The Effect of Extended Anaerobic Treatments on the Chromosomes of *Vicia faba*[*,‡]

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ABSTRACT

The effects of extended anaerobic treatments on *Vicia faba* lateral root-tip chromosomes were determined. It was observed that aberrations resulted from these treatments, and that the frequency varied from root to root as well as from experiment to experiment. It was suggested that the inconsistency observed might be due to variation in the abilities of different roots to produce energy via fermentation routes. If this were true, an inhibition of fermentation would result in a more consistent aberration frequency. A fermentation inhibitor, NaF, was used in combination with extended anaerobic treatments. The observed frequency of aberrations after the combined treatments was generally higher and considerably less variable. Although other hypotheses might account for the NaF effect, the hypothesis most compatible with the evidence is that the effect is due to energy deprivation. The experimental results are discussed in terms of the aforementioned effect and in terms of three alternative hypotheses for the production of chromosomal aberrations as a consequence of a lack of energy. It is concluded that damage might result from a build-up of normal cellular compounds to abnormally high concentrations which would act directly or indirectly on the chromosomes, from the breakdown of DNA as an energy source, or simply as a result of the fact that the chromosome needs energy to remain intact.

INTRODUCTION

Interest in the effects of nitrogen on various cytological systems has a fairly long historical development. Most of the early work was concerned with the effect of nitrogen treatments on mitosis, and the observations were quite consistent in that tissues subjected to anaerobic environments uniformly exhibited a lack of mitotic activity (1, 2). Steinitz (3) later reported treating barley seedlings with nitrogen for periods of time varying from 12 to 144 hours. She observed, in addition to cell death, chromosome fragments, metaphase clumping of chromosomes, and sticky anaphase bridges. In more recent years, nitrogen treatments have been routinely used to modify the action of radiation and chemical mutagens on chromosomes (4–7), the objective being to obtain a more complete understanding of the role of oxygen in induced cellular damage.

The effects of presence and absence of oxygen have been variously explained as influencing the rejoining of radiation-induced chromosome breaks via ATP production (8), breakage via ATP production (7), breakage as a result of altering the effective geometry of the ion path by radiochemical reactions leading to radical formation (9), and breakage due to changing the chromosomal sensitivity by the oxidation and reduction of chromosomal iron (10). The oxygen effect has not yet been fully explained and an additional element of confusion has been injected into the picture by Conger's and Fairchild's observation (11) that oxygen alone is capable of inducing chromosomal aberrations.

The somewhat unexpected results obtained

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by Beatty *et al.* (12–14) seem, at first glance, to contradict results derived from earlier studies. Using radiation in combination with extended anaerobic pretreatments (up to 400 minutes in He_2), they showed that marked increases in aberration frequency are realized when the anaerobic pretreatment period is longer than 200 minutes. They have accepted the hypothesis of Wolff and Luippold (8) that the oxygen effect is a dual phenomenon. That is, that the level of O_2 (up to 21 per cent) governs in part the frequency of breakage and in part the frequency of rejoining. The rejoining process is, according to this hypothesis, a system requiring energy (usually provided by oxidative phosphorilation) for its action but it is much less damaged during radiation in the absence of O_2 than in its presence. Essentially then, the presence of high O_2 concentration during radiation results in a correspondingly high breakage frequency and more damage to the rejoining system (as compared to radiation in N_2) but the actual act of rejoining following breakage requires energy and therefore oxygen. Lack of oxygen during radiation therefore "protects" the rejoining system and results in rapid rejoining if O_2 is presented immediately following radiation, but if energy production is blocked by continued anoxia the breaks will not rejoin, remaining open until energy is available.

A different role for oxygen in determining radiation sensitivity has recently been offered by Ebert *et al.* (15) and Read (16). From experiments in which O_2 was retained at levels prevalent in air, but in which inert gases were used at higher than usual pressures, they have concluded that O_2 confers a degree of sensitivity by occupying critical sites in the nucleus and on the nuclear membrane (a lipide-water surface). When O_2 is replaced from these sites by the inert gases radiation sensitivity is reduced. The fact that there is a close parallel between these results and the ability of gases under pressure to replace O_2 in lipide systems reinforce their belief in the "site" hypothesis.

