Plant Physiol. (1981), 67, 910–916
0032-0889/81/67/0910/07/$00.50/0

Induction and Characterization of Chlorate-resistant Strains of *Rosa damascena* Cultured Cells[1]

Received for publication April 22, 1980 and in revised form November 7, 1980

TERENCE M. MURPHY AND CATHERINE W. IMBRIE
Department of Botany, University of California, Davis, California 95616

ABSTRACT

The sensitivity of *Rosa damascena* cultured cells to chlorate was measured by plating samples of suspensions in agar containing $NaClO_3$. This sensitivity depended on the age of the cultures that were plated. Chlorate-resistant colonies isolated from 5- to 7-day cultures retained their resistance through many generations of growth in medium lacking $NaClO_3$; they also retained resistance when mixed with sensitive cells. Treating cell aggregates with ultraviolet (UV) light (254 nanometers), or UV light (360 nanometers) in the presence of 4'-methoxymethyltrioxsalen, increased the proportion that was resistant to $NaClO_3$. However, the amount of increase was low (three times) and required very specific doses of UV light. The UV treatments did not select for chlorate-resistant cells over chlorate-sensitive cells. The data suggested that UV had induced mutations leading to chlorate resistance. Approximately 15% of the resistant strains did not grow on medium containing nitrate as the sole nitrogen source. These strains lacked ability to reduce chlorate to chlorite. This observation supports the current idea that chlorate toxicity depends on the activity of nitrate reductase. Approximately 85% of the resistant strains grew on medium containing nitrate as the sole nitrogen source. These strains lost catalase activity following chlorate treatment, indicating that they took up and reduced chlorate. These strains have a mechanism for tolerating chlorate and its reduction products, rather than avoiding them.

The lethal effect of $NaClO_3$ on higher plants has long been recognized, but the mechanism of chlorate toxicity remains a mystery. Harvey (8) suggested that chlorate oxidized "respiratory chromagens," but presented no evidence beyond the observation that treated tissue turned black. Neller (13) observed decreased catalase in roots of chlorate-treated *Convolvulus*, but the time between treatment and assay was such (7 months) that the loss of catalase seems more a reflection of chlorate toxicity than an intermediary step.

The chemical reduction of chlorate is one important step in its mechanism of action. The first suggestion of this came from comparisons of chlorate uptake and chlorate residues in sensitive and resistant plants (Yamasaki, 1929, quoted in ref. 1). Work with mutant strains of microorganisms and cultured plant cells supported this suggestion. Mutants of *Escherichia coli* selected for resistance to chlorate lacked nitrate reductase (15), an enzyme later shown to reduce chlorate (6). Similar selection experiments were reported for *Nostoc muscorum* (17), *Aspergillus nidulans* (3), *Chlamydomonas reinhardii* (18), and cultured tobacco callus (11). In tobacco, Müller and Grafe (11) isolated nine strains on the basis of chlorate resistance. Seven of these strains lacked detectable nitrate reductase and did not grow on medium containing nitrate as the sole nitrogen source. Two other strains had very little nitrate reductase and grew very poorly on nitrate medium. It seems that the presence of nitrate reductase, which presumably reduces chlorate to chlorite, is necessary for the full toxic effect of chlorate to be expressed.

If strains of plant cells that possess both nitrate reductase and chlorate resistance were located, they might provide clues to further steps in the mechanism by which chlorate kills normal cells. Cove (3) discovered a group of chlorate-resistant mutants of *A. nidulans* that could utilize NO_3^-; at least three separate genes were involved, but these mutants were very rare. One group of chlorate-resistant mutants of *N. muscorum* (17) retained nitrate reductase activity (inferred, not measured) and lacked nitrogenase activity. Here we report that when strains of *Rosa damascena* cultured cells are selected for chlorate resistance, only a minor fraction lacks the ability to grow on medium containing nitrate as the sole nitrogen source and probably lacks the ability to reduce chlorate to chlorite; the major fraction retains the ability to grow on nitrate medium and possesses the ability to transform chlorate to toxic products.

MATERIALS AND METHODS

Cultures. The culture of *Rosa damascena* Mill. var "Gloire de Guillan" has been described previously (12). Small aggregates of cells were grown in suspension in 25 or 50 ml of liquid medium. The cells were subcultured by 1:10 dilution every 7 days. The media used were adapted from ref. 14: MXG contained 5 mM Na glutamate and 5 mM $NaNO_3$ as nitrogen sources; MX2 contained 10 mM $NaNO_3$ as sole nitrogen source. Cultures were rotated 100 rev/min at 26 C in the dark.

Plating Assay. Cells to be assayed for plating ability were diluted in growth medium to a concentration of 1,000 to 50,000 aggregates/ml. One ml of suspension was mixed in a 15 × 100 mm standard plastic Petri dish with 5 ml of medium containing 0.7% purified agar. The Petri dish was sealed with paraffin film and incubated at 26 C for 28 days. At the end of the incubation period, colonies larger than 1.0 mm in diameter were counted. In untreated plates, the number of colonies was proportional to the number of aggregates plated (over at least the range 100–5,000 aggregates per plate). When used, chlorate was autoclaved in water, then added to sterile medium. The numbers of colonies appearing in plates containing chlorate were always expressed relative to an untreated control.

Viability and Growth Assay. Small samples of suspensions were assayed for viability with FDA.[2] Dry weight increases in liquid

[1] Supported in part by National Science Foundation Grant PCM 76-19954. A preliminary communication of a portion of these data was presented at the 4th International Congress of Plant Cell and Tissue Culture, Calgary, Alberta, 20–25 August 1978.

