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EVALUATION OF CHLORATE AS A TOOL FOR STUDYING NITRATE ASSIMILATION IN PLANTS

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ABSTRACT

EVALUATION OF CHLORATE AS A TOOL FOR STUDYING NITRATE ASSIMILATION IN PLANTS

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The absence of a long-lived radioisotope of nitrogen is the major obstacle to advances in understanding nitrogen transport in plants. Chlorate behaves as an analogue of nitrate in the nitrate reductase (NR) reaction, but its role as a competitor with nitrate at the level of absorption remains obscure. The objective of this study was to evaluate ^{36}Cl-chlorate as a nitrate analogue in transport studies with the XD strain of cultured tobacco cells (Nicotiana tabacum L. cv. Xanthi) grown on nitrate, or an efficient urea-assimilating variant (14U cells) grown on urea.

Chlorate uptake by 14U cells was pH dependent with a maximum at pH 5.5. 14U cells concentrated chlorate against a gradient at pH 5.5 but not at pH 6.5. Chlorate did not induce a chlorate uptake system. The rate of chlorate uptake increased with chlorate concentration. Kinetic analysis revealed both a saturable uptake system, functioning below 1 mM chorate and a linear system, responsible for rate increases at chlorate concentrations above 1 mM. The former system is shared with nitrate, while the latter is shared with chloride but not with nitrate. The nitrate-inducible nitrate transport system appears to be distinct from the high affinity chlorate transport system, in that

nitrate transport is insensitive to chlorate, and chlorate transport is constitutive. It was concluded that chlorate is not an ideal analogue of nitrate for transport studies in tobacco cells.

Tobacco cells' NR could not discriminate between chlorate and nitrate. Their mutual inhibition at the level of reduction was competitive. The affinity of the enzyme for nitrate was an order of magnitude greater than that for chlorate. Chlorite was undetectable as a product of chlorate reduction. Its apparently rapid conversion to chloride may explain the intrinsic chlorate tolerance of XD cells. Chlorate, unlike nitrate, could not induce NR activity.

Nitrate was antidotal to chlorate toxicity in tomato seedlings. Unexpectedly, these plants were more chlorate tolerant when grown on nitrate than on any source of reduced nitrogen. Alternative nitrogen sources had dramatic effects on tomato seedling root development. γ-Amino butyric acid in particular elicited a light dependent periodicity of root hair position which was not abolished by concomitant feeding of additional nitrogen compounds.

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3-APSA	3-amino propane sulfonic acid
AS	ammonium succinate
BA	6-Bencylaminopurine
CCCP	Carbonyl cyanide, m-chlorophenyl- hydrazone
Cl-less N-less M1D	N-less M1D in which the chlorides of calcium and potassium had been substituted by equimolar amounts of calciumsulfate and potassium phosphate respectively
2,4-D	2,4-dichlorophenoxyacetic acid
EGTA	Ethyleneglycol-bis-(β-amino-ethyl ether) N,N'-tetra-acetic acid
GABA	γ-amino butyric acid
HEPES	N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid
IAA	Indole-3-acetic acid
I_{50}	The concentration resulting in a 50% reduction in growth rate with respect to the control
K_i	Dissociation constant
Km	Michaelis constant, substrate concentration at which velocity is half-maximal
M1D	A modification of White's medium in which nitrate is the sole nitrogen source
MES	2(N-morpholino) ethanesulfonic acid
NADH	Nicotinamide adenine dinucleotide,reduced form
N-less M1D	M1D medium in which the nitrates of calcium and potassium have been replaced by equimolar amounts of their respective chlorides
N-less T_{12} medium	T_{12} medium in which the nitrates of ammonium and potassium have been replaced by equimolar amounts of KCl
NR	Nitrate reductase
S	Substrate
[S]	Substrate concentration

T_{12} medium	Culture medium consisting of the major salts of Murashige and Skoog supplemented with Nitsch's vitamins, hormones (IAA, 2, 4-D and BA), inositol and sucrose
TLC	Thin layer chromatography
14U	Urea-assimilating variant strain of XD cells
Urea-M1D	N-less M1D medium to which a filter-sterilized urea solution was added to give a final urea concentration of 3 mM
v	velocity
Vmax	maximum velocity
XD	Strain of cultured tobacco cells (*Nicotiana tabacum* L. cv. Xanthi)

INTRODUCTION

Soil nitrogen is comprised of two distinct pools with regard to its availability to higher plants: a large inaccessible pool of organic compounds and a small readily accessible pool of inorganic forms (Scarsbrook, 1965). The latter is in continuous flux as soil microorganisms generate accessible nitrogen from atmospheric nitrogen by nitrogen fixation, or from the relatively inaccessible pool by mineralization (ammonification and nitrification). Simultaneously, nitrogen is removed from the relatively accessible pool by leaching and by assimilation and denitrification by plants, animals and bacteria (Harmsen *et al.*, 1965). The steady-state concentration of accessible nitrogen in the soil is determined by its exchange between these sources and sinks.

The steady-state concentration of accessible nitrogen of any given field decreases with cultivation since the nitrogen fixation rate in the soil is insufficient to replenish the nitrogen which is removed annually by harvesting. After several years of cultivation, nitrogen fertilizer must be applied to maintain soil fertility at a level allowing adequate crop productivity. It can thus be inferred that,in general, current crop cultivars do not take up nitrogen as rapidly from low steady-state concentrations of accessible nitrogen in unfertilized, intensively cultivated fields as they can from the high concentrations in fertilized fields. One approach to overcoming

this problem, besides adding increasingly costly nitrogen fertilizer to cultivated soils each year, is to increase the efficiency with which crop plants take up nitrogen at low concentrations of accessible nitrogen. Because nitrogen fixation is inhibited by high concentrations of accessible nitrogen, the lower steady-state concentration would have the important benefit of increasing the rate of natural replenishment of soil nitrogen.

Inorganic nitrogen in most arable soils is mostly found in the nitrate form, regardless of past fertilization practices. Soil ammonium from whatever source is rapidly oxidized to nitrate, in most soils, by nitrifying bacteria. Accordingly, nitrate is the most important source of available nitrogen for cultivated plants. Since nitrate is anionic, it is not bound by the anionic soil clays. Thus, the total supply of nitrate in the root zone is available (Scarsbrook,1965), though subject to leaching losses.

Nitrate nitrogen is incorporated into plant cell constituents by the following pathway:

$$NO_3^-(\text{outside}) \xrightarrow{\text{Nitrate Uptake}} NO_3^-(\text{inside}) \xrightarrow{\text{Nitrate Reductase}} NO_2^- \xrightarrow{\text{Nitrite Reductase}} NH_4^+ \longrightarrow \text{AMINO ACIDS}$$

Nitrate reductase is thought to be the rate-limiting enzyme in the pathway (Schrader, 1978). This assumption is based on the observation that plants and cultured plant cells can accumulate nitrate considerably in excess (50-100x) of the ambient concentration (Heimer and Filner, 1971; Jackson *et al.*,1976) but do not accumulate nitrite. If nitrate reductase were not highly regulated, *i.e.*, if the amount of enzyme were independent of the environment, the accumulation of nitrate, and lack of accumulation of nitrite would be strong evidence that the enzyme is rate-limiting in the pathway. Alternatively, the enzyme might be actively modulated by regulatory

mechanisms, so as to adjust nitrate reductase amounts or activities to achieve reaction rates compatible with the plants' overall ability to use the nitrogen in nitrate. That is, the rate at which nitrogen is assimilated may be the cause rather than the effect of a given level of nitrate reductase activity. This model is supported by the observation that added glucose enhances the level of nitrate reductase without affecting the total nitrate in corn root tissues (Aslam and Oaks, 1975). These results were attributed to a redistribution of root nitrate, resulting in a greater portion being present in the metabolic pool, thus enhancing nitrate reductase activity (Aslam and Oaks, 1975). Therefore the concentration of nitrate in the metabolic pool, which is primarily determined by the rate of nitrate uptake, could be the limiting factor in nitrogen assimilation. Chantarotwong and coworkers (1976) and Eisele and Ullrich (1977) have also concluded that, under normal conditions, the rate of *in vivo* nitrate reduction is more a function of the rate of nitrate uptake than of the amount of nitrate reductase present in the cell or tissue.

Consequently, it is not unreasonable to propose that plants capable of taking up nitrate more efficiently, from soils containing low steady state concentrations of accessible nitrogen, may be able to grow more rapidly than less efficient absorbers of nitrate under such suboptimal conditions. Therefore, it is important to examine the characteristics and regulation of nitrate uptake in order to evaluate the feasibility of selecting these more efficient nitrate absorbers.

Experiments on the mechanism of absorption of nitrate and ammonium ions have been less extensive and less conclusive than experiments on assimilation of most other macronutrient ions (Carter and Lathwell, 1967; Furner and Sung, 1982; Kochian and Lucas, 1982; Nissen, Schiff and Hodson, 1973; 1973; Siegel, 1977; Smith, 1975, 1980). At first glance, this seems

surprising in view of the great importance of nitrogen in soil fertility and the plant nutrition. For the investigation of ion absorption by plant tissues, radioisotopes have been indispensible because of the ease and extreme sensitivity of radioassay methods and because they provide a means of distinguishing among different pools and fluxes of the same element. However, there simply isn't a generally useful radioisotope of nitrogen. Because ^{13}N has a half-life of only 10.05 minutes, one must have access to a nuclear reactor or particle accelerator in which ^{13}N can be produced, and one is limited to rather short-term experiments. As a result, experimenters have more often used the stable isotope ^{15}N, the assay of which, by mass spectrometry, is more cumbersome, expensive and difficult to adapt to high throughputs of samples, and less sensitive than most radioassay procedures. Still more often, researchers have used chemical methods of analysis which limit them to following depletion or accumulation of chemical amounts. These methods also do not approach the radioassay in ease and sensitivity, especially for short-term experiments.

Another special aspect of absorption of nitrate ions is inherent in the biology of the process and its metabolic consequences. Other mineral nutrients such as phosphorus and sulfur, are incorporated into organic metabolites, but none equal nitrogen in the extent, variety and complex consequences of its transformations. For this reason, absorption of the nitrate ion does not necessarily lead to accumulation of the ion within the cells.

Experimentally, reduction of nitrate can be inhibited by means of tungstate. Incorporation of tungstate, rather than molybdate, into the nitrate reductase molecule renders it non-functional for reduction, and tungstate treatments often result in an increase in nitrate accumulation by the tissue(Chantarotwong *et al.*, 1976; Eisele and Ulrich, 1977; Flores and

Guerrero, 1980a; Goldsmith *et al.*, 1973;Heimer *et al.*, 1969; Heimer and Filner, 1971; Jackson *et al.*, 1973; Minotti and Jackson, 1970; Radin, 1975; Rao and Rains, 1976a; Schloemer and garret, 1974; Yoder and Scheffer, 1973). There are instances, however, where tungstate treatment decreased the accumulation of nitrate (Heimer *et al.*, 1969; Rao and Rains, 1976b). Moreover, even when increased accumulation does occur, the uptake process may still be restricted (Jackson, 1978; Rao and Rains, 1976b); it can only be concluded that uptake is not as restricted as is reduction.

Use of tungstate to separate nitrate uptake from nitrate reduction may also be complicated by indirect effects. Decapitated corn seedlings grown in the absence of nitrate, upon exposure to nitrate, showed the typical pattern of apparent induction (Ezeta and Jackson, 1975; Jackson *et al.*, 1973). The presence of tungstate in the uptake solution resulted in the initial slow phase of uptake being extended. It also slightly lowered the rate after the accelerated phase had been initiated. It clearly cannot be assumed that tungstate affects only one component (reduction) of the nitrate assimilation pathway in root tissue (Jackson, 1978). Tungstate may at times restrict nitrate uptake (perhaps indirectly), but it seldom seems to abolish it completely.

In spite of these limitations, valuable information about the nitrate uptake systems of higher plants has still been obtained. Roots of whole plants and plant cells previously grown in the absence of nitrate exhibit negligible or low uptake rates upon first exposure to nitrate; the rate increases steadily, reaching a constant rate about 5 times greater than the initial rate. This suggests that the nitrate transport across the plasmalemma of the root cells is induced by nitrate, the substrate for the transport process. This pattern is in contrast to that commonly observed for uptake of other essential elements by

root tissue. Such systems are ordinarily characterized by relatively linear rates. The increase in activity of the nitrate transport system in response to the substrate, nitrate, was discovered by Heimer and Filner (1971) in a study done on the XD strain of cultured tobacco cells. Since then, induction of the nitrate uptake system by nitrate has been demonstrated in corn (Ezeta and Jackson, 1975; Jackson *et al.*, 1973; Neyra and Hageman, 1975) barley (Chantarotwong *et al.*, 1976; Rao and Rains, 1976a), cotton and tobacco (Jackson *et al.*, 1972), *Wolffii arrhiza* (Swader *et al.*, 1975) dwarf bean (Breteler, 1979), cell suspension cultures of tobacco (Behrend and Mateles,1975), *Penicillium chrysogenum* (Goldsmith *et al.*, 1973), *Neurospora crassa* (Schloemer and Garret, 1974), and *Lemna gibba* (Ullrich *et al.*, 1981). The nitrate transport system of cultured tobacco cells was totally absent from cells grown on urea (Heimer and Filner, 1971). Breteler *et al.* (1979) found that, in dwarf bean plants, started nitrate uptake at about 30 to 40% of the fully induced rate immediately after addition of nitrate to the medium. The preexisting nitrate uptake was attributed to either constitutive uptake or to the physico-chemical processes of diffusion and adsorption into the apparent free space.

The nitrate uptake system is highly specific for nitrate and is not induced by other anions (*e. g.* sulfate, chloride, phosphate, borate or molybdate) at concentrations commonly employed in standard nutrient solutions. Chlorate does not induce the nitrate transport system either (Jackson,1978). However, it is not at all certain that nitrate is the only inducer; nitrite will induce nitrate transport in tobacco cell suspension cultures (Heimer, 1975).

Inhibitors of RNA and protein synthesis restrict development of the accelerated uptake phase in higher plants (Ezeta and Jackson, 1975; Jackson,

1975; Jackson *et al.*, 1973; Neyra and Hageman, 1975; Rao and Rains, 1976a) the fungi *Neurospora crassa* (Schloemer and Garret, 1974) and *Penicillium chrysogenum* (Goldsmith *et al.*, 1973). This is suggestive of an inducible transport system which turns over and which requires continual protein synthesis to sustain maximal transport rates. However, these metabolic inhibitors have multiple effects on the components of the nitrate metabolic pathway and detrimental effects on the transport of other ions which do not show the apparent induction uptake pattern (Jackson, 1978). Therefore, the use of these compounds does not give unequivocal evidence for synthesis of a specific proteinaceous component of the nitrate transport system during the apparent induction period. Consequently it is not possible at this time to conclude with certainty that the increasing nitrate uptake rate observed in root tissues or cultured cells upon first exposure to nitrate represents a true induction phenomenon, rather than the enhanced activity of a preexisting system.

Plant cells maintain a negative electrical potential between their cytoplasm and the ambient medium (Higinbotham, 1973) but they can accumulate nitrate considerably in excess (50-100x) of the ambient concentration (Heimer and Filner, 1971; Jackson *et al.*, 1976b). Transport of nitrate against a strong electrochemical potential gradient, as well as detrimental effects on nitrate accumulation, of low temperature, anaerobiosis, and inhibitors of oxidative phosphorylation clearly indicate a dependence on metabolic energy for sustaining nitrate uptake (Huffaker and Rains, 1978). However, movement of nitrate is not restricted to the inward direction accross the plasmalemma, even when the system is operating effectively. ^{15}N-nitrate-fed ryegrass and wheat released previously absorbed ^{14}N-nitrate to the ambient solution, concurrently with an inward flux of the ^{15}N-nitrate

(Jackson *et al.*, 1976b; Morgan *et al.*, 1973). These data have been interpreted in terms of a "pump and leak" model (Morgan *et al.*, 1973). Its essential features include a passive bidirectional pathway, an active uptake mechanism, and a pathway for recycling of endogenous nitrate within unstirred layers from the passive pathway to the active uptake site.

Low ambient hydrogen ion concentrations decrease nitrate transport and the process is restricted above pH 6 (Lycklama,1963; Rao and Rains, 1976a). However, the response to a decrease in pH below pH 6 is not uniformly observed. In some cases, the decline in nitrate uptake is not evident until pH values of 4.5 or below are attained (Minotti *et al.*, 1969b). In the work of others, the rate of nitrate uptake started to decrease below pH 6 (Lycklama,1963).

Once the nitrate uptake system is fully developed, the rate of nitrate uptake depends upon the concentration of nitrate in the medium. The uptake mechanism follows Michaelis-Menten saturation kinetics in a range of concentrations up to 1 mM or higher. The reported apparent Km values for nitrate uptake represent a wide range: A value of 0.3 mM was reported for *N. crassa* (Schloemer and Garret, 1974) while it was less than 0.01 mM in *P. chrisogenum* (Goldsmith *et al.*, 1973), 0.1-0.2 mM in corn (Honert and Hooymans, 1955; Neyra and Hageman, 1975), 0.033 mM in perennial ryegrass (Lycklama, 1963), 0.6 mM in rice (Fried *et al.*, 1965), 0.11-0.2 mM in barley (Chantarotwong *et al.*, 1976Rao and Rains, 1976a), 0.4 mM in *Wolffii arrhiza* (Swader *et al., 1975)* and 0.4 mM in the XD strain of cultured tobacco cells (Heimer and Filner, 1971). Behrend and Mateles (1975) found a Km of 0.7 mM for the same tobacco cell line cultured on a different medium. Lancaster, 1976 (cited by Huffaker and Rains, 1978) reported the Km values for a number of annual range grass species: *Avena fatua L.* (0.015 mM), *Bromus mollis L.*

(0.015 mM) and *Lolium multiflorum Lam.* (0.03 mM). He suggested that the Km value reflects the distribution of the plants in the field since *L. multiflorum* (Km=0.03 mM) requires relatively high soil fertility to compete with the other grasses. *B. mollis* (Km=0.015 mM) on the other hand, has been found to occupy less fertile soils. However, Van de Dijk (1981) and Van de Dijk *et al.*, (1982), indicated that the Km value is not important for the distribution of different species in the field: *Urtica dioica L.* (Km=11 μM) is a species from nutrient-rich sites; *Plantago lanceolata L.* (Km=20 μM) occurs at nutrient-poor sites together with *Hypochaeris radicata ssp. radicata* (Km=13 μM); *Plantago major ssp major L.* (Km=15 μM) is intermediate between *U. dioica* and *P. lanceolata*; *Hypochaeris radicate L. ssp ericetorum* (Km=17 μM) occurs at extremely poor sites.

In the case of *Wolffia arrhiza* (Swader *et al.*, 1975), *Oryza sativa* (Fried *et al.*, 1965) and *Arabidopsis thaliana* (Doddema and Telkamp, 1979), when the rate of nitrate uptake was plotted against nitrate concentration in the medium, biphasic absorption curves were obtained. Such absorption kinetics have been interpreted as reflecting two mechanisms, I and II, of ion absorption (Epstein, 1972). Mechanism I acts at low ion concentrations with a high affinity for the ion. Mechanism II has a low affinity for the ion and becomes operative at higher ionic concentrations (1-50μM). This biphasic mechanism of ion absorption has been criticized extensively (Borstlap, 1981). To get such a pattern the second uptake system must be sigmoidal. This is not generally realized. If both show Michaelis-Menten kinetics there would be no inflection. More precise measurements are required to demonstrate unequivocally the existence of more than one nitrate uptake system whether they are in series (two mechanisms: one operating at the plasmalemma and the other at the tonoplast) or in parallel (two independent mechanisms operating at the

plasmalemma). In the case of *Arabidopsis thaliana* the chlorate resistant mutant BI (Oddema and Telkamp, 1979) also showed the dual-phase relationship. However, phase II did not follow Michaelis-Menten kinetics and the uptake rate of nitrate in the phase II concentration range was considerably lower in the BI mutant than in the wild type. It was concluded that the mutation in BI disturbed phase II of the uptake system, without affecting phase I. Thayer and Huffaker (1982), using ^{13}N have measured initial rates of nitrate transport in *Klebsiella pneumoniae* at concentrations between 1μM and 1 mM and their results indicated the presence of two transport processes; 4.9μM and 4.2 mM were the Km values for the high- and low-affinity systems respectively. A number of genetic studies on *Aerobacter* (*Klebsiella*) sp. (Stouthamer, 1967a, b), *Pseudomonas* sp. (Hartinsgsveldt and Stouthamer, 1973; Sias and Ingraham, 1979; Sias *et al.*, 1980) and *Escherichia coli* (Venables, 1972) have not revealed a class of mutants that would be considered permease negative.

A restriction in net nitrate uptake occurs when intact plants have been exposed to prolonged high nitrate concentrations, and net leakage from the roots can occur even to nitrate-containing solutions (Jackson *et al.*, 1976b; Minotti and Jackson, 1970; Minotti *et al.*,1969a). The ineffective plasmalemma transport may be caused directly by a limitation in the energy supply or indirectly by an increase in the root cytoplasmic ammonium pool as the carbohydrate supply to the root system is curtailed (Jackson *et al.*, 1976a; Minotti and Jackson, 1970). Also, it has been shown that high levels of endogenous nitrate restrict net nitrate uptake in excised barley roots and disks of storage tissue (Cram, 1973; Smith, 1973). Glass (1976) has proposed a relevant model for allosteric regulation of potassium transport in excised barley roots, the effect being exerted by cytoplasmic , not ambient, potassium.

Ambient ammonium has been shown to restrict nitrate uptake by roots of wheat (Jackson *et al.*, 1976a; Lycklama, 1963; Minotti *et al.*, 1969b), rice (Fried *et al.*, 1965; Sasakawa and Yamamoto, 1978; Shen, 1969), barley (Rao and Rains, 1976a) and corn (Butz *et al.*, 1975; Ivanko and Ingrersen, 1971), and cell cultures of rose (Mohanty and Fletcher, 1976) and tobacco (Heimer and Filner, 1971). Similar restricting effects have been observed with *Spirodella* (Ferguson and Bollard, 1969), fungi (Goldsmith *et al.*, 1973; Schloemer and Garret, 1974), various green algae (Pistorius *et al.*, 1978; Syrett and Leftley, 1976; Thacker and Syrett, 1972; Tischner and Lorenzen, 1979), blue-green algae (Flores *et al.*, 1980b; Ohmori *et al.*, 1977) and bacteria (Thayer and Huffaker, 1982). None of the studies with root systems, however, has revealed the mechanism of control exerted by ammonium. The effect appears to be an indirect one, rather than direct competition with nitrate for uptake. Recent experiments carried out with the blue-green algae (Flores *et al.*, in preparation, cited by Losada *et al.*, 1981) have shown that in the presence of specific inhibitors of ammonium assimilation (methionine sulfoximide to block glutamine synthetase, and azaserine to block glutamate synthase) ammonium does not inhibit nitrate uptake. These results suggest feedback regulation by the organic products of ammonium assimilation. Inhibitory effects of various amino acids indicate end product regulation of the nitrate transport system of tobacco cell cultures (Heimer and Filner, 1971, 1970). Inhibition, inactivation, or repression may all be involved.

Relatively little nitrate needs to be absorbed for the apparent induction of nitrate uptake (Heimer and Filner, 1971) or root nitrate reductase (Oaks *et al.*, 1972). Transfer of fully induced tissue to solutions lacking nitrate results in a rapid decline of *in vitro* nitrate reductase activity of tobacco cells (Heimer and Filner, 1971) and root tissues (Jackson *et al.*, 1973; Oaks *et al.*, 1972).

These effects occur even when the tissue contains large quantities of nitrate at the time of transfer. It is clear that compartmentalization of nitrate exists in most plant tissues and that the storage nitrate pool (presumably in vacuoles (Martinoia *et al.*, 1981)), cannot readily supply the metabolic nitrate pool (presumably in the cytoplasm) for sustained nitrate reductase induction (Aslam *et al., 1976;* Ferrari *et al.*, 1973). Information on the distribution of nitrate between storage and metabolic pool is limited. Aslam *et al.* (1976) reported data suggesting the existence of two mechanisms for regulating the metabolic nitrate pool: a) a transfer from the storage pool which requires light; and b) a transfer from the external medium which requires either glucose or light.

In order to facilitate further studies on the characteristics and regulation of nitrate uptake, it would be desireable to make use of a non-metabolizable labelled analogue of nitrate as in K^+ uptake studies using rubidium ($^{86}Rb^+$) (Nissen, 1973). Chlorate is a chemical analogue of nitrate and was used extensively as a non-selective herbicide during the 1930's to 1950's (Crafts, 1962). Its toxicity decreases with increasing soil fertility. In water culture, nitrate is the most effective nutrient for protecting plants from chlorate absorption. In general, the more successfully a plant competes with weeds, the more sensitive it is to chlorate (Crafts, 1962). These observations suggest that nitrate and chlorate may be taken up by the same transport system, and that varietal differences in nitrate uptake should be paralleled by differences in chlorate uptake.

Åberg (1947) proposed that chlorate was not itself toxic to the plants, but rather as an analogue of nitrate, it was rendered toxic by conversion to chlorite via the catalytic action of nitrate reductase. Several independent lines of evidence support this hypothesis of chlorate toxicity: 1) Resting

anaerobic suspensions of *Escherichia coli* catalyze the reduction of chlorate to chlorite in the presence of lactate and methylene blue; reduction of chlorate is competitive with nitrate and *vice versa* (Goksøyr, 1952). 2) Nitrate reductase isolated from *A. aerogenes* and *M. denitrificans* (Pichinoty, 1969), *Chlorella vulgaris* (Solomonson and Vennesland, 1972), *Chlamydomonas reinhardtii* and *Chlorella pyrenoidosa* (Rhodes and Filner, 1979) and *Lycopersicum esculentum* (Hofstra, 1977) catalyze the chlorate dependent oxidation of NADH. 3) Genetic selection for chlorate resistance concomitantly selects for nitrate reductase deficiency in *A. aerogenes* (Stouthamer, 1967), *Escherichia coli* (Piechaud *et al.*, 1967), *Chlamydomonas reinhardtii* (Nichols and Syrett, 1978), *Aspergillus nidulans* (Core, 1976), *Arabidopsis thaliana* (Braaksma and Feenstra, 1975; Oostindiër-Braaksma and Feenstra, 1973). *Hordeum vulgare* (Kleinhofs *et al.*, 1980), *Pisum sativum* (Feenstra and Jacobsen, 1980), allodihaploid *Nicotiana tabacum* cultured cells (Müller and Grafe, 1978), diploid *Rosa damascena* cultured cells (Murphy and Imbrie, 1981), haploid *Datura innoxia* cultured cells (King and Khanna, 1980), haploid *Hyoscyamus muticus* mesophyll protoplasts (Strauss *et al.*, 1981) and haploid *Nicotiana plumbaginifolia* protoplasts (Márton *et al.*, 1982).

Cove (1976) however, has suggested an alternative hypothesis for the mechanism of chlorate toxicity in *Aspergillus nidulans*. Chlorate, as an analogue of nitrate, interacts with the product of a positive regulator gene (*nir* A) for nitrate assimilation. This leads to reduced catabolism of nitrogen-containing compounds, producing symptoms of nitrogen starvation. Cove's proposal is based on the following findings: a) chlorate is much more toxic to wild-type strains with some nitrogen sources than with others, b) chlorate is not toxic with certain nitrogen sources which can also act as carbon sources, when 1% D-glucose is replaced by alternative carbon sources and c) not all

mutations in the *cnx* or *nia* D genes which abolish nitrate reductase lead to chlorate resistance.

All mutants isolated at the plant level were found to have some nitrate reducing capacity (Feenstra and Jacobsen, 1980; Kleinhofs *et al.*, 1980; Oostindier-Braaksma and Feenstra, 1973). Fully deficient lines, however were obtained in cultured allodiploid *Nicotiana tabacum* cells by chlorate selection (Müller and Grafe, 1978). In this species only two types of deficient mutants were recovered, *nia* (structural gene for the nitrate reductase apoprotein) and *cnx* (molybdenum-containing cofactor). However, in *Aspergillus* and *Neurospora* the utilization of nitrate is controlled by at least 10 genes (Marzluf, 1981). In the case of *Nicotiana plumbaginifolia*, Márton and coworkers (1982) selected 7 clones defective in the nitrate reductase apoenzyme and 4 clones defective in the molybdenum-containing cofactor. The cofactor defective mutants could be divided into two groups. In 2 of the clones (NX1 and NX9) nitrate reductase activity was partially restored by the addition of high (0.2-1mM) molybdate to the culture medium. The other 2 clones (NX21 and NX24) did not restore nitrate reductase activity upon addition of molybdate. They are not allelic but complemented with the NX1 and NX9 clones in somatic hybrids. Therefore, NX21 and NX24 are not allelic with the other cofactor defectives either, and are types not yet described in flowering plants.

Three soybean mutants (LNR-2) have been isolated by selecting for chlorate resistance which lacked "constitutive", or non-nitrate induced nitrate reductase activity usually evident in urea-grown soybeans. However, all had inducible activity when grown on nitrate. In the LNR-2 mutant, the lesion was a single recessive mutation (Ryan *et al.*, 1982).

Murphy and Imbrie (1981) reported that when strains of *Rosa*

damascena cultured cells are selected for chlorate resistance, only a minor fraction (15%) 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 (85%) retains the ability to grow on nitrate medium and possesses the ability to transform chlorate to toxic products. These strains lost catalase activity following chlorate treatment, indicating that they took up and reduced chlorate. These observations suggest the operation of a mechanism for tolerating chlorate and its reduction products, rather than avoiding them.

A common theme of both Åberg's and Cove's hypotheses of chlorate toxicity is the nature of chlorate as an analogue of nitrate. This together with Craft's observations of the antidote effect of nitrate in relieving chlorate toxicity symptoms in chlorate-treated plants suggests that plants are in general unable to discriminate between these two molecules in certain key metabolic and regulatory processes involving nitrate.

If plants are indeed unable to discriminate between chlorate and nitrate at the level of uptake, then ^{36}Cl-chlorate could become a powerful tool in studies of nitrate transport in plant cells, thus overcoming the lack of a readily available long-lived radioisotope of nitrate. Furthermore, if nitrate transport into roots is rate-limiting for growth, then it might be possible to make use of chlorate as a screening tool to select for genetic material with superior nitrate transport properties.

Screening of large populations and manipulation of nutrient availability is easier with cell cultures than with whole plants. I therefore decided to investigate in cultured cells the following questions: 1) How good an analogue of nitrate is chlorate in the process of nitrate transport? A vital question that arises when transport studies are carried out with cultured cells is: 2) Does

the nitrate transport system of cultured cells from a given cultivar reflect the relative transport capability of roots of that cultivar?

The XD cell line of tobacco cells was the system of choice in which to seek the answer to the first question. This cell line has been maintained for about 20 years in the laboratory of P. Filner and laboratories scattered around the world (e.g., Japan, Germany , Israel). In this time about 100 papers with data on the XD cells have appeared. Among them, considerable data have been collected regarding XD cell biochemistry and physiology relevant to this study. The regulation of nitrate uptake (Heimer and Filner, 1971), nitrate compartmentalization within the cell (Ferrari *et al.*, 1973) and nitrate reductase (Behrend and Nateles, 1975; Filner, 1966; Zielke and Filner, 1971) have been characterized. Regenerative activity of this cell line was demonstrated after 14 years (T. Rice, personal communication), 17 years (Traynor and Flashman, 1981), and 19 years (Jacobs and Zabala, in progress). A particularly useful feature of this cell line was its ability to grow with urea as a sole nitrogen source. This allows measurements of chlorate uptake without the interference of nitrate accumulated during the growth period previous to the uptake assays.

The genus *Lycopersicon*, which includes the cultured tomato and its wild relatives, was chosen as the system in which to investigate the second question for several reasons: 1) rapid and uniform seed germination, 2) rapid growth and convenient seedling size, 3) well-developed genetics and physiology, 4) amenability to tissue culture and 5) availability of a large store of variable germplasm. On the other hand, tobacco is severely deficient in 1), 2) and 3).

It should be noted that plants have been observed to activate or derepress nitrate uptake as nitrate concentrations become suboptimal. Jackson *et al.*

(1976b) reported that the rate at which wheat seedlings take up nitrate from a 1mM solution increased about 5-fold as the concentration of nitrate on which the plants were precultured was decreased from 15mM to 0.25mM. In another study, activity of the sulfate uptake system of cultured tobacco cells also increased as sulfate became less available (Hart and Filner, 1969; Smith, 1975).

There is also the possibility that plants might increase their root surface area (size) under suboptimal nitrate concentration conditions (Jackson *et al.*, 1976b). This would bring about an increase in the number of nitrate "carriers" and consequently an increase in Vmax (Frith and Nichols, 1975). These responses in either root surface area or nitrate uptake affinity may operate down to nitrate concentrations at which diffusion becomes rate-limiting. If this were indeed the case, then devising *in vitro* selections for cells displaying enhanced nitrate scavenging ability would be unwarranted.

PART I: NITRATE AND CHLORATE UPTAKE SYSTEMS IN CULTURED TOBACCO CELLS

MATERIALS AND METHODS

Plant Material

Two tobacco cell lines were used in this study. the XD cell line was initiated from pith explants of *Nicotiana tabacum* L. cv. Xanthi-nc by P. Filner in 1961. Since its isolation this cell line has been maintained on M1D (a minimal medium in which nitrate is the sole nitrogen source). under these conditions, XD cultures grow with a exponential doubling time of 2 days and reach stationary phase after 10 days (Filner, 1965).

The second cell line, 14U, arose spontaneously from the XD cell line after two years of serial subculturing of the cells in a medium with urea as the sole source of nitrogen (Yamaya and Filner, in preparation). The cells were cloned when urease was 4 times the level in XD cells, and they were subcloned from a variant of the clone in which urease had increased further. The urease activity of 14U cells is 14 times that of the wild type XD and they retain the high urease level in the absence of urea (Yamaya and Filner, in preparation). The mechanism giving rise to this permanent change is still under investigation. The growth characteristics of 14U cells grown in urea-M1D are very similar to those of XD cells grown on nitrate, having an exponential doubling time of 2.2 days and an exponential phase lasting about 10 days (Yamaya and Filner, in preparation).

Growth Media

The XD cells were maintained in M1D, a modification of White's medium described by P. Filner (1965). It contains 2.5 mM nitrate as the sole nitrogen source and limiting nutrient. The cells cease growing on this medium when they exhaust the nitrogen supply.

Nitrate-less M1D (N-less M1D) medium was made by replacing the nitrates of calcium and potassium with equimolar amounts of their respective chlorides. As a result, the final chloride concentration of this N-less M1D medium becomes 2.55 mM.

A variation of the N-less M1D medium called chloride-less nitrate-less M1D (Cl-less N-less M1D) medium was developed for experiments where the effects of chloride were investigated. Cl-less N-less M1D medium was prepared by substituting the chlorides of calcium and potassium with equimolar amounts of calcium sulfate and potassium phosphate respectively. These changes increased the sulfate concentration from 312 mM and phosphate from 12 mM in M1D to 398 and 178 mM, respectively in the Cl-less N-less M1D.

All media were adjusted to pH 6.2 with NaOH before autoclaving at 20 psi for 20 minutes.

Urea-M1D medium was prepared by adding to sterile N-less M1D an aliquot of a filter-sterilized urea solution (ultra pure grade, Schwarz /Mann). The final urea concentration in the urea-M1D medium was 3 mM.

As in the case of urea, when supplemented media were required, sterile solutions of the supplements were diluted into sterile media. The supplement solutions were adjusted to the desired pH prior to filter sterilization.

Growth Conditions

Stock cultures were grown in 1 litre erlenmeyer flasks containing 500 ml of liquid medium on a horizontal platform reciprocal shaker with a displacement of 4 cm at 80 cycles per minute. The shaker was kept in a controlled room at 28 ° C with a light regime of 12 hour day/12 hour night (the cells were not green, and growth was unaffected by light).