It seemed important, therefore, in the light of the mass of data representing studies on the effect of oxygen or its lack on radiation-induced cellular damage, to examine the effect of anaerobic environments and actual intracellular anoxia, produced by long anaerobic treatments, on chromosome behavior.

Materials and Methods

The experimental material used in these experiments consisted of lateral root tips of the broad bean (*Vicia faba*): the varieties Seville Long pod and Claudia Aquadulce were obtained from Carters Tested Seeds, Ltd., London, England. The roots were grown in shell vials in the dark at 25°C. for 6 days following an initial 24-hour period of soaking. The tap water was changed daily during growth and recovery (time between treatment and fixation). Twenty-four hours before treatment the roots were placed in a 17°C. incubator. Most treatments were performed at 17°C. and recovery took place at 17°C. in the dark. The treatment vials have been described by Kihlman (7). The treatments designed to produce anoxia consisted of bubbling a constant stream of commerical, prepurified tank nitrogen through the vials for the total treatment time. Wherever a chemical treatment was combined with anoxia, three methods were used: (1) the chemical was introduced in aerated water and then followed by N_2 so that its penetration and initial effect would occur in the presence of O_2; (2) it was introduced at the same time as N_2; (3) N_2 was bubbled through the chemical in solution to remove any O_2 and the chemical was introduced while the roots were in N_2. The chemicals used in the experiments discussed herein are sodium fluoride (NaF), 2,4-dinitrophenol (DNP), sodium azide (NaN_3), iodoacetate, parachloromercuricbenzoate (PCMB), sodium arsenite, adenosinetriphosphate (ATP), adenosinediphosphate (ADP), and pyruvate. X-rays were also used as a treatment agent (20 R.P.M.), total dose of 100 r at 100 kv. and 10 ma.).

The root tips were fixed after recovery in cold 3:1 alcohol-acetic acid, and slides prepared as Feulgen-squashes. When chromatid exchanges and iso-chromatid breaks were to be scored, the roots were treated with 0.05 per cent colchicine for 3 to 4 hours before fixation; otherwise, they were fixed directly and anaphase bridges and fragments were scored.

RESULTS

Beatty *et al.* (12, 13), and Beatty and Beatty (14), reported observing, as a result of radiation, a pronounced increase in breakage frequency in *Tradescantia* microspore chromosomes when in helium for 400 minutes as against short exposures in helium. These experiments were repeated on *Vicia* root-tip cells and the results appear in Table I. It is evident that x-ray breakage is increased materially over the short N_2 treatment as a consequence of a 3-hour pretreatment in N_2. It is also clear that the rate of breakage observed after 3 hours in N_2 is considerably higher than the rate occurring spontaneously. The frequencies obtained by radiating after 3 hours pretreatment in N_2 are about equalled by adding the frequencies

TABLE I

Comparison of the Effects of Nitrogen and X-rays Alone and in Combination Treatments

Treatment	No. cells scored	Abnormal metaphases	Deletions	Isochromatids	Exchanges
		per cent	*per cent*	*per cent*	*per cent*
Untreated	200	1.0	0	1.0	0
Air X-ray (100 r)	300	63.0	27.7	57.7	19.0
N_2 (5 min.) X-ray (100 r)	300	30.7	12.7	18.3	6.3
N_2 (3 hrs.) X-ray (100 r)	300	48.7	23.0	36.3	12.0
N_2 (3 hrs.)	200	28.0	7.5	20.0	8.0

TABLE II

The Effect of Recovery in Oxygen on Anaerobically Induced Breakage

Expected order of effect assuming imbalance theory	Expected order assuming oxygen starvation theory	Observed order	Abnormal anaphases
			per cent
1	3	3	2
2	2	2	8
3	1	1	19

Treatment

1 = 3 hours N_2 followed by 2 hours O_2.

2 = 3 hours N_2 followed by 2 hours air.

