6	Nitrogen	5½	400	6	1.8	2.8
"	4	10	6	Air	19	300	62	71.6	17
"	4	10	6	Nitrogen	19	300	4	2.6	0.8
MH	2	12	6	Air	24	300	66.3	66.8	12.8
"	2	12	6	Nitrogen	24	300	20.3	18.3	2.8
"	2	12	6	Air	48	200	38	30	11.5
"	2	12	6	Nitrogen	48	200	19	14	3.5
DEPE	2	12	6	Air	24	200	44	44	7.5
"	2	12	6	Nitrogen	24	200	57	44	17
"	2	12	6	Air	48	200	65	54	32.5
"	2	12	6	Nitrogen	48	200	65	68	19

TABLE II

The Influence of Sodium Azide and 2,4-Dinitrophenol (DNP) on the Effect of Maleic Hydrazide (2×10^{-4} M, 12°C., pH 6, 2 Hours)

Pretreatment	Chemical added to treatment solution		Recovery period	No. of metaphases analyzed	Abnormal metaphases	Aberrations per 100 cells	
	Formula abbreviation	Concentration				Isolocus breaks	Exchanges
		M	*hrs.*		*per cent*		
—	—	—	24	100	77	98	22
—	NaN_3	4×10^{-4}	24	100	3	3	0
—	—	—	48	200	59	55.5	19.5
—	NaN_3	4×10^{-4}	48	200	14.5	8	6
—	—	—	24	100	64	64	16
2×10^{-4} M DNP for 30 min. at 17°C.	DNP	10^{-4}	24	100	1	1	0
—	—	—	48	100	32	32	6
2×10^{-4} M DNP for 30 min. at 17°C.	DNP	10^{-4}	48	100	4	4	0

due to the fact that the appearance of the aberrations is considerably delayed. This is indicated by a greater frequency of breaks in the MH-DNP series at 48 hours, which could be due to a compensatory effect. In the case of DEPE, pretreatments with DNP seem to result only in a delayed ap-

TABLE III

The Effect of Pretreatments with Dinitrophenol (10^{-4} M, 17°C., 2 Hours) on the Production of Chromosome Aberrations by EOC (10^{-2} M, 12°C., 2 Hours), MH (2×10^{-4} M, 12°C., pH 6, 2 Hours), and DEPE (2×10^{-4} M, 12°C., 2 Hours)

Mutagen	Pretreatment	Recovery period	No. of metaphases analyzed	Abnormal metaphases	Aberrations per 100 cells	
					Isolocus breaks	Exchanges
		hrs.		*per cent*		
EOC	—	22	200	35.5	39.5	1
"	DNP	22	170	1.2	1.2	0
MH	—	24	100	68	65	13
"	DNP	24	100	12	11	2
"	—	48	100	43	41	9
"	DNP	48	100	7	4	3
DEPE	—	24	100	34	30	6
"	DNP	24	100	18	16	2
"	—	48	100	50	46	21
"	DNP	48	100	71	67	45

TABLE IV

The Effect of Posttreatments with Dinitrophenol (10^{-4} M, 17°C., 2 Hours) on the Production of Chromosome Aberrations by EOC (10^{-2} M, 12°C., 2 Hours), MH (2×10^{-4} M, 12°C., pH 6, 2 Hours), and DEPE (2×10^{-4} M, 12°C., 2 Hours).

Mutagen	Posttreatment	Recovery period	No. of metaphases analyzed	Abnormal metaphases	Aberrations per 100 cells	
					Isolocus breaks	Exchanges
		hrs.		*per cent*		
EOC	—	24	100	42	45	5
"	DNP	24	100	36	42	2
MH	—	24	100	64	72	13
"	DNP	24	100	19	15	5
"	—	48	100	50	48	13
"	DNP	48	100	57	63	18
DEPE	—	24	100	34	23	13
"	DNP	24	100	3	3	2
"	—	48	100	83	103	70
"	DNP	48	100	62	58	27

pearance of chromosome abnormalities, as evidenced by the compensatory rise in aberrations found at 48 hours. Similar results have previously been obtained by Loveless (17) in a study of the effect of DNP pretreatments on

the production of chromosome aberrations by DEPE. However, the DEPE-DNP series (Table IV) provide data which suggest that, if a delay is involved, the delay is greater if the DNP is given as a posttreatment than as a pretreatment.

The results to be described were derived from experiments planned on the basis of Novick's recent discovery that theophylline under anaerobic conditions is inactive as a mutagen in the bacterium *Escherichia coli* (18). Novick and Szilard have been studying the mutagenic effect of methyl xanthines

TABLE V

The Effect of Guanosine (2.5×10^{-3} M), Adenosine (10^{-2} M), and Caffeine (10^{-2} M) on the Production of Chromosome Aberrations by EOC (10^{-2} M, 12°C., 2 Hours).

Fixations were made 22 hours after the treatments.