[2] Abbreviation: FDA, fluorescein diacetate; MOMT, 4'-methoxymethyltrioxsalen.

cultures were measured by standard techniques (19).

Radiation and Illumination Treatments. For irradiation with far UV light (254 nm), 3 ml of cell suspension were irradiated in 15 × 60 mm Permanox (UV-transparent) Petri dishes (Eurolab) with periodic agitation, using a short wave UV lamp with a CS 7-54 filter. The fluence rate incident on the suspension was 1.4 w/m^2.

Plates to be photoreactivated were exposed to white fluorescent light, 200 to 300 w/m^2, for periods of 48 h or less. Controls (and plates of all experiments in which photoreactivation was not explicitly mentioned) were placed in the dark immediately.

For irradiation with near UV light (360 nm peak), 8 ml of cell suspension were irradiated in 15 × 100 mm standard plastic Petri dishes (transmission <1% below 285 nm), 12 cm from two F40BLB fluorescent tubes, with constant agitation. The fluence rate was 4.26 w/m^2. MOMT, when present, was added before irradiation at 5 μg/ml.

Nitrate, Chlorate, and Nitrate Reductase Assays. For measurement of nitrate concentration, 5 ml samples of suspension were acidified, frozen and thawed three times, then centrifuged (30 min × 16,000*g*) and filtered. Nitrate was measured by high pressure liquid chromatography (21). Chlorate in the medium was measured according to White (22). Nitrate reductase was extracted and assayed according to Wright and Murphy (24) but using the improved buffer of Kuo *et al.* (10) and omitting the G25 chromatography.

Catalase Extraction and Assay. Packed cells were resuspended in 3 ml of 10 mM K-phosphate, pH 7.6, homogenized with a Polytron tissue homogenizer, and centrifuged for 10 min at 16,000 *g*. One-ml samples of the supernatant solution were assayed for catalase using a spectrophotometric test for substrate H_2O_2 (4).

RESULTS

Toxicity of Chlorate in the Plating Assay. Rose-cell aggregates that were plated in agar/MXG medium and incubated for 2 to 4 weeks at 26 C formed discrete colonies with 30 to 50% efficiency. The addition of $NaClO_3$ to the plating medium strongly reduced the plating efficiency of the aggregates (Fig. 1). In MXG medium, 33 mM $NaClO_3$ inhibited the growth of over 90% of potential colonies. In MX2 medium (nitrate, no glutamate as nitrogen source), the cells were even more sensitive, and 10 mM $NaClO_3$ inhibited the growth of over 90% of potential colonies.

NaCl was toxic, but much less than $NaClO_3$: 33 mM NaCl had no effect on plating efficiency, and 100 mM NaCl reduced plating efficiency by only 60% (Fig. 1). The data indicate that the major effect of $NaClO_3$ results specifically from the chlorate ion, and is not an effect of Na^+ ion, ionic strength, or osmolality.

The effect of higher concentrations of $NaClO_3$ on plating efficiency did not depend on plating density. In three experiments, different numbers of aggregates (varying over approximately a 10-fold range) were plated on MXG medium containing 56 mM $NaClO_3$. Within each experiment, differences in the number of colonies growing per aggregate plated were not significant (data not shown).

The sensitivity of the plating efficiency of rose cells to $NaClO_3$ did depend on the age of the cultures which were plated (Fig. 2). Suspensions from 2-, 3-, and 4-day-old cultures were relatively insensitive to $NaClO_3$; suspensions from 5-, 6-, and 7-day-old cultures were more sensitive. The changes in sensitivity might correspond to any of a number of physiological changes that take place in these cells during the growth of the cultures (12, 14); under our conditions, for instance, cell division stops rather abruptly at 5 days.

$NaClO_3$ did more than just inhibit colony formation; it was lethal, as indicated by FDA assays performed on cells maintained in suspension. In 4-day-old cultures, most cells (over 75%) in all aggregates were alive and showed good structure with bright nuclei and sharp cytoplasmic strands. Six h after addition of 10 mM $NaClO_3$, half of the aggregates contained fewer than 75% live cells. After 48 h, most of the aggregates had fewer than 25% live cells. Control cultures showed only slight decreases in viability over the 48-h period. We observed no effect of culture age on the sensitivity of the cells to $NaClO_3$ using this assay, but this was probably due to the limited precision of the assay. Also, the FDA assay does not accurately reflect colony-forming ability (23).

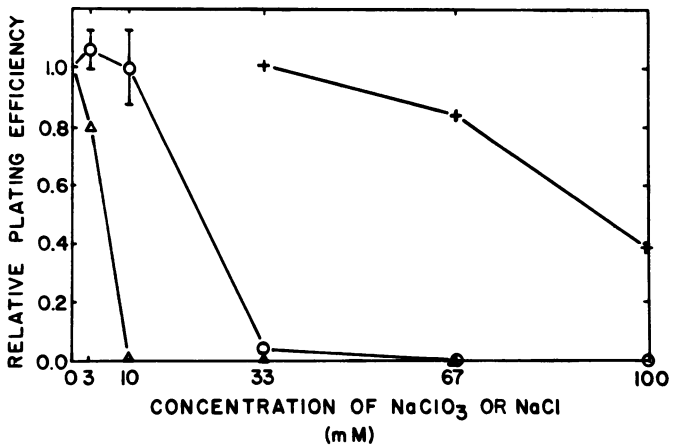

FIG. 1. Effects of NaCl and $NaClO_3$ on the relative plating efficiency of rose cell aggregates. In all experiments, plating efficiency was expressed relative to controls without $NaClO_3$ or NaCl. (○), $NaClO_3$ in MXG (medium with nitrate and glutamate as nitrogen sources); (△), $NaClO_3$ in MX2 (medium with nitrate as sole nitrogen source); (+), NaCl in MXG. Error bars indicate standard errors from four to six experiments.