Cell suspensions were subcultured by inoculating 500 ml portions of fresh media with 25-ml aliquots of 10-day-old stationary phase cultures using wide bore pipettes. The fresh weight of the initial inoculum was 0.5 to 1 g/l. Experiments were always conducted with exponentially growing cultures, 4 to 6 days following subculture.

Measurement of Nitrate and Chlorate Uptake

When cells growing in M1D were used to study the mechanism of nitrate and chlorate uptake, exponential phase cultures were combined and harvested by vacuum filtration onto Whatman No 1 filter paper. Cells were then rinsed twice with N-less M1D, being kept moist at all times. The cells were then resuspended in fresh N-less M1D. Accumulated nitrate was allowed to diffuse out of the cells into the N-less medium for 30 minutes. After this period of time, the cells were again harvested and rinsed as described above and resuspended in fresh N-less M1D at a density of approximately 8 grams of cells per litre. 25-ml aliquots were then transferred to 125-ml erlenmeyer flasks by means of a wide bore pipet. Solutions of nitrogen sources and other compounds were diluted into the 125-ml flasks to give the appropriate final concentration of the respective ions. Two replicate flasks were prepared per data point. Experimental cultures were grown on the same shaker as described for

maintenance of the stock cultures. The uptake reaction was stopped by harvesting the cells by filtration onto a glass microfibre filter of 2.1 cm diameter. The cell pellet was rinsed twice with 5 ml of fresh N-less M1D, weighed and transferred to scintillation vials when determinations of uptake of ^{36}Cl-chlorate were to be made. Each vial also contained 5 ml of the scintillation cocktail described by Trombolla (1970), consisting of 30% toluene, 70% triton X-100, 8 g/l PPO and 0.35 g/l dimethyl POPOP. When measurements of nitrate uptake were to be made, the cell pellets were weighed and transferred to 30-ml Kjeldahl flasks for digestions as described below.

When 14U cells growing in Urea-M1D were used, the harvesting procedure was changed to filtration by gravity. This was due to the observation made by Thom *et al.* (1981) that vacuum filtration temporarily damaged active transport in plant cell systems in general and in the XD cells in particular. This observation of Thom et al., (1981) was readily verified, whereupon the harvesting procedure was changed to avoid the temporary injury. Thus, 5 day old 14U cell cultures were harvested by gravity filtration onto a 202 μm nylon mesh cloth and rinsed twice with N-less M1D. The harvested cells were carefully transferred to a graduated cylinder and the cell suspension brought to the desired volume by the addition of N-less M1D medium. The cell suspension was then transferred to a 3 or 6 litre fernbach flask to which aliquots of urea and a hydrogen ion buffer had previously been added. The buffer consisted of 20 mM 2[N-morpholino] ethanesulfonic acid (MES) and 20 mM N-2-hydroxyethylpiperazine N´-2-ethanesulfonic acid (HEPES) (final concentrations). The pH of the buffer solution was adjusted to 5.5 or 6.5 by the addition of crystalline Tris HCl. 25 ml of the supplemented cell suspensions were transferred to 125 ml erlenmeyer flasks to which

solutions containing the anion of interest had been added.

All the manipulations described above for the 14U cells were carried out under aseptic conditions in a laminar flow hood.

The remaining steps in the processing of the 14U samples were carried out as described for the XD cells.

Synthesis and Purification of ^{36}Cl-Chlorate

^{36}Cl-chlorate can be synthesized by electrolysis of commercially available H^{36}Cl. Under alkaline conditions possible partial reactions during the electrolytic process include the following:

$$Cl^- + 2OH^- \longrightarrow ClO^- + H_2O + 2e^- \quad 0.90\ v$$

$$ClO^- + 2OH^- \longrightarrow ClO_2^- + H_2O + 2e^- \quad 0.59\ v$$

$$ClO_2^- + 2OH^- \longrightarrow ClO_3^- + H_2O + 2e^- \quad 0.35\ v$$

$$ClO_3^- + 2OH^- \longrightarrow ClO_4^- + H_2O + 2e^- \quad 0.17\ v$$

The lifetime of the radioisotope ^{36}Cl is 3.1×10^5 years and the observed mode of decay is β^-, β^+, EC. The decay energies are : 0.712 Mev (β^-) and 1.14 Mev (β^+, EC),i.e., higher than ^{14}C (0.156 Mev) but lower than ^{32}P (1.710 Mev). The low specific activity of the radioisotope, 500,000 cpm/μmole represents the only disadvantage for short term uptake studies since the measured rate of chlorate uptake by tobacco cells (see Part II) is 4 nmoles / h / g fr. wt. under unrestricted conditions.

^{36}Cl-chlorate was synthesized and purified as described by Trombolla (1970) with some modifications. H^{36}Cl (New England Nuclear; specific activity 0.23 μCi/μmole) was diluted to 0.02 M in a total volume of 7 ml of distilled water. The solution was made alkaline by addition of 2 M NaOH to a

final concentration of 0.023 M. The solution was electrolyzed at room temperature (20° C) for 3.5 hours at 50 mA constant current and approximately 12 volts. The apparatus used for electrolysis consisted of a 10 ml glass beaker covered with a plastic cap through which two platinum wire electrodes were inserted. The spacing between the electrodes was 1.5 cm. After electrolysis, the electrolysate was rotary evaporated at 30° C to a final volume of 0.2 ml and applied to the base line of a plastic thin layer chromatography plate coated with MN 300 cellulose. The products of electrolysis were chromatographed in a solvent composed of 80 ml 1-butanol, 40 ml pyridine, 80 ml H_2O and 5 ml ammonia. The bands corresponding to the three products of the electrolysis (perchlorate, chlorate and chloride) were localized by autoradiography of the plate. Chlorite was undetectable. The bands for ^{36}Cl-perchlorate, ^{36}Cl-chlorate and ^{36}Cl-chloride have Rf's of 0.73, 0.54 and 0.34 respectively. The identity of those compounds with their respective standards was demonstrated by developing the spots on a TLC plate with a solution of aniline in HCl (50 g of pure aniline hydrochloride in 1 l of 1:3 hydrochloric acid) (Welcher, 1947). Chlorate develops a bluish green colour and chlorite a spot which is violet for about half a minute before turning bluish green. The reaction with chlorite develops soon after the spraying, whereas the chlorate spot is not visible until the paper is practically dry. Chloride and perchlorate give no colour with this reagent (Goksøyr, 1952). Perchlorate was detected by spraying first with saturated aqueous sodium acetate and, once dried, overspraying with 0.2% aqueous methylene blue. Perchlorate becomes violet against a light blue background (Harrison and Rosenblatt, 1964)

^{36}Cl-chlorate was extracted from the chromatogram by incubating the TLC plate fragments overnight in a minimum volume of water. The recovery

of label as chlorate after 3.5 hours was approximately 30% and the remainder was ^{36}Cl-perchlorate (68%) and ^{36}Cl-chloride (2%). Purity of chlorate was better than 99%. The ^{36}Cl-chlorate solution was stored at 4° C. Rechromatography of the preparations, and analysis of chloride and chlorite content over a period of 6 months indicated that the ^{36}Cl-chlorate preparations were stable to storage. In most experiments ^{36}Cl-chlorate was supplied to cells at a maximum specific activity of 500,000 cpm / μmole at a level of 7,000 cpm/ml medium or 14 μM. In addition, unlabelled chlorate was added to achieve higher chemical concentrations, with proportionally lower specific radioactivities.

The time course of the electrolysis of H^{36}Cl was determined by removing 20-μl samples from the electrolysis every 30 minutes for 4 hours. Three microliters from each one of those samples were chromatographed as described above. Strips corresponding to each time point were cut into 1 cm fragments corresponding to the Rfs of the electrolysis products. Each fragment was then counted by scintillation spectrometry. Optimum recovery of ^{36}Cl-chlorate was achieved after 1.5 hours of electrolysis (Figure 1). However, for routine synthesis of ClO_3^-, electrolysis was allowed to proceed for 3.5 hours, as suggested by Trombolla (1970).

Kjeldahl Procedure

Nitrate incorporated into XD cells was determined by measuring ^{15}N abundance, using mass spectrometry after conversion of nitrogen to N_2. An estimation of the total nitrogen in the plant cell was achieved by a two-step Kjeldahl procedure (Nelson and Sommers, 1973). 0.2 to 0.6 g fresh weight of tobacco cells were placed in a 30 ml Kjeldahl flask to which 2 ml of a salicylic acid-H_2SO_4 mixture (1 g salicylic acid per 40 ml of conc. H_2SO_4) were added.

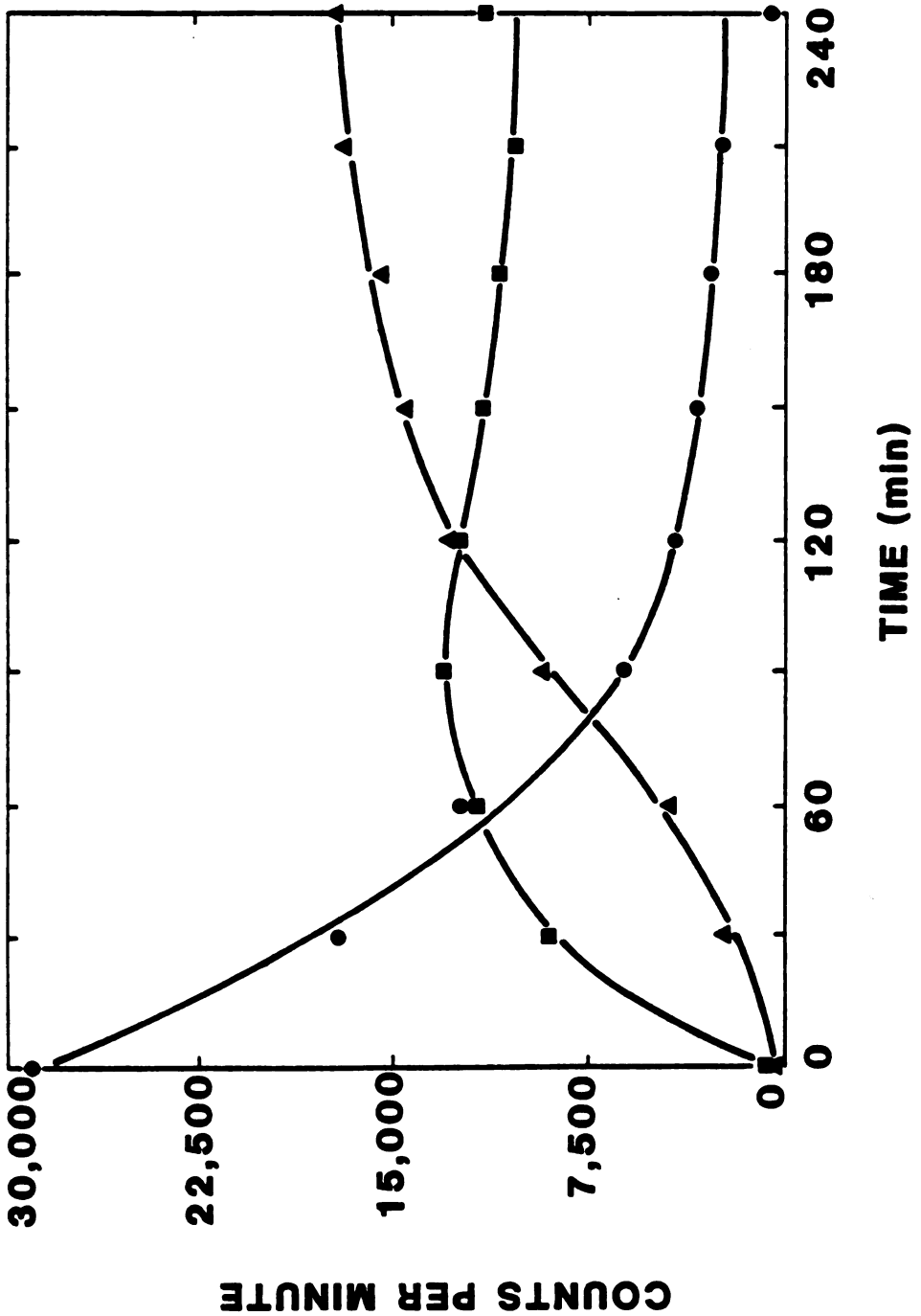

Figure 1: Time course of product formation during $H^{36}Cl$ electrolysis under alkaline conditions. (▲) = $^{36}ClO_4^-$; (■) = $^{36}ClO_3^-$; (●) = $^{36}Cl^-$.

After allowing the mixture to stand at 25° C for 2 hours, 0.25 g of sodium thiosulphate was added followed by cautious heating over a burner until frothing ceased. The tube was then removed from the heating block and cooled to 25° C. 4 ml of a 5 M H_2SO_4 solution containing 20 g per litre of sodium selenate and 20 g per litre of $CuSO_4 \cdot 5H_2O$ were then added. The tube was swirled to mix the sample and digestion reagents and then returned to the burner. The samples were digested at the boiling point of the mixture 60 minutes past the time of clearing, removed from the heating block, and allowed to cool to room temperature. At this point, all forms of nitrogen are converted to ammonium. The digest was them diluted with distilled water to a final volume of 15 ml. Ammonium in the digest was then released in a steam distillation apparatus by addition of 15 ml of a 5 N NaOH solution. The ammonia and water vapors were condensed and collected in an erlenmeyer flask containing 2 ml of 0.1 M $H_2 SO_4$. The excess water was evaporated to dryness on a hot plate. The concentrated $(NH_4)_2SO_4$ solution was rediluted in 2 ml of water and the amount of ammonium determined by a direct colorimetric method described by McCulfough (1967). One ml each of the diluted $(NH_4)_2SO_4$ solution, reagent A (10 g/l phenol and 50 mg/l of sodium nitroprusside), and reagent B (5 g/l NaOH, 53.7 g/l $Na_2HPO_4 \cdot 12\ H_2O$ and 10 ml of 10-14% sodium hypochlorite) were mixed and incubated at 70° C for 5 minutes. The absorbance was measured at 625 nm in a Gilford spectrophotometer.

Determination of ^{15}N Atom Percent

The usual way of introducing a sample into an isotope ratio mass spectrometer is as follows: 1) A vacuum-proof manifold vessel containing the ammonium sample is attached to both the mass spectrometer inlet system and

to a preevacuated reservoir of lithium hypobromite. 2) The sample is then preevacuated by opening it to the vacuum system intercalated between the mass-spectrometer reservoir and the sample's vessel. 3) The vacuum system is closed and the ammonium sample is allowed to react with an aliquot of lithium hypobromite drawn in from its reservoir. 4) The reaction mixture is frozen by surronding the sample vessel with liquid nitrogen. 5) The N_2 gas formed in the reaction between ammonium sulfate and lithium hypobromite is then drawn through the mass spectrometer reservoir to the analyzer. 6) ^{15}N percent abundance is then measured and corrected with that of the residuals previously measured.

This procedure has two major disadvantages. Large amounts of sample are required and the glassware of such an inlet system is expensive, fragile and cumbersome.

A procedure was devised for handling large numbers of samples quickly with inexpensive disposable glassware. One-half ml of the $(NH_4)_2SO_4$ solution produced in the total nitrogen determination, was injected into a preevacuated glass vial (10x36 mm; 1 ml) covered with a rubber septum. The injection of the sample was done by allowing the vacuum in the vial to draw in the sample, minimizing contamination by air. The ammonium was then oxidized to nitrogen gas by injection into the vial (as described for the sample) of 0.5 ml lithium hypobromite solution (60 ml cold 10% w/v lithium hydroxide (analytical grade) to which 2 ml of analytical grade bromine was added). The vial was then immersed in liquid nitrogen and as soon as the solution froze, another rubber septum was placed over the first one (Figure 2). The air space between the two septa permitted the removal of the air located between the needle used to inject the sample into the mass spectrometer (MAT GD 150) and the valve which connects to the reservoir. Once that air was removed by

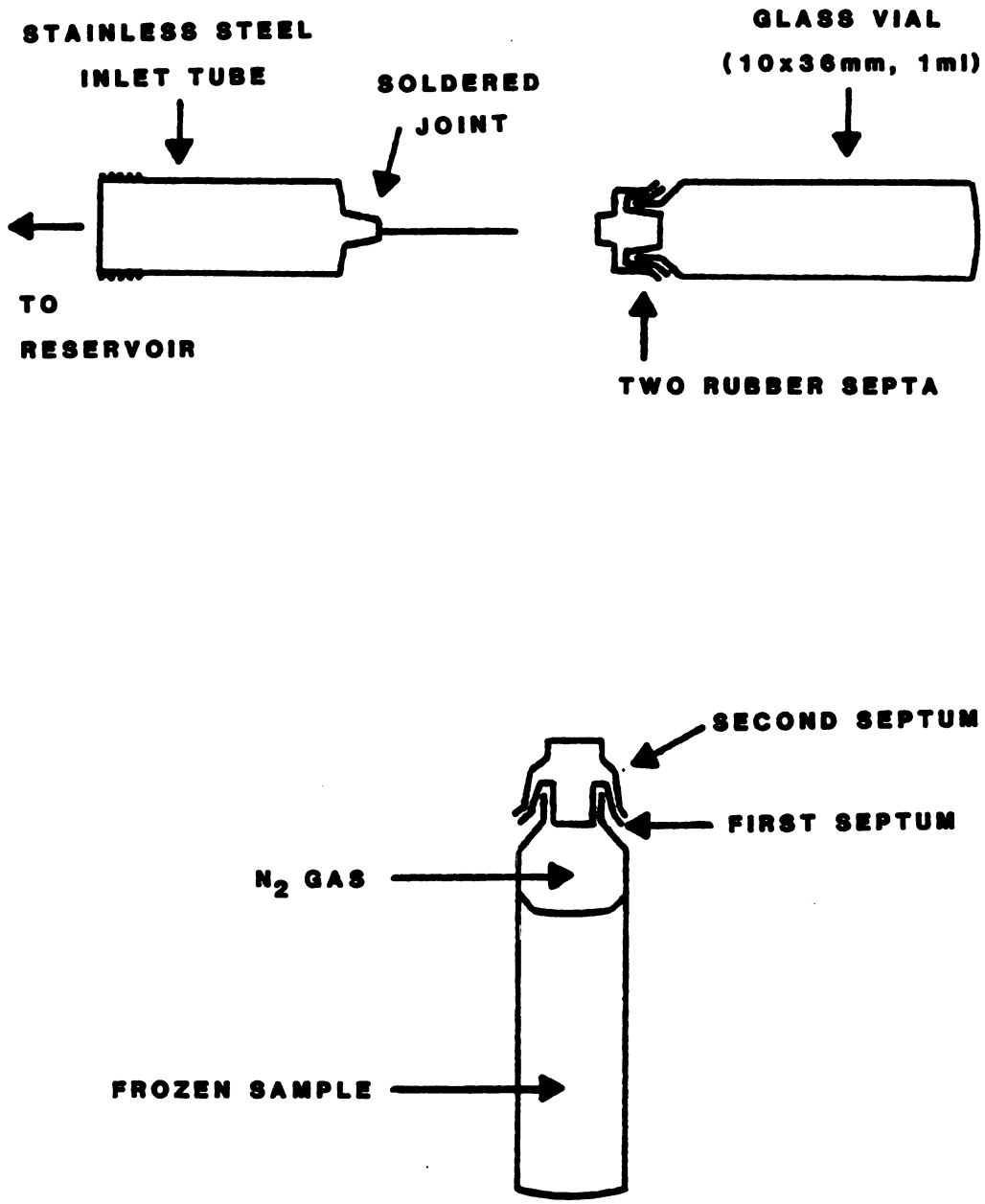

Figure 2: Diagram of apparatus devised for introduction of samples into the MAT GD 150 Mass Spectrometer.

the instrument's vacuum system, the needle was forced through the first septum into the head space containing $^{15}N_2$. The valve to the reservoir was opened for 6 seconds and the isotope ratio of ^{15}N and ^{14}N of the nitrogen gas measured. The spectra of both the residuals and a 92-93% ^{15}N atom percent of a $(^{15}NH_4)_2SO_4$ standard sample were used to determine a factor to correct the error due to air contamination of each individual sample (see example in Table 1). ^{15}N abundance was then calculated in one of two ways: 1) For samples with low abundance, and therefore with low probability of containing ^{15}N-^{15}N (mass 30) molecules, the following expression was used (Hauck and Bremner, 1976): ^{15}N atom % $= 100/2R + 1$ where R represents the quotient of the residual-oxygen corrected peak areas of ^{14}N-^{14}N (mass 28) divided by that of ^{14}N-^{15}N (mass 29). The ^{15}N atom$\%_{excess}$ is then determined by subtracting the ^{15}N atom$\%_{atmospheric}$(0.37) from the ^{15}N atom$\%_{observed}$. 2) For samples with high ^{15}N abundance and therefore with high probability of containing ^{15}N-^{15}N (mass 30) molecules, the expression ^{15}N atom % $= 100/0.5R' + 1$ was used (Hauck and Bremner, 1976). R' corresponds to the quotient of the residual-oxygen corrected peak areas of ^{14}N-^{15}N (mass 29) over that of ^{15}N-^{15}N (mass 30). The ^{15}N atom$\%_{excess}$ is then determined as described above for low ^{15}N abundance samples.

^{15}N enriched potassium nitrate (^{15}N atom% = 99) was purchased from BIO-RAD laboratories.

Determination of Nitrate

Nitrate in the cell's soluble compartment and in the external medium was measured by the method described by Lowe and Hamilton (1967), based on the ability of soybean nodule bacteroids to reduce nitrate to nitrite.

Bacteroid suspensions prepared by Y. Heimer in 1969 and kept frozen at

Table 1: Method of determination of ^{15}N atom % abundance from mass spectrometric data.

1) Determination of correction factor (F)

	PEAK AREAS AT:			
	Mass 28	Mass 29	Mass 30	Mass 32
Residuals	a	b	c	d
Standard (92.5% ^{15}N)	e	f	g	h

$\Delta 28 = e - a$
$\Delta 32 = h - d$

Correction Factor, $F = \frac{\Delta 28}{\Delta 32} = \frac{e - a}{h - d}$

2) Correction for residuals and oxygen

	PEAK AREAS OF:			
	Residuals	Samples	Correction for Residuals	Correction for Oxygen
Mass 28	A	G	$i = G - A$	$K = i - (F \times l)$
Mass 29	B	H	$j = H - B$	$L = j - (F \times \frac{l}{133})$
Mass 30	C	I	$k = I - C$	$M = k - (F \times \frac{l}{266})$
Mass 32	D	J	$l = I - D$	

3) Determination of ^{15}N abundance

For samples low in ^{15}N	For samples high in ^{15}N
^{15}N abundance (28/29)	^{15}N abundance (29/30)
$R = K/L$	$R' = L/M$
$Q = 100/(2R + 1)$	$Q = 100/(0.5R' + 1)$

-20° C were used for this analysis. The assay mixture was composed of the following: 0.5 ml of 0.2 M potassium succinate buffer pH 6.8, 0.3 ml of the nitrate containing solution and 0.2 ml of undiluted bacteroid suspension. The assay tubes were incubated for 30 minutes at 40° C. The reaction was stopped by the addition of 1 ml of 1% w/v sulfanilamide in 3N HCl. The diazonium salt formed with the nitrous acid is reacted with 1 ml of 0.02% N-(1-naphthyl)-ethylenediamine dihydrochloride to give a colored diazoamino compound (Azo, Benzene 1-Naphthalene, 4-Sulphamyl, 4'- N- Ethylene-1,2-Diamine Hydrochloride) that absorbs at 540 nm. Before reading the absorbancy the assay mixtures were cleared by centrifugation at 15,000 x g for 10 minutes (Snell and Snell, 1949).

For the assay described above, nitrate was extracted from the cells' soluble compartment with ethanol. Cells were separated from the ethanol by filtration and the nitrate recovered by evaporating the alcohol in a rotoevaporator at 38 ° C.

RESULTS

Changes in the Internal and External Nitrate Pools of XD Cells in Culture

The XD cell line was initially used to characterize the cellular nitrate and chlorate uptake systems for two reasons. First, some features of its nitrate metabolism had already been established (Heimer and Filner, 1971; Ferrari *et al.*, 1973) and second, as mentioned in Materials and Methods, this cell line has proven to be a stable experimental system in the past.

Previously reported indications of the analogy between the nitrate and chlorate ions in physiological systems prompted us to examine the nitrate status of the XD cells during their growth cycle as well as the effect it could have on the analysis of chlorate uptake.

Stationary-phase XD cells were subcultured into M1D and the nitrate concentration of both the cells' nitrate pool and medium was determined over the cells' growth cycle. The results are summarized in Figure 3. The initial medium nitrate concentration of 2.5 mM decreased to a negligible level by the time the culture entered the stationary phase. Nitrate accumulated in the cellular pool as the cells divided exponentially, reaching a maximum concentration of 24 μmoles nitrate per g fr. wt. at the mid-exponential phase of the culture cycle. As the cells entered stationary phase under these conditions of nitrogen limitation, the level of cellular nitrate was reduced, depleting the intracellular pool, since no further accumulation was taking place. This indicated that exponential cultures, ideally suited for physiological studies, contained high levels of nitrate in a free pool.

In order to determine the effect that this nitrate pool had on chlorate uptake, the following experiment was performed. Late exponential phase cells were transferred to N-less M1D and the uptake of chlorate was measured with time. In addition, the amount of nitrate in the medium and in the cells' nitrate pool was concurrently determined. The results, shown in Figure 4 reveal a rapid release of the cells' nitrate into the medium. This is in agrement with the results reported by Ferrari *et al.* (1973). Since my working hypothesis presumed that chlorate and nitrate shared a common transport system, preincubation and washing of the cells in N-less medium was clearly indicated. A preincubation period of 30 minutes was chosen followed by

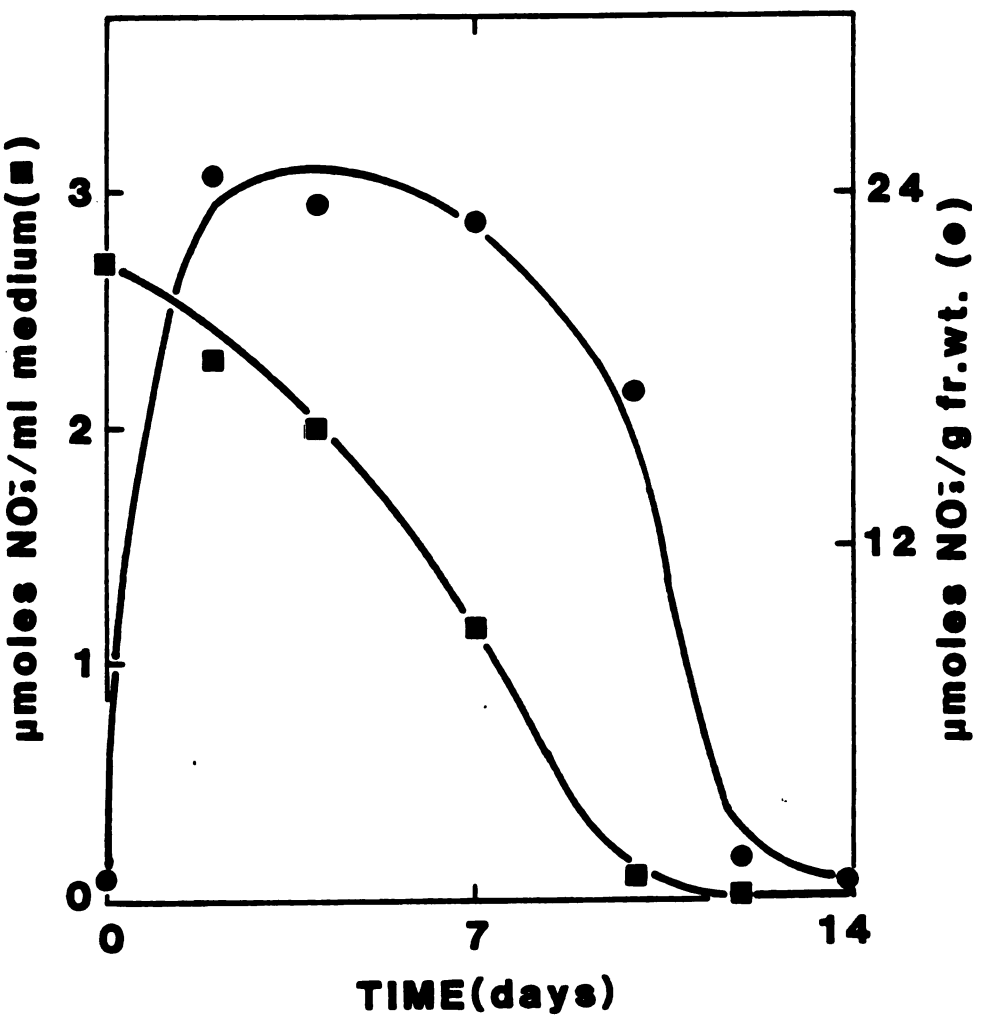

Figure 3: Intra- and extracellular nitrate levels in XD cells over the culture cycle. (●) = NO_3^- content of cells (µmoles / g fr. wt.); (■) = medium NO_3^- (µmoles / ml).

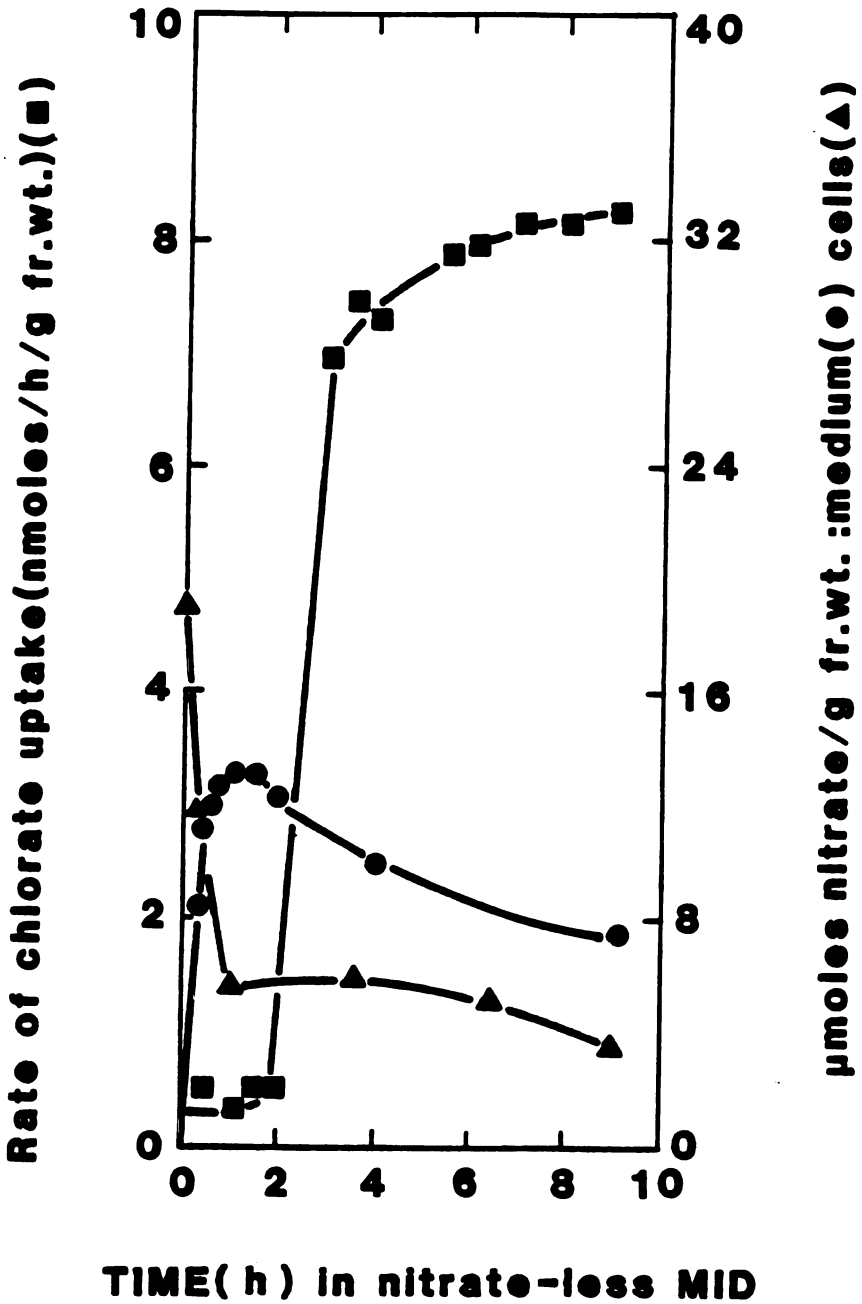

Figure 4: Leakage of NO_3^- from XD cells cultured in the presence of $^{36}ClO_3^-$. (■) = rate of $^{36}ClO_3^-$ uptake, nmoles / h / g fr. wt.; (●) = medium NO_3^-, μmoles / g fr. wt.; (▲) = cell NO_3^-, μmoles / g fr. wt..

rinsing in N-less medium and transfer of the cells to experimental solutions.

The onset of chlorate accumulation followed a lag of approximately 2 hours. The rate of accumulation then increased to a constant value after 4 hours. The lag phase of chlorate uptake could be explained by the observation made by Thom *et al.* (1981) that vaccum filtration temporarely damaged active transport in plant cell systems. They studied arginine uptake in *Nicotiana tabacum* XD cell line following both gentle (gravity) and vigorous (vacuum) filtration. Arginine uptake after vigorous filtration showed a lag of approximately two hours, whereas gentle filtration resulted in no appreciable lag in transport.

As this report appeared well into the progress of our studies, gravity filtration was not adopted until the beginning of the experiments with 14U cells (see below).

Figure 5 shows the accumulation of chlorate in exponential (2-day-old) and early stationary phase (10-day-old) XD cells with time. These chlorate uptake measurements were carried out after a preincubation period of 30 minutes in N-less medium to correct for contamination by nitrate leaking out of the cells. This correction reduced but did not eliminate the initial lag in chlorate uptake (Figures 7 and 14).

Changes in Km and Vmax of Chlorate Uptake with Changes in Cell Age and with Time in Inducing Medium (Nitrate-less MID).