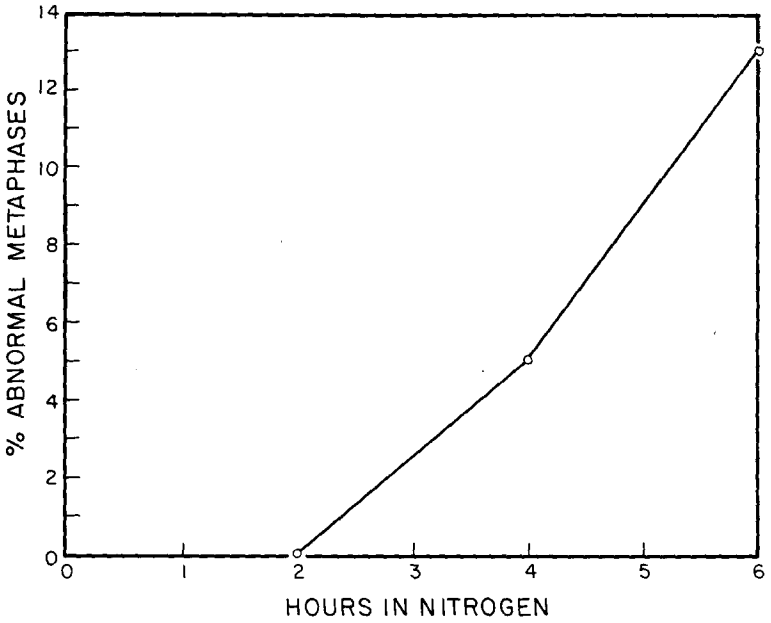
3 = 5 hours N_2.

resulting from the pretreatment alone and the radiation after pretreatment in N_2 for 5 minutes. The effect of the 3-hour treatment in N_2 is about the same as observed after radiation during the short N_2 treatment. These results are not in agreement with Beatty *et al.* (12, 13, 14).

To determine whether the observed effect of anoxia actually resulted from oxygen starvation rather than from the induction of an unstable state which results in chromatid breaks in the presence of O_2 during recovery (this might be thought of diagrammatically as follows: chromosome + anoxia → unstable state + oxygen → reaction → breakage), lateral root tips were treated with (1) 3 hours of N_2 followed by 2 hours of O_2; (2) 3 hours of N_2 followed by 2 hours of air; (3) 5 hours of N_2. Assuming that the observed damage was a result of oxygen starvation alone, the most effective treatments in descending order would probably be 3, 2, 1. If the imbalance theory were correct, one would expect the order to be 1, 2, 3. The expected results and those observed appear in Table II. Fixations were made after 24 hours and 48 hours recovery to make certain that mitotic inhibition did not account for the difference in results. The observed order was the same in both cases and it indicates that the observed effect of anoxia is the result of oxygen starvation. Fixations were also made immediately after 3 hours and 5 hours in N_2. There were very few cells in division, mitosis having been almost completely inhibited, but those observed were all normal as far as could be determined visually.

To ascertain whether the damage observed was proportional to the time in N_2, root tips were treated with N_2 for 2, 4, and 6 hours, and fixed after 24 hours recovery. The results appear in Fig. 1.

Since the breakage frequency observed after the 6-hour treatment in N_2 (Fig. 1) was lower than originally observed after 3 hours in N_2 (Table II), it seemed desirable to determine the variability of the anoxia effect. Treatment times in N_2 of 2, 4, 5, 6, and 8 hours were used. The 5- and 6- hour treatments were done twice on separate occasions; the 8-hour treatments 6 times. The results are listed in Table III, which also includes an experiment using He_2 instead of N_2 in order to determine whether extended treatments with another so-called inert gas would produce the same results as nitrogen. The results were quite variable; the highest frequency of aberrations was obtained as a consequence of an 8-hour treatment in N_2. Some variability between treatments is probably to be expected if the effect is due to a lack of energy brought about by a stoppage of oxidative metabolism, *i.e.*, different roots differ in their ability to produce ATP through fermentation. Some of the variability, however, may be due to two other phenomena. Most of the roots showing a lower frequency of aberrations contain many pyknotic and presumably dead cells. These have been most severely affected by the N_2 treatment and consequently do not contribute to the scorable figures. This belief is reinforced by the observation that the occurrence of high aberration frequencies coincides with a high incidence of mitotic figures. This condition appears to result from



TEXT-FIG. 1. The influence of anaerobic treatment time on aberration frequency.

the first burst of divisions following inhibition by the extended anaerobic treatments. The time that the burst occurs varies from root to root on any given bean as well as from bean to bean. Some beans, after 8 hours in nitrogen, have 80 per cent of their lateral root tips dead, while others show no observable external damage.