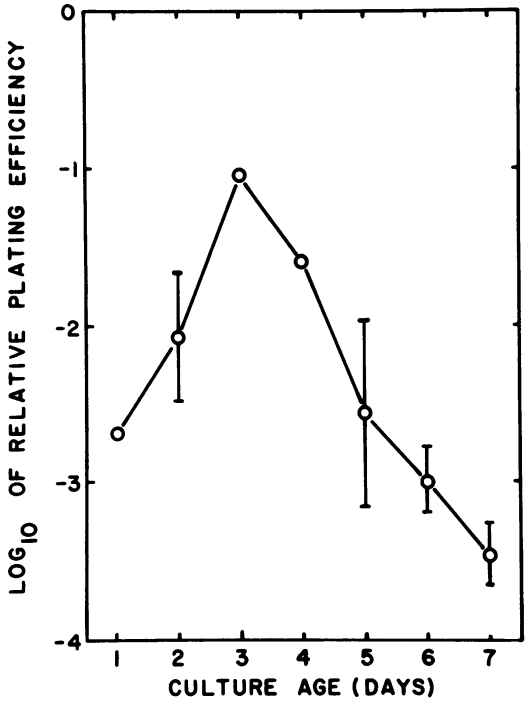

FIG. 2. Effects of $NaClO_3$ (56 mM, MXG medium) on the relative plating efficiency of rose cell aggregates from cultures of different ages. Plating efficiency is expressed relative to controls without $NaClO_3$. Error bars indicate standard errors from five to six experiments; points without error bars represent single determinations. The data in the graph do not take into account four experiments with 5-day cultures and two experiments with 7-day cultures that indicated zero plating efficiency. The timing of "culture age" began when 7-day cultures were diluted 1/10 into fresh MXG medium.

Isolation of Chlorate-resistant Clones. When 5- to 7-day cultures were plated on 56 mM $NaClO_3$ and incubated for more than 2 weeks, the colonies which grew appeared to be uniquely resistant to the effects of the salt. This was because the colonies grew very large (more than 2 mm diam.), whereas the mass of aggregates did not grow at all. The colonies that grew were randomly placed (Fig. 3), suggesting that their growth did not result from localized environmental conditions.

To confirm that the growth of colonies in 56 mM $NaClO_3$ represented resistance, rather than statistical or experimental accident: (*a*) The colonies were transferred to agar containing 67 mM $NaClO_3$ and observed for further growth. In most experiments, 70 to 100% of the colonies continued to grow. In a few experiments, those in which large numbers of colonies had grown, few of the colonies that were transferred continued to grow. Colonies that did not grow generally turned brown. (*b*) The surviving colonies were dispersed in liquid MXG medium (no $NaClO_3$) and allowed to grow through two or more culture cycles (more than seven generations). Suspensions were replated on agar containing 28 and 56 mM $NaClO_3$. Approximately 85% (43/51) of the colonies tested showed increased resistance in this plating assay (Table I). The degree of resistance varied among colonies.

Several strains that were demonstrated to be resistant to $NaClO_3$ were maintained in nonselective liquid culture over a period of 7 to 10 months (more than 120 generations). Most strains retained their resistance (Table I).

Four of the resistant strains that had been cultured for 7 months were reisolated from single colonies growing in plates containing 56 mM $NaClO_3$. As expected the reisolates were resistant to $NaClO_3$ in further plating experiments (Table I). The degree of resistance still varied among the strains. Strain 60-4, for instance, was highly resistant to $NaClO_3$, even at 56 mM; strain 30-5 was marginally resistant to $NaClO_3$, and only at 28 mM. In earlier experiments in which cultures were grown for a few generations (e.g. 10) in the absence of selective agent, the variation in resistance could have occurred because cultures were mixtures containing different proportions of resistant and sensitive cells. It is highly unlikely that the same occurrence would be repeated, especially because a long period of growth in nonselective conditions will tend to lower the proportion of aggregates containing mixtures of resistant and sensitive cells. The conclusion from this experiment is that different selected strains have different degrees of resistance to $NaClO_3$.

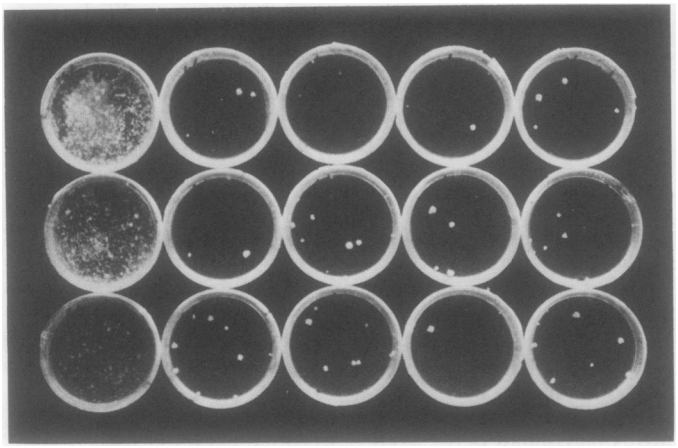

FIG. 3. Growth of rose cell aggregates in MXG agar (left column) and in MXG agar containing 56 mM $NaClO_3$ (right four columns). Cells in the top row were unirradiated; cells in the middle row received 42 J/m^2; cells in the bottom row received 126 J/m^2 of UV light (254 nm). Concentrations of cell aggregates were chosen so that control plates in each row had about 1700 colonies.