Pretreatment	Treatment	No. of metaphases analyzed	Abnormal metaphases	Aberrations per 100 cells	
				Isolocus breaks	Exchanges
			per cent		
—	EOC	100	32	36	2
2.5×10^{-3} M guanosine for 30 min. at 17°C.	EOC + guanosine	100	40	39	8
10^{-2} M adenosine for 30 min. at 17°C.	EOC + adenosine	100	7	6	0
—	EOC	200	34.5	40	2.5
—	EOC + adenosine	200	26.5	26	2
—	EOC + caffeine	200	13.5	12.5	0.5
—	Caffeine	100	3	2	1

and other purines (19) and have found that the ability of theophylline to induce mutations of *E. coli* from phage sensitivity to phage resistance is completely inhibited by the addition of adenosine, guanosine, or inosine to the treatment solution (20). Since bacteria grown anaerobically are known to contain significant amounts of adenosine, Novick concludes that the protective effect of anoxia can be understood on the basis of the presence of adenosine (18). The experiments described hereafter were done to find out if the effect of anoxia on the production of chromosome aberrations by EOC could be explained in a similar way.

The results, listed in Table V, show that the effect of 10^{-2} M EOC was not altered by the presence of 2.5×10^{-3} M guanosine. Adenosine, however, in a

concentration of 10^{-2} M, significantly reduced the effect of EOC. The antagonistic effect of adenosine was particularly pronounced when the roots were treated with adenosine not only during the EOC treatment but also for 30 minutes before the EOC treatment. Thus, both the production of chromosome aberrations in *Vicia* by the trimethylxanthine derivative EOC, and the mutagenic effect of the dimethylxanthine theophylline in *E. coli*, are reduced by anoxia as well as by the presence of adenosine.

The antagonistic effect of adenosine does not necessarily indicate that the primary action of EOC is interference with nucleoside metabolism, since the mutagen caffeine is actually a more efficient antagonist (Table V).

During the experiments it was discovered that caffeine and adenosine increase considerably the solubility of EOC in water, caffeine again being the more effective. In the presence of caffeine the solubility of EOC is nearly doubled. The fact that purine derivatives are able to increase the solubility of other compounds has been known for some time, the phenomenon having been studied by several authors, in particular Weil-Malherbe (21).

It seems very likely that the ability of adenosine to reduce the frequency of chromosome aberrations produced by EOC is a result of an interaction between the molecules similar to that responsible for the solubilizing effect. (Probably the ones involved are of the same kind as those concerned in the formation of molecular complexes.)

This conclusion, however correct in explaining the preceding phenomena, does not necessarily apply to the antagonism described by Novick. In the cytological experiments the antagonistic effect of adenosine on EOC was observed only with equimolar concentrations of adenosine and EOC. Novick is able to counteract the mutagenic effect of theophylline by very small amounts of adenosine or guanosine, a fact which is difficult to explain by the complex formation hypothesis, unless the ribosides are assumed to accumulate in the cell until they are equal in concentration to theophylline.

Although the EOC-adenosine antagonism may be due in part to a molecular interaction of the kind suggested, it does not exclude the possiblity of adenosine being involved in the protective effect of anoxia even though such an explanation seems an unlikely one. Even when equimolar concentrations are used, the protective effect of adenosine is considerably less than that of anoxia. Little is known about the intracellular concentrations of adenosine in bean roots under aerobic and anaerobic conditions, but it seems doubtful that it ever could be as high as is necessary to reduce markedly the effect of EOC.

DISCUSSION

The frequencies of chromosome aberrations resulting from treatments by EOC and DEPE were observed to be little influenced by the hydrogen ion

concentration in the treatment solutions. MH, on the other hand, is considerably less effective at neutral pH values than in acid solutions.

Previously, Naylor and Davis (22) observed an influence of pH on the effect of MH on root tip respiration of a number of plants. At pH 6 the respiration was not in general affected by MH in concentrations from 0.05 to 0.4 per cent. A marked inhibition was obtained, however, at pH 4. Naylor and Davis considered as a possibility that the hydrogen ion concentration affects the penetration of MH into the individual cells. MH is a weak acid, and it may be that it enters the cell most readily in the undissociated form. The explanation of the pH effect by Naylor and Davis seems to account for the results obtained in the present study.

The fact that the effect of MH and DEPE increases markedly with increasing temperature during treatment could also be explained as a penetration phenomenon, since the permeability of cell walls is very sensitive to temperature changes, being much higher at high temperatures (23). It is also quite possible in the case of DEPE at least, that the effect is initiated by a primary chemical reaction which is dependent on temperature.

The influence of temperature on the effect of EOC is more complex than on that of MH and DEPE. Up to 12°C. the effect of temperature on the production of chromosome aberrations by EOC is similar to that of MH and DEPE, *i.e.*, the frequency of aberrations increases with increasing temperature. A further increase in temperature results in a decrease in the frequency of aberrations (Text-fig. 1). Since the toxic effect of EOC increases with rising temperature, it was proposed that the decrease in frequency of aberrations was a result of those cells most affected by the treatment being killed before entering mitosis (24). Subsequent experiments showed, however, that this explanation was untenable since a similar effect of temperature was obtained in cases where no serious toxic effect could be observed (8). At present, no satisfactory explanation can be given to the unusual temperature effect in the case of EOC.