Nitrate uptake rates have been reported to be higher in nitrogen-starved than in nitrogen-fed plants (Jackson *et al.*,1976a and 1976b). Thus, the sensitivity of the uptake system to nitrate depends upon the system's preexisting nitrogen nutritional status. It was therefore pertinent to know whether chlorate uptake was affected by prior nitrogen status. The kinetics of

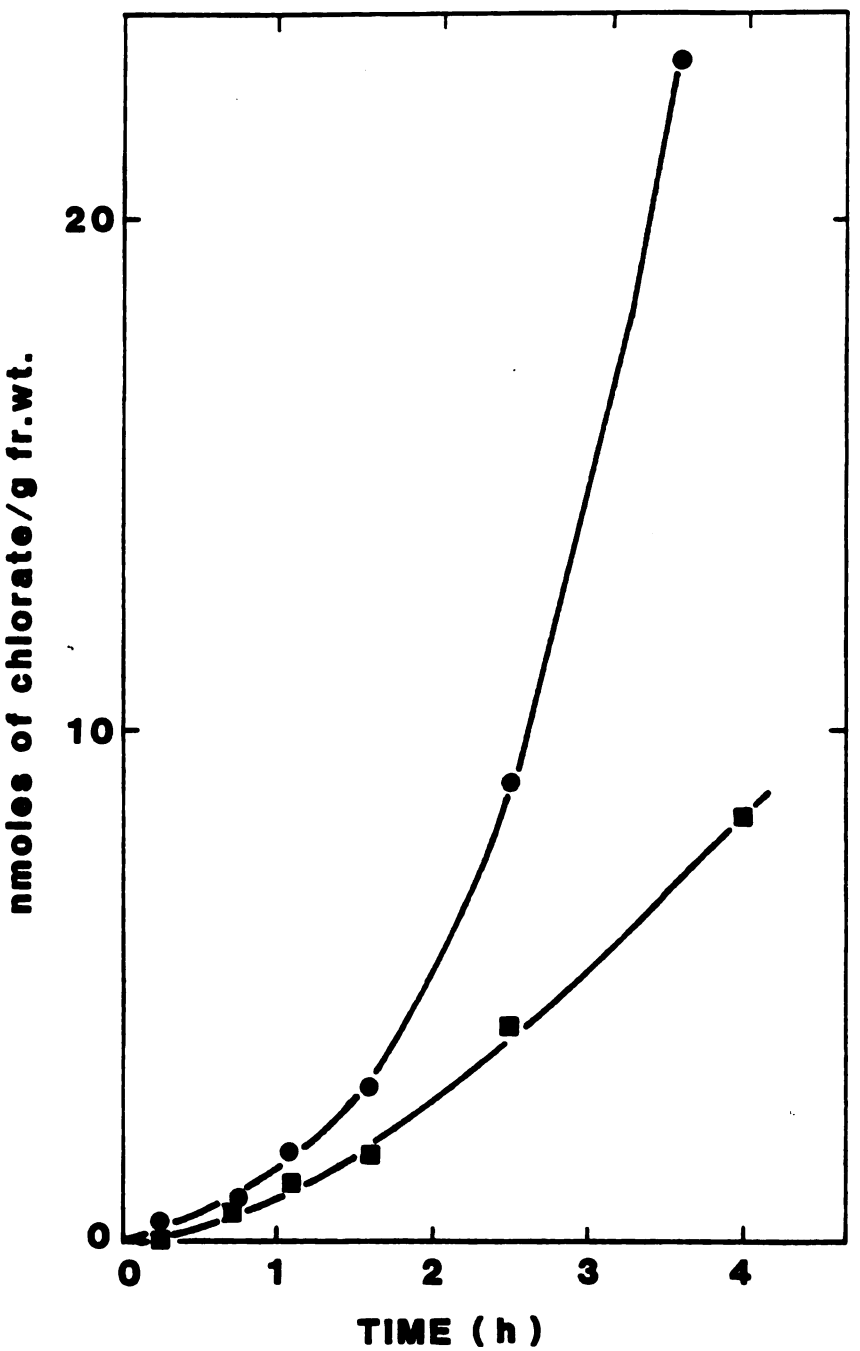

Figure 5: Culture-cycle dependence of chlorate uptake by XD cells. Accumulation of $^{36}Cl^-$ in exponential (●) and stationary (■) phase XD cells was determined after incubation with $K^{36}ClO_3$ for the times indicated.

chlorate uptake of cells preincubated in N-less MID for 0, 2, and 4 hours were measured. This preincubation period was in addition to the standard 30 minute rinse in N-less medium. XD cells growing in M1D were harvested by vacuum filtration, rinsed twice with N-less M1D and resuspended in fresh N-less M1D. Accumulated nitrate was allowed to diffuse out of the cells into the N-less medium for 30 minutes. The cells were then harvested, rinsed and resuspended in fresh N-less M1D at a density of approximately 8 g of cells per litre. 0.2 g of cells were then transferred to 125-ml Erlenmeyer flasks. The cells were incubated with ^{36}Cl-chlorate (7,000 cpm/ml of medium) and unlabelled potassium chlorate (0.1 to 3.2 mM) to give a range of specific activities (60,800-2,165 cpm/μmole). ^{36}Cl-chlorate taken up into the cells was measured after 2 hours of incubation. This analysis was repeated with cells of different ages and the results are summarized in Table 2. Figure 6 shows an example of a Lineweaver-Burk plot from which some of the Km and Vmax values reported in Table 2 were generated. This particular set of plots corresponds to the 4-day-old cells after 0, 2 and 4 hours of nitrogen starvation. The apparent affinity of the uptake system for chlorate increased with time in N-less medium, regardless of culture age. Three reasons can be proposed for this effect. First, nitrogen starvation could indeed derepress or activate a nitrate, and therefore a chlorate, transport system. Second, injury of the cells by vigorous (vacuum) harvesting could have a negative effect on the uptake system during the first hours following transfer to N-less medium. The greater affinity demonstrated by the transport system after 2 or 4 hours may simply reflect recovery from such injury (Thom *et al.*, 1981). Finally, nitrate continues to leak out of the cells following the second transfer to N-less medium (see Figure 4; also Ferrari *et al.*, 1973). This nitrate may compete with chlorate for a shared uptake system, resulting in the initially higher Km

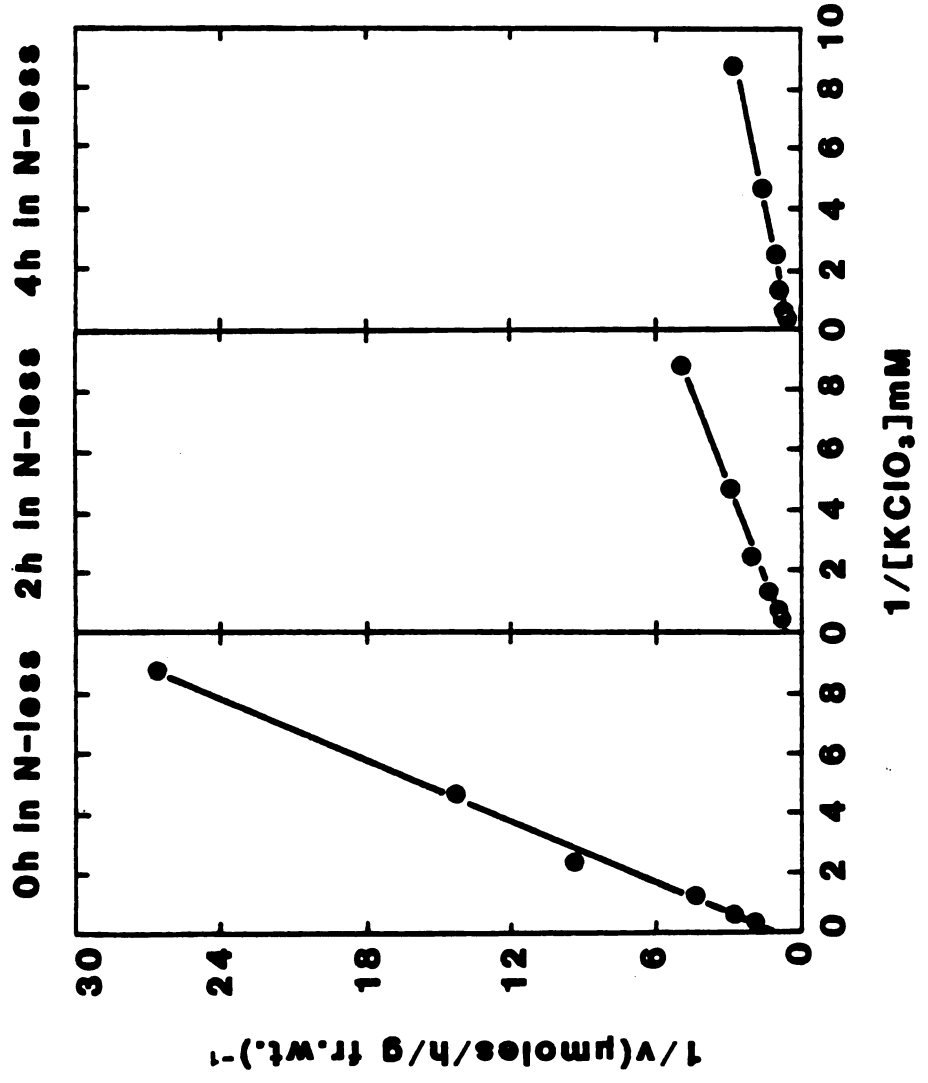

Figure 6: Effect of nitrogen starvation on kinetic characteristics of chlorate uptake by XD cells. All cultures were exponential phase.

Table 2. Effect of nitrogen starvation on kinetic characteristics of chlorate uptake in XD cells.

Age of Culture (d)	Fresh Weight (g)	Preincubation in N-less Medium (h)	$V_{max_{36}}$ (μmoles of ClO_3^-/ h/g fr.wt.)	K_m (mM)
4	2.9	0	.793	2.252
		2	1.431	.697
		4	2.509	.651
7	5.9	0	.988	2.520
		2	1.604	.643
		4	1.785	.562
10	25.0	0	.137	.570
		2	.209	.430
		4	.255	.399
12	28.9	0	.139	.776
		2	.365	.536
		4	.407	.239
14	30.3	0	.120	.630
		2	.117	.238
		4	.198	.183

values.

The effect of nitrogen starvation on nitrate uptake was also examined. Six-day-old cells were preincubated in N-less medium for 0, 2, and 4 hours before the addition of ^{15}N-KNO_3 at concentrations ranging from 0.1 to 1.6 mM. Cells were harvested after 2 hours of incubation, and the ^{15}N abundance of the total cellular nitrogen was determined by mass spectrometry. The double reciprocal plots of the data obtained from those measurements are shown in Figure 7. The Km and Vmax values obtained from these measurements are presented in Table 3. Whereas the chlorate uptake system reflected changes in the state of nitrogen nutrition of the cells, the nitrate uptake system displayed no systematic effect. The Km values in all instances are close to 0.1 mM.

Effect of Nitrate on Chlorate Uptake.

In our first attempt to evaluate directly the interaction between chlorate and nitrate uptake, we analyzed the changes in the kinetic parameters of chlorate uptake caused by the addition of nitrate to the incubation medium. XD cells growing in M1D at a density of 2 g fr. wt./l were harvested and transferred to N-less M1D where they were incubated for a period of 30 minutes at a cell density of 26 g fr. wt./l. Cells were then harvested and resuspended in 600 ml of fresh N-less medium at a cell density of approximately 9 g fr. wt./l. This culture was divided into three portions of 100, 100 and 400 ml. A tracer amount of ^{36}Cl-chlorate (14.5 μM) was added to each of the 100-ml portions. To one of these, nitrate was also added to give a 1 mM final concentration. Ten-ml aliquots were removed from each culture every 30 minutes during a 4.5-hour incubation period. Determination of radioactive incorporation into the cells at each time point gave a time course of chlorate

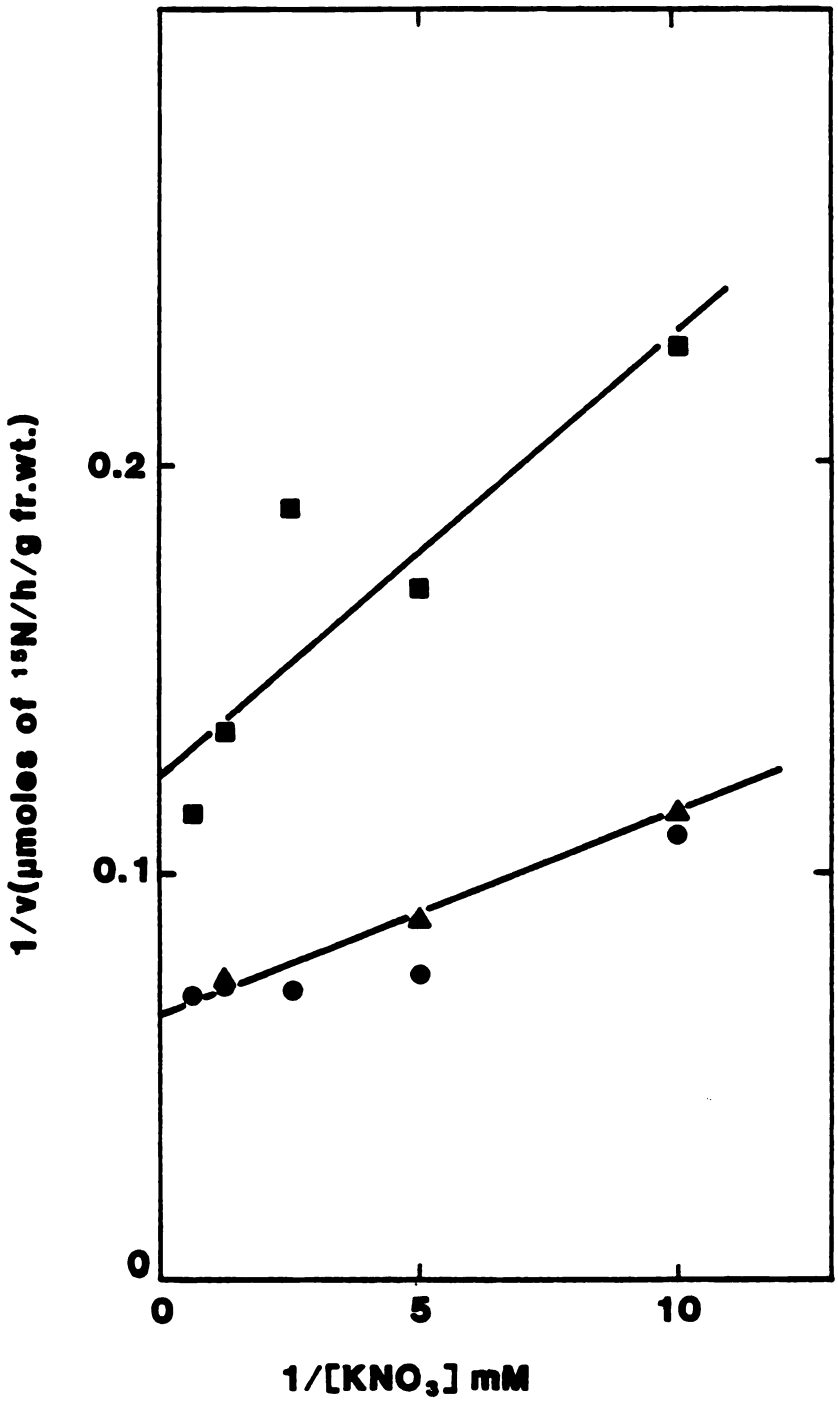

Figure 7: Effect of nitrogen starvation on kinetic characteristics of nitrate uptake in XD cells. (■) = 0 h, (●) = 2 h, and (▲) = 4 h.

Table 3: Effect of nitrogen starvation on kinetic characteristics of nitrate uptake in XD cells.

Time in N-less M1D (h)	Km (mM)	Vmax (μ moles ^{15}N/h/g fr. wt.)
0	0.08	7.9
2	0.10	15.9
4	0.06	15.4

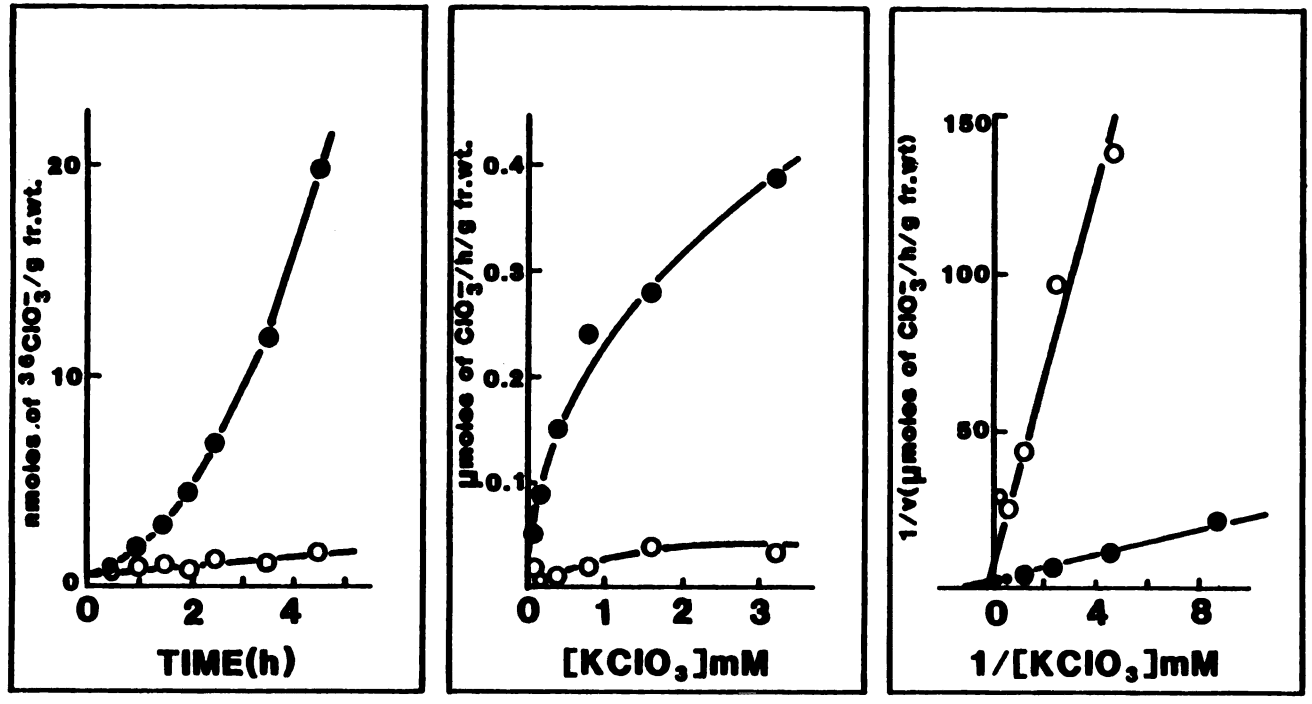

Figure 8: Effect of nitrate on the kinetics of chlorate uptake in XD cells. A: Time course of chlorate accumulation in the presence of 1 mM KNO_3 (O) and in the absence of nitrate (●). B: Concentration dependence of chlorate uptake in the presence of 1 mM KNO_3 (O) and in the absence of nitrate (●). C: Lineweaver-Burk plot of chlorate uptake in the presence of 1 mM KNO_3 (O) and in the absence of nitrate (●).

uptake in the presence or absence of nitrate (Figure 8A).

The third portion (400 ml) of the initial culture was set aside on inducing medium (N-less M1D) for 4 hours. From this culture, two series of 25-ml batches were made. Each series consisted of a range of non-radioactive chlorate concentrations (0.1 to 3.2 mM) and a constant initial input of labelled chlorate (7000 cpm/ml). In addition, one series was brought to 1 mM KNO_3, while the other remained N-free. The amount of chlorate accumulated by each culture was measured after 2 hours of incubation (Figure 8B).

Nitrate appears to have a strong inhibitory effect on chlorate uptake. 1 mM nitrate decreased 10-fold the amount of chlorate incorporated into the cells after 4.5 hours (Figure 8A). The effects of nitrate on the apparent Km (20 mM with 1 mM KNO_3 *versus* 1 mM without KNO_3) and Vmax (0.6 μmoles of $KClO_3$ / h / g fr. wt.) for chlorate uptake, deduced from the Lineweaver-Burk double reciprocal plot (Figure 8C) suggest that nitrate may act as a competetive inhibitor of chlorate uptake in this system. However, this conclusion must remain tentative, since the scatter of points on the $+KNO_3$ line does not permit a precise determination of whether the $+ KNO_3$ and $-KNO_3$ lines intersect on the ordinate.

Concurrent Determination of Kinetic Characteristics of Nitrate Inhibition of Chlorate Uptake and of Chlorate Inhibition of Nitrate Uptake.

To investigate further the relationship between chlorate and nitrate uptake systems, an experiment was designed to determine the K_i of inhibition of chlorate uptake by nitrate, as well as the K_i of nitrate uptake by chlorate. These measurements were made in the same experiment by using simultaneously ^{36}Cl-chlorate and ^{15}N-nitrate in the following way. A 7-day-old XD culture, treated as previously described for uptake studies, was divided

into an experimental block of 25-ml batches and incubated for 3 hours in N-less medium. To each 25-ml batch ^{36}Cl-chlorate was added to give a final concentration of 7000 cpm/ml to different subsets of the experimental block, non-radioactive chlorate was added to final concentrations of 0 to 3.2 mM. Likewise, final ^{15}N-KNO_3 concentrations of 0.02 to 2 mM were generated in the orthogonal subsets of the block. Each member of this grid of chlorate and nitrate concentrations was incubated for 2 hours and then divided into 2 equal portions. One portion was used to measure ^{36}Cl-chlorate accumulation and the other to determine ^{15}N atom % by mass spectrometry.

Figure 9 shows the results obtained from the ^{36}Cl-chlorate determinations. The increase in the slope of the double reciprocal plot (and therefore the increase in the Km) resulting from an increase in nitrate concentration and the fact that the ordinate intercept remains constant (Vmax $\simeq$ 0.6 μmole of chlorate / h / g fr. wt.) once again indicates that KNO_3 behaves as a competitive inhibitor of chlorate uptake.

A Dixon plot (Figure 10) of 1/v *versus* [inhibitor, KNO_3] at different substrate concentrations gives a series of straight lines with slopes inversely proportional to the substrate ($KClO_3$) concentration. The intersection of these lines on the abscissa at [KNO_3] = -0.1 mM gives a K_i value of 0.1 mM. The K_i value for nitrate inhibition of chlorate uptake is identical to the Km obtained for nitrate uptake after 2 hours of nitrogen starvation (see Table 3).

On the other hand, when the 1/v values obtained from measurements of ^{15}N abundance in the other portion of each sample were plotted against 1/S (S = [KNO_3]) for each $KClO_3$ concentration, no pattern characteristic of inhibition was apparent (Figure 11).

The effect of chlorate on nitrate transport was reexamined to confirm the results described above, using a slightly different experimental approach.

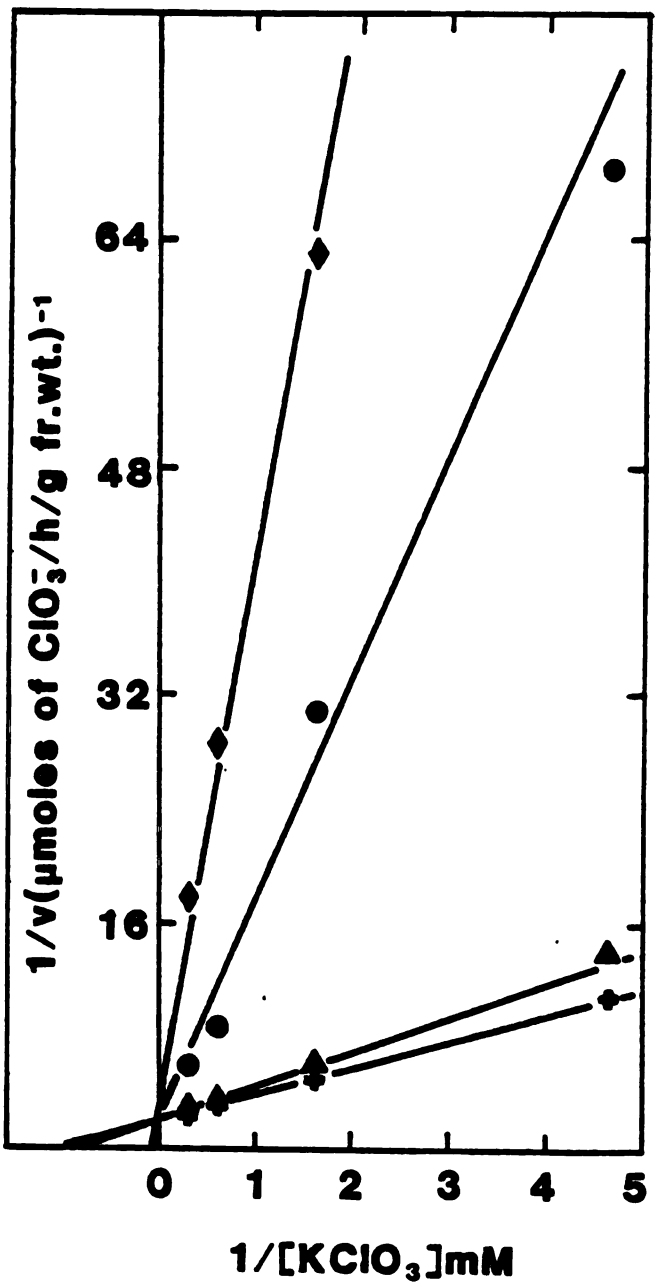

Figure 9: Lineweaver-Burk plot of nitrate inhibition of chlorate uptake in XD cells. $[NO_3^-]$ = 0.75 (◆), 0.25 (●), 0.05 (✚), 0 (▲) mM.

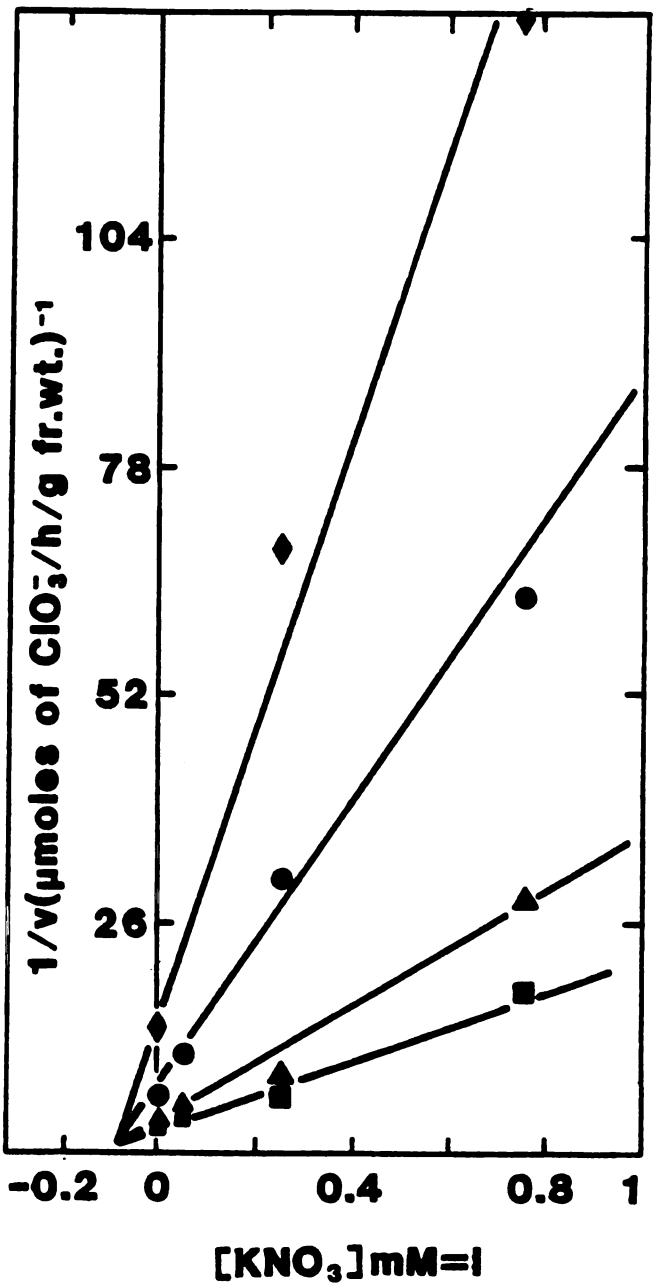

Figure 10: Dixon plot of nitrate inhibition of chlorate uptake in XD cells. $[ClO_3^-]$ = 0.21 (◆), 0.61 (●), 1.61 (▲), and 3.21 (■) mM. K_i = 0.1 mM.

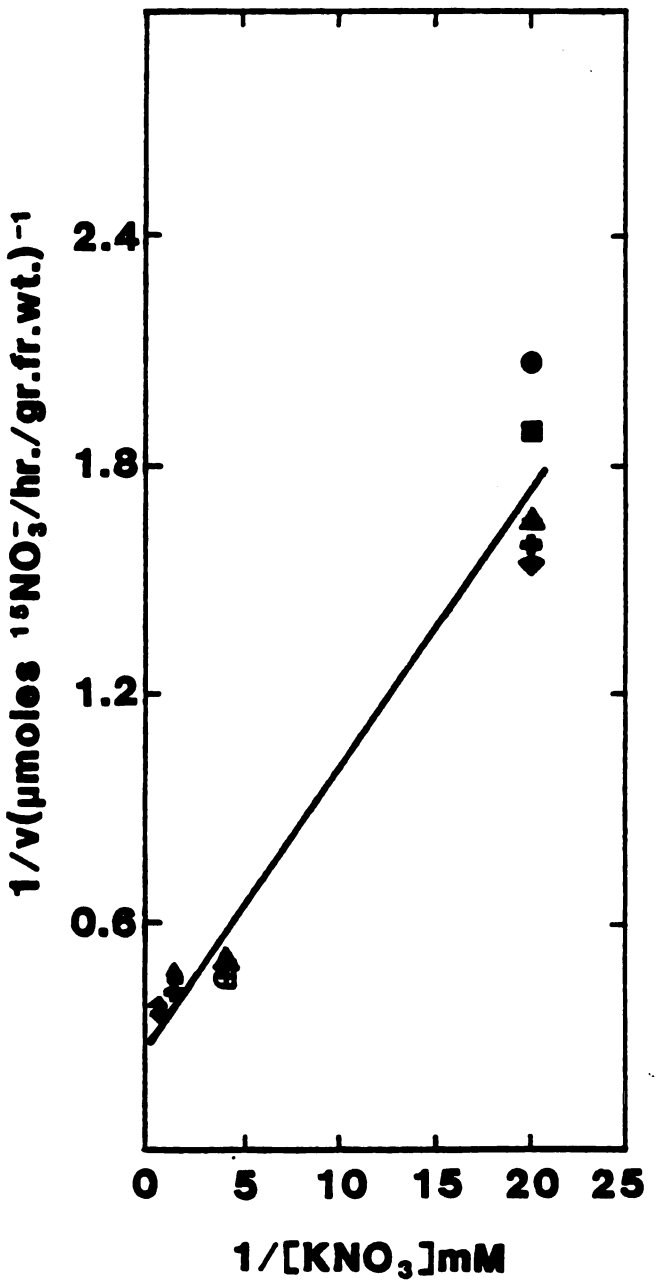

Figure 11: Lineweaver-Burk plot of chlorate inhibition of nitrate uptake in XD cells. (●) = 1.5 mM, (■) = 3.2 mM, (▲) = 0.6 mM, (✚) = 0.02 mM, and (◆) = 0 mM $KClO_3$.

Five-day-old cells preincubated in N-less medium were transferred to N-less medium containing 0.5 mM ^{15}N-KNO_3. The culture was divided into 25-ml portions to which $KClO_3$ was then added to give final concentrations ranging from 0 to 20 mM. After 2 hours, the cells were harvested and prepared for ^{15}N atom% measurements. In this instance, the ^{15}N abundance of the remaining medium after harvesting the cells was also measured. This determination was used to correct for the change in ^{15}N abundance caused by leakage of ^{14}N from the cells' nitrate pool. The percent decrease in medium ^{15}N abundance ranged from 17 to 28%.

The results obtained after correcting for the decrease in the medium ^{15}N abundance are shown in Figure 12. 1/v values plotted against inhibitor concentration gave an almost horizontal line indicating that chlorate only slightly inhibits the uptake of nitrate, thus supporting our previous finding.

Inhibition of Nitrate Uptake by Chlorate in Urea-Grown Cells.

The rate of chlorate and nitrate incorporation into XD cells previously maintained in urea-M1D medium, upon transfer to N-less M1D containing either chlorate or nitrate, is the same for each of the two anions during the intial 2.5 hours. After this time, the rate of nitrate uptake increases rapidly while the chlorate accumulation maintains the intial rate for a longer period of time (David Rhodes, personal communication). From these results, it was hypothesized that XD cells have a constitutive uptake system that does not discriminate between chlorate and nitrate while there is an inducible system with a higher affinity for nitrate. If this were true, then nitrate uptake should be inhibited by chlorate only during the first 2.5 hours upon transfer of nitrate-free cells to nitrate supplemented medium.

This hypothesis was tested by transferring the urea-grown cells to M1D

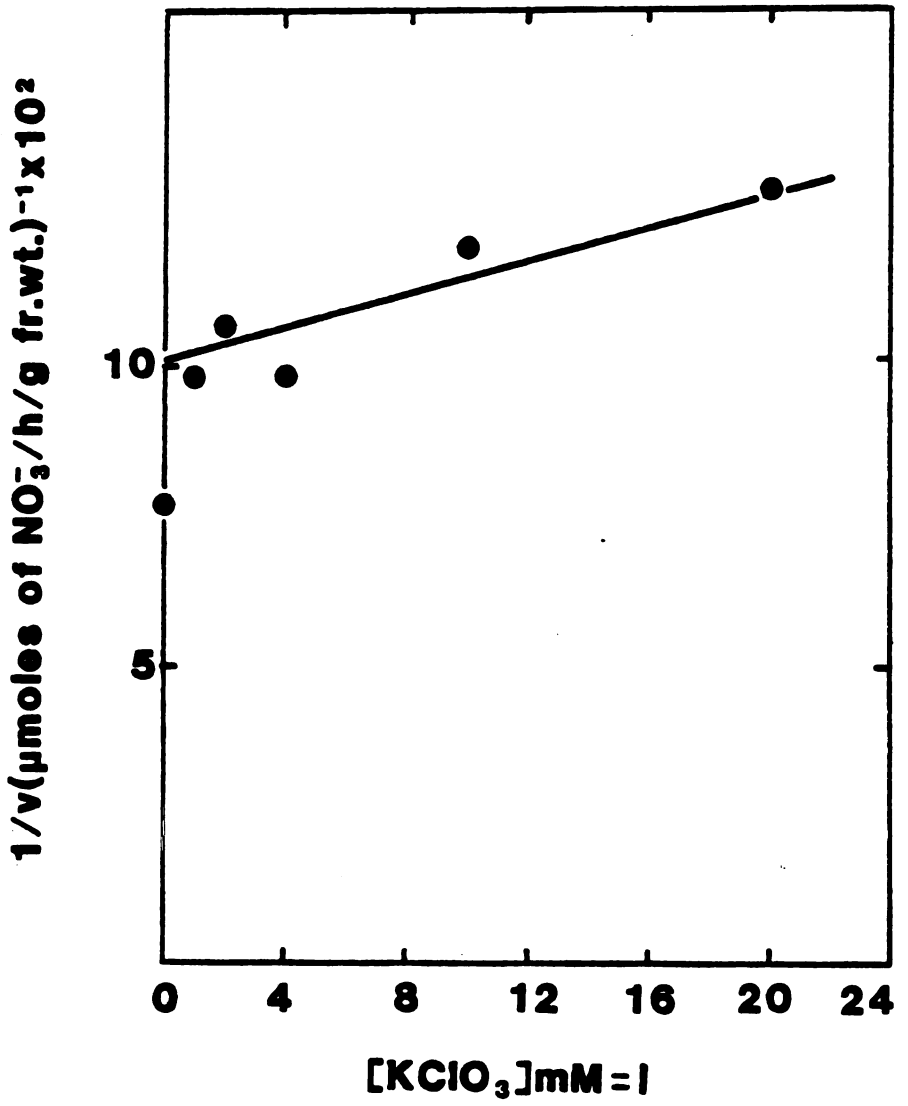

Figure 12: Dixon plot of chlorate inhibition of nitrate uptake in XD cells grown in nitrate. [KNO_3] = 0.5 mM.

containing 0.5 mM ^{15}N-KNO_3. This suspension was then divided into 25-ml batches to which chlorate was added to final concentrations of 0 to 10 mM. ^{15}N abundance in cells harvested at 2.5 hours following inoculation was determined by mass spectrometry.

A plot of the reciprocal of the intial velocity values *versus* the inhibitor concentration ($KClO_3$) gave a line characteristic of inhibition in support of the proposed hypothesis (Figure 13). Chlorate inhibited nitrate uptake when the measurements were done during the time period when chlorate and nitrate accumulation were equivalent.