Table I. *Stability of the Chlorate-resistance Trait*

The mutants listed were isolated in June, 1978 as discrete colonies on solidified medium containing 56 mM chlorate. They were dispersed and grown in MXG liquid medium, without chlorate, subculturing by 1:10 dilution into fresh medium every 7 days.

Strain	Chlorate in Test	Test Date			
		8-29-78	3-9-79	5-4-79	6-6-79
	mM				
Parental	28	0.058[a]	0.032		0.052
	56	0.0046	0.030		0.0078
0-1	28	0.20	0.67	0.64[b]	
	56	0.061	0.23	0.31[b]	
10-2	28	0.49	0.78		
	56	0.062	0.24		
10-6	28	1.03	0.18	0.85[b]	
	56	0.74	0.16	0.22[b]	
30-5	28	0.16	0.13	0.24[b]	0.46[b]
	56	0.071	0.0086	0.0015[b]	0.0006[b]
30-6	28	0.96	0.36		1.7
	56	0.69	0.30		0.76
60-4	28	1.4	0.63		1.0[b]
	56	0.82	0.18		1.0[b]

[a] All data represent plating efficiencies relative to zero-chlorate controls.
[b] Reisolate from single colony on solid medium containing 56 mM chlorate.

The chlorate resistance of three selected strains, out of four tested, was also expressed when cells were assayed for viability with FDA (data not shown). A 24-h treatment with 10 mM $NaClO_3$ in liquid medium significantly lowered the viability of the parental strain and one selected strain, 4-17-0-2. The other selected strains (30-6, 4-17-0-1, 4-17-0-4) showed less severe responses to the $NaClO_3$: in each case the viability of $NaClO_3$-treated suspension was not significantly different from that of the control incubated without chlorate.

Induction of Chlorate Resistance by UV Light. Treatments with UV light were tested for their ability to induce resistance to $NaClO_3$ in the plating assay. The motivation for these experiments was 2-fold. First, we wished to determine whether these treatments, known to be mutagenic in microorganisms (5) and in a variety of differentiated plant cells (2, 20), were also mutagenic in cultured plant cells. Second, by applying the test to the chlorate system, we wished to obtain evidence that the acquisition of chlorate resistance was in fact a mutational event.

The first treatment involved far-UV light (254 nm). Samples of cell suspension irradiated with far-UV light showed a dose-dependent loss of colony forming ability (Fig. 4A). Illumination of plates with white fluorescent light for 48 h after plating reduced the inactivating effect of the UV light, indicating a photorepair of UV damage. The effect of the white light was greater for low doses of UV light than for high doses. Since the best-known photorepair system works through DNA photolyase (7), which splits pyrimidine dimers in DNA, the results suggest that UV is damaging DNA by inducing the formation of pyrimidine dimers, and that the UV treatment is thus potentially mutagenic.

Samples were irradiated with 14, 42, 84, 126, or 252 J/m^2 before plating on $NaClO_3$. The fraction of aggregates resistant to $NaClO_3$ appeared to rise with all UV doses, except 252 J/m^2 (Table II). However, it was highest with 42 J/m^2. At this dose, the increase in the frequency of resistance averaged 3-fold; in nine experiments, it ranged from 1.8- to 12-fold.

The second treatment involved near-UV light (360 nm) in conjunction with a photosensitizing agent, MOMT. MOMT is a psoralen; psoralens intercalate between DNA base pairs, and in the presence of UV light they link covalently to pyrimidines. UV/

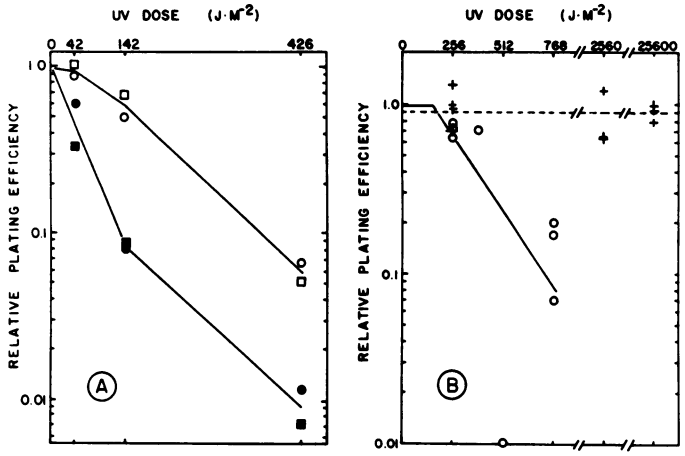

FIG. 4. Effects of UV radiation treatments on the relative plating efficiency of rose cell aggregates. (A), Far UV light (254 nm). (●▲), plates incubated in the dark. (○□), plates incubated in fluorescent light for 48 h then dark. Different symbols (○●, □■) represent different experiments. (B), Near UV light (black light). (○), MOMT (5 μg/ml) added before irradiation. (+), no MOMT. Plating efficiencies are expressed relative to unirradiated controls.