The pH effect in the case of MH and the temperature effect in the case of MH and DEPE can readily be explained as effects on penetration of these compounds, although conclusive evidence still is lacking. Whether the oxygen effect could be explained in this way too should also be considered.

It is now a well known fact that the absorption of ions in root tips is dependent on cytochrome respiration (25). MH is an acid which, at the pH used, is partly ionized. It is, therefore, conceivable that its absorption is dependent on respiration. There is no evidence, however, that the entrance of a neutral, non-metabolic organic molecule, such as EOC, should be due to anything other than a passive diffusion process, dependent only on such factors as the lipoid solubility and size of the molecule and the structure and chemical composition of the cell wall. The fact remains that the only effect

of EOC that seems to be inhibited by anoxia is the effect on the chromosomes. The toxic effect of EOC at high temperatures is actually increased by anoxia (Kihlman, unpublished data). It can, therefore, be concluded that the oxygen effect, at least in the case of EOC, is not one of interference with its penetration into the cell.

On the basis of the results of his studies on the mutagenic effect of methyl xanthines in bacteria, Novick (18) has suggested that these compounds are unable to induce mutations under anaerobic conditions because bacteria grown anaerobically contain significant concentrations of adenosine. This riboside had previously (20) been found to be an effective antagonist against the mutagenic effect of methyl xanthines in *Escherichia coli.*

Although adenosine proved to be an efficient inhibitor of the effect of EOC on bean root chromosomes, the influence of anoxia on the production of chromosome aberrations in *Vicia* cannot be explained by this theory (discussed on page 551).

The facts obtained are understandable, however, if it is assumed that oxidative phosphorylation is involved in the chain of events that results in chromosome aberrations after treatments with EOC.

It is tempting to explain the influence of oxygen on the MH effect in a similar way. That is to say that oxidative phosphorylation is involved in the chain of events that leads to chromosome breakage after treatment with MH as well as EOC. It is quite conceivable that this is the case. But the possibility that oxygen influences the MH effect as a direct result of influencing the penetration of MH into the cell (as was previously noted) cannot be excluded.

In the case of DEPE there is no evidence to indicate that the effect is dependent on oxidative phosphorylation, although the possibility of this being the case should not be overlooked. DEPE is an alkylating agent and the action of such an alkylating agent "is initiated by reaction at some cellular site probably either an ionized acid or unionized amino group" (17). This primary chemical reaction is independent of oxidative phosphorylation. The chromosomal aberrations themselves, however, may be a result of secondary reactions which are dependent on oxidative metabolism. Provided that the primary reaction product remains in the cell, aberrations would be produced as soon as oxidative phosphorylation is resumed. The only effect expected after inhibiting oxidative phosphorylation would then be a delayed appearance of the aberrations; that is, the effect which has been observed.

The involvement of a step requiring respiration energy in the production of chromosome aberrations may be, in fact, a general phenomenon. Irradiating *Vicia* seeds which had been soaked in water for 24 hours and studying the effect in the first root tip mitosis, Wolff (26) has found that the frequency of chromosome exchanges, produced by a fractionated dose of x-rays, can

be modified by keeping the seeds in anaerobic conditions or treating them with inhibitors of oxidative phosphorylation between the irradiations.

Recently Allfrey, Mirsky, and Osawa (27) have discovered that isolated calf thymus nuclei are able to generate energy-rich phosphate by some oxidative mechanism, which, like that in mitochondria, is sensitive to cyanide and DNP. Assuming that such an oxidative synthesis of energy-rich phosphate occurs also in the nuclei of bean root tips, it would probably be the process which is involved in the production of chromosome aberrations, and not the well known oxidative phosphorylation localized in the mitochondria.

SUMMARY

In the present paper, the results of a study on the influence of temperature, hydrogen ion concentration, and oxygen tension on the production of chromosome aberrations in *Vicia* root tips by maleic hydrazide (MH), di-(2, 3-epoxypropyl)ether (DEPE), and 8-ethoxycaffeine (EOC), are described.

Variations in the hydrogen ion concentration of the treatment solutions did not significantly influence the effect of EOC and DEPE. In contrast, the MH effect was considerably diminished by raising the pH from 4.7 to 7.3.

A marked increase in the frequencies of aberrations produced by DEPE and MH was obtained by raising the temperature from 3° to 25°C. The effect of EOC increased with rising temperature up to 12°C. With a further rise in temperature the effect of EOC decreased, so that at 25°C. it was of about the same magnitude as at 3°C.

The effect of EOC was completely inhibited, and that of MH partly so, when during the treatment (1) oxygen was excluded from the solution, (2) respiration was inhibited by azide or cyanide, or (3) phosphorylation was uncoupled from respiration by 2, 4-dinitrophenol (DNP). Pretreatments with DNP had a similar effect, but posttreatments did not influence the frequencies of aberrations. The effect of DEPE was unchanged by anoxia. Pre- or posttreatments with DNP did not change the total number of aberrations produced by DEPE, but the appearance of the effect was considerably delayed.

The results are discussed.

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