Nitrate transport is inducible by nitrate in XD cells (Heimer and Filner, 1971; Behrend and Matales, 1975). Grown in urea, XD cells are therefore uninduced for nitrate transport (Heimer and Filner, 1971). When XD cells are transferred from urea to nitrate medium, the induction of their nitrate transport systems should cause them to behave in this situation exactly like nitrate-maintained XD cells. Such a result would strengthen our interpretation of the findings reported above. This experiment was performed by preincubating XD cells, which had been previously grown on urea, for 6 hours in nitrate medium (0.5 mM KNO_3). Cells were then harvested and incubated in N-less M1D for 30 minutes, harvested again and split into 25-ml portions. ^{15}N-nitrate (0.5 mM) and chlorate (0 to 10 mM) were added as previously described. After two hours of incubation, cells were harvested and ^{15}N atom % determined. The values obtained were corrected for abundance changes of medium ^{15}N as before. The results shown in Figure 14 are equivalent to those shown in Figure 12 for cells maintained on nitrate. This agreement reinforces the hypothesis of a dual system for nitrate uptake, of which one carrier is constitutive and shared with chlorate, while the other is inducible and essentially exclusive for nitrate.

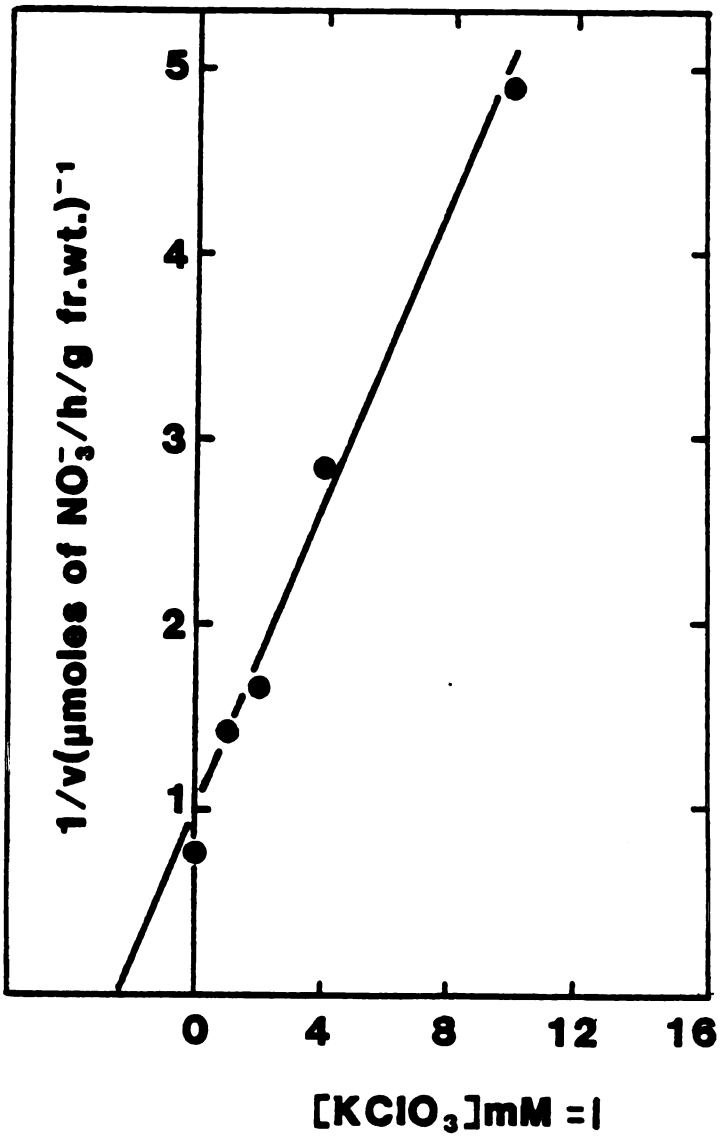

Figure 13: Dixon plot of chlorate inhibition of nitrate uptake in XD cells grown in urea. [KNO_3] = 0.5 mM.

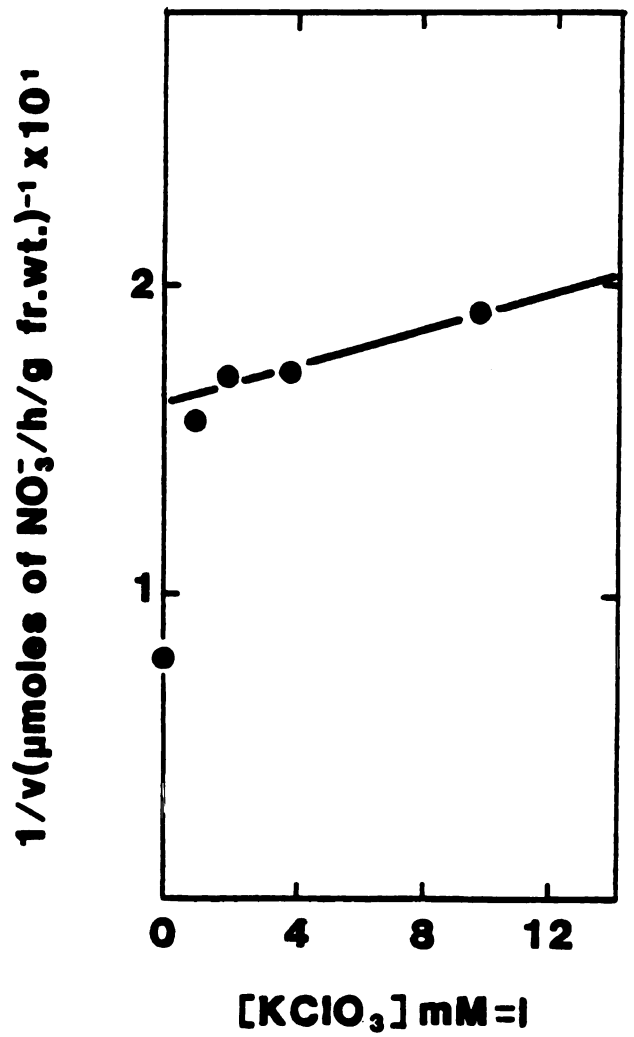

Figure 14: Dixon plot of chlorate inhibition of nitrate uptake by 14U cells pretreated in nitrate induction medium. [KNO_3] = 0.5 mM.

Effects of Ammonium and Cycloheximide on Chlorate Uptake.

The effect of ammonium on chlorate accumulation was examined since it is widely recognized that ammonium prevents nitrate utilization (Buth *et al.*, 1975; Heimer and Filner, 1971; Minotti *et al.*, 1969a; and, Rao and Rains, 1976a). If nitrate and chlorate uptake share common uptake systems, then ammonium should affect both systems similarly. Cycloheximide was used to investigate a protein synthesis requirement or a high turnover rate of the carrier involved in chlorate uptake.

Exponential-phase cells were washed and resuspended in 200 ml of N-less M1D to which ^{36}Cl-chlorate had been added. Five- ml aliquots were harvested every 30 minutes, and the number of counts incorporated was measured. After 3 hours, two 50-ml batches of the cell suspension were removed. Ammonium succinate was added to one to a final concentration of 1 mM. Succinic acid was added together with ammonium because it has been shown previously that plant cells cannot grow on ammonia in the absence of an organic acid (Heimer and Filner, 1971; Ohta *et al.*, 1981). A solution of cycloheximide to a final concentration of 20 μg/ml [sufficient to completely inhibit protein synthesis in XD cells (Heimer and Filner, 1971)] was added to the second batch. The remaining cells were used to continue measuring chlorate incorporation as a control. 5-ml aliquots were harvested from each one of the three cultures every 30 minutes, and chlorate accumulation was measured by liquid scintillation counting.

Ammonium succinate does not inhibit chlorate accumulation in the cells but rather seems to have a stimulatory effect (Figure 15). It is well documented that ammonium uptake by either cultured plant cells or roots brings about a release of protons, increasing the acidity of the medium (Ohta

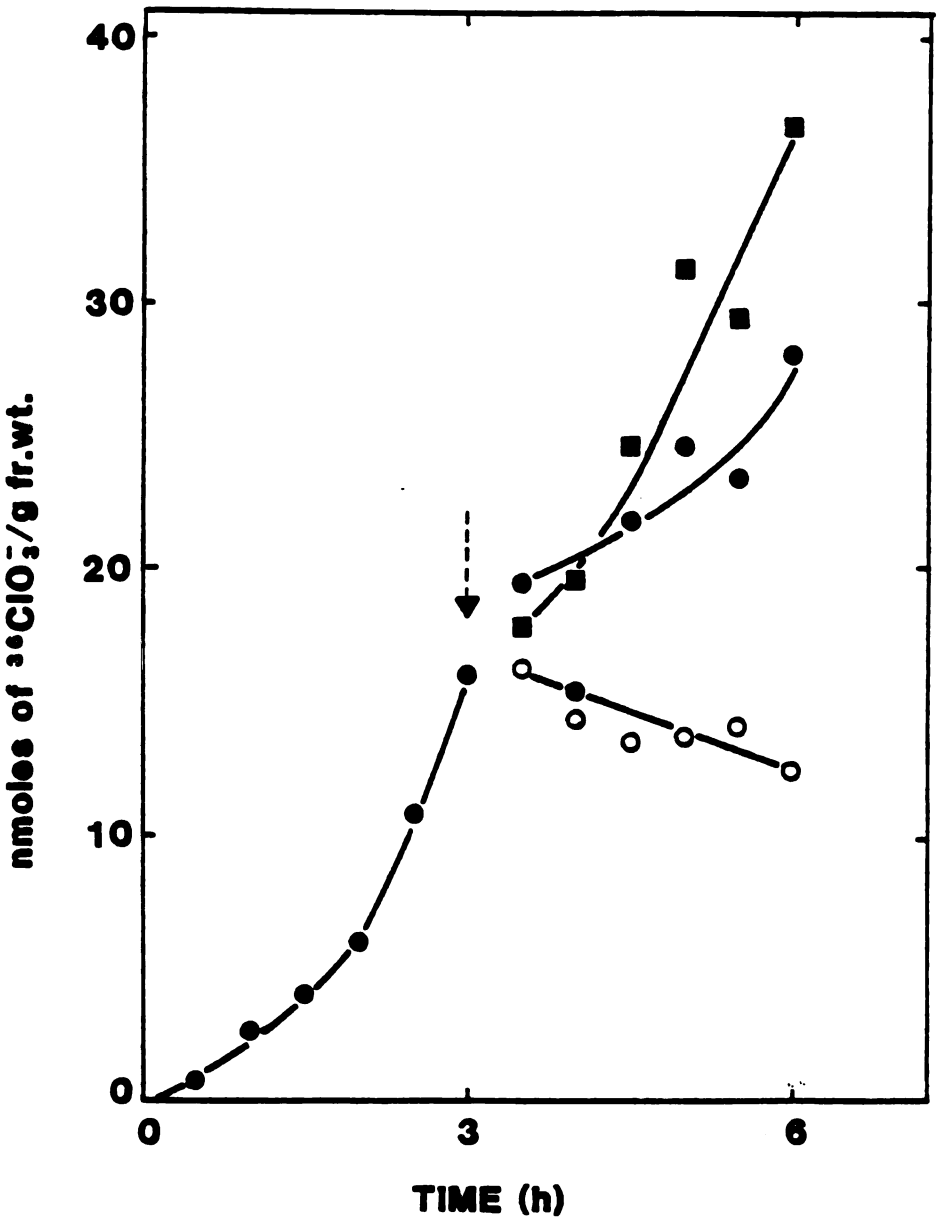

Figure 15: Effect of ammonium and cycloheximide on chlorate uptake in XD cells. Arrow indicates time of addition of ammonium succinate (to 1 mM,[■]) or cycloheximide (to 20 μg / ml [O]). (●) = untreated control.

et al., 1981; Raven and Smith, 1976). As discussed below, the optimum pH for chlorate uptake is 5.5. The medium used for this experiment had a pH of 6.2. Therefore, a decrease of the medium pH by ammonium incorporation into the cells could explain an increase in the rate of chlorate uptake.

Cycloheximide inhibited chlorate uptake very rapidly, and no appreciable incorporation of label took place after its addition. It would appear that not only did cycloheximide inhibit $^{36}ClO_3^-$ uptake but also allowed leakage of ^{36}Cl-chlorate back into the medium. In a period of 3 hours, ^{36}Cl-chlorate content decreased from 16 nmoles / g fr. wt. to 12.4 nmoles / g fr. wt. The high sensitivity of the system to cycloheximide is an indication that the carrier for chlorate may have a rapid rate of turnover. This is surprising since the chlorate uptake system is constitutive. However, there are reports in the literature (Jackson, 1978) of other effects of cycloheximide besides inhibiting protein synthesis. The drug may have inhibited the uptake system directly. It should be pointed out that manipulation of the cultures at 3 hours resulted in a chlorate uptake lag in all treatments, including the chlorate-only control.

Chlorate Uptake in 14U Tobacco Cells.

At this stage of the study, the 14U cell system became available, with growth rate characteristics on urea identical to the wild type XD cells on nitrate. The XD cell cultures grow 20% less rapidly on urea than they do on nitrate. The 14U cell line simplified procedures by obviating the need to eliminate nitrate contamination from the internal cell pool. The method of harvesting the cells was also changed from vacuum filtration to a more gentle one (gravity filtration) for reasons already described.

The discovery that chlorate uptake has a sharp pH optimum at 5.5 and

the transport rate at pH 6.5 is one-fifth that at pH 5.5 (M. Guy, personal communication) made it desirable to repeat the chlorate uptake measurements using buffered incubation media at two different pHs: 5.5 and 6.5 (Figure 16). After 20 hours of uptake, cells incubated at pH 5.5 accumulated five times more chlorate (48 nmoles / g fr. wt.) than those incubated at pH 6.5 (9.4 nmoles / g fr. wt.). Comparing those two values with the amount of chlorate per ml in the external medium (15 nmoles / ml), tobacco cells concentrated chlorate against a gradient at pH 5.5 but not at pH 6.5. It should also be noted that the plots of chlorate accumulation by 14U cells grown on urea do not show the lag phase seen with the XD cells grown on M1D. The possibility remains that intrinsic properties of the cell line, rather than the harvesting procedure, are responsible for the observed lag in chlorate uptake in the XD cells *versus* the 14U cells, where no lag was observed.

The rate of chlorate uptake increases with increasing chlorate concentration, giving complex uptake kinetics (Figure 17). This indicates the existence of two systems of chlorate uptake: A saturable component functioning at concentrations below 1 mM and a linear one which accounts for net increases at concentrations above 1 mM. This linear portion of the curve could be the result of diffusion of chlorate into the cells such that the rate would be proportional to the external chlorate concentration. Another poissibility is the existence of a second potentially saturable transport system with low affinity for chlorate.

Figure 18 shows the ratio of chlorate accumulated (values obtained from Figure 17) over the amount of chlorate in the external medium at different chlorate concentrations. At pH 5.5, those ratios remain above 1, supporting the previous observation that chlorate at pH 5.5 is taken up against a concentration gradient.. On the other hand, at pH 6.5, this ratio is close to or

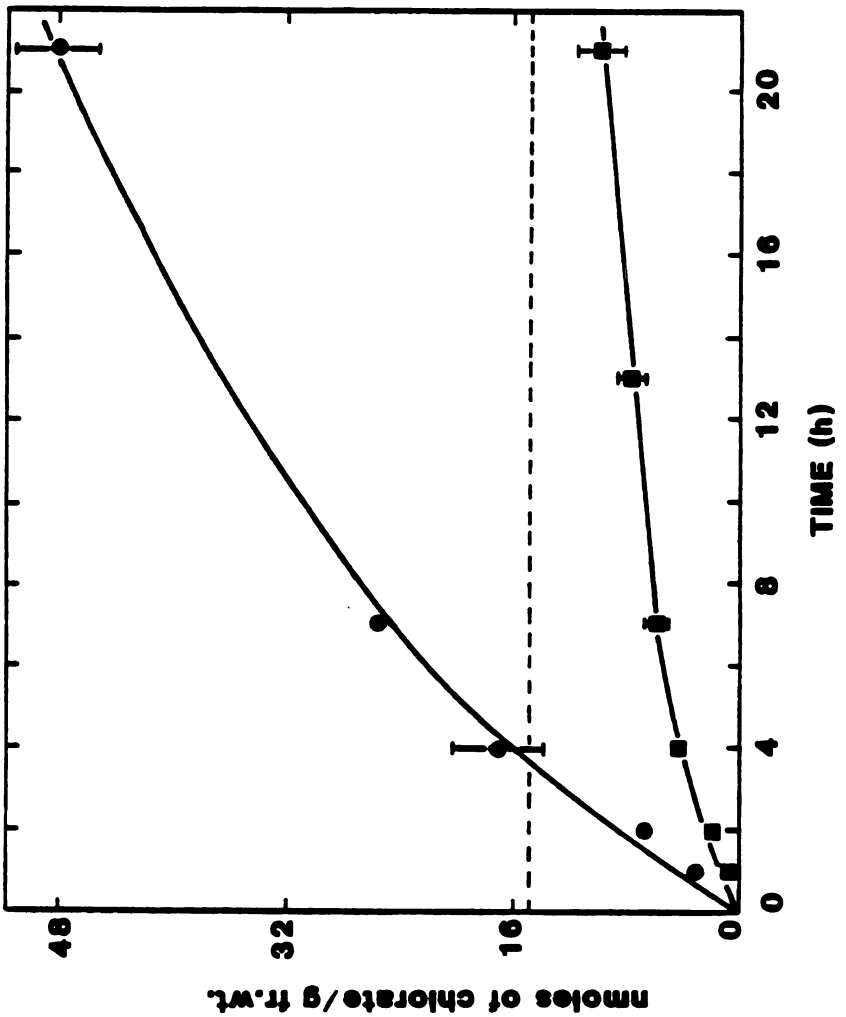

Figure 16: pH dependence of chlorate accumulation in 14U cells. Dashed line indicates point of equivalence between intracellular and ambient chlorate concentration. (●) = pH 5.5; (■) = pH 6.5.

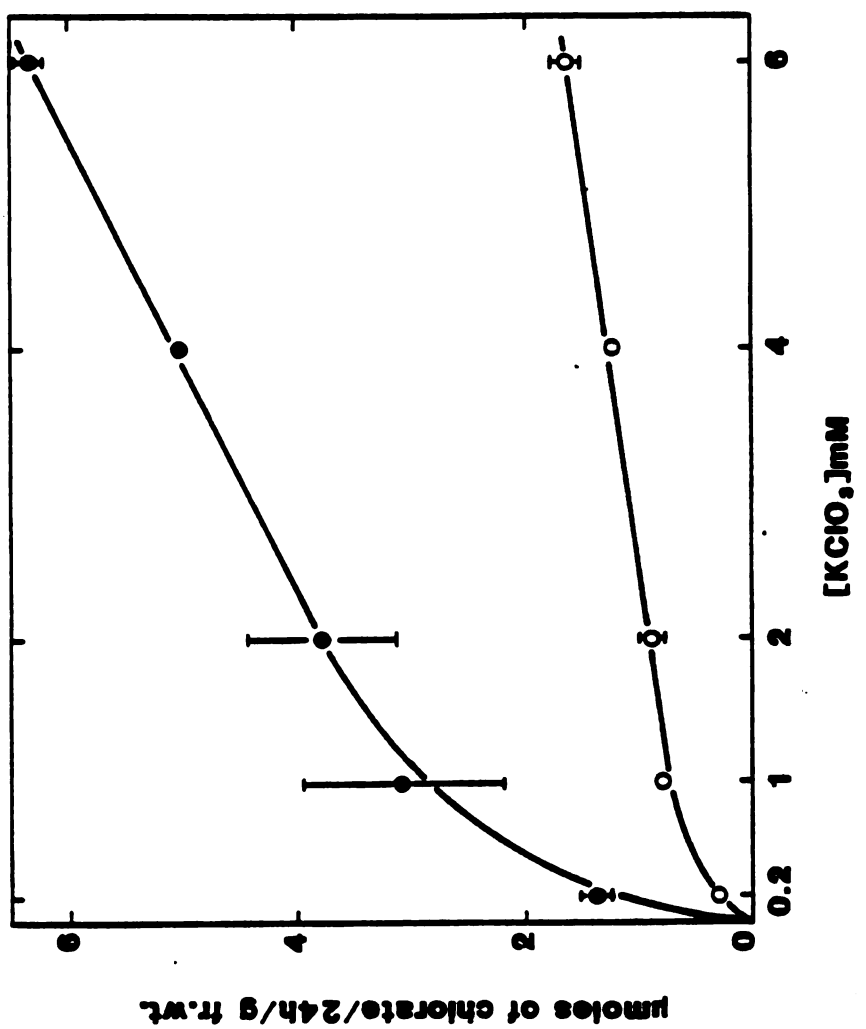

Figure 17: pH dependence of chlorate uptake in 14U cells. (●) = pH 5.5; (○) = pH 6.5.

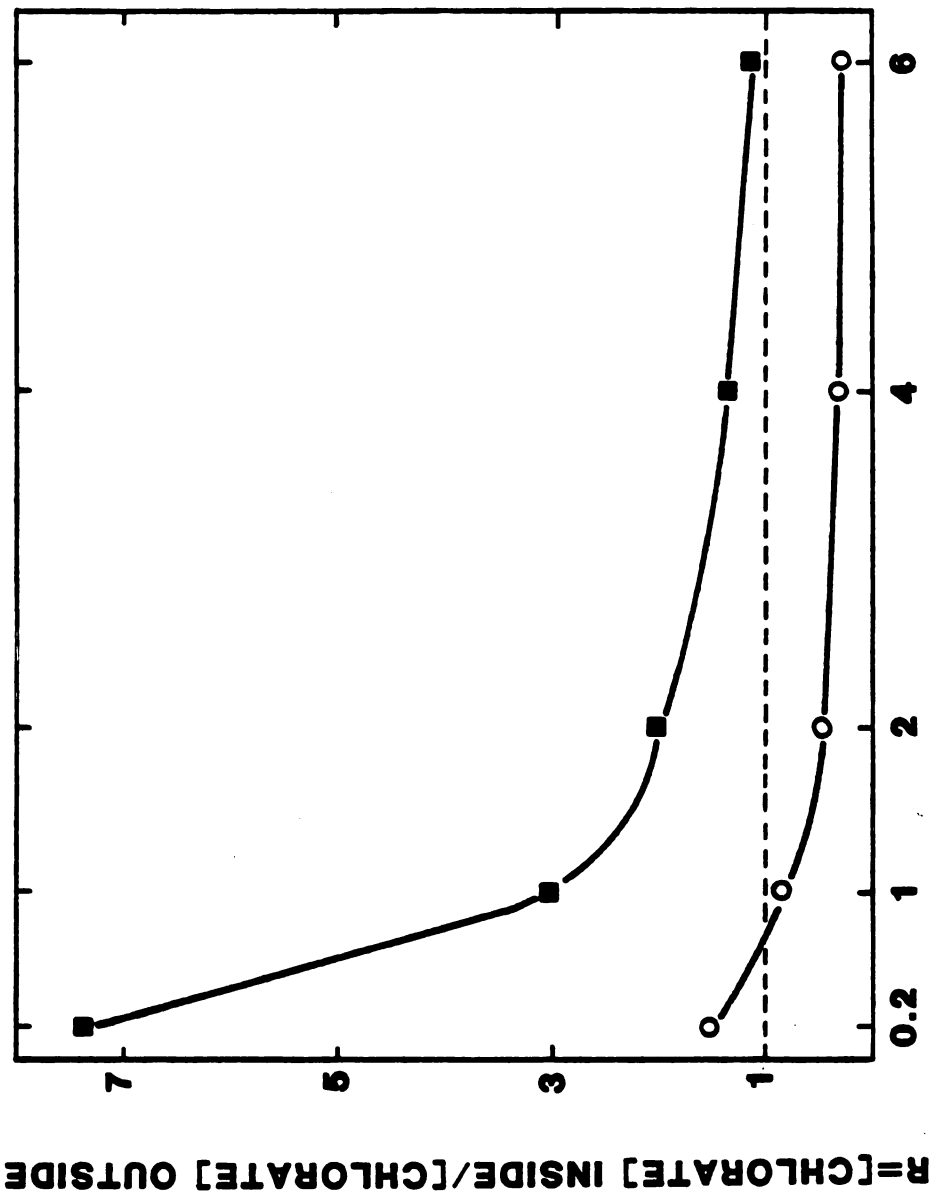

Figure 18: pH dependence of intracellular : extracellular chlorate concentration ratio in 14U cells.
(■) = pH 5.5; (O) = pH 6.5.

below 1. This suggests that active uptake or cotransport of chlorate occurs at pH 5.5, but at pH 6.5, chlorate enters passively, perhaps by facilitated diffusion. Protons could conceivably play a role in the active process at pH 5.5.

Inhibition of Chlorate Uptake by Nitrate in 14U Tobacco Cells.

The pH dependence of inhibition of chlorate uptake by nitrate was examined in light of the above results. Nitrate concentrations ranged from 0 to 30 mM. Nitrate inhibited chlorate uptake at pH 5.5 (Figure 19) and 6.5 (Figure 20). The curved portion of each plot of v *versus* [S] becomes a straight line when the rate of chlorate uptake is measured in the presence of 15 mM nitrate. Apparently, 15 mM nitrate completely inhibits the saturable component of chlorate transport at chlorate concentrations below 1 mM (the high affinity system) but does not inhibit transport at high chlorate concentrations (low affinity system or diffusion). The latter is evident from the parallel lines above 1 mM chlorate. Subtraction of the low affinity or the diffusion component from the v *versus* [s] curves in Figures 19 and 20 reveals the saturation kinetics of the high affinity system (see insets). From these plots, the Km and Vmax for chlorate uptake by the two systems at the two pHs were determined (see Table 4).

Effect of Chloride Ion on Chlorate Uptake.

To further analyze the discriminatory power of the chlorate uptake system, the effect of chloride on chlorate accumulation was examined. KCl at 15 mM was added to the incubation medium (urea-M1D) which already contained 2.5 mM KCl. The results at pH 5.5 are shown in Figure 21. Surprisingly, chloride dramatically inhibited chlorate incorporation into the cells. However, of greater interest is the observation that chloride inhibits the

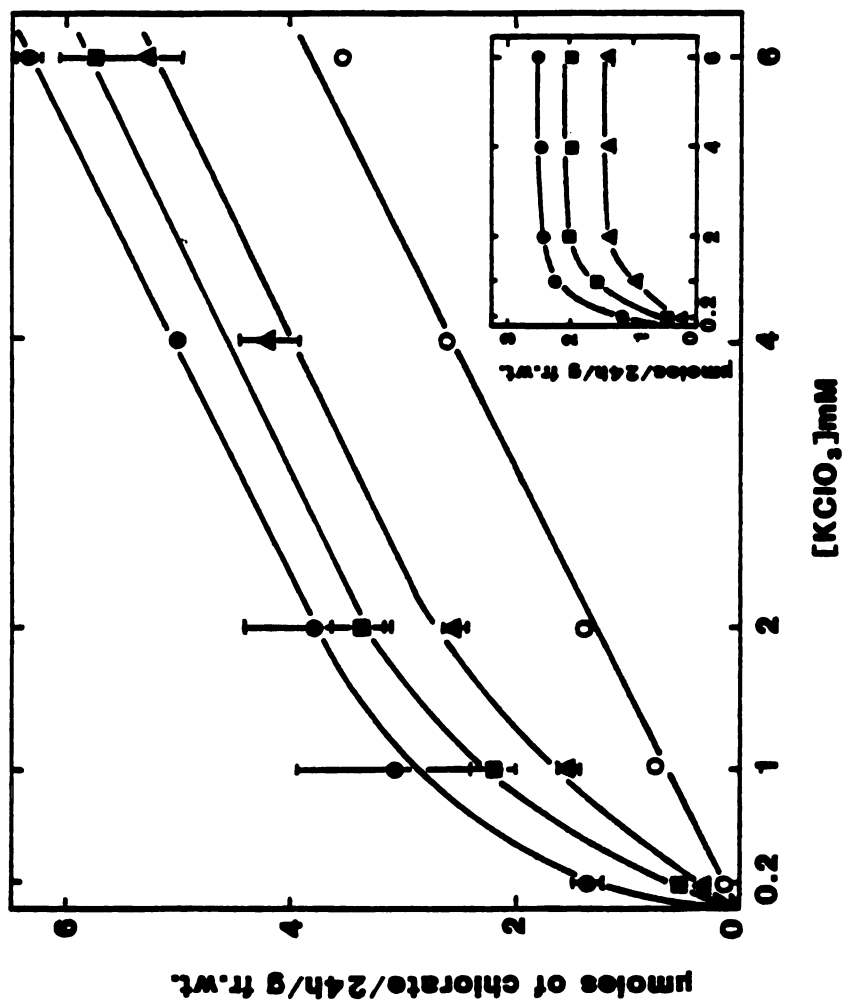

Figure 19: Nitrate inhibition of chlorate uptake at pH 5.5 in 14U cells. $[NO_3^-] = 0$ (●), 2.5 (■), 5 (▲), and ≥15 (O) mM. Insert shows curves resulting from subtraction of non-saturable component from all curves.

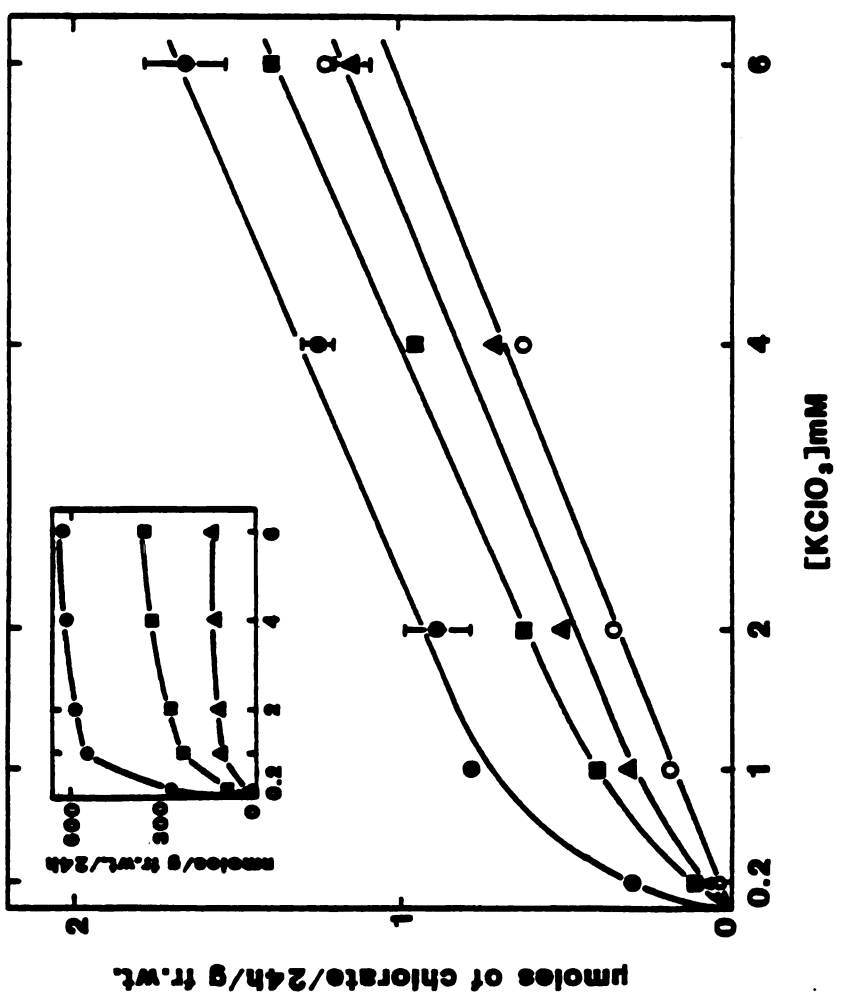

Figure 20: Nitrate inhibition of chlorate uptake at pH 6.5 in 14U cells. $[NO_3^-] = 0$ (●), 2.5 (■), 5 (▲), and 15 (O) mM. Insert shows curves resulting from subtraction of non-saturable component from all curves.

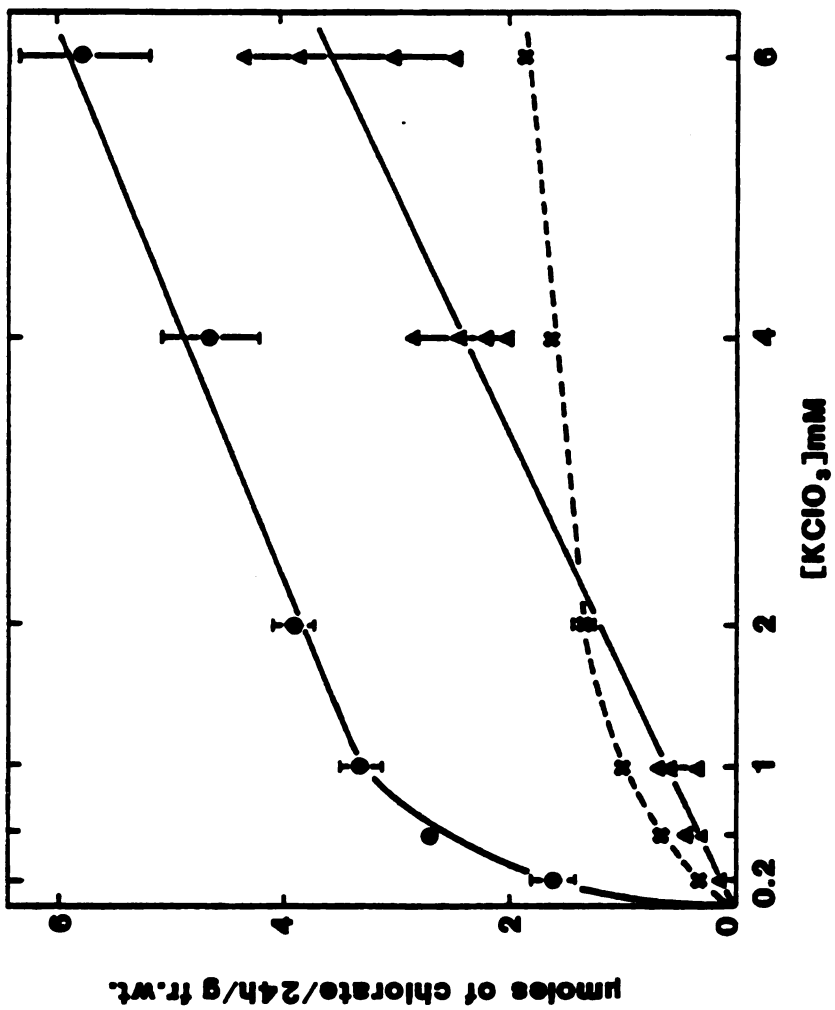

Figure 21: Effect of Cl^- on ClO_3^- uptake by 14U cells. (●) = 0 NO_3^- + 2.5 mM KCl; (▲) ≥ 15 mM NO_3^- + 2.5 mM KCl; (✱) = 0 NO_3^- + 17.5 mM KCl.

linear component of chlorate uptake, but not the high-affinity system. This is precisely opposite the effect that nitrate has on the two components of chlorate uptake. Chloride inhibition at high chlorate concentrations cannot be explained by a diffusion process. Therefore, the linear component of chlorate uptake appears to be an uptake system with low affinity for chlorate.

Since chloride inhibits chlorate uptake and N-less M1D medium contains 2.5 mM chloride, the previous values obtained for chlorate uptake in this N-less M1D medium may represent an inhibited transport system. In order to correct for this possible error, chlorate uptake was measured in cells incubated in a chloride-free nitrogen-free medium (Cl-less N-less M1D) to which urea, chlorate and chloride were added. Indeed, chlorate transport by the low affinity system was enhanced at both pHs 5.5 and 6.5 (Figures 22 and 23). In the experiment plotted in Figure 22, all $CaCl_2$ and KCl were simply omitted from the medium. However, in the following experiment (Figure 23), the missing cations were returned to the medium in the form of $CaSO_4$ and K_2HPO_4. This nutritional alteration was probably responsible for the differences in uptake rates between similar treatments in the two experiments.