In order to ascertain whether the variability was due to a difference in ability of different roots to produce energy through glycolysis in the absence of oxidative metabolism, a fermentation inhibitor (NaF) was used in combination with nitrogen. It would be expected that under these conditions the frequency of aberrations would be generally higher and somewhat more consistent since the factor of variability (fermentation) has been removed. The results of three experiments using combination treatments and one using NaF alone are in agreement with this hypothesis and appear in Table IV. Combination treatments were fixed after 40 hours recovery, the first divisions occurring at about that time.

Another possible method of determining whether the effects observed after extended periods of anoxia result from a lack of energy is to examine the influence of temperature on the effectiveness of anoxia. At low temperature, when the cellular activity and need for energy is greatly diminished, the effect should be considerably less since the supply of energy itself would not be depleted at the normal rate. To test this hypothesis, an experiment utilizing the combined treatments (which produced more consistent results) of N_2 plus NaF for 8 hours was performed at different temperatures. The results (Table V) are compatible with this hypothesis.

TABLE III

Inconsistency of Effects Produced by Varying Times of Anaerobic Treatments as Well as Variation between Treatments of the Same Duration

Treatment	No. cells scored	Abnormal metaphases	Deletions	Isochromatids	Exchanges
		per cent	*per cent*	*per cent*	*per cent*
N_2-2 hrs.	100	0	0	0	0
N_2-4 hrs.	100	5	2	9	1
N_2-5 hrs.	100	21	5	10	6
N_2-5 hrs.	100	35	10	30	10
N_2-6 hrs.	100	7	2	5	0
N_2-6 hrs.	100	13	0	7	7
N_2-8 hrs.	100	59	12	44	25
N_2-8 hrs.	100	3	1	1	1
N_2-8 hrs.	100	12	4	4	5
N_2-8 hrs.	100	11	2	11	3
N_2-8 hrs.	100	3	0	2	1
N_2-8 hrs.	100	25	4	23	9
He_2-5 hrs.	200	8	4	4	0

TABLE IV

The Influence of NaF on Anaerobically Produced Aberrations

Treatment	No. cells scored	Abnormal metaphases	Deletions	Isochromatids	Exchanges
		per cent	*per cent*	*per cent*	*per cent*
N_2 plus NaF 8 hrs.	200	48	17	60	34
N_2 plus NaF 8 hrs.	200	38	7	37	25
N_2 plus NaF 8 hrs.	200	41	15	52	37
NaF alone 8 hrs.	500	0	0	0	0

TABLE V

The Influence of Temperature on Aberrations Produced by Combined Treatments of N_2 and NaF

Treatment	No. cells scored	Abnormal metaphases	Deletions	Isochromatids	Exchanges
		per cent	*per cent*	*per cent*	*per cent*
N_2 plus NaF 8 hrs.-4°C.	100	4	1	2	1
N_2 plus NaF 8 hrs.-10°C.	100	4	1	3	0
N_2 plus NaF 8 hrs.-14°C.	100	4	1	2	2
N_2 plus NaF 8 hrs.-22°C.	100	44	17	46	18

It was of interest to know at what time during the combined treatment the addition of NaF was producing its effect. Two types of experiment were performed. In the first, combination treatments of varying durations were compared to N_2 treatments alone for the same duration. The results (Table VI) indicate that the effect occurs after 4 hours of the combined treatment and does not increase thereafter. The second type of experiment utilized a treatment of 4 hours in N_2 during which NaF was added for times varying from 1 to 4 hours. The results (Table VII) indicate again that the effect occurs only after 4 hours of combined treatment.