Table II. *Frequency of $NaClO_3$-resistant Plating Units in Suspensions of R. damascena Aggregates Irradiated with Far-UV (254 nm) Light or MOMT plus near-UV light*

UV dose	No. Experiments	No. Viable Aggregates[a]	No. Resistants[b]	Frequency of Resistance[c]
J/m^2				
		Far UV		
0	14	2.10×10^5	196	9.3×10^{-4}
14	4	2.33×10^4	37	16×10^{-4}
42	9	5.55×10^4	157	28×10^{-4}[d]
84	4	2.32×10^4	31	13×10^{-4}
126	7	3.97×10^4	77	20×10^{-4}
252	1	4.08×10^3	0	$<2.4 \times 10^{-4}$
		MOMT (5 μg/ml), Near-UV		
0	5	2.84×10^4	5	1.76×10^{-4}
256	3	2.77×10^4	3	1.04×10^{-4}
383	3	3.14×10^4	16	5.09×10^{-4}
511	2	4.71×10^4	0	$<2.1 \times 10^{-5}$
767	4	2.22×10^4	0	$<4.5 \times 10^{-5}$

[a] The numbers of "viable aggregates" were estimated by growth on plates without $NaClO_3$.

[b] "Resistants" grew on plates containing 56 mM $NaClO_3$ and, when transferred, on plates containing 67 mM $NaClO_3$.

[c] Relative SE for "frequency of resistance" was about 30%.

[d] Value differs from 0 UV value at the 1.0% level of confidence.

psoralen treatments are known to be mutagenic in bacterial systems (16). The UV/psoralen treatment killed rose cell aggregates at 767 J/m² though not at 256 J/m² (Fig. 4B). Treatments with UV light alone, or with MOMT alone, did not kill the cells.

The UV/MOMT treatment increased the number of chlorate-resistant aggregates observed in rose cell suspensions only at 383 J/m² (Table II). The highest stimulation was by a factor of 3. In this series of five experiments, the frequency of chlorate-resistant colonies appearing in unirradiated suspensions was lower than observed before. We do not know the reason for this variation, but it was not a matter of culture age.

The increase in the frequency of resistance associated with the UV treatments could represent an induction of newly resistant cells or a selection for resistance. The following reconstruction experiment was designed to test the possibility that chlorate-resistant cells were UV-resistant and were thus selected by the radiation treatment. Samples of parental (sensitive) and a resistant strain were mixed, irradiated with UV light (42 J/m², 254 nm), and plated in agar with, and without 56 mM $NaClO_3$. The results were compared to those obtained with the unmixed strains, irradiated and plated in the same way. In two experiments, there were increases in the numbers of chlorate-resistant colonies over those expected (the amounts of increase were 20 and 60%); in two experiments, the numbers of chlorate-resistant colonies observed equaled those expected; in one experiment, the number of chlorate-resistant colonies observed was less (by 55%) than that expected. We conclude that the far-UV treatment did not systematically select for chlorate resistance.

The idea that 42 J/m² of far-UV light induced the formation of new resistant cells is supported by the observation that in five of the nine experiments the fraction of the total number of aggregates, as well as of the viable aggregates, that showed chlorate resistance was higher in irradiated suspensions than in controls.

If UV was acting as a mutagenic agent in these cells, it showed some unusual characteristics. First was the sharp optimum in the dose response relationship; second was the relative ineffectiveness of the optimum treatment, yielding no more than an average 3-fold increase in the frequency of resistance (Table II). Studies with bacteria and fungi generally show dose response relationships in which the proportion of mutants per viable cell increases, then plateaus, with increasing UV dose (5) though some slight decreases have been observed with very high doses (survival 10^{-5}) (25); in these experiments, 100-fold increases in mutant frequency are not uncommon. In higher plant cells, the decrease in the proportion of chlorate-resistant aggregates per viable aggregate observed with superoptimal UV doses could reflect the sensitivity of DNA repair processes to high doses of UV, demonstrated in carrot protoplasts (9). Thus, even the unusual aspects of UV effects on the frequency of chlorate resistance are consistent with the idea that UV acts as a mutagen in this system.

Do the Chlorate-resistant Cells Lack Nitrate Reductase? Because chlorate resistance in other organisms has also been associated with loss of nitrate reductase, we initially expected that resistance to chlorate in rose cells would also be related to nitrate reductase. One indirect test of our expectation involved an attempt to select against chlorate-resistant cells. We reasoned that cells which lacked nitrate reductase would be selected against in medium which contains nitrate as sole nitrogen source (MX2). If a major fraction of chlorate-resistant cells lacked nitrate reductase, then the property of chlorate resistance should occur less frequently in MX2-grown cultures. MX2-grown suspensions were compared to MXG (nitrate plus glutamate)-grown suspensions in the plating assay (56 mM $NaClO_3$ in MXG/agar). The results of five out of six experiments did not support the idea that growth in MX2 selects against chlorate resistance. Overall, the frequency of chlorate resistance was greater in MX2-grown suspensions than in MXG-grown suspensions by a factor of 2.7. It may have been that the lack of reduced nitrogen (glutamate) tended to induce, or select for, some function that led to chlorate resistance. We presume that the function would not involve the lack of nitrate reductase, since cells growing in MX2 need nitrate reductase.