In the absence of chloride, there was a four-fold reduction in the pH sensitivity of chlorate uptake (Figures 22 and 23).

To determine whether the KCl effect (Figures 21, 22, and 23) was due to chloride or to the counterion K^+, the inhibitions of chlorate transport by the K^+ and Na^+ salts of chloride were compared. No differences were observed in the degree of inhibition caused by the two salts at either pH 5.5 or 6.5 (Figure 24). Therefore, chloride, not the counterion K^+ is responsible for the inhibition.

The sensitivity of the chlorate uptake system was examined further by

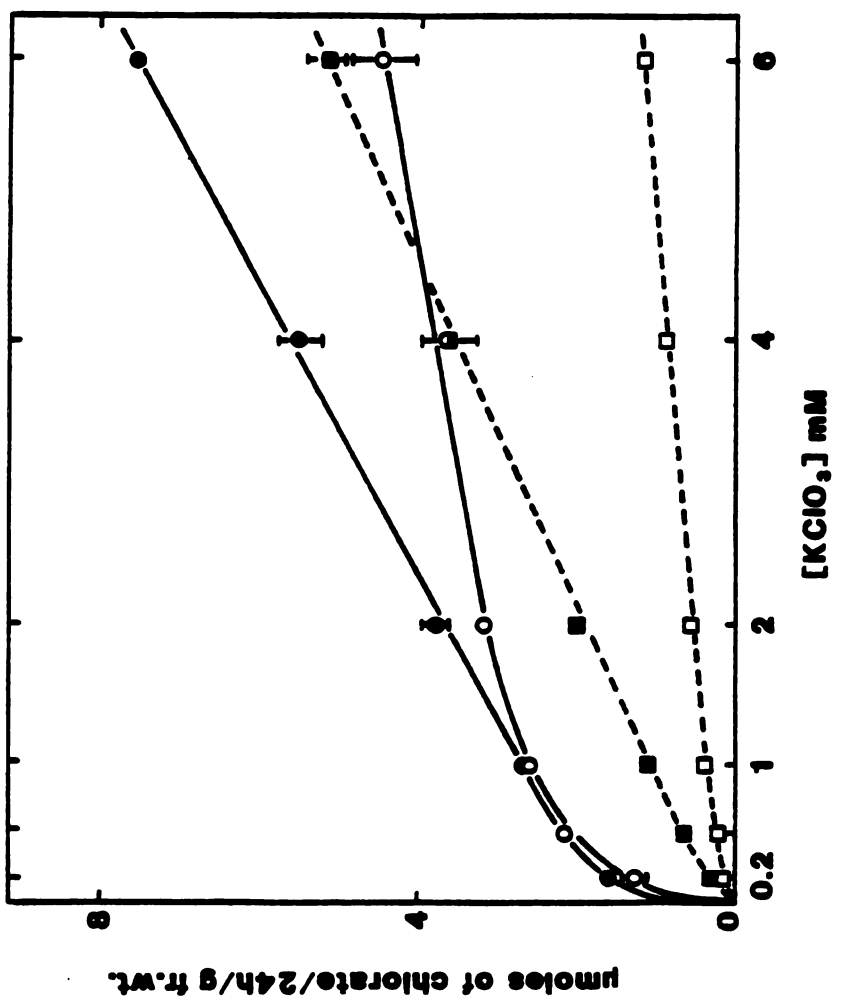

Figure 22: Combined effects of pH and Cl^- on ClO_3^- uptake in 14U cells. (●) = 0 KCl, pH 5.5; (○) = 2.5 mM KCl, pH 5.5; (■) = 0 mM KCl, pH 6.5; (□) = 2.5 mM KCl, pH 6.5.

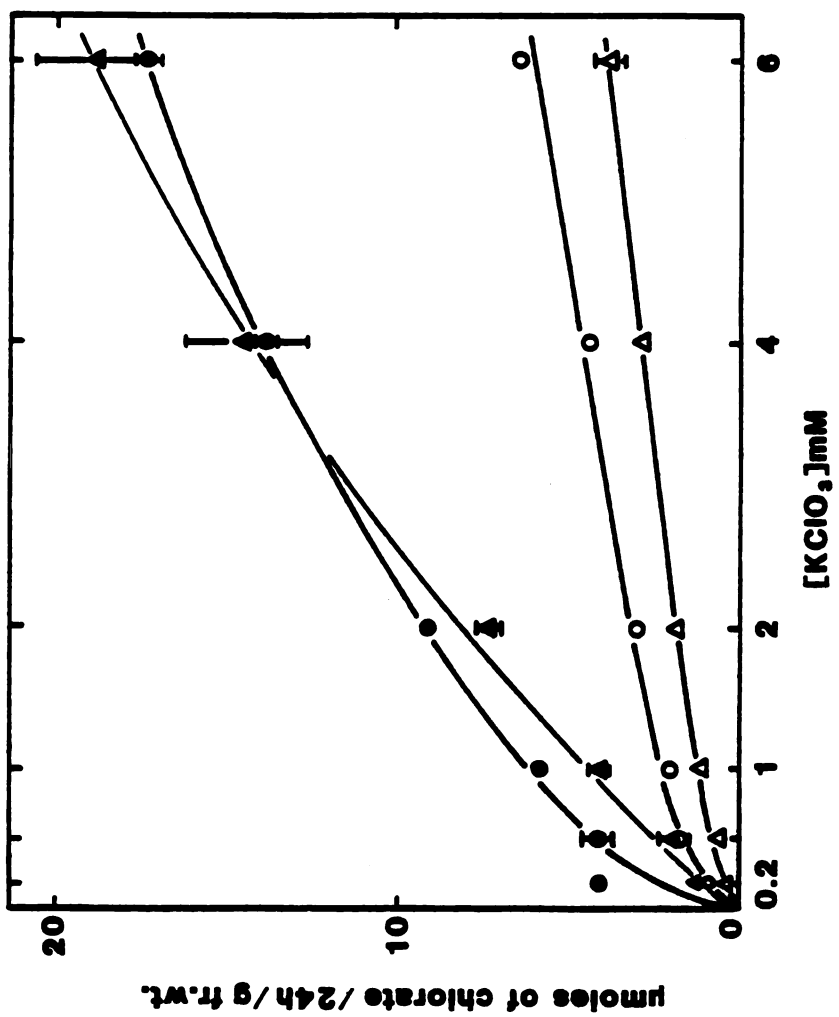

Figure 23: Combined effects of Cl^- and pH on ClO_3^- uptake in 14U cells. (▲) = 0 KCl, pH 6.5; (●) = 0 KCl, pH 5.5; (O) = 6 mM KCl, pH 5.5; (Δ) = 6 mM KCl, pH 6.5.

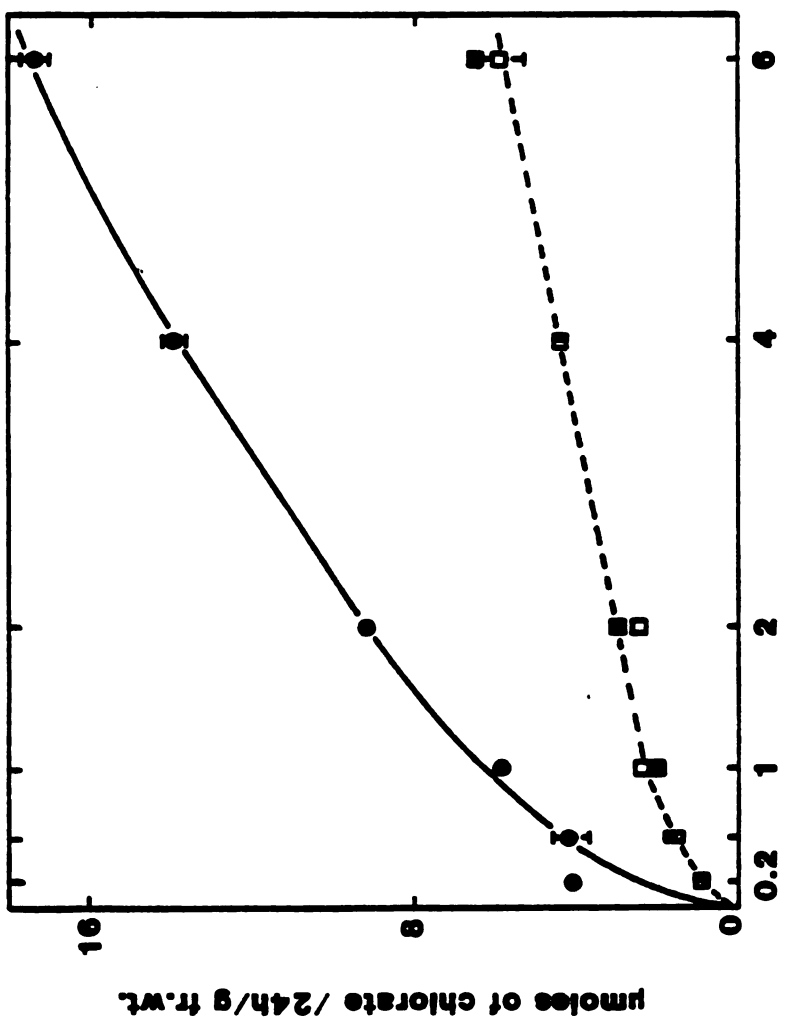

Figure 24: Effect of counterion on Cl^- inhibition of ClO_3^- uptake in 14U cells. All pH 5.5. (●) = 0 KCl; (■) ≐ 6 mM KCl; (□) = 6 mM NaCl.

evaluating the effect of perchlorate on chlorate transport. Guy (personal communication) had shown that the kinetic characteristics of perchlorate uptake were identical to those of chlorate uptake in 14U cells. The results shown in Figure 25 indicated that the high-affinity component of chlorate uptake was inhibited by perchlorate at both pHs 5.5 and 6.5, suggesting an interaction between the two ions at the uptake level.

The results presented thus far suggest that chloride inhibits chlorate uptake at the chlorate low-affinity transport system. An experiment was designed to determine the nature of the inhibition. Chlorate uptake was measured as a function of increasing chloride concentrations (3 to 9 mM KCl) (Figure 26). Three millimolar KCl, the lowest concentration tested, completely inhibited the chlorate low affinity transport system, suggesting either that the affinity of the chlorate low affinity transport system for chloride is very high or that chloride inhibits chlorate uptake by a mechanism other than competitive inhibition.

In order to determine whether the previously observed inhibition of chlorate transport had been influenced by the presence of chloride in the medium, the effect of two nitrate concentrations (2.5 and 15 mM) on chlorate uptake was measured in the new Cl-less N-less M1D medium. As in previous experiments, nitrate inhibited chlorate uptake (Figure 27). With nitrate at 15 mM, the high affinity system for ClO_3^- transport was completely inhibited, and a linear rate of chlorate uptake was observed throughout the range of chlorate concentrations, with the slope being 30% lower at 15 mM NO_3^- than at 0 or 2.5 mM. This means that NO_3^- can inhibit the low affinity system too, but rather weakly.

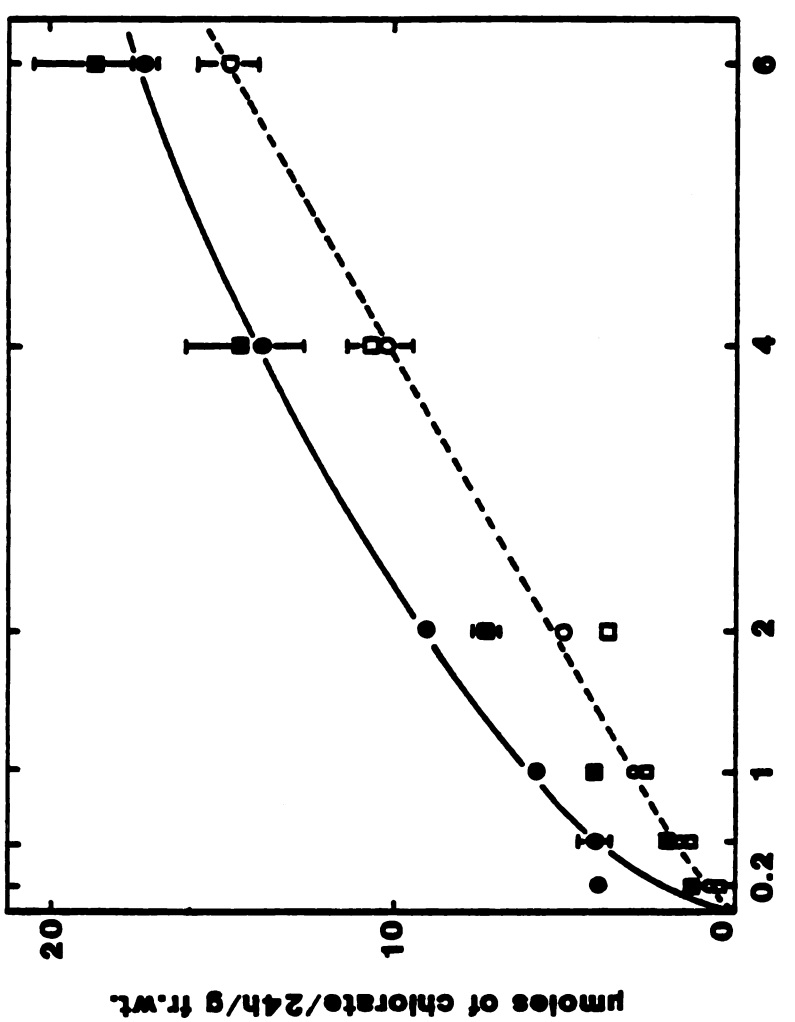

Figure 25; Effect of ClO_4^- on ClO_3^- uptake in 14U cells. No Cl^- was present in any treatment. (■) = 0 ClO_4^-, pH 6.5; (●) = 0 ClO_4^-, pH 5.5; (○) = 6 mM ClO_4^-, pH 5.5; (□) = 6 mM ClO, pH 6.5.

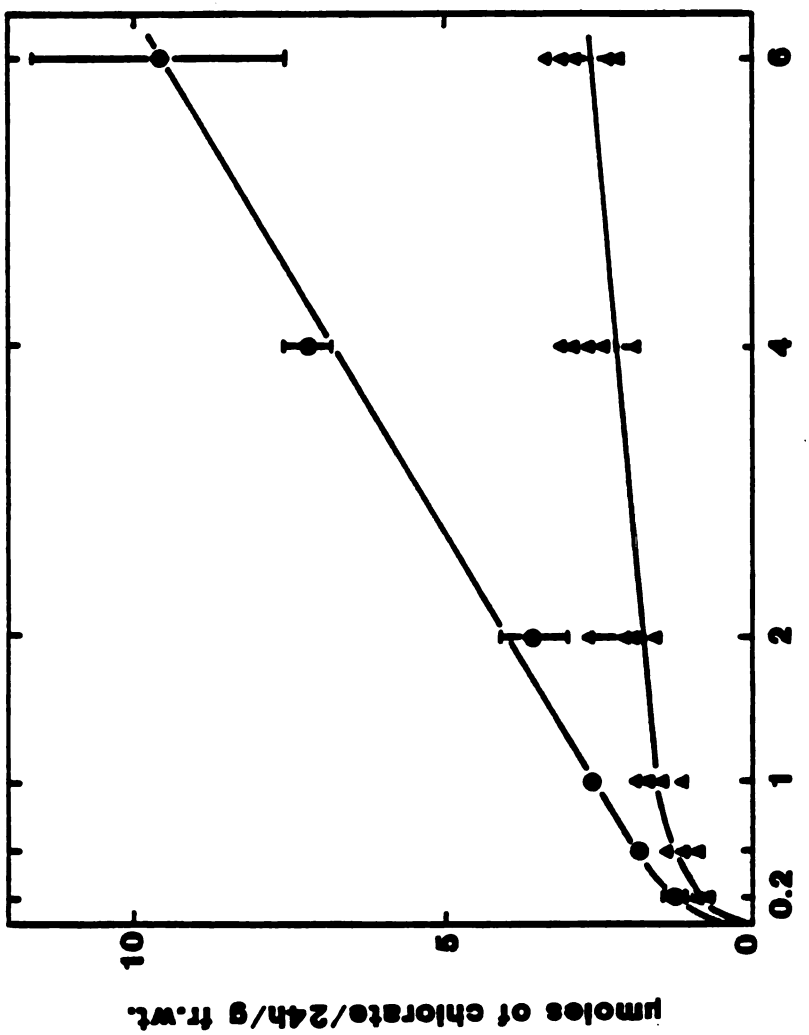

Figure 26: Effect of Cl^- on ClO_3^- uptake by 14U cells. (●) = 0 KCl; (▲) ≥ 3 mM KCl.

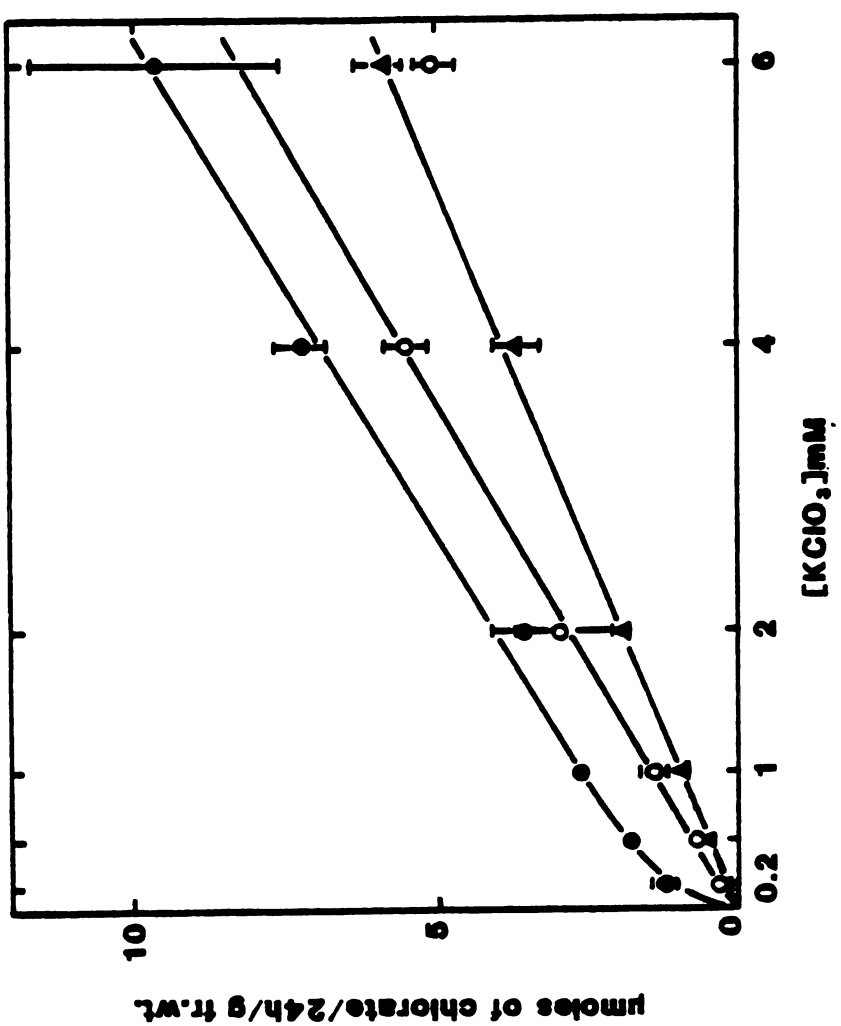

Figure 27: Effect of NO_3^- on ClO_3^- uptake in 14U cells in the absence of Cl^-. (●) = 0 KNO_3; (○) = 2.5 mM KNO_3; (▲) = 15 mM KNO_3.

Characteristics of Chloride Uptake System.

The interaction of chloride and chlorate described above raised the question of whether they share a common (low affinity) transport system.

In contrast to chlorate uptake, chloride transport at pHs 5.5 and 6.5 displays saturation kinetics.(Figure 28). The double reciprocal plot (Figure 29) for chloride transport suggests the existence of two uptake systems.

Chlorate at 6 mM lowered the Vmax of chloride uptake at both pHs, though proportionally more at pH 5.5 (Figure 28). Since chloride transport is relatively insensitive to pH, the greater inhibition of chloride uptake by chlorate at pH 5.5 than at 6.5 can be accounted for by the pH dependence of chlorate transport. Chlorate uptake at pH 5.5 was 4 times that at pH 6.5 in the presence of 2.5 mM chloride (Figure 22) and chlorate was 3.2 times more effective as an inhibitor of chloride uptake (Figure 28) under conditions in which chlorate uptake would have been proceeding 4 times more rapidly (Figure 22).

In addition, a comparison between the double reciprocal plots (Figure 29) of chloride transport in the presence and absence of chlorate, suggests that chlorate preferentially inhibits a low-affinity chloride uptake system. That is, at low 1/[S], the + and - chlorate plots converge, but are parallel at high 1/[S]. Therefore, it appears that chloride is taken up via separate high and low-affinity systems. Only the low affinity transport system is inhibited by chlorate. The type of inhibition is unclear, but seems not to be competitive (Figure 29). Table 4 summarizes the properties of chlorate and chloride transport systems.

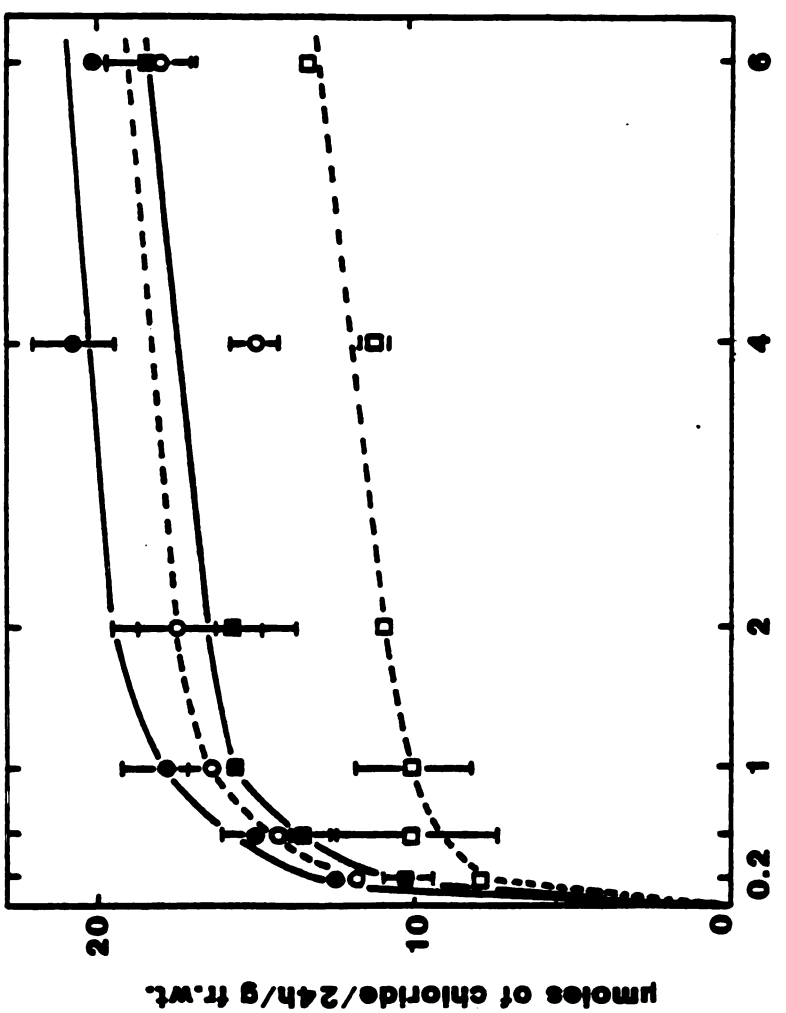

Figure 28: Effect of chlorate and pH on Cl^- uptake in 14U cells. (●) = 0 ClO_3^-, pH 6.5; (○) = 6 mM ClO_3, pH 6.5; (■) = 0 ClO_3^-, pH 5.5; (□) = 6 mM ClO_3^-, pH 5.5.

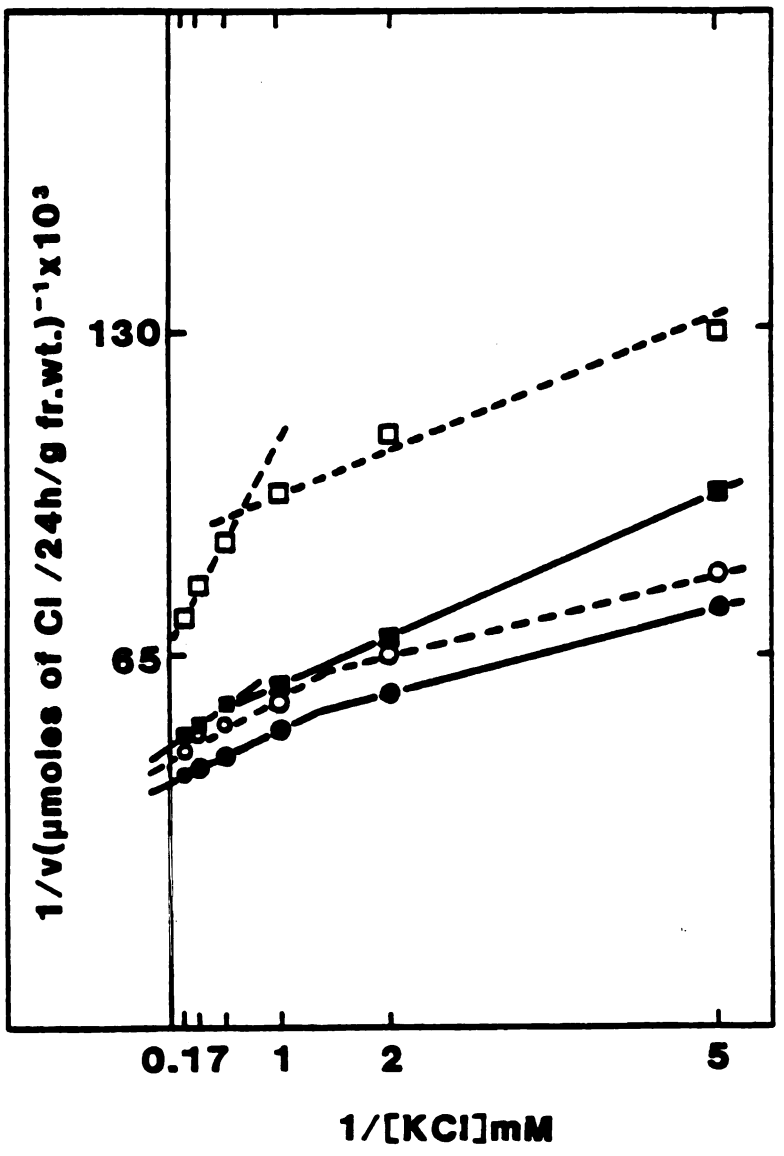

Figure 29: Lineweaver-Burk analysis of effect of pH and ClO_3^- on Cl^- uptake in 14U cells. (□) = 6 mM ClO_3^-, pH 5.5; (■) = 0 ClO_3^-, pH 5.5; (○) 6 mM ClO_3^-, pH 6.5; (●) = 0 ClO_3^-, pH 6.5.

Table 4. Properties of chlorate and chloride transport systems.

	System I		System II		System III	
	pH 5.5	pH 6.5	pH 5.5	pH 6.5	pH 5.5	pH 6.5
ClO_3^- V5mM (μ mole ClO_3^-/g fr.wt./24 h)	2.45	0.63	3.91	0.52	-	-
ClO_3^- Km (mM)	0.33	0.29	>>5	>>5	-	-
Cl^- V max (μ mole Cl^-/g fr.wt./24 h)	-	-	18.9	21.74	18.2	19.23
Cl^- Km (mM)	-	-	0.27	0.21	0.15	0.10
Inhibition by nitrate of ClO_3^- transport	yes	yes	slight	slight	-	-
Inhibition of Cl^- of ClO_3^- transport	no	no	yes	yes	-	-
Inhibition by ClO_3^- of Cl^- transport	-	-	yes	yes	no	no

PART II: REDUCTION OF CHLORATE BY NITRATE REDUCTASE

MATERIALS AND METHODS

Preparation of Enzyme Extracts

Cells were harvested by vacuum filtration onto Whatman No. 1 filter paper, rinsed with deionized distilled water and weighed. The cells were resuspended in a phosphate buffer consisting of 0.1 M K_2HPO_4 pH 7.5, 1 mM EGTA, 1 μM L-cysteine free base (Trinity, 1981). Five milliliters of the buffer were used per gram of cells. In experiments employing urea, the concentration of L-cysteine was increased to 5 mM. The cells were homogenized at 4° C with 30 strokes of a motor driven teflon-glass homogenizer. The soluble enzymes were separated from cell debris by centrifugation at 12,000 x g in a Sorvall SS-34 rotor for 20 minutes at 4°C. Nitrate reductase was precipitated from the supernatant solution by 1 hour incubation in 50% saturated ammonium sulfate pH 7.5, followed by centrifugation at 12,000 x g for 20 minutes at 4°C. The pellet was resuspended in a volume of phosphate buffer (described above) equal to that to which ammonium sulfate had been added in the earlier step. Low molecular weight substances were removed from the extract by centrifugation of 2 ml aliquots through 4 ml columns of Sephadex G-25-80 resin (particle size: 20-80μ) in a 5 ml disposable plastic syringe. The centrifugation was performed at 3400 rpm for 15 seconds in a clinical centrifuge at 4°C, with each syringe hanging in a 15ml conical centrifuge tube, in a swinging bucket rotor.

Determination of Nitrate and Chlorate Reductase Activities

Nitrate reductase activity was measured as the rate of nitrite formation at 25°C in the presence of 0.1 ml 0.1 M KNO_3, 0.1 ml 1 mM NADH and 0.5 ml 0.1 M K_2HPO_4 buffer pH 7.5. Final volume of 1 ml was reached by addition of 0.2 ml of enzyme extract and 0.1 ml of water (Wray and Filner, 1970). The latter was substituted by 0.1 ml of different chlorate solutions when the effect of chlorate on nitrate reduction was examined. The amount of nitrite formed after 30 minutes of incubation was determined colorimetrically as described above (pages 25 - 26). A reaction mixture stopped at zero time was used as the blank.

Chlorate reductase was followed as the rate of ^{36}Cl-chlorite and ^{36}Cl-chloride formation at 25°C in the presence of 50 µl of approximately 2.6 mM ^{36}Cl-chlorate and 50 µl of 1 mM NADH. A final volume of 0.5 ml was attained by the addition of 0.4 ml of enzyme extract. When the effect of nitrate on chlorate reduction was examined, the volume of each of the above components was tripled and 50 µl of different nitrate solutions added. The reaction was stopped after 30 minutes of incubation as described below.

Determination of ^{36}Cl-Chlorite and ^{36}Cl-Chloride

^{36}Cl-chlorite produced by chlorate reduction was chemically reduced to ^{36}Cl-chloride by the addition of 0.5 ml 100 mM KI and 0.5 ml 4 M acetic acid to 0.5 ml assay mixtures (Goksøyr, 1952). The chloride produced was precipitated with unlabeled carrier chloride by the addition of 0.5 ml of 20 mM NaCl (as carrier) and 3 ml of 100 mM $Ag(NO_3)_2$, thereby forming insoluble AgCl. In parallel tubes, enzymatically formed ^{36}Cl-chloride was precipitated by the addition of 0.5 ml 20 mM NaCl, 1 ml of H_2O and 3 ml of 100 mM $Ag(NO_3)_2$. The silver chloride precipitates were collected by centrifugation at

12,000 x g for 10 minutes, washed with 5 ml of water, and recentrifuged. The $^{36}Cl^-$ in the precipitate was solubilized by addition of 1 ml of 1 M NaCN, thereby forming soluble NaCl and AgCN. Scintillations were counted in 5 ml of the scintillation cocktail described by Trombolla (1970). ^{36}Cl-chlorite was determined by the difference in counts between the chloride specific precipitate and the chlorite and chloride precipitate.

Determination of Total Protein

Total protein was determined by the method of Lowry et al (1951). A 1-ml aliquot of the enzyme extract was precipitated with an equal volume of 20% (w/v) trichloroacetic acid. After 2 hours at room temperature, the precipitate was collected by centrifugation and rinsed twice with 95% ethanol. After drying under nitrogen gas, the precipitate was dissolved in 1 N NaOH and assayed by the Lowry procedure. Bovine serum albumin was used as a standard.

RESULTS

Inhibition of Cell Growth by Chlorate

According to Åberg's hypothesis, chlorate is rendered toxic upon its reduction to chlorite by the enzyme nitrate reductase. While it is true that this hypothesis has been very useful in providing a rationale for the successful selection of nitrate-reductase-less mutants of bacteria, fungi, algae and higher plants based on chlorate resistance, the actual mechanism of chlorate action

has not been completely established. Prior to this work, it has not been proven that nitrate reductase of higher plants catalyzes the reduction of ClO_3^-. The XD and 14U cell lines, together with ^{36}Cl-chlorate, provided a suitable system in which to examine details of the mechanism of chlorate toxicity proposed by Åberg (1947). According to the hypothesis, XD cells growing on a medium with nitrate as the sole nitrogen source and containing high levels of nitrate reductase should be more sensitive to chlorate than the 14U cells growing on a medium with urea as the sole nitrogen source and containing much lower levels of nitrate reductase. To our additional advantage, these two cell lives have equal growth rates in their respective media, greatly simplifying comparisons of their growth rates under different conditions.

The toxic effect of chlorate on the XD and 14U cells was determined by measuring the inhibition of cell growth in the following way. Growth tests were begun by diluting 25 ml of stationary-phase cultures into 225 ml of M1D or urea-M1D medium containing $KClO_3$ at concentrations ranging from 0 to 100 mM. The pH of both media, M1D and urea-M1D,had been adjusted to 6.2. Since the results of our uptake experiments established a pH optimum for chlorate uptake of 5.5, an additional series of XD cultures was prepared in a medium of pH 5.5. No buffers were added to maintain pH during the period of the growth test. The cultures were kept on a reciprocal shaker in the dark at 28°C. Periodically the cells from 2 flasks from each treatment were harvested by vacuum filtration and their fresh weights determined. The pH of the medium remaining after harvesting the cells was measured in some instances.