Several other inhibitors were tried with and without N_2. Only a low level of effectiveness was observed. These data are listed in Table VIII. In many cases the low frequency of breakage may be attributable to the lethal effect, but it

TABLE VI

Effects Produced by Varying Times in N_2 and NaF

Treatment	No. cells scored	Abnormal metaphases	Deletions	Isochromatids	Exchanges
		per cent	*per cent*	*per cent*	*per cent*
N_2-2 hrs.	100	0	0	0	0
N_2 plus NaF 2 hrs.	100	1	0	1	0
N_2-4 hrs.	100	2	1	1	0
N_2 plus NaF 4 hrs.	100	41	8	33	21
N_2-6 hrs.	100	7	1	4	2
N_2 plus NaF 6 hrs.	100	33	8	46	39
N_2-8 hrs.	100	2	0	2	0
N_2 plus NaF 8 hrs.	100	27	1	38	14

TABLE VII

Effect of Duration of NaF Treatments in Combination with 4 Hours Nitrogen

Treatment	No. cells scored	Abnormal metaphases	Deletions	Isochromatids	Exchanges
		per cent	*per cent*	*per cent*	*per cent*
Total N_2 time—4 hrs. NaF present 4th hr.	100	2	1	1	1
Total N_2 time—4 hrs. NaF present 3rd and 4th hrs.	100	1	0	1	0
Total N_2 time—4 hrs. NaF present 2nd, 3rd, and 4th hrs.	100	7	2	4	1
Total N_2 time—4 hrs. NaF present 4 hrs.	100	26	2	21	7

may also be that lethality occurs without accompanying damage to chromosomes. In all cases excepting DNP and NaN_3 many of the cells on any given slide were dead.

In an attempt to show that the observed damage after the N_2 and NaF combined treat-

TABLE VIII

Comparison of the Radiomimetic Effects of Several Respiratory Inhibitors

Treatment	No. cells scored	Abnormal metaphases	Deletions	Isochromatids	Exchanges
		per cent	*per cent*	*per cent*	*per cent*
NaN_3 (5×10^{-4}) 8 hrs.	100	7	1	2	4
N_2 plus NaN_3 (5×10^{-4}) 8 hrs.	100	4	2	2	1
NaF (10^{-2}) plus NaN_3 (5×10^{-4}) 8 hrs.	100	1	0	1	0
Arsenite (10^{-4}) 8 hrs.	100	0	0	0	0
Iodoacetate (10^{-3}) 8 hrs.		Lethal			
DNP (10^{-4}) 8 hrs.	100	3	1	2	0

ments was clearly the result of energy deprivation, the energy-deprived cells were treated with ATP and ADP. At low concentration (1×10^{-5} M) no change in effect was observed. At high concentration (1×10^{-3} M, 1×10^{-4} M) these compounds were lethal. There is no reason to believe, however, that intact ATP or ADP penetrated the cells.

DISCUSSION

As was stated in the introduction, the intent of this study was to ascertain the effect of extended anaerobic treatment on chromosome structure. Beatty *et al.* (13) had previously observed that *Tradescantia* microspores treated for 400 minutes in He_2 exhibited no aberrations but that this treatment in conjunction with radiation at low intensities (2 R.P.M.) increased the radiation-induced damage to an appreciable extent over that induced following short exposures in helium. These results have not been confirmed in the present study utilizing N_2 as the treatment gas and *Vicia faba* as the treated organism. The extended treatments in N_2 resulted in chromatid aberrations in the chromosomes of *Vicia faba* lateral root tips, although the frequency observed was quite variable from root to root and from experiment to experiment (Table III). The increased damage observed after combining anaerobic treatments with x-ray is a function of adding the damage produced by x-rays after short N_2 pretreatment to the damage produced by long anaerobic treatments alone (Table I). That this is true is indicated by the fact that the increase seen in the combined treatments (x-ray plus extended anoxia) is as inconsistent as is the damage observed after extended anoxia alone (Merz, unpublished observations). There is no reason to assume, however, that the same effects are operating in *Tradescantia* microspores. The aberrations observed were chromosomal in the *Tradescantia* experiments, but there are few, if any, chromosomal aberrations seen in *Vicia faba* lateral root tips; the aberrations scored in this study were all of the chromatid type. It is not inconceivable, however, that the increase observed in *Tradescantia* microspore chromosomal aberrations after radiation and anoxia combined is the result of anoxia-induced sensitization that appears in *Vicia* as actual breaks. That is to say, the cellular conditions at the time when chromosome aberrations are induced in *Tradescantia* are different from those at the time when chromatid aberrations are induced in *Vicia faba* lateral root tips, and it is possible to conceive of an effect that produces observable damage in one and non-observable damage in the other.