We also tested the abilities of rose cell strains, selected for chlorate resistance, to grow on MX2 medium and to reduce nitrate. Figure 5 shows the results of growth tests performed on the parental strain and on five chlorate-resistant strains. Over the 7-day growth period, the parental strain grew 75% as much in MX2 as in MXG medium; the amount of growth in MX2 medium remained constant over three successive weekly transfers. The strains that had been selected for chlorate resistance fell into two groups. One group showed growth in MX2 similar to that found in the parental strain. The other group grew less in MX2 and showed a trend toward progressively less growth in three succes-

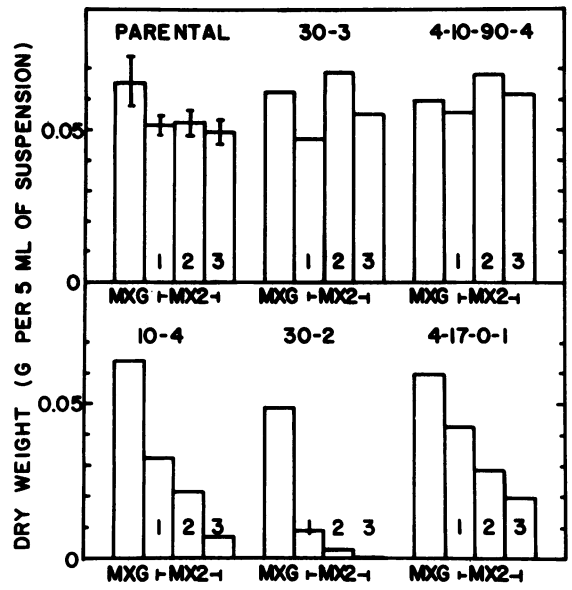

FIG. 5. Growth of the parental strain and five strains selected for chlorate resistance on MXG medium (nitrate, glutamate) and MX2 medium (nitrate as sole nitrogen source). Ordinate gives dry weight after 7 days growth in liquid suspension: zero time values were 0.006 ± 10%. Error bars for the parental strain indicate standard errors from eight experiments. The three bars labeled MX2 represent three successive weeks of culture: MX2-1 was inoculated from MXG; MX2-2, from MX2-1; MX2-3, from MX2-2.

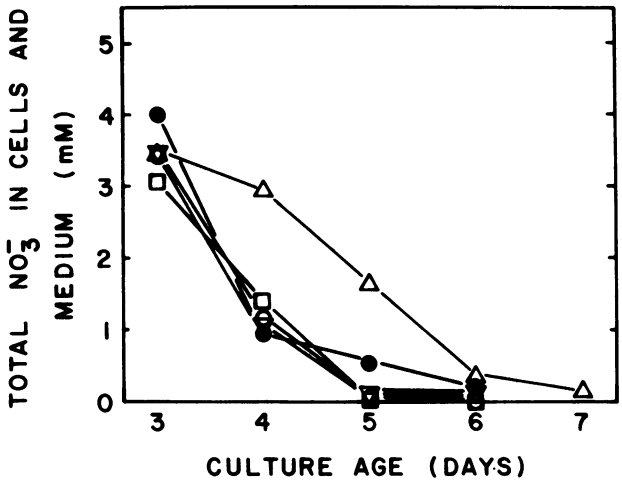

FIG. 6. Nitrate reduction by suspension cultures of the parental strain (●) and four strains selected for resistance to chlorate, BI (○), CI (□), OH (△), 4-17-0-4 (▽). Strains were inoculated into MXG medium (5 mM NO_3^-) on day zero.

sive weekly transfers. Among 53 strains tested as described above, 45 fell into the first group and eight fell into the second group. Thus, the majority of chlorate-resistant strains had the ability to take up and reduce enough NO_3^- for nearly normal growth.

To confirm that strains which grew on MX2 were actually reducing a significant amount of nitrate, we measured *in vivo* nitrate reduction in seven strains, all of the first group, and in the parental strain. Nitrate reduction was estimated by the loss of nitrate from medium plus cell extract. Reduction of nitrate occurred from day 3 to day 6 (Fig. 6) with some variation among strains and experiments. The amount of total nitrate removed from the system was over 90%. Extracts of 5-day cultures of the parental strain and four resistant strains were assayed for nitrate reductase *in vitro*. Specific activities in the resistant strains were low (strain BI, 9.7 nmol/mg protein·h; CI, 11.5; OH, 3.5; 4-17-0-4, 4.6) but higher than was found in parental cells (<0.7 nmol/mg protein·h).

The fact that chlorate-resistant strains of rose grow in nitrate medium and can be shown to reduce nitrate does not mean that these strains produce chlorite. The strains could fail to take up chlorate, or their nitrate reductases could lack chlorate-reducing activity. Alternatively, the strains could produce toxic amounts of chlorate-reduction products, but tolerate these products in some way. It proved difficult to investigate chlorate uptake and reduction by direct assays. The amount of chlorate removed from medium by cells of the parental strain proved to be too small to measure, even during the period when most of the nitrate in the medium was taken up. Likewise, the low nitrate reductase activity observed *in vitro* suggested that chlorate reductase activity would not be measurable.

An indirect assay for chlorate uptake and reduction was devised following observations that chlorite and hypochlorite, but not chlorate, oxidize the heme groups of horseradish peroxidase. We speculated that the same effect would hold for a related enzyme, catalase, inside the rose cells. Figure 7 shows inactivation of catalase in crude rose-cell extracts by $NaClO_3$, $NaClO_2$, and NaClO. Catalase activity was inactivated 50% or more by 3×10^{-4} M $NaClO_2$ or NaClO, but only 35% by 3×10^{-2} M $NaClO_3$. These results matched the loss of heme absorption (400 nm) from peroxidase under the same conditions. At room temperature, the inactivation went to completion in 10 to 20 min. Untreated control extracts were stable, retaining 85–95% activity over 2 h.

When the cells of parental strain were treated with 10 mM $NaClO_3$ for 24 h (from 4 to 5 days of culture), the specific activity of catalase appearing in a crude-extract was reduced to 11% of the control value (Fig. 8). A similar result was found for three strains, 30-6, 4-17-0-2, and 4-17-0-4, which were able to grow on MX2 medium. The data suggest that these strains, which possess nitrate-reducing activity, also take up and reduce chlorate to a form which inactivates the intracellular enzyme, catalase. In contrast, the loss of catalase was not statistically significant in a fourth chlorate-resistant strain, 4-17-0-1, which showed reduced growth on MX2 medium (Fig. 5) and was thought on that basis to lack nitrate reductase activity.