The XD cells growing on a medium containing nitrate at pH 5.5 and 6.2 were more sensitive to chlorate than were the 14U cells grown on urea at pH 6.2 (Figures 30 A, B, C and 31). The I_{50} values deduced from the dose response curves of Figure 31 were 54 mM for the 14U cells and 16 and 17.5 mM for the

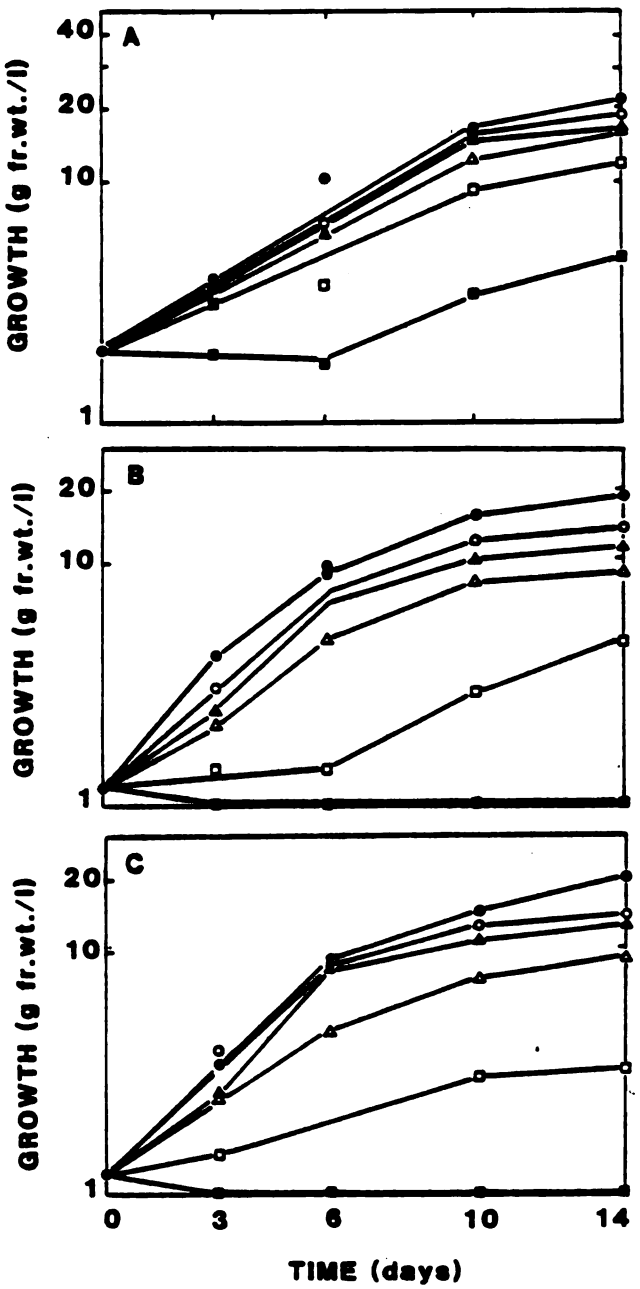

Figure 30: Effect of chlorate on growth of (A) 14U cells in urea-M1D, pH 6.2; (B) XD cells in M1D, pH 6.2; (C) XD cells in M1D, pH 5.5. [$KClO_3$] = 0(●), 1 (○), 3 (▲), 10 (△), 30 (□), 100 (■) mM.

XD cells at pH 5.5 and 6.2, respectively. Chlorate seems to be slightly more toxic to the XD cells at pH 5.5 than at pH 6.2. This pH effect might have been more prominent had the cultures been grown in buffered media. The pH of the M1D medium increased with the age of the culture (Table 5) shifting chlorate uptake to a rate characteristic of cells grown under less acidic conditions. The difference in sensitivity to chlorate between the XD and 14U cells lines may have been overestimated. The fresh weight of the initial inoculum was 2 g/l for the 14U cells and 1.2 g/l in the case of the XD cells. As will be seen below chlorate toxicity seems to depend upon the size of the initial inoculum.

Another characteristic of the response of a tobacco cell culture to chlorate is the culture's eventual recovery from growth inhibition. Cultures in the 100 mM and 30 mM treatments in figures 30A and 30B, respectively, attained growth rates similar to that of the control after a 6-day lag. This recovery phenomenon becomes more prominent in experiments described below.

Chlorate Reduction by Nitrate Reductase of Tobacco Cells

The reduction of chlorate by NR was indicated by chlorate-dependent NADH oxidation catalyzed by extracts of the alga *Chlorella* (Solomonson and Vennesland, 1972) or tomato plants which contain NR activity (Hofstra, 1977). In this laboratory, enzyme-catalyzed reduction of $^{36}ClO_3^-$ has been measured directly by following $^{36}ClO_2^-$ + $^{36}Cl^-$ formation after reduction of ^{36}Cl-chlorate by partially purified NR from the algae *Chlamydomanas* and *Chlorella* (Rhodes and Filner, 1979). In the present study, the latter approach was used to demonstrate chlorate reduction by NR of tobacco cells. Previous attempts to apply a colorimetric assay for chlorite formation to the tobacco cell system had been unsuccessful (P. Filner, personal communication).

A desalted enzyme extract from exponential-phase XD cells had a NR

Table 5: Effect of chlorate on pH of culture medium. 14U = 14U cells grown in urea-M1D, pH 6.2; $XD_{6.2}$ = XD cells grown in M1D, pH 6.2; $XD_{5.5}$ = XD cells grown in M1D, pH 5.5

Time	[$KClO_3$] mM																	
(days)	0			1			3			10			30			100		
	14U	$XD_{6.2}$	$XD_{5.5}$	14U	$XD_{6.2}$	$XD_{5.5}$	14U	$XD_{6.2}$	$XD_{5.5}$	14U	$XD_{6.2}$	$XD_{5.5}$	14U	$XD_{6.2}$	$XD_{5.5}$	14U	$XD_{6.2}$	$XD_{5.5}$
0	6.1	6.4	5.4	6.3	6.3	5.3	6.2	6.4	5.2	6.3	6.3	5.4	6.2	6.2	5.2	6.2	6.1	5.3
10	5.7	6.1	6.1	5.9	6.5	6.3	5.8	6.6	6.4	5.8	6.5	6.4	5.9	6.6	6.6	6.0	5.4	5.9
14	5.6	6.0	6.0	5.7	6.4	6.4	5.4	6.6	6.4	5.8	6.5	6.5	6.0	6.5	6.9	6.2	6.0	5.8

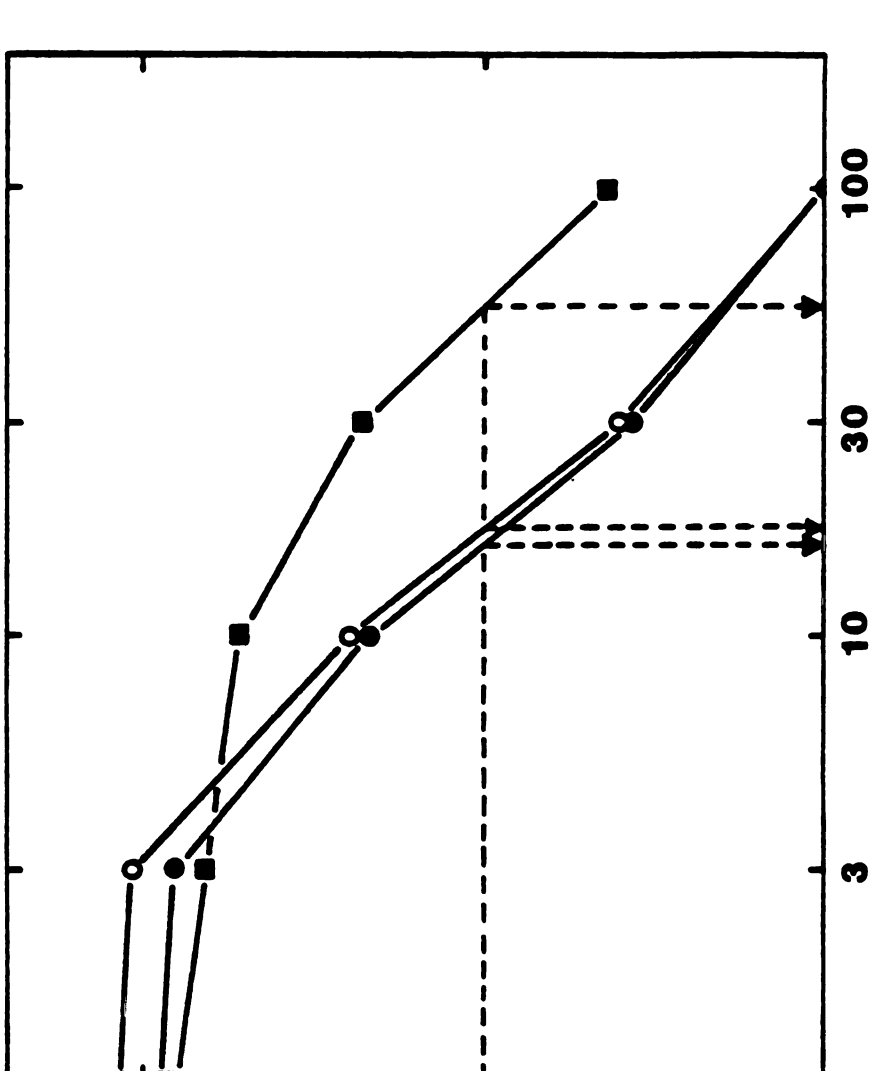

Figure 31: Dose-response curves of chlorate effect on 14U and XD cell culture growth. (■) = 14U in urea-M1D, pH 6.2; (○) = XD in M1D, pH 6.2; (●) = XD in M1D, pH 5.5.

activity of 31 nmoles / min / g fr. wt. and catalyzed chlorate reduction with an initial rate of 0.75 nmoles / min / g fr. wt. This rate decreased rapidly during the assay (Figure 32). This could have been due 1) to progressive depletion of substrate [Km (ClO_3^-) = 3.5 ± 1.7mM; Table 12] or 2) inhibition of NR by chlorite. Indeed, NR inhibition by chlorite has been previously demonstrated in *Chlorella* (Solomonson and Vennesland, 1972). However, upon analysis of radioactive label in silver chloride precipitates before and after conversion of chlorite to chloride by chemical reduction with KI in acetic acid, no evidence for the formation of ^{36}Cl-chlorite was found. All the radioactivity was found to be ^{36}Cl-chloride (Table 6).

On the assumption that chlorite could be converted to chloride via an enzymatic reduction and that, as an analog of chlorite, nitrite could compete in this reaction, we determined whether nitrite could inhibit chlorite reduction, therefore allowing the detection of chlorite. From previous experiments it had been determined that the maximum amount of chlorite which could be formed by chlorate reduction under our assay conditions was 2.86 μM. Nitrite was added to the assay mixture at concentrations ranging from 0 to 50 μM. After 30 minutes of incubation, the radioactivity in the silver chloride precipitates before and after conversion of chlorite to chloride showed that nitrite did not have any effect on the recovery of counts as chlorite (Table 7). Alternatively, it seemed possible that the origin of chloride in these assays is via the non-enzymatic reduction of the powerful oxidant chlorite (Rhodes and Filner, 1979).

To evaluate the generality of this phenomenon, chlorite formation upon reduction of chlorate by crude extracts from soybean nodule bacteroids was measured. Soybean nodule bacteroids are a source of abundant and easily accessible nitrate reductase (Lowe and Hamilton, 1967). 21% of the initial

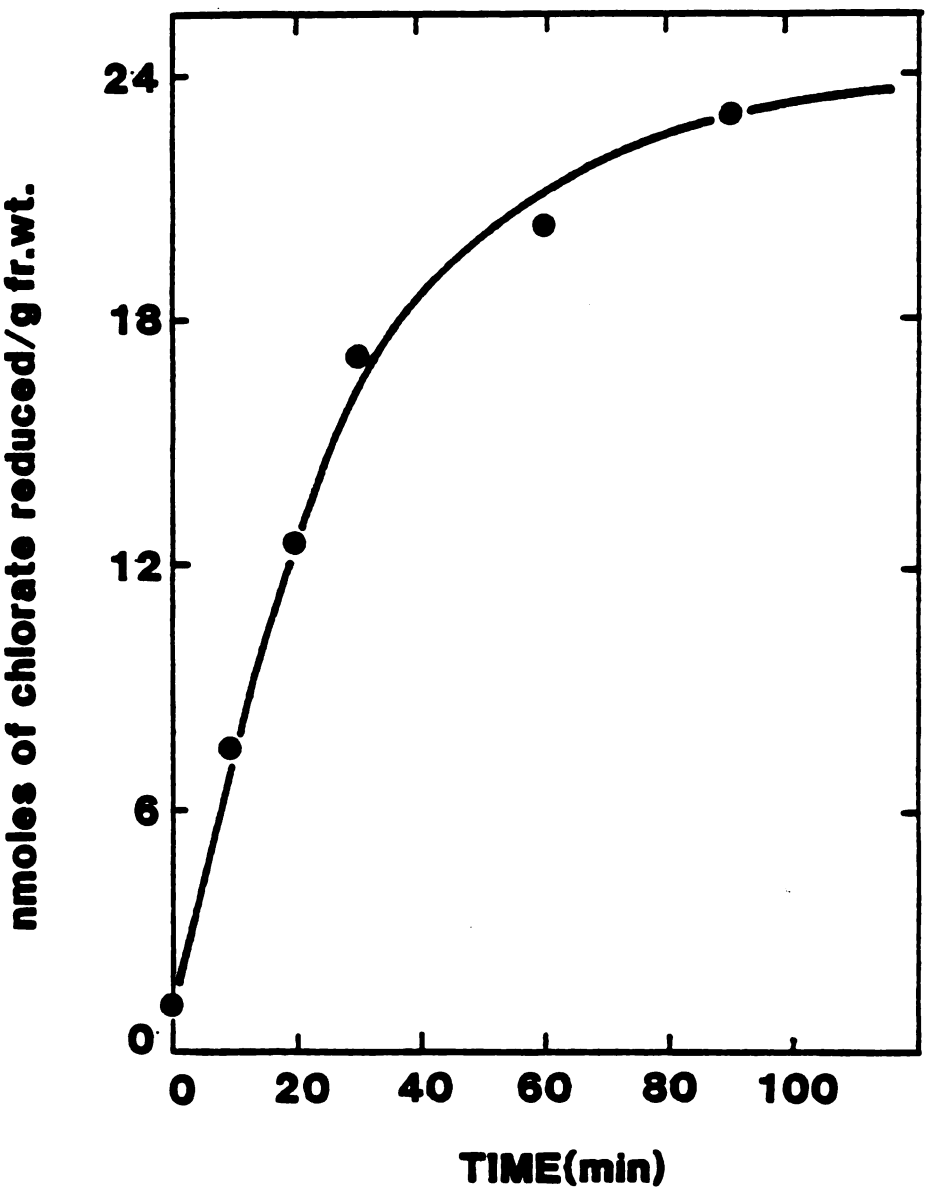

Figure 32: Time course of chlorate reduction by extracts of XD cells.

Table 6: Substrate dependence of chlorate reduction by XD cell extracts. Assay mixture consisted of 292 μM $^{36}ClO_3^-$ (73,088 cpm), 0.1 mM NADH and 0.4 ml enzyme extract.

Assay Mixture	Incubation Time (min)	Cl^- + ClO_2^- (cpm)	Cl^- (cpm)
Complete	0	141	N.D.
Complete	90	1054	1114
-NADH	90	118	N.D.
-Enzyme	90	N.D.	89

Table 7: Effect of nitrite on recovery of chlorite from chlorate reduction products of XD cell extracts.

$[NaNO_2]$ μM	$Cl^- + ClO_2^-$ (cpm)	Cl^- (cpm)
0	726	739
1.5	666	730
3.0	670	695
10.0	716	667
25.0	635	604
50.0	590	544

Assay mixture:	283 μM $^{36}ClO_3^-$ M (77957 cpm)
	0.1 mM NADH
	0.4 ml enzyme extract
	50 μl NaNO2 (0, 16.5, 33, 110, 275, 550 μM)
Incubation time:	30 minutes.

^{36}Cl-chlorate was reduced after a period of 30 minutes. 19% of that 21% was recovered as ^{36}Cl-chlorite (Table 8).

Table 8: Chlorate Reduction by Bacteroid Nitrate Reductase

Assay Mixture	Incubation Time (min)	$ClO_2^- + Cl^-$ (cpm)	Cl^- (cpm)	ClO_2^- (cpm)
Complete	30	16,424	13,314	3110

Assay mixture: 305 μM $^{36}ClO_3^-$ (76,347 cpm)
0.05 M potassium succinate
0.1 ml crude soybean bacteroid extract

In the results obtained with the tobacco cell extracts, only 1.4% (1054 cpm / 73,088 cpm x 100%) of the initial chlorate present was reduced. Using the bacteroid data as a guide, 19% of this reduction product would mean a recovery of only 200 cpm, too small an amount to be detected accurately in the assay used.

No chlorate reduction activity was detectable in a desalted enzyme extract prepared from exponential-phase XD cells growing in urea M1D with a NR activity of only 46 pmoles / min / g fr. wt. (Table 9). These results support the view that NR is directly involved in chlorate reduction.

Table 9: Chlorate Reduction by NR from Urea and Nitrate Grown XD Cells

N-Source	ClO_3^- Reduction (cpm / h / g fr. wt.)
Urea	0
KNO_3	795

Assay Mixture: 300 μM $^{36}ClO_3^-$ (75,000 cpm)
0.01 mM NADH
0.4 ml enzyme extract

Kinetic Characteristics of Chlorate and Nitrate Reduction By Nitrate-Reductase Preparations

To measure the kinetic parameters of chlorate and nitrate reduction as well as their mutual inhibitory effects, it was desirable for comparative purposes to utilize for each measurement samples taken from the same enzyme preparation. Crude extracts containing NR can be stored at -20°C in the modified buffer (see MATERIALS AND METHODS) for up to two weeks without loss of NR activity. In Table 10, the changes with time in storage of the enzyme activity of a crude extract preparation are shown. These data indicate not only the maintenance of activity but indeed a slight increase (P. Trinity, 1981).

Crude extracts still retained trace amounts of nitrate after the 50% saturated ammonium sulfate precipitation step. Desalting of the enzyme

preparation was required to avoid such contamination. As indicated in Table 10, the enzyme ativity was enhanced by passage through a G-25 Sephadex column. In general, the total protein recovery from a 4-ml G-25 Sephadex column is 60% (Yamaya and Filner, 1981). It has been shown that NR in the XD cells is present in an active and inactive form and that the ratio between the two forms changes during the cell's growth cycle (Trinity, 1981). The data in Table 10 suggest that a low molecular-weight inhibitor of NR, present in crude extracts, is removed by Sephadex G-25 chromatography. The possibility of co-extraction of an inhibitor is supported by a further observation. In one experiment, the enzyme extract was concentrated two-fold to increase its activity. When nitrate reduction by the 2X extract was measured under saturating substrate conditions, a plateau was reached after 15 minutes (Figure 33A). The enzyme activity in unconcentrated extracts is linear up to 40 minutes. In a similar experiment, the specific activity of NR was shown to decrease with increasing concentrations of enzyme in the assay mixture (Figure 33B). The desalted extract did not show such a trend (Figure 33A and 33B).

The Km's and Vmax's of chlorate and nitrate reduction were calculated several times using fresh and stored desalted extracts from exponential-phase XD cells. To measure the kinetics of nitrate reduction, KNO_3 was used as the substrate at concentrations ranging from 0.02 to 0.2 mM. To determine the Km and Vmax of chlorate reduction, ^{36}Cl-chlorate was added to the assay mixture to a final concentration of 0.3 mM. Unlabelled $KClO_3$ was added to the reaction mixture to final concentrations ranging from 0.3 to 2.0 mM. Corresponding ^{36}Cl-chlorate specific activities ranged from 500,000 to 67,525 cpm/μmole.

Tobacco nitrate reductase has a higher affinity for nitrate (Km values

Table 10: Stability of NR extracted from 14U cells. Assay mixture consisted of 0.1 ml 0.1 M KNO_3, 0.1 ml 1 mM NADH, 0.5 ml 0.1 M K_2HPO_4 pH 7.5, 0.2 ml enzyme extract, 0.1 ml H_2O. Crude extracts were stored at -20°C.

Time in storage (days)	Enzyme act. crude ext. (nmole/min/g fr.wt.)	Enzyme act. desalted ext. (nmol/min/g fr.wt.)
0	66	72
1	72	76
2	72	82
3	71	79
4	69	78
5	70	78

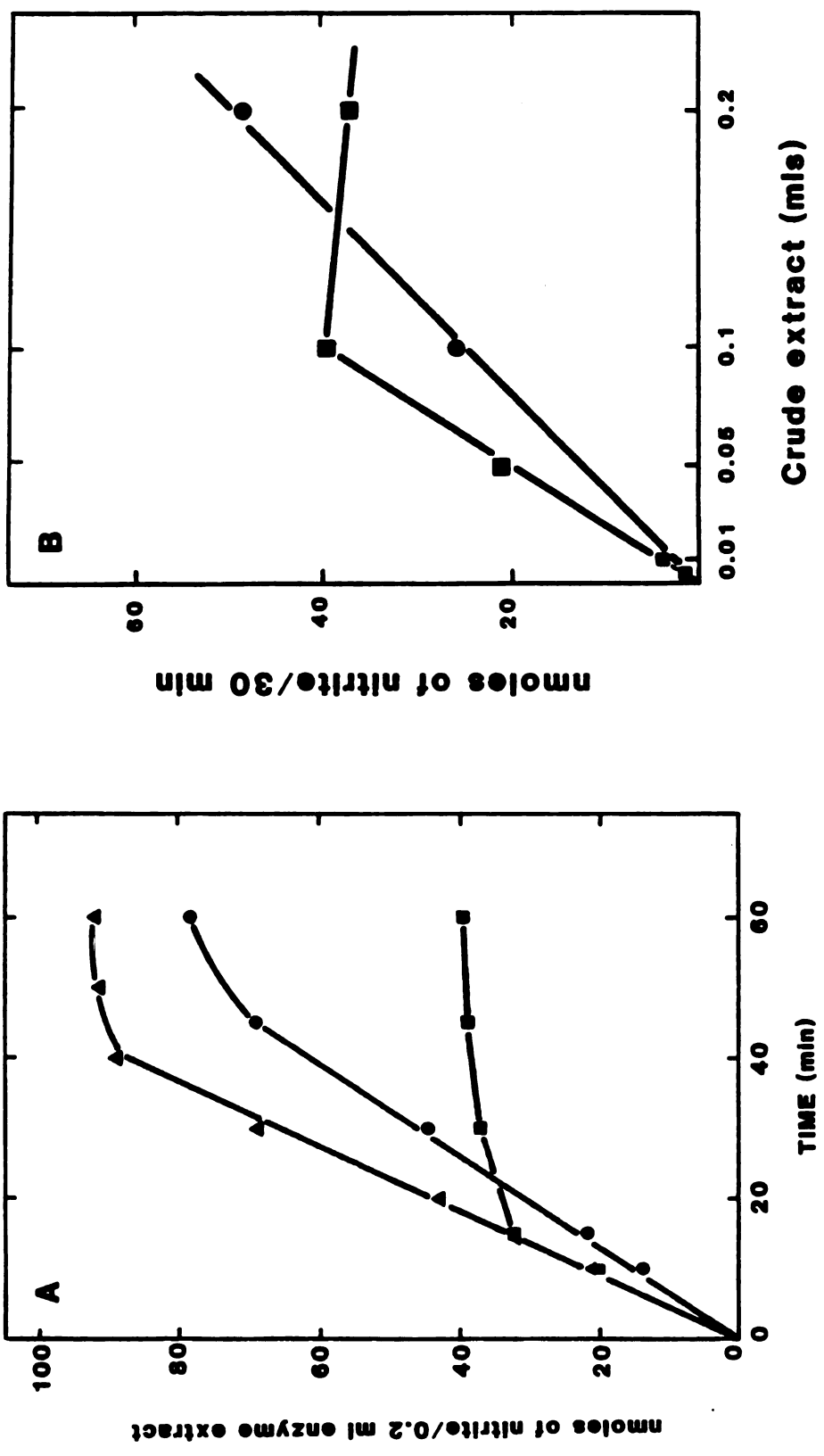

Figure 33: (A) Time dependence of nitrate reduction by extracts of XD cells. (B) Concentration dependence of nitrate reductase activity from XD cells. (▲) = 1x crude extract, (■) = 2x crude extract, and (●) = 2x desalted extract.

Table 11: Kinetic parameters of nitrate and chlorate reduction by XD cells. Each row represents an independent experiment.

NITRATE REDUCTION			CHLORATE REDUCTION		
Km (mM)	Vmax (nmol/min/g fr.wt.)	$K_i(ClO_3^-)$ (mM)	Km (mM)	Vmax (nmol/min/g fr.wt.)	$K_i(NO_3^-)$ (mM)
-	-	-	6.0	10.2	-
0.21	46	6.8	-	-	-
0.28	39	-	-	-	-
0.28	53	7.5	5.4	11.0	0.20
0.18	31	8.9	6.6	7.0	0.20
0.24	58	5.0	2.3	8.0	0.13
0.26	39	-	-	-	-

ranged from 0.18 to 0.28 mM) than for chlorate (Km values ranged from 2.3 to 6 mM) (Table 11). Determination of Km's for chlorate reduction was difficult because the chlorate concentrations used to determine the Km values were below the Km. An example of the double recriprocal plot from which the Km and Vmax values of chlorate reduction were calculated is shown in Figure 34. The Km values for nitrate and chlorate fall in the same range as data found for tomato plants, green algae and several bacteria (Table 12).

Inhibition by Chlorate of Nitrate Reduction

We have shown so far that tobacco NR preparations reduce nitrate and chlorate anions. To determine whether or not these two substrates compete for the same enzyme, the effects of chlorate on nitrate reduction and nitrate on chlorate reduction were examined. The inhibition of nitrate reduction was first analyzed by incorporating KNO_3 at 0.08 to 0.4 mM and $KClO_3$ from 0 to 16 mM in the assay mixture. The reciprocal of the reaction velocity was then plotted against the reciprocal of nitrate concentration for each $KClO_3$ concentration. As the concentration of chlorate was increased in the assay, there was an increase in the Km for nitrate reduction (Figure 35). These results indicated that chlorate competitively inhibited nitrate reduction. The K_i value of this inhibition was determined in several experiments and ranged from 5 to 8.9 mM. These values are in the same range as the higher Km values obtained for chlorate reduction as expected for a competitive inhibitor. On this basis, the low Km ClO_3^- values of 2.3 (Table 10) might be considered erroneous. An example of a Dixon plot from which these K_i values were generated is shown in Figure 36.

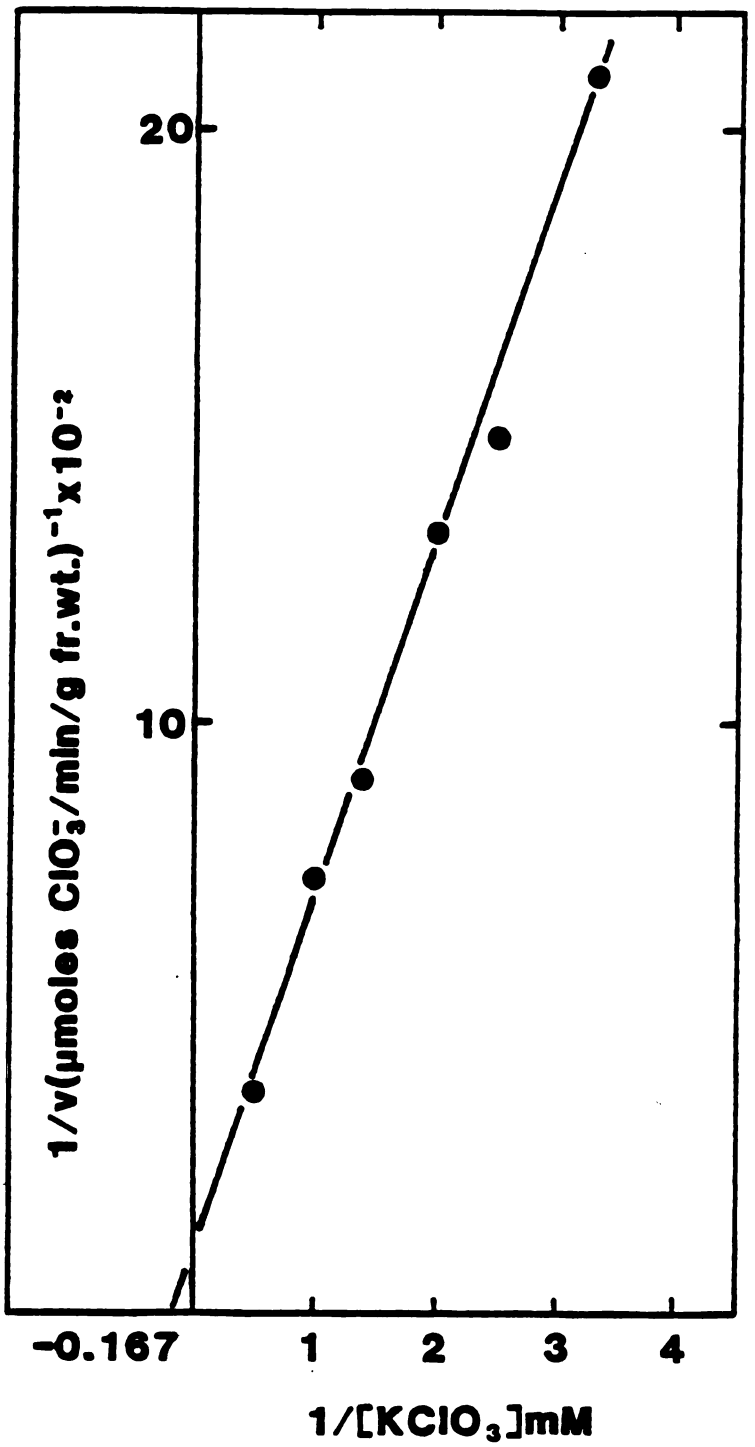

Figure 34: Lineweaver-Burk plot of chlorate reduction by XD cell extracts.

Table 12: Michaelis constants for nitrate and chlorate reductases of various species.

Species	Km(NO_3^-)	Km(ClO_3^-)	References
Lycopersicon esculentum leaves	0.15	4.0	Hofstra, 1977
Chlamydomonas reinhardtii	-	3.4	Rhodes and Filner, 1979
Chlorella pyrenoidosa	-	3.6	Rhodes and Filner, 1979
Chlorella vulgaris	0.084	1.2	Solomonson and Venesland, 1972
Pseudomonas multivorans	0.19	4.84	Pichinoty, 1969
Micrococcus denitrificans	0.54	2.65	Pichinoty, 1969
Aerobacter aerogenes	0.45	1.55	Pichinoty, 1969
Escherichia coli	0.78	2.5	Pichinoty, 1969

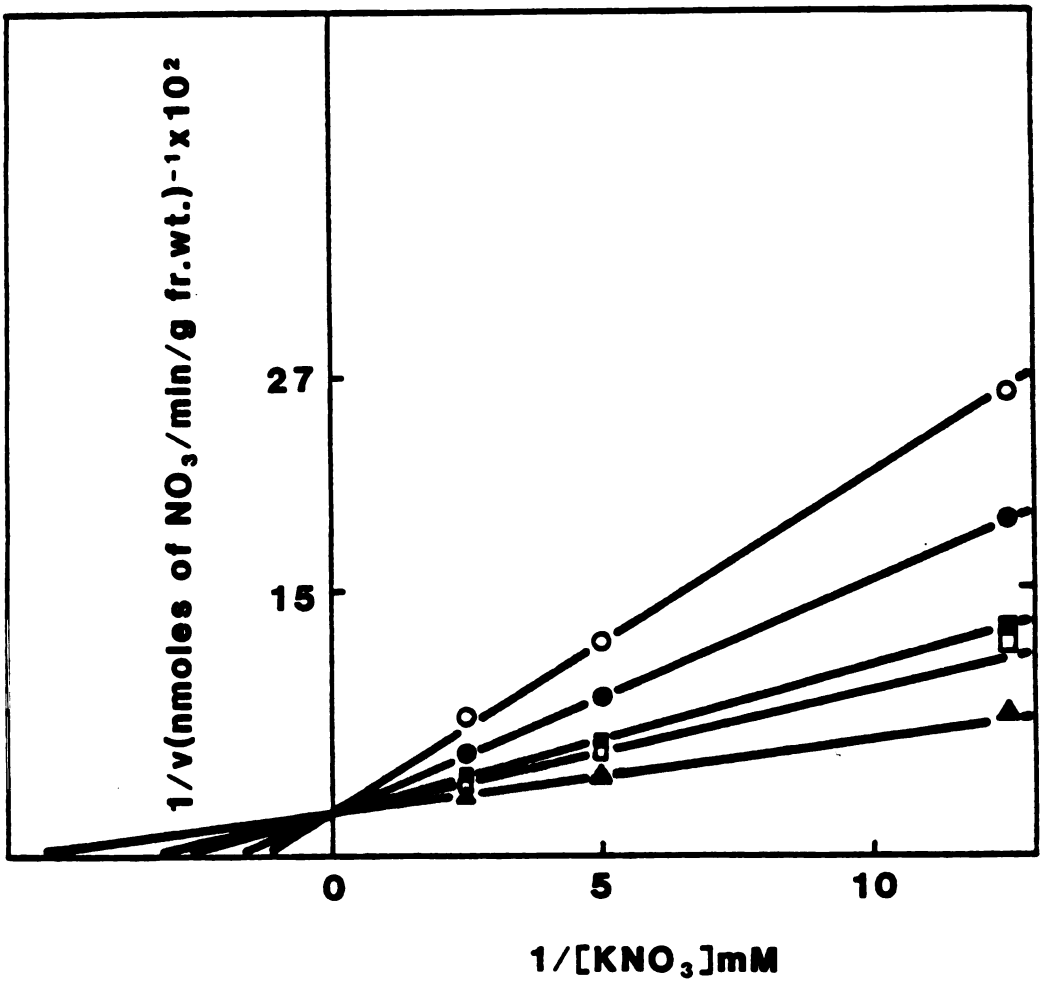

Figure 35: Lineweaver-Burk plot of inhibition by chlorate of nitrate reduction in XD cells. Intersection of lines on ordinate is indicative of competitive inhibition. [$KClO_3$] = 0 (▲), 2 (□), 4 (■), 8 (●) and 16 (○) mM.

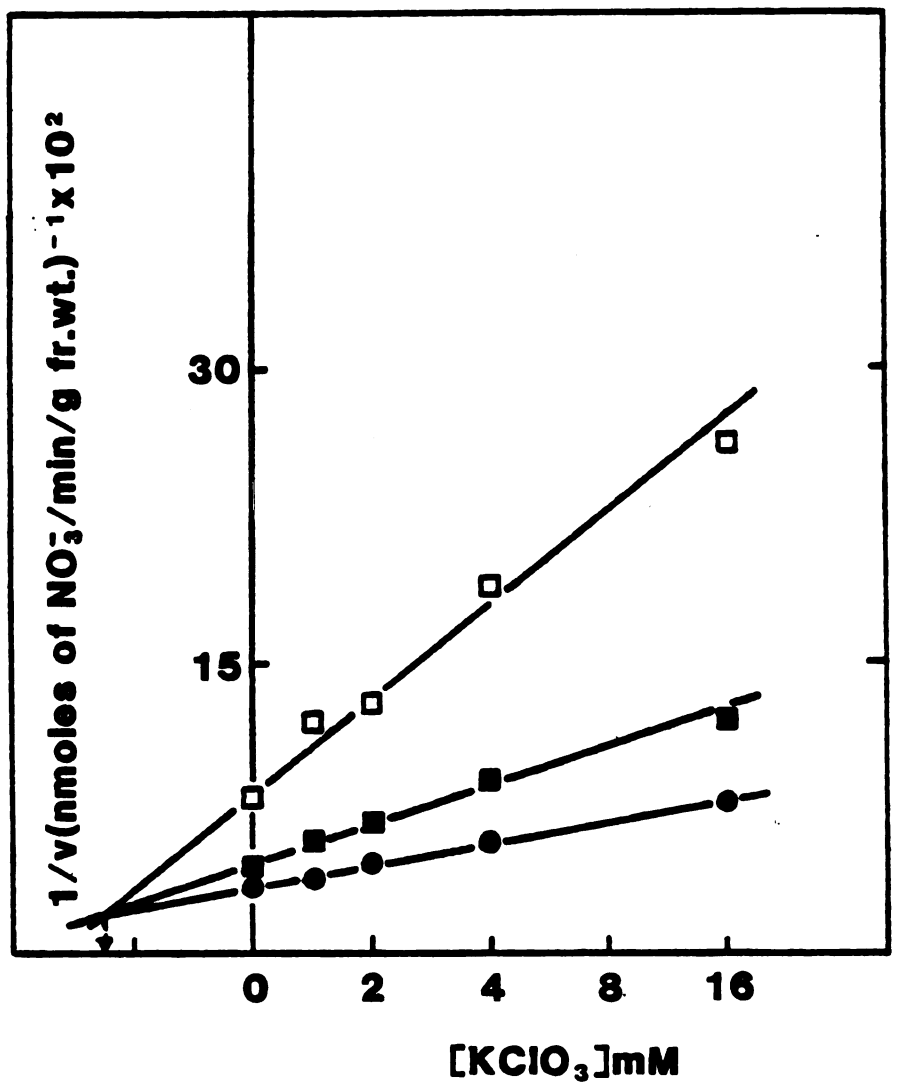

Figure 36: Dixon plot of chlorate inhibition of nitrate reduction in XD cells. K_i = 5 mM $KClO_3$. [KNO_3] = 0.08 (□), 0.2 (■), and 0.4 (●) mM.