The decrease in variability between experiments of the same kind as a result of the addition of NaF, an inhibitor of fermentation, to treatments of anoxia (Table IV) indicates that the observed variability may be due to the variation in the ability of different roots to produce energy *via* glycolysis, and that the effect observed is a consequence of energy starvation. The same decrease in variability seen when NaF is added to extended anaerobic treatments is observed when these combined treatments are used in conjunction with x-ray treatments, and compared to x-ray in short duration anoxia exposures (Merz, unpublished observations). The increase is always attributable to the damage resulting from the combined effects of anoxia and NaF. No synergism is evident.

The effect of NaF combined with anoxia can be explained in terms other than that related to a lack of energy. It can be argued that NaF has a direct effect upon the chromosomes and only penetrates the nuclear membrane after long periods of anoxia and consequent membrane breakdown. It seems unlikely, however, that

NaF does not penetrate in an oxygen environment since the NaF treated tissue showed inhibition of mitotic activity, chromosome stickiness, and clumping of chromosomes shortly after NaF was introduced in oxygenated water. It is also conceivable that NaF produces its effect as a result of its reaction with a reduced compound and therefore is effective only when introduced after long anaerobic treatments. This does not, however, seem to be the case since the effect produced by NaF is as much a function of the length of time it is present as an agent as it is of the duration of anoxia, *i.e.*, 4 hours of N_2, including 1, or 2, or 3 hours of NaF, does not produce the effect but 4 hours of N_2, including 4 hours of NaF, does (Tables VI, VII). The explanation of the observed damage in terms of energy deprivation seems, then, to be the most acceptable.

There are several explanations for a lack of cellular energy resulting in observable damage. It is clearly possible that during the process of metabolic inhibition some compounds, present in small amounts normally, begin to pile up as they are not utilized. These compounds, which in their normal concentrations are not radiomimetic, may at abnormally high concentrations become mutagenic. This is true of such metabolically vital elements as the trace metals which, when presented to the cell in concentrations far above normal, produce chromatid aberrations (17).

There are two other hypotheses which seem reasonable although there is little evidence at present to support them. Under extreme conditions of energy deprivation, it seems conceivable that the cell might break down DNA for use as an energy source. There is no evidence as to what this breakdown would involve chemically or that it would occur under the experimental conditions set up in this study. It has been stated, however, by Beers (18) that his data support the thesis that the primary biological role of RNA may well be that of a readily available high energy source. Assuming this to be so, it is not altogether unreasonable to suggest the possibility of DNA being used as an energy source under conditions requiring energy production for cell viability. Repair of such breakdown during recovery might well result in chromatid exchanges and fragments which would probably be present in higher frequencies in the first divisions following recovery than in subsequent divisions. This is what occurs experimentally; divisions subsequent to the first burst are almost entirely free of damage. One would also expect that a continuation of DNA breakdown would result in cell death and it has been observed that any increase in treatment time results in the death of most cells examined.

The other explanation is that the chromosome itself requires energy in order to maintain itself as a complete entity in the same sense as it requires energy in the duplication process. Lack of energy would result then in fragmentation of the chromosome to some extent. One might expect that at first the process would involve breaks in specific localized areas where the energy required for a particular bond is needed to a greater degree than in other areas. As the process continued chromosomes would shatter. An alternative hypothesis would involve a threshold of energy below which all the bonds requiring energy would break, resulting in many fragments in all affected cells. There is no evidence, however, of any localization of chromosomal damage; the breaks were observed to occur at random in areas known to be heterochromatic as well as euchromatic, and including the nucleolar organizer region and centromeres. The complete fragmentation or shattering of chromosomes, although not a commonly observed condition, was occasionally observed, and particularly in those roots containing cells with the highest frequency of aberrations (shattered chromosomes are not included in determining this frequency).

The evidence in general is compatible with all three hypotheses though slightly more so with the latter two. There is, however, not enough evidence at this time to make a decision between the alternative hypotheses presented.

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