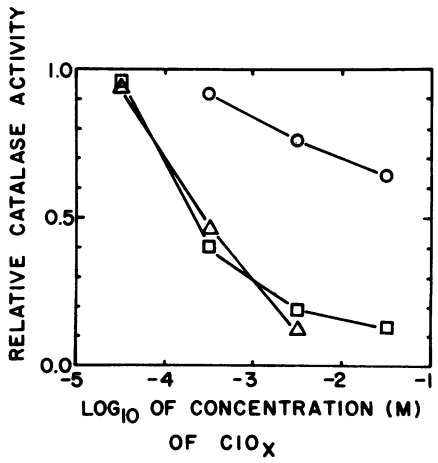

FIG. 7. Effect of sodium salts of chlorate (○), chlorite (□), and hypochlorite (△) on the *in vitro* activity of catalase. Catalase was assayed as a loss of H_2O_2 (A_{240}) catalyzed by crude extracts of parental strain rose cells. The ordinate gives the fraction of activity remaining 20 min after the addition of the indicated concentration of salt. No addition, or the addition of water, had less than a 3% effect. All points represent means of three experiments.

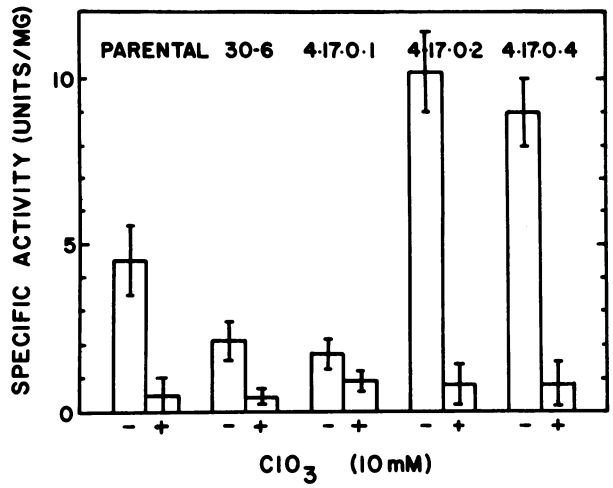

FIG. 8. Effect of sodium chlorate on the *in vivo* catalase activity of the parental strain and four strains selected for chlorate resistance. Chlorate (10 mM) was added to 4-day-old cultures. Samples of the 4-day-old cultures and of 5-day-old cultures were extracted and assayed for catalase activity and protein concentration. (−), Control, average of 4-day and untreated 5-day samples; error bars indicate standard errors from seven experiments. (+), Chlorate-treated 5-day samples; error bars indicate standard errors from two experiments.

DISCUSSION

We believe that the balance of information suggests that the chlorate-resistant strains which we isolated and maintained were genetic, rather than nongenetic, variants of the parental population. The evidence in support of this assertion includes: (*a*) stability in the absence of selection (Table I); (*b*) increase in frequency of resistance caused by UV, a known mutagen (Table II); (*c*) random variation in the frequency of variants in different unselected cultures (*e.g.* Table II); (*d*) variation among different strains of selected cells in their degrees of chlorate resistance (Table I) and types of chlorate resistance (Fig. 5). However, epigenetic or physiological changes can also lead at least to a short term chlorate resistance. This is shown by the variation in frequency of resistance as a function of culture age (Fig. 2). Not only was the frequency at 3 to 4 days very high, but the decrease that occurred over the period between 3 and 7 days cannot be explained by a dilution of mutant cells. Thus, the peak of resistance observed at 3 to 4 days of culture represented a reversible physiological change in the cells. The possibility of such a change casts doubt on the nature of resistance that appeared at the other times. In the absence of direct evidence, the genetic basis of chlorate resistance cannot be considered as proven. A demonstration of chlorate resistance in plants regenerated from our strains and attempts at formal genetic analysis of the trait must wait for the development of techniques for regeneration of plants from rose callus.

The study of chlorate-resistant strains has provided useful information about the mechanism of chlorate toxicity. The dependence of chlorate toxicity on intact nitrate reductase genes has been supported by the observation of many independent instances in which cells of species of *Nostoc*, *Escherichia*, *Aspergillus*, *Nicotiana*, and *Chlamydomonas* simultaneously become chlorate-resistant and nitrate-nonutilizing. The existence of strains, resistant to chlorate, which lack the ability to grow on medium in which the sole nitrogen source is nitrate ion, suggests that in rose, as in other species, the toxicity of chlorate depends on the presence of nitrate reductase.

In light of Müller and Grafe's report (11), it is surprising that most of our strains that were resistant to chlorate retained full ability to grow on a medium in which the sole nitrogen source was nitrate. There are two general strategies by which such strains might have developed their resistance. One strategy is avoidance of toxic substances, through limiting uptake of chlorate, sequestration of chlorate or its reduction products in the vacuole, or chemical detoxification of the reduction products. A second strategy is tolerance of toxic substances through protection of specific, sensitive cell components, or induced independence of sensitive cell components. Experiments using catalase as an intracellular indicator for the presence of toxic chlorate reduction products suggested that three strains adopted the second strategy. In these strains, catalase was inactivated (Fig. 8) by a chlorate treatment which had little or no effect on the viability of the cells. Since these cells did not die, the loss of catalase did not depend on cell death. *In vitro* experiments (Fig. 7) suggested that the loss observed *in vivo* (Fig. 8) resulted from contact of catalase with chlorite or hypochlorite. Thus, these strains tolerated, rather than avoided, the toxic reduction products of chlorate.