Inhibition by Nitrate of Chlorate Reduction

The effect of nitrate on chlorate reduction was analyzed by supplementing the assay mixture with 500 μM $^{36}ClO_3^-$, 4, 8 and 16 mM unlabelled chlorate and 0 to 0.4 mM KNO_3 (final concentrations). As in the case of chlorate inhibition of nitrate reduction, nitrate competitively inhibited chlorate reduction. The K_i values obtained for inhibition by nitrate were 0.13 and 0.2 mM, very close to the Km values for reduction of nitrate (see Table 11). An example of a Dixon plot of these data is shown in Figure 37.

Induction of Nitrate Reductase by Nitrate And Chlorate

Hofstra (1977) attempted, without success, to study the induction of nitrate reductase by chlorate using *Lycopersicon esculentum* leaf discs. Hackenthal et. al (1965) examined the induction of NR by a series of organic anions structurally related to nitrate in *Bacillus cereus*. Among the anions tested, perchlorate but not chlorate induced the enzyme. In our attempt to evaluate the use of chlorate as an analog of nitrate for metabolic studies, we made use once again of the 14U tobacco cells growing in urea M1D to re-examine the induction of NR by chlorate.

The cells from 3 one-liter flasks containing 500 mls of a 5 day old culture in urea-M1D were harvested and transferred to 3 other flasks containing 500 ml of fresh urea M1D medium. KNO_3 and $KClO_3$ were added to flasks number 1 and 2 respectively to give final concentrations of 6 mM. The cells from the third flask were untreated controls. Starting at time 0, 100 ml of the cell suspension was removed from each culture every 2 hours. The cells were harvested and crude enzyme extracts were assayed for nitrate reductase activity. The results summarized in Table 13 show an induction of NR activity

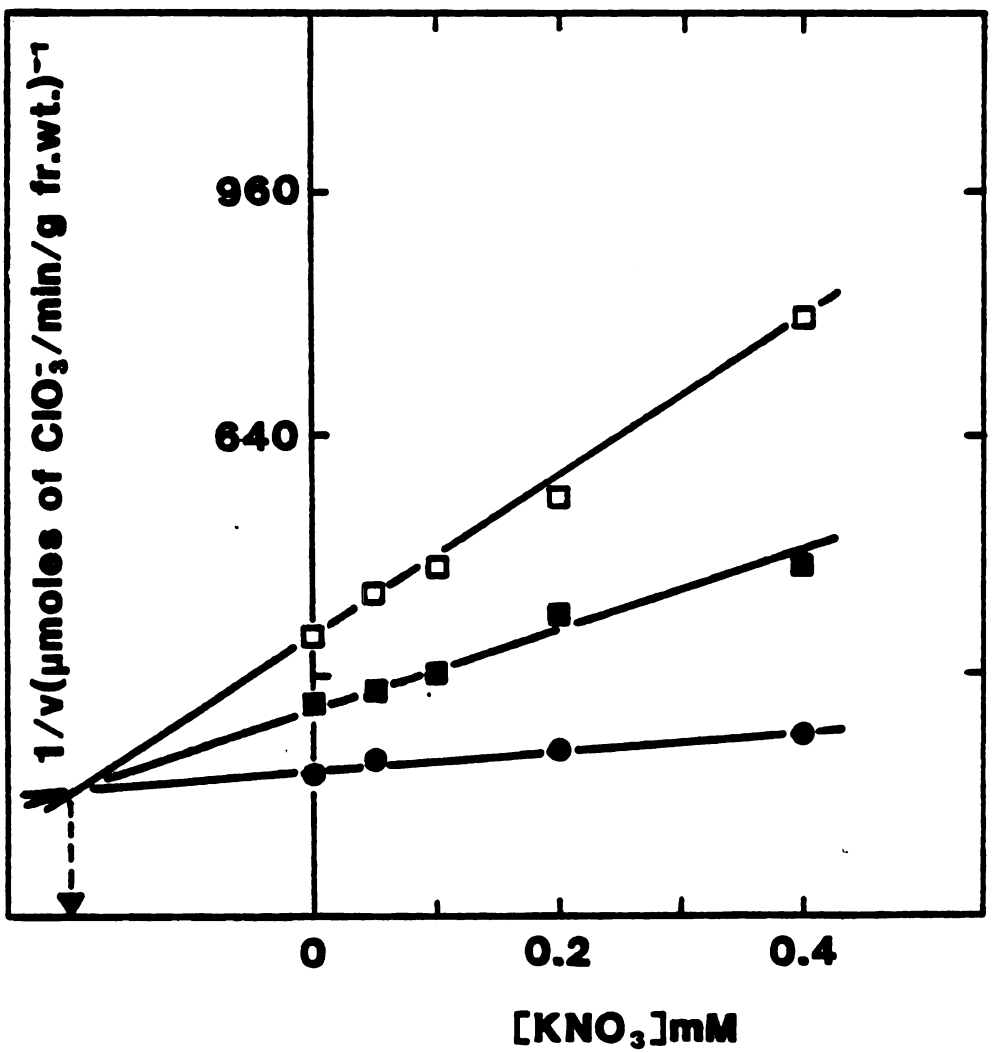

Figure 37: Dixon plot of nitrate inhibition of chlorate reduction in XD cells. K_i = 0.2 mM KNO_3. [$KClO_3$] = 4 (□), 8 (■) and 16 (●) mM.

Table 13: Induction of NR activity in 14U cells by nitrate, chlorate and urea. 6 mM KNO_3 and 6 mM $KClO_3$ were added in their respective treatments to basal urea-M1D medium employed throughout.

	NITRATE REDUCTASE ACTIVITY		
Time (h)	KNO3 nmoles/h/g fr wt.	KClO3 nmoles/h/g fr.wt.	UREA nmoles/h/gfw
0	10	0	22
2	179	48	132
4	287	0	8
6	410	11	0
8	315	27	54

by nitrate but not by chlorate or urea. The NR activity values obtained after an induction period of 6 hours in the case of nitrate are low (410 nmoles / h / g fr. wt.) compared to those reported by Heimer and Filner (1971) (1,000 nmoles / h / g fr. wt.). This difference and the irregular values of enzyme activity obtained from control cells and chlorate treatment could be explained by the presence of phenolics in the crude extracts. In this experiment, the concentration of cysteine free base used in the extraction buffer was the same as the one used to prepare crude extracts from XD cells growing in M1D medium. The amount of phenolics seems to be higher in the tobacco cells grown in urea judging from the color of the extracts. The discrepancy between our results and those of Heimer and Filner could also be due to a difference in cell type. For their induction studies, XD cells were grown in urea-M1D for a short period of time (5 days). However, in our experiments we used the 14U cells, that had been maintained on urea for over one hundred generations.

To reconcile the old and current data, we measured the growth and NR induction response of 14U cells growing in urea-M1D and urea-M1D + 2.5 mM nitrate, at a range of chlorate concentrations. Such an experiment should provide an estimate of the degree of chlorate sensitivity as a function of induced NR enzyme activity.

Ten milliliters of stationary phase 14U cells were subscultured into two series of 500 ml flasks containing 200 ml urea-M1D medium and $KClO_3$ at concentrations ranging from 0 to 100 mM. To one of the series, KNO_3 was added to a final concentration of 2.5 mM. Two flasks per treatment were harvested 4, 6, 8 and 12 days after the start of the experiment and the fresh weights determined. The levels of NR were measured at days 0, 4, 6, 8 and 12 for the 0 and 3 mM chlorate treatments, as indicated in Table 14.

The results of the growth tests are shown in Figures 38 and 39. Chlorate

Table 14: Effect of medium nitrogen source, chlorate and stage of culture cycle on nitrate reductase activity extractable from 14U cells. NR specific activities are expresed in terms of nmoles NO_2^- g fr. wt. of tissue, above, and in terms of nmoles NO_2^-/mg protein, below.

		NIRATE REDUCTASE ACTIVITY				
Medium	[$KClO_3$] (mM)	TIME (days) 0	4	6	8	12
U-M1D	0	15 ± 3	39 ± 16	30 ± 13	27 ± 11	3 ± 2
U-M1D	3	15 ± 3	45 ± 17	45 ± 10	36 ± 12	1 ± 1
U-M1D+NO_3^-	0	15 ± 3	1,105 ± 501	279 ± 113	91 ± 35	49 ± 6
U-M1D+NO_3^-	3	15 ± 3	771 ± 111	218 ± 78	65 ± 8	22 ± 7

nmoles/g fr.wt./h

		NIRATE REDUCTASE ACTIVITY				
Medium	[$KClO_3$] (mM)	TIME (days) 0	4	6	8	12
U-M1D	0	3 ± 1	4.5 ± 2	4 ± 2	6 ± 3	0.9 ± 0.6
U-M1D	3	3 ± 1	4.5 ± 2	4.5 ± 2	6 ± 2.5	0.3 ± 0.4
U-M1D+NO_3^-	0	3 ± 1	119 ± 24	40 ± 13	24 ± 9	17.5 ± 2.3
U-M1D+NO_3^-	3	3 ± 1	71 ± 17	25 ± 7	12 ± 1	7 ± 3

nmoles/mg prot/h

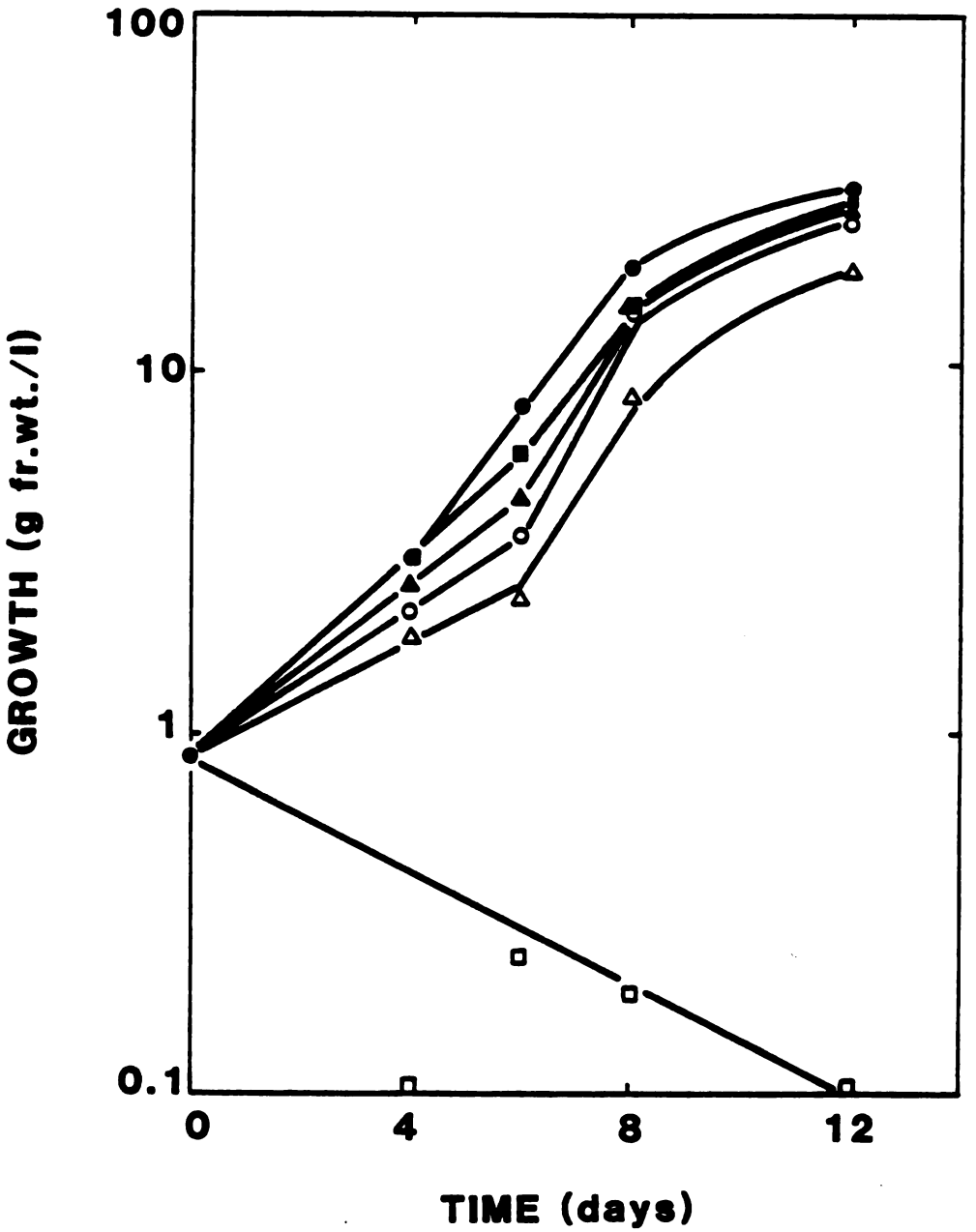

Figure 38: Effect of chlorate on growth of 14U cells in urea-M1D. [$KClO_3$] = 0 (●), 1 (▲), 3 (■), 10 (○), 30 (△), and 100 (□) mM.

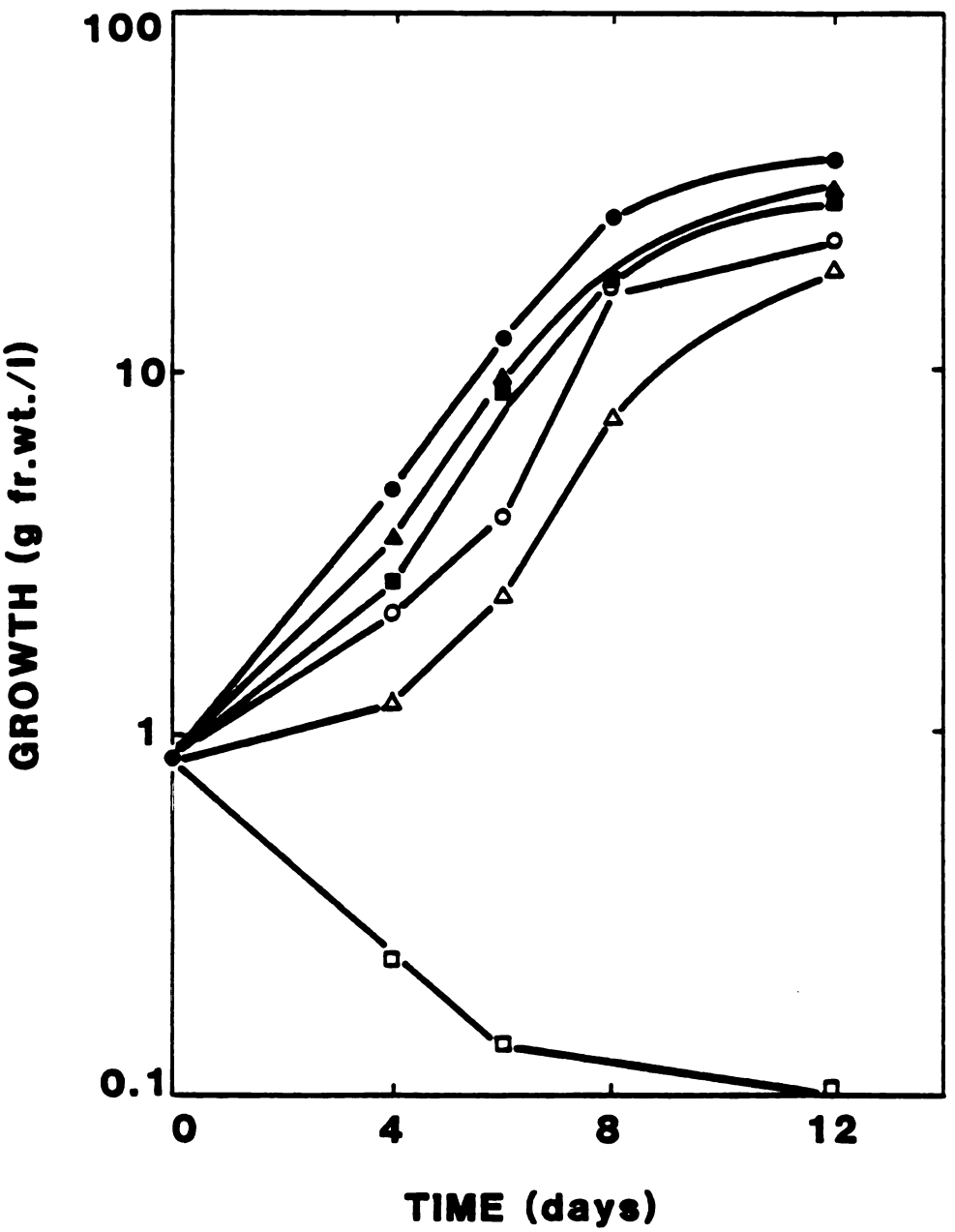

Figure 39: Effect of chlorate on growth of 14U cells in urea-M1D + 2.5 mM KNO_3. [$KClO_3$] = 0 (●), 1 (▲), 3 (■), 10 (○), 30 (△) and 100 (□) mM.

imposed a lag, after which the cells grew at rates similar to those of the controls. When the initial growth rate, expressed as a percent of the control rate, is plotted against $KClO_3$ concentrations (Figure 40), a slight increase in sensitivity to chlorate is observed when nitrate is present in the medium. This increase corresponds to increased NR activity in these cells (Table 14). The relationship is reversed however, when the log phase growth rates, rather than initial rates, are used (Figure 41). In addition, if growth is measured as total fresh weight accumulation after 8 days, the urea and nitrate-grown cells once again appear more tolerant of chlorate (Figure 42). The control cells in this experiment (14U cells growing in urea-M1D) appeared to be more sensitive to chlorate than were similar controls in the experiment shown in Figures 30 and 31. Differences in inoculum size could explain this discrepancy. In the earlier experiment, the average fresh weight of the initial inoculum was 2.0 g/l, while in the current experiment it was 0.86 g/l.

The enzyme analysis summarized in Table 12 revealed the previously established induction of nitrate reductase activity by nitrate. Three millimolar chlorate reduced the level of NR induction by nitrate, perhaps by inactivation of the enzyme by the products of chlorate reduction (Solomonson and Vennesland, 1972). 3 mM chlorate in the nitrate-less medium did not induce NR activity. The fully induced NR levels seen in Table 15 are now comparable to those reported for XD cells by Heimer and Filner (1971). Our inability to achieve such levels in previous experiments (Table 13) could have been due to 1) the protection afforded by higher cysteine levels in Heimer and Filner's conditions, or 2) a metabolic state in their cultures more ripe for nitrate induction, engendered by previous maintenance on nitrate as a sole nitrogen source.

The sensitivity to chlorate of the 14U cells in urea-M1D + 2.5 mM KNO_3

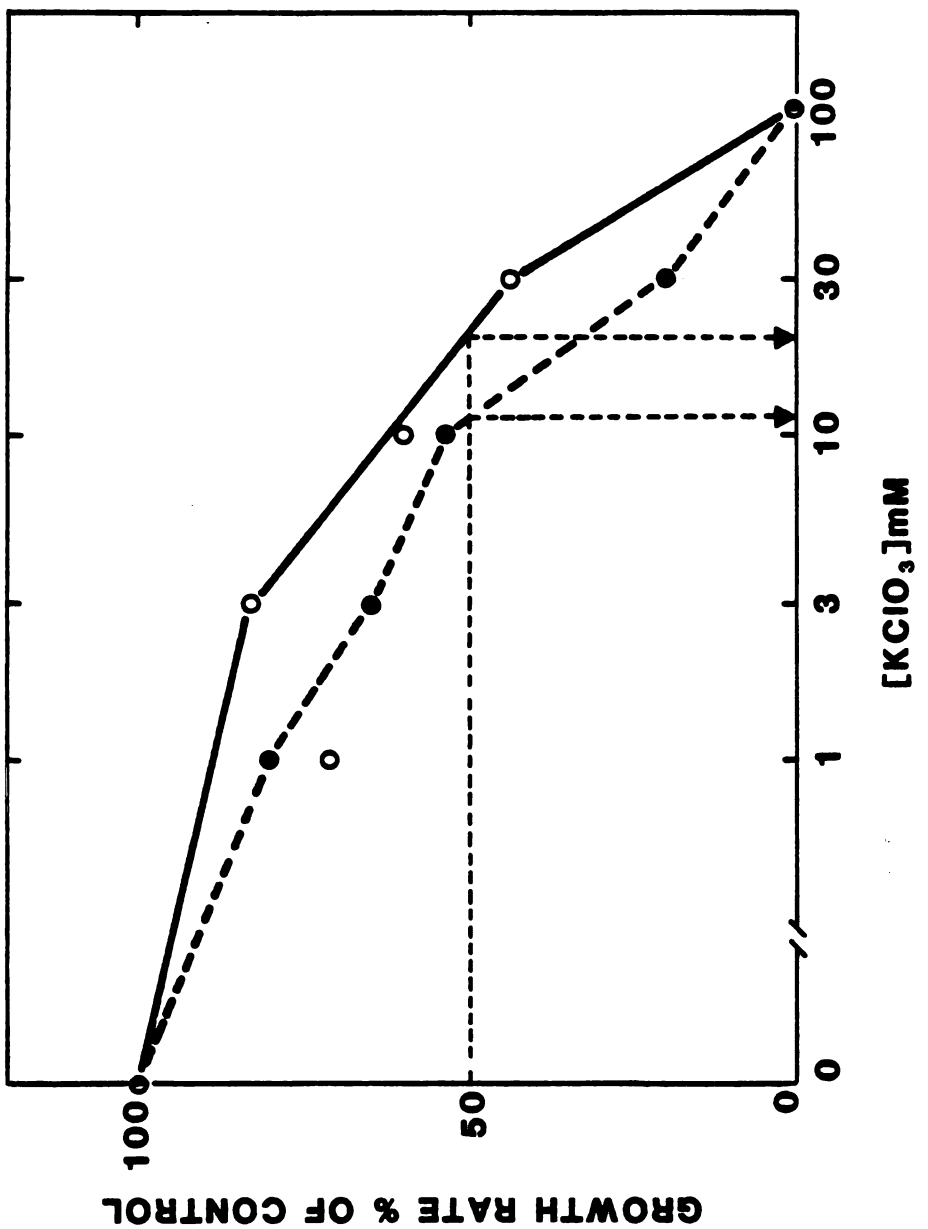

Figure 40: Dose-response curve of chlorate effect on initial growth rates of 14U cells. (O) = urea-M1D; (●) = urea-M1D + 2.5 mM KNO_3. Data taken from Figures 38 and 39.

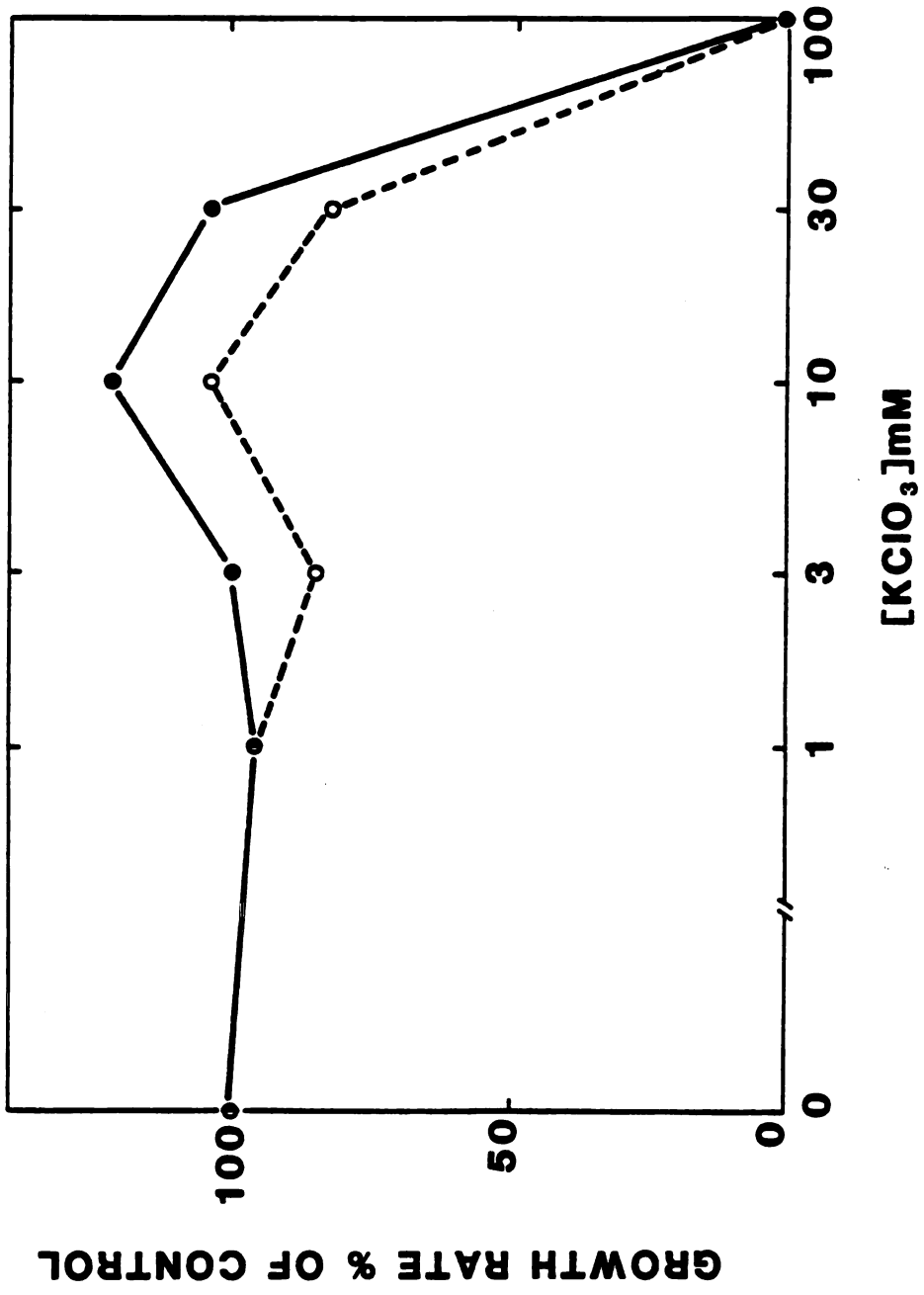

Figure 41: Dose-response curve of chlorate effect on exponential growth rates of 14U cells. (O) = urea-M1D; (●) = urea-M1D + 2.5 mM KNO_3. Data taken from Figures 38 and 39.

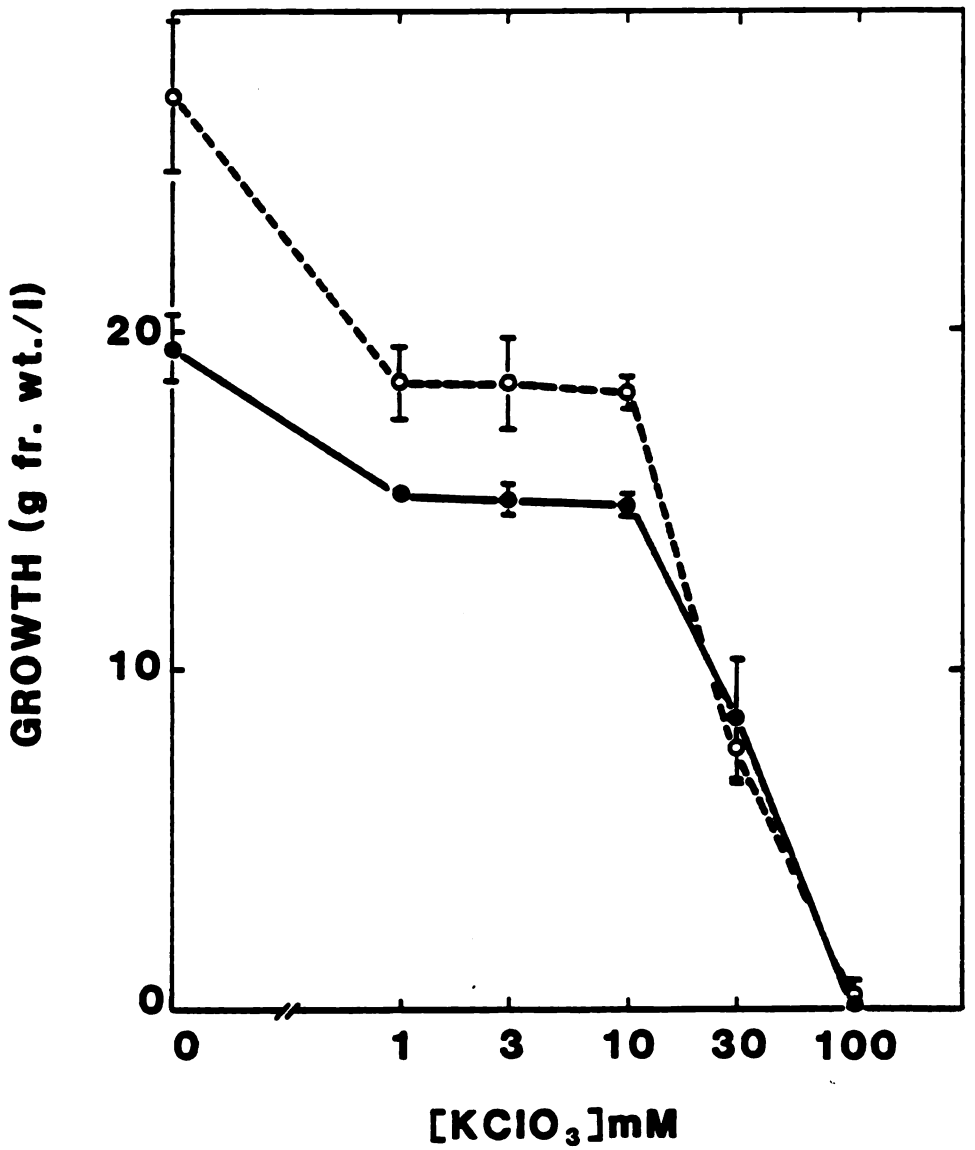

Figure 42: Dose-response curve of chlorate effect on fresh weight accumulation in 14U cell cultures. Fresh weights were measured after 8 days of growth. (●) = urea-M1D; (○) = urea-M1D + 2.5 mM KNO_3. Data taken from Figures 38 and 39.

although greater than that of the 14U cells in the absence of KNO_3, was not as great as that of XD cells in M1D medium. Either some clonal characteristic of the 14U cells or the medium (urea-M1D) itself may have been protecting the cells from more acute chlorate toxicity. To test those hypotheses, an experiment was designed in which sensitivity to chlorate was measured in the 14U cells in several media: 1) M1D, 2) urea-M1D and 3) M1D with a preincubation period in M1D. The latter treatment was added to test for the elimination of any enhancement of protection against chlorate provided by an excess of urea (or its reduction products). The sensitivity to chlorate of the XD cells in M1D was also measured for comparative purposes. Figure 43 shows the dose-response curve obtained by plotting the logarithmic growth rate (as percent of control) for each treatment against $KClO_3$ concentration. 14U cells showed higher sensitivity to chlorate in M1D than in urea-M1D and the degree of sensitivity was similar to that of the XD cells in M1D. Therefore, these results exclude the possibility of the 14U cells become intrinsically more tolerant to chlorate than the XD cells. When 1-3 mM $KClO_3$ was present in M1D-grown 14U cultures, the growth rate was higher than that of the control (0 mM $KClO_3$). This could be an artifact of experimental manipulation. It may mean that chlorate stimulates growth at low concentrations. This growth stimulation by chlorate has been reported for cultures of *Datura innoxia* (King and Khanna, 1980). Also, Doddema and Telkamp (1979) reported enhanced uptake of nitrate by *Arabidopsis* plants in the presence of very low concentrations of chlorate.

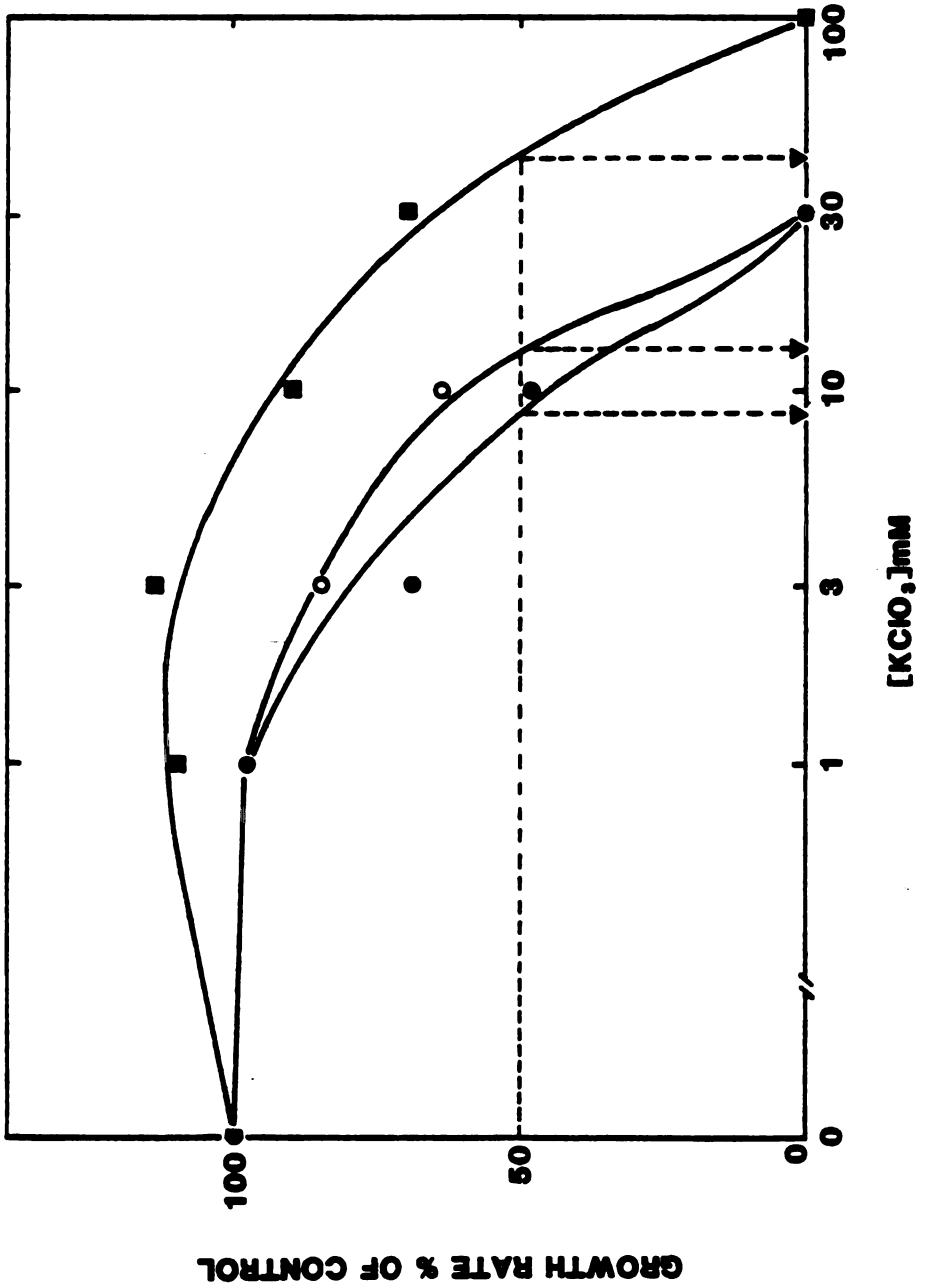

Figure 43: Dose-response curves of chlorate effect on growth rates of 14U and XD cells. (●) = 14U in M1D; (■) = 14U in urea-M1D; (▲) = 14U in M1D, preincubated in M1D; (○) = XD in M1D.

Effects of Urea, Chloride and Ammonium Ions on Tobacco Cells' Sensitivity to Chlorate.

The medium constituents that may confer some type of protection from ClO_3^- toxicity upon the 14U cells are chloride, urea or the products of urea metabolism. As mentioned above, the chloride content of the urea-M1D medium is higher than that of the M1D medium. Since the results of the uptake experiments (Part I) showed that chloride inhibits chlorate uptake at high chlorate concentrations, it might be expected that this inhibition results in the protection of the 14U cells from chlorate toxicity. Urea (and the products of its reduction) was the only other addition to the standard M1D recipe in urea-M1D.

The growth of XD and 14U cells in their respective maintenance media, modified as indicated, was measured to determine the effect of urea and chloride ions on the cell's sensitivity to chlorate. Stationary phase XD cells were subcultured into M1D medium supplemented with either 2.5 mM KCl or 3 mM urea at chlorate concentrations ranging from 0 to 100 mM. Stationary phase 14U cells were subcultured into urea-M1D, Cl-less N-less M1D + 3 mM urea or N-less M1D + 2.5 mM KNO_3 each at chlorate concentrations ranging from 0 to 100 mM. The fresh weight of the cells was measured after 8 days. The results obtained are plotted as a percent of control's fresh weight *versus* chlorate concentration (Figure 44 and 45).

Chloride ions conferred a slight protection against chlorate to the XD cells when added to the M1D medium. However, removal of chloride ions from urea-M1D did not increase the sensitivity of 14U cells to chlorate. On the other hand, when the M1D medium was supplemented wih urea, the XD cells were less sensitive to chlorate. Also, when urea was replaced by 2.5 mM

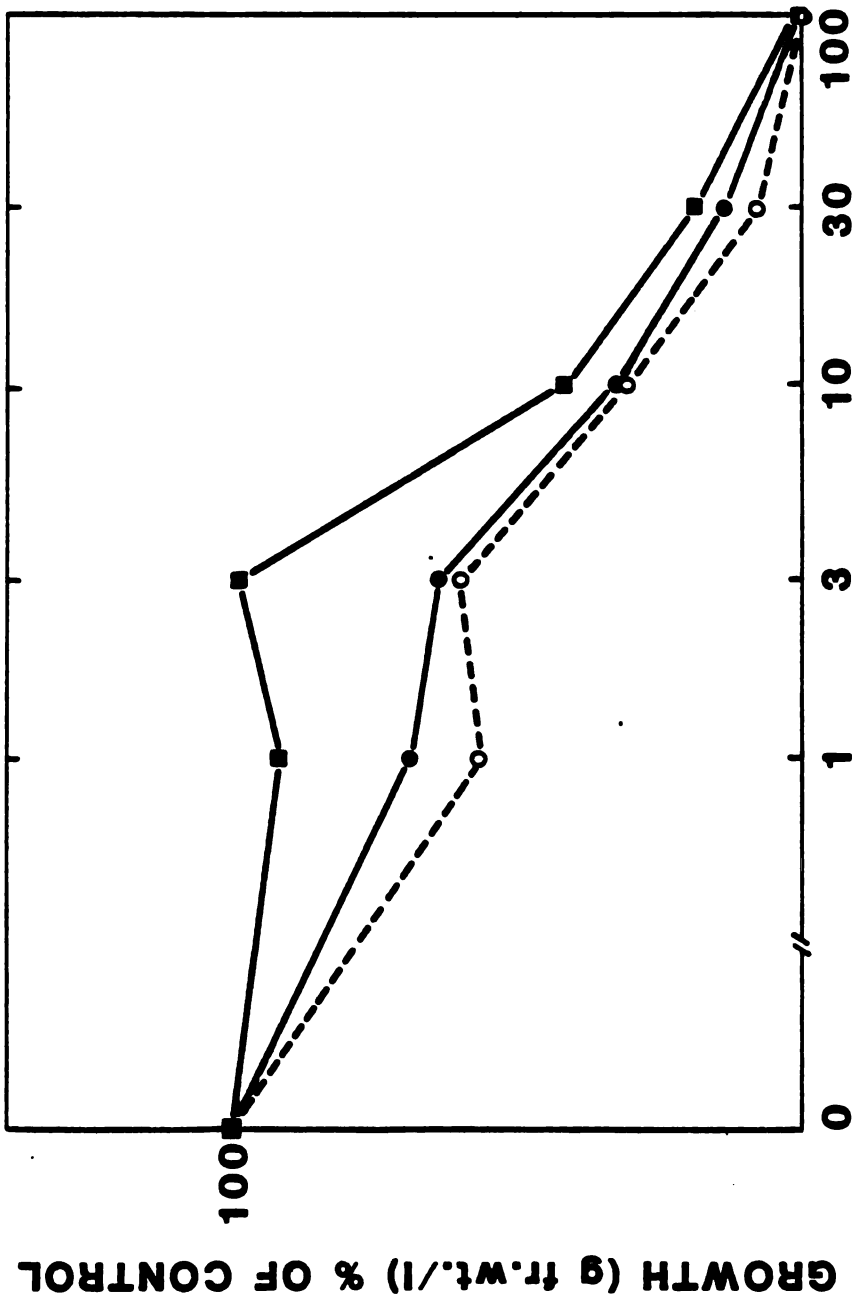

Figure 44: Dose-response curves of effect of chlorate on fresh weight accumulation in XD cell cultures. (○) = M1D; (●) = M1D + 2.5 mM KCl; (■) = M1D + 3 mM urea.

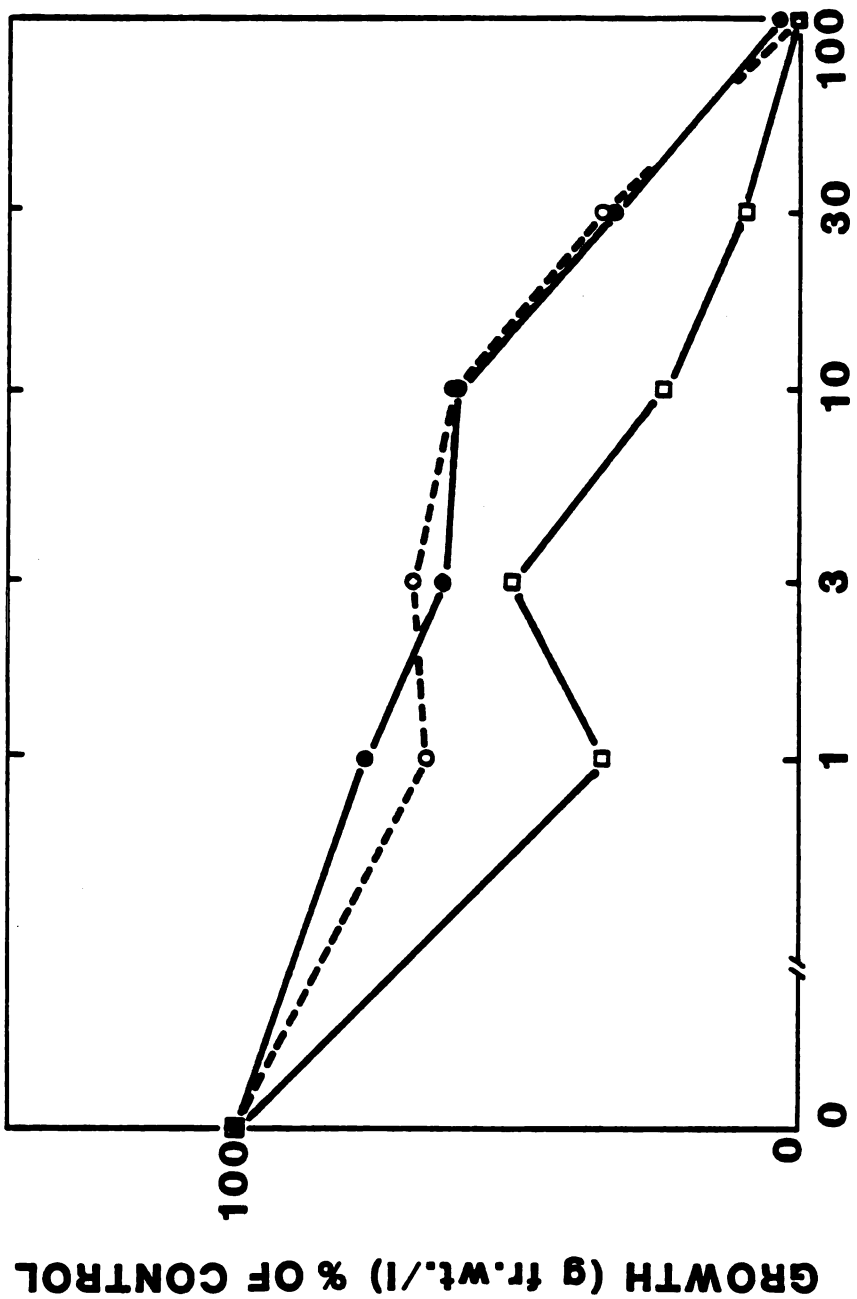

Figure 45: Dose-response curves of effect of chlorate on fresh weight accumulation in 14U cell cultures. (O) = N-less M1D + 3 mM urea; (●) = Cl-less N-less M1D + 3 mM urea; (□) = N-less M1D + 2.5 mM KNO_3.

nitrate in the urea M1D medium, the 14U cells became more sensitive to the toxic effect of chlorate.

These results suggest that urea by itself, or the products of its reduction, rather than chloride ions, may be responsible for the observed protection.

To further investigate the urea effect, an experiment was designed in which the growth response of the XD cells to chlorate was measured in M1D medium supplemented with increasing concentrations of either urea (0, 3, 6 and 9 mM) or ammonium succinate (0, 3, 6 and 9 mM NH_4Cl + 0, 1.5, 3 and 4.5 mM succinic acid, respectively). As an additional treatment, the response of the XD cells to chlorate in N-less M1D supplemented with 3 mM NH_4Cl plus 1.5 mM succinic acid was also measured.

The results of this experiment did not agree with our previous observation. Urea did not protect the XD cells against chlorate at any of the concentrations tested (Figure 46). Ammonium succinate on the other hand protected the cells against chlorate toxicity (Figure 47) in a curious fashion. At 3 mM ammonium succinate XD cells were less sensitive (than controls) to chlorate at any of the tested concentrations. However, ammonium succinate at 6 and 9 mM concentrations protected the cells differently depending upon the chlorate concentration. Protection was greatest with 6 and 9 mM ammonium succinate at 10 mM $KClO_3$. This same effect was observed when the XD cells were grown on N-less M1D plus 3 mM ammonium succinate (Figure 48). The pH of the medium measured in several instances after harvesting the cells (values recorded on figures next to corresponding data points) follow the same pattern as the one observed for the protection response against chlorate toxicity. The basis for this apparently synergistic interaction of chlorate and ammonium succinate has yet to be resolved.

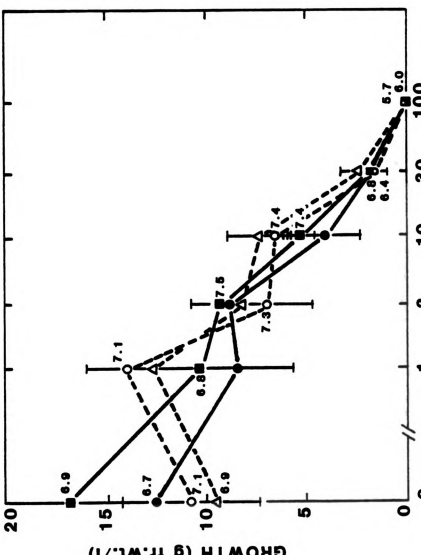

Figure 46: Dose-response curves of effect of chlorate on fresh weight accumulation by XD cells grown in urea-supplemented M1D. (●) = M1D; (△) = M1D + 3 mM urea; (■) = M1D + 6 mM urea; (○) = M1D + 9 mM urea.

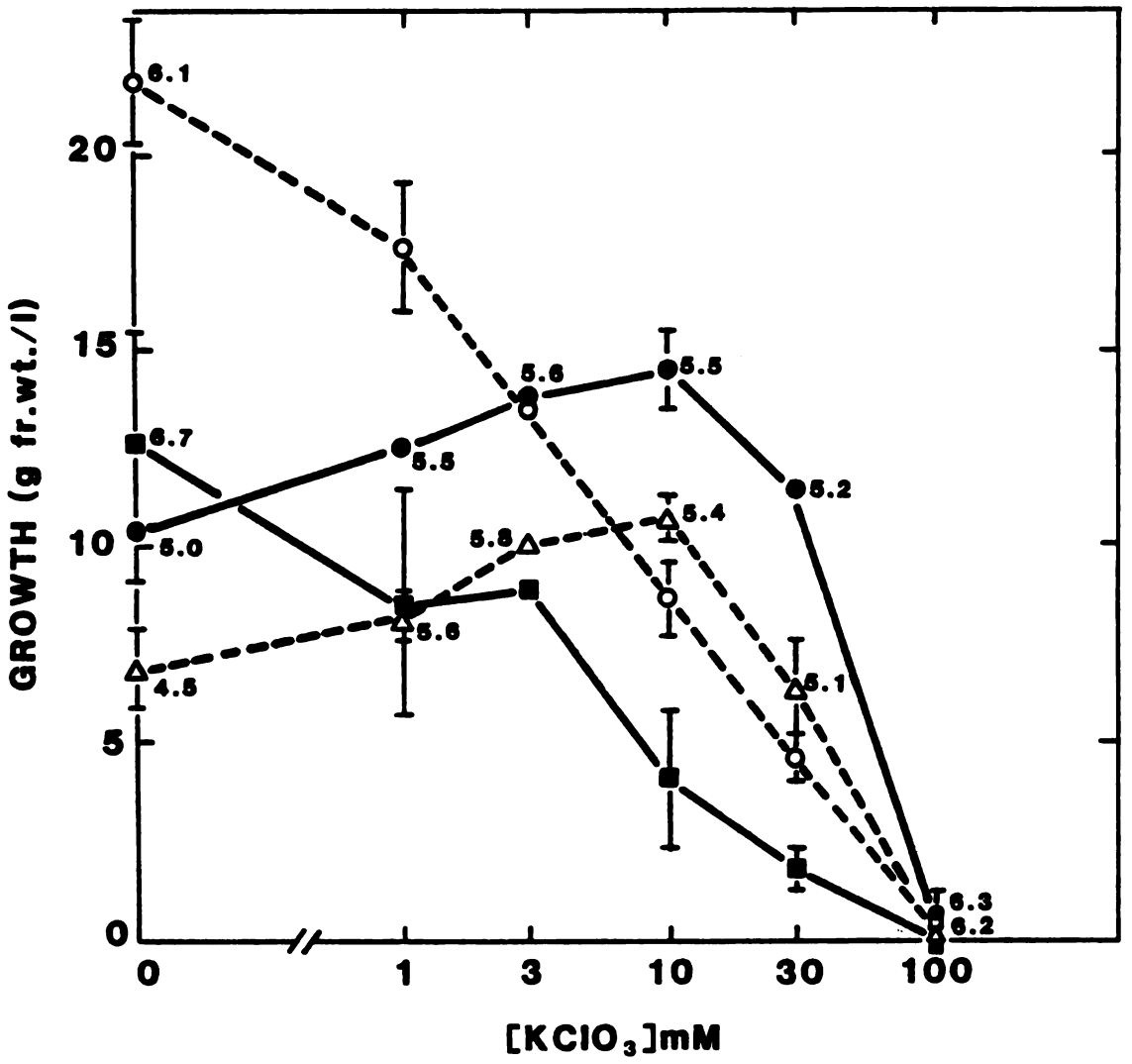

Figure 47: Dose-response curves of effect of chlorate on fresh weight accumulation in XD cell cultures supplemented with ammonium succinate. (○) = M1D + 3 mM ammonium succinate (AS); (△) + M1D + 6 mM AS; (●) = M1D + 9 mM AS; (■) = M1D control.

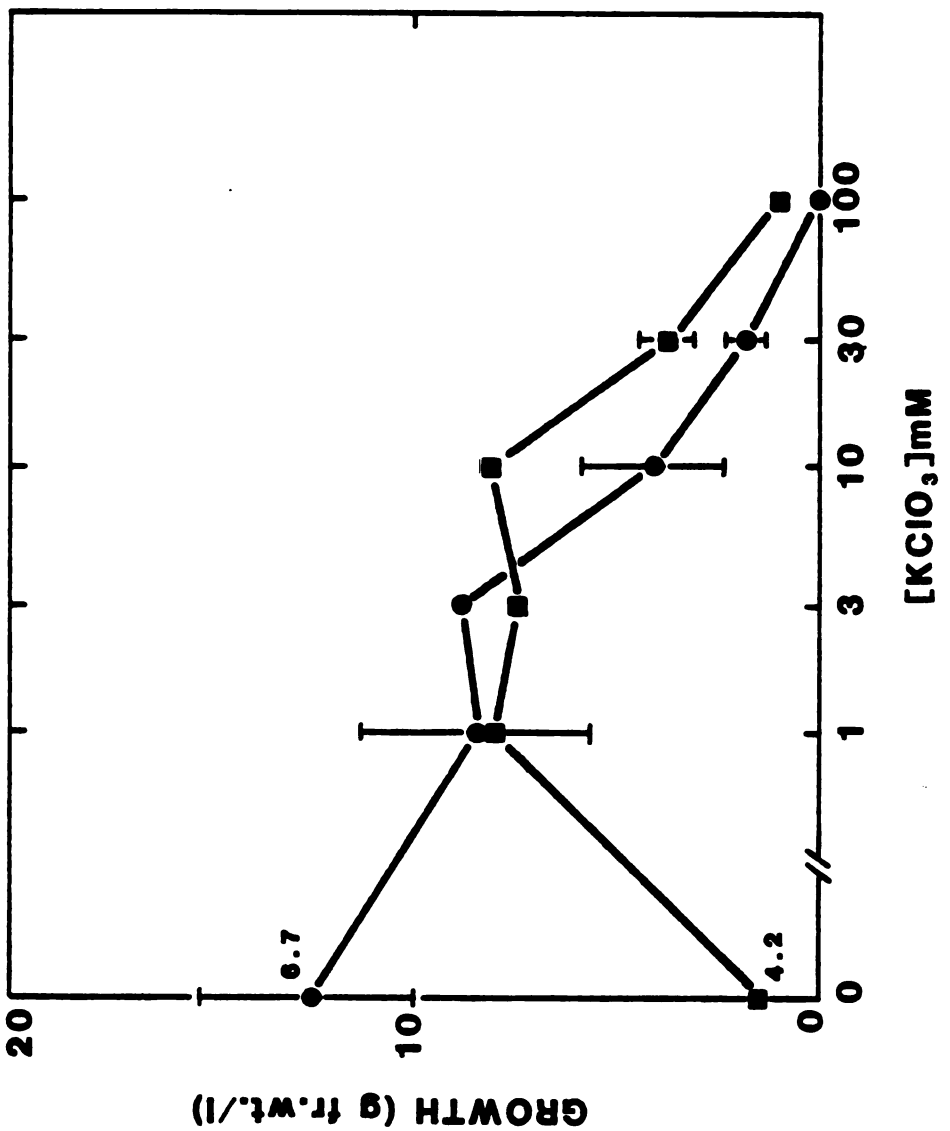

Figure 48: Dose-response curves of effect of chlorate on fresh weight accumulation in XD cell cultures in which ammonium succinate replaced nitrate. (●) = M1D; (■) = N-less M1D + 3 mM ammonium succinate.

PART III: NITROGEN NUTRITION AND CHLORATE UPTAKE IN TOMATO SEEDLINGS

MATERIALS AND METHODS

Plant Material.

An objective of the present work was to evaluate the correlation between nitrate uptake affinity and adaptation to nitrogen poor soils, in a number of species or cultivars. To judge the feasibility of cell culture selections for altered nitrate uptake, the developmental consistency of this character must be compared in whole plants and cultured cells of an individual cultivar.

Tomato plants were chosen for this part of our study for several reasons: 1) rapid and uniform seed germination, 2) rapid growth and convenient seedling size, 3) well-developed genetics and physiology, 4) amenability to tissue culture and 5) availability of a large store of variable germplasm. On the other hand, tobacco is severely deficient on 1), 2) and 3).

J. Fobes (ARCO-PCRI, Dublin, California) kindly supplied seeds of the following genotypes: *Lycopersicon chilense*, drought resistant plants with an extensive root system native to very sandy soils poor in nitrogen. *L. pimpinellifolium*, which competes well in corn fields. *L. pennellii*, drought resistant plants with small root systems surviving in very sandy soils. *L. esculentum* cv VF-36, a cultivated tomato. S.E. Barsel (Department of Botany and Plant Pathology, Michigan State University) generously supplied seeds of *L. esculentum* cv. VFN-Bush, also a cultivated tomato.

Initiation and Maintenance of Cultures.

Callus cultures were induced from radicle, hypocotyl and cotyledonary leaf explants of each one of the tomato species and cultivars described above. Seeds were surface sterilized by soaking them for 30 minute in 10% commercial bleach plus 1% sodium lauryl sulfate (with periodic agitation) followed by 6 five minute rinses in sterile distilled water. The seeds were then germinated over sterile Whatman number 1 filter paper discs wetted with 2 ml of sterile water. Seeds were germinated at room temperature (22° C to 25° C) on the laboratory bench top. Portions of the cotyledons, hypocotyls and radicle were dissected from 7 day old seedlings and plated on T_{12}, M1D and M1D supplemented with the hormones of T_{12} medium (see media formulae below). Callus developed from these explants was subcultured onto fresh medium every 2 weeks thereafter. Cultures of the genotypes mentioned above were initiated in January, 1981. From these callus cultures, suspension cultures were made by introducing 3 to 6 grams of friable callus of each cell line into 50 ml of liquid medium in a 125 ml erlenmeyer flask. Cultures were maintained in a regime of 12 h light and 12 h dark at 28° C on a reciprocal shaker. Subculture was achieved by diluting 25 ml of the cell suspension into 200 ml of fresh medium. The subculture schedule varied, depending on medium and genotype.

T_{12} medium (developed by S.E. Barsel) consisted of the major and minor salts of Murashige and Skoog (1962) supplemented with Nitsch's vitamins (0.5 mg/l thiamine, 2 mg/l glycine, 5.0 mg/l nicotinic acid, 0.5 mg/l pyridoxine, 0.5 mg/l folic acid and 0.05 mg/l biotin), hormones (3 mg/l IAA, 2 mg/l 2,4-D and 0.1 mg/l BA), 100 mg/l inositol and 30 g/l sucrose. Solid medium contained 0.9% agar. The pH of the medium was adjusted to 5.8 with KOH and/or HCl

prior to autoclaving for 15 minutes at 20 pounds pressure.

Nitrate-less T_{12} medium was made by replacing NH_4NO_3 and KNO_3 with 9 mM KCl. For the experiments, this medium was supplemented with one or more of the following nitrogen sources: 1) NH_4Cl (20 mM) + Na succinate (20 mM), 2) Urea (3mM) and 3) Arginine (3mM).

M1D and urea-M1D medium were prepared as described in MATERIALS AND METHODS of PART I. Arginine-M1D medium was prepared by dilution of an arginine solution into nitrogen-less M1D medium to give a final arginine concentration of 3 mM. Solid media contained 0.9% agar and the pH was adjusted to 6.2 with NaOH and/or HCl prior to autoclaving for 15 minutes at 20 psi.

Tomato explants were also plated on a modified M1D medium in which the hormones had been substituted by those of the T_{12} medium.

Supplemented media were always made by addition of filter-sterilized supplements to autoclaved basal media.

Culture of Tomato Seedlings.

Tomato seeds were surface disinfested and germinated as described above. The petri dishes containing the germinating seeds were sealed with parafilm and stored in the dark at room temperature. When the radicles had reached 1 to 2 cm in length, the seedlings were transfered in a laminar flow hood, to test tubes where they were grown hydroponically, as described below.

Half strength modified Hoagland's solution (Wilson *et al*,.1978) was supplied for the growth of seedlings. N-free Hoagland's solution was made by replacing the nitrates of calcium and potassium with equimolar amounts of their respective chlorides. The pH of the solution was adjusted to 5.4, 5.5 or 6.5 with NaOH and/or HCl. In several instances, MES (20 mM) was added to

stabilize pH. Twenty-ml aliquots of medium were dispensed into 25mm x 200 mm test tubes. A filter paper support platform was then inserted into the test tubes prior to sealing with foam plugs. Sterilization was carried out by autoclaving for 15 minutes at 20 psi.

The support platform was constructed by folding a Whatman No. 4 filter paper disc (7 cm in diameter) over the mouth of a 23 mm diameter test tube. A small hole was punctured in the center of the paper platform for insertion of the seeling radicle and an additional hole was punctured half way to the edge of the platform to be used for the addition of media supplements.

When supplemented media were required, sterile solutions of the supplements were diluted into sterile media. The supplemental solutions were adjusted to the desired pH prior to filter sterilization.

The seedlings were transfered to the test tubes by inserting the seedling radicle through the center hole of the paper platform. This assembly was then pushed down with sterile forceps until the skirt of the platform was in contact with the liquid medium. The bottom part of the test tube was covered with aluminum foil to darken the root environment. The seedlings were grown under gro-lights (126 microeinsteins M^{-2} sec^{-1}) with a photoperiod of 14 hours followed by 10 hours of darkness. The temperature inside the test tube, recorded with a thermister, was 23° C during the dark period and 27° C during the light. Three replicates were prepared per data point.

The seedlings were harvested at one week intervals and their growth was determined by fresh and dry weight measurements. Fresh tissues were dried in a 60° C oven for 24 hours. After removal of the seedlings, the pH of the liquid media was also measured. Root size and morphology were recorded by spreading the roots over cellophane sheets and photocopying them on a Xerox 8200. The length of main and lateral roots was measured with a planimeter

(Arin, W. Germany).

Compartmental Analysis.

L. esculentum cv VF-36 seedlings were exposed to N-free Hoagland's solution supplemented with 3 mM glutamine, 20 mM MES and ^{36}Cl-chlorate for a "loading" period of 10 hours. They were then transfered to a solution containing an identical nutrient solution lacking ^{36}Cl-chlorate for a subsequent "unloading" period of 2 hours. The nutrient solution was replaced every 4 minutes at the beginning of the "unloading" period. This interval was gradually increased to 32 minutes by the end of the "unloading" period. Such a scheme minimized reabsorption of the radioisotope The radioactivity in 3 ml aliquots from each of the rinses was counted in 12 ml of the scintillation fluid described by Trombolla (1970). Following the final rinse, the seedlings were blotted with paper towelling, the roots and shoots separated and the fresh weight of each portion measured. The amount of radioactivity remaining in these tissues was determined by counting in 15 ml of the scintillation cocktail.

Measurements of Chlorate Uptake by Tomato Seedlings.

Chlorate uptake was measured in three week old *L. esculentum* cv. VF-36 seedlings grown on N-free Hoagland's solution supplemented with either 3 mM glutamine or 3 mM γ-amino butyric acid (GABA). ^{36}Cl-chlorate, $KClO_3$ and KNO_3 solutions were delivered to the nutrient medium with a sterile syringe through the lateral hole in the paper platform. After 24 hours, the roots were blotted with a Kimwipe and rinsed for 10 minutes in fresh medium (minus chlorate and nitrate). The seedlings were then blotted dry in paper toweling and their fresh weight determined. The roots and shoots were

counted separately in the scintillation cocktail described by Trombolla (1970).

RESULTS

Nitrogen Nutrition of Tomato Tissue Cultures.

All explants from all genotypes developed friable calluses when grown on T_{12} and ammonium succinate-T_{12}. These calluses gave rise to finely divided cell suspensions when transfered to liquid T_{12} or liquid ammonium succinate-T_{12}. *L. esculentum* cv. VF-36 suspensions in liquid ammonium succinate-T_{12} medium grew with a doubling time of about 2 days (Figure 49). This particular cell line had been subcultured 13 times at 10 day intervals prior to these growth measurements.

Tomato cells grown in M1D would provide a direct comparison with results obtained with tobacco cells. However, tomato seedling explants on M1D and M1D supplemented with T_{12} hormones became necrotic and showed no signs of growth.

To avoid nitrate carryover from the tomato cells' maintenance medium, we sought a combined nitrogen source other than nitrate on which to maintain tomato cells stock cultures. Ammonia was unacceptable because of its interference with nitrate transport.

Tomato calluses of all cultivars developed from root explants and maintained for one year on T_{12}, were transferred to arginine-T_{12} and urea-T_{12}. At this time callus tissue from the same source was evaluated on M1D, urea-M1D and arginine-M1D. Although most of the callus tissue became

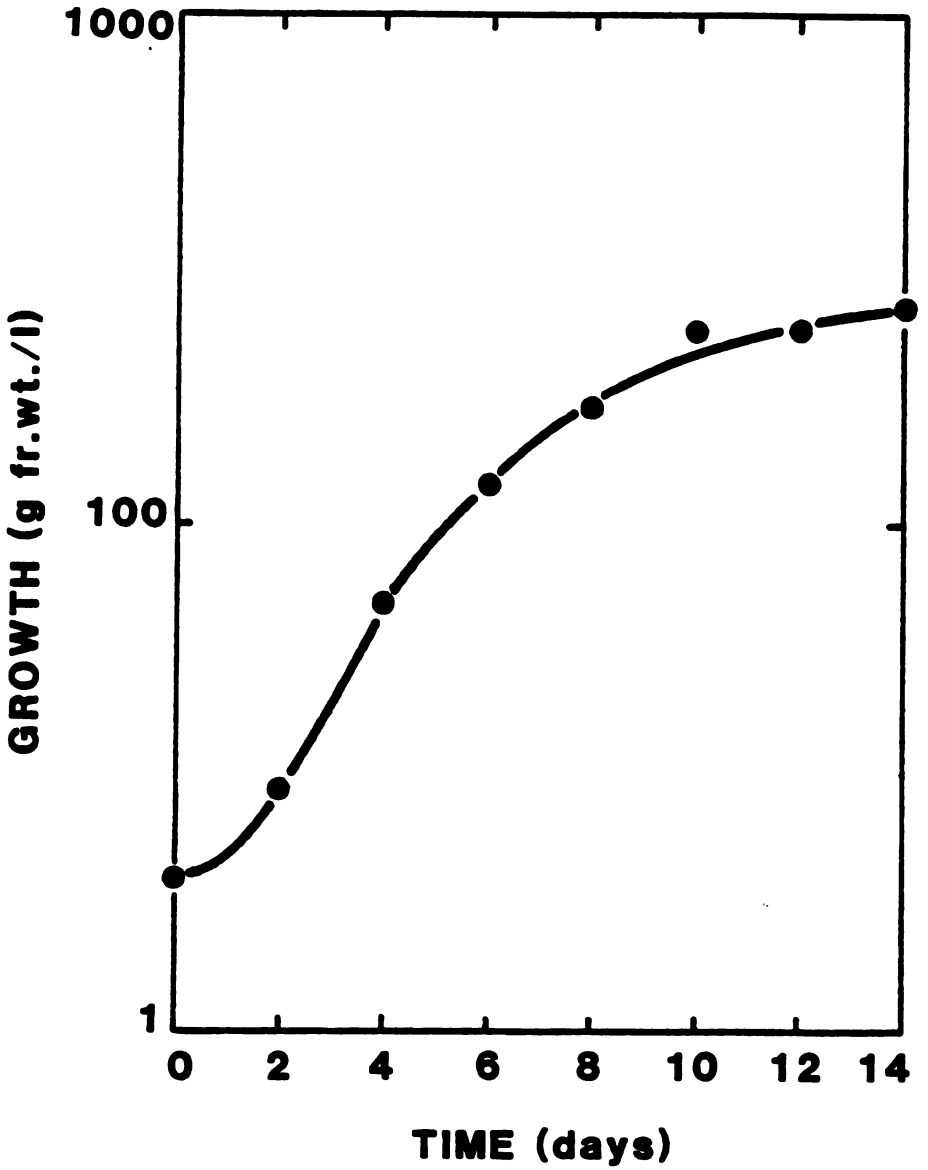

Figure 49: Growth of batch suspension cultures of *L. esculentum* cv. VF-36.

necrotic on all media tested, upon repeated transfer to the same media, rare colonies arose. Those colonies were then picked and serially transferred, selecting for cells able either to metabolize the supplied nitrogenous compounds or grow on a minimal medium (M1D). The current status of these cultures is shown in Table 15.

L. esculentum cv. VF-36 calluses grown on urea-M1D medium were transfered to liquid urea-M1D medium and a cell suspension was obtained. After 8 subcultures at 13 day intervals growth kinetics of the cell suspension were determined. A doubling time of 3.8 days was computed from the growth curve shown in Figure 50.

Chlorate Toxicity in Tomato Cells.

Hofstra (1977) reported that tomato plants were insensitive to chlorate when grown on ammonia as the sole nitrogen source. This finding agrees with the current belief that chlorate becomes toxic only after its reduction to chlorite by the enzyme nitrate reductase (Åberg, 1947). *L. esculentum* cv. VF-36 seedlings should therefore display a reduced sensitivity to chlorate when grown on media containing nitrogen sources other than nitrate. However I found that ammonia-grown seedlings were more sensitive to chlorate than those grown on nitrate (see below). In addition, lower concentrations of chlorate were required to cause growth inhibition in tomato seedlings comparable to that in tobacco cells (see below). The latter could be accounted for by chlorate being concentrated by the seedlings via transpiration. Nevertheless, an evaluation of the cell-level chlorate response of *L. esculentum* cv. VF-36 in ammonium succinate-T_{12} was needed to establish the relative sensitivity of cultured tomato cells to chlorate toxicity.

Ten ml aliquots of *L. esculentum* cv. VF-36 tomato cells growing in

Table 15: Callus growth response of tomato species to different medium nitrogen sources. Root, shoot or leaf tissues from tomato germplasm sources indicated were qualitatively evaluated for callus response on indicated nitrogen sources. Overall evaluation of performance was made after 6 to 8 passages in culture: (+++) = prolific growth; (++) = fair growth; (+) = poor growth; (-) = no growth; (NT) = no tested.

Table 15: Callus growth response of tomato species to different medium nitrogen sources.

Genotype	Source of Explant	MEDIA M1D	Urea-M1D	Arg-T_{12}	Urea-T_{12}	Am.Suc.-T_{12}	T_{12}
L.esculentum cv VF36	root	+ +	+ +	+	+ +	+ + +	+ + +
	shoot	NT	NT	NT	NT	+ + +	+ + +
	leaf	NT	NT	NT	NT	+ + +	+ + +
L.esculentum cv VFN-Bush	root	+ +	NT	+	+ +	+ + +	+ + +
	shoot	NT	NT	NT	NT	+ + +	+ + +
	leaf	NT	NT	NT	NT	+ + +	+ + +
L. Pennellii	root	+ +	+ +	(tiny colonies)	+ +	+ + +	+ + +
	shoot	NT	NT	NT	NT	+ + +	+ + +
	leaf	NT	NT	NT	NT	+ + +	+ + +
L. Pimpinelli-folium	root	(tiny colonies)	NT	-	(tiny colonies)	+ + +	+ + +
	shoot	NT	NT	NT	NT	+ + +	+ + +
	leaf	NT	NT	NT	NT	+ + +	+ + +
L. chilense	root	NT	NT	NT	NT	+ + +	+ + +
	shoot	NT	NT	NT	NT	+ + +	+ + +
	leaf	NT	NT	NT	NT	+ + +	+ + +