The mechanism by which chlorite or hypochlorite kills cells is still unknown. One might predict that substances such as chlorite and hypochlorite might inactivate many essential cell components simultaneously. However, the fact that certain cells can tolerate treatments implies that the lethal damage must be reasonably specific. The tolerant strains should be very useful in identifying the sensitive components, and in determining the mechanism of tolerance.

Acknowledgments—Judith Murphy, Elizabeth Hess, Carol Hamilton, Kathy Bishop, and Clyde Wilson provided excellent technical assistance at various stages of this study. Dr. Steven Morrison helped us with the nitrate analyses. The work was begun at the Botanical Laboratories, University of Leicester, with the advice and encouragement of Professor H. E. Street, now deceased.

LITERATURE CITED

1. BRIAN RC 1976 The history and classification of herbicides. *In* LJ Andus, ed, Herbicides: Physiology, Biochemistry, Ecology, Ed 2 Vol 1 Chap 1. Academic Press, London, pp 1–37
2. CAMPBELL WF 1975 Effects of enhanced UV-B radiation on *Trandescantia* inflorescences. *In* AJ Grobecker, ed, Impacts of Climatic Change on the Biosphere, Part 1 Chap 4 Appendix P. CIAP Monograph 5. Department of Transportation, Washington, DC, pp 4–279 to 4–287
3. COVE DJ 1976 Chlorate toxicity in *Aspergillus nidulans*: the selection and characterization of chlorate resistant mutants. Heredity 36: 191–203
4. DECKER LA, ed 1977 Worthington Enzyme Manual. Worthington Biochemical Corp, Freehold, NJ, pp 63–65
5. DOUDNEY CO 1976 Mutation in ultraviolet light-damaged microorganisms. *In* SY Wang, ed, Photochemistry and Photobiology of Nucleic Acids, Vol 2, Biology. Academic Press, New York, pp 309–374
6. FORGET P 1974 The bacterial nitrate reductases: solubilization, purification, and properties of the enzyme A of *Escherichia coli* K12. Eur J Biochem 42: 325–332
7. HARM H 1976 Repair of UV-irradiated biological systems: photoreactivation. *In* SY Wang, ed, Photochemistry and Photobiology of Nucleic Acids, Vol 2, Biology. Academic Press, New York, pp 219–263
8. HARVEY RB 1931 The action of toxic agents used in the eradication of noxious plants. J Am Soc Agron 23: 481–489
9. HOWLAND GP 1975 Dark-repair of ultraviolet-induced pyrimidine dimers in the DNA of wild carrot protoplasts. Nature 254: 160–161
10. KUO T, RL WARNER, A KLEINHOFS 1980 Stabilization of extractable nitrate reductase from barley leaves. Plant Physiol 65: S-31
11. MÜLLER AJ, R GRAFE 1978 Isolation and characterization of cell lines of *Nicotiana tabacum* lacking nitrate reductase. Mol Gen Genet 161: 67–76
12. MURPHY TM, CM HAMILTON, HE STREET 1979 A strain of *Rosa damascena* cultured cells resistant to ultraviolet light. Plant Physiol 64: 936–941
13. NELLER JR 1931 Effect of chlorates upon the catalase activity of the roots of bindweed. J Agr Res 43: 183–189.
14. NASH DT, ME DAVIES 1972 Some aspects of growth and metabolism of Paul's scarlet rose cell suspensions. J Exp Bot 23: 75–91
15. PUIG J, E AZOULAY, F PICHINOTY 1967 Étude génétique d'une mutation a effet pléiotrope chez l'*Escherichia coli* K12. CR Acad Sci Paris 264D: 1507–1509
16. SEKI T, K NOZU, S KONDO 1978 Differential causes of mutation and killing in *Escherichia coli* after psoralen plus light treatment: monoadducts and cross-links. Photochem Photobiol 27: 19–24
17. SINGH HN, KC SONIE 1977 Isolation and characterization of chlorate-resistant mutants of the blue-green alga *Nostoc muscorum*. Mut Res 43: 205–213
18. SOSA FM, T ORTEGA, JL BAREA 1978 Mutants from *Chlamydomonas reinhardii* affected in their nitrate assimilation capability. Plant Sci Lett 11: 51–58
19. STREET HE, ed 1977 Plant Tissue and Cell Culture. Univ of California Press,

Berkeley, p 93
20. SWANSON CP, LJ STADLER 1955 The effect of ultraviolet radiation on the genes and chromosomes of higher organisms. *In* A Hollaender, ed, Radiation Biology, Vol 2. McGraw-Hill, New York, pp 249–284
21. THAYER JR, RC HUFFAKER 1980 Determination of nitrate and nitrite by high-pressure liquid chromatography: comparison with other methods for nitrate determination. Anal Biochem 102: 110–119
22. WHITE RG 1965 Handbook of Ultraviolet Methods. Plenum Publishing Co, New York, p 227
23. WITHERS LA, HE STREET 1977 Freeze preservation of cultured plant cells. III. The pregrowth phase. Physiol Plant 39: 171–178
24. WRIGHT LA, JR, TM MURPHY 1975 Photoreactivation of nitrate reductase production in *Nicotiana tabacum* var. *Xanthi*. Biochim Biophys Acta 407: 338–346
25. ZELLE, MR, A HOLLAENDER 1955 Effects of radiation on bacteria. *In* A Hollaender ed, Radiation Biology, Vol 2. McGraw-Hill, New York, pp 365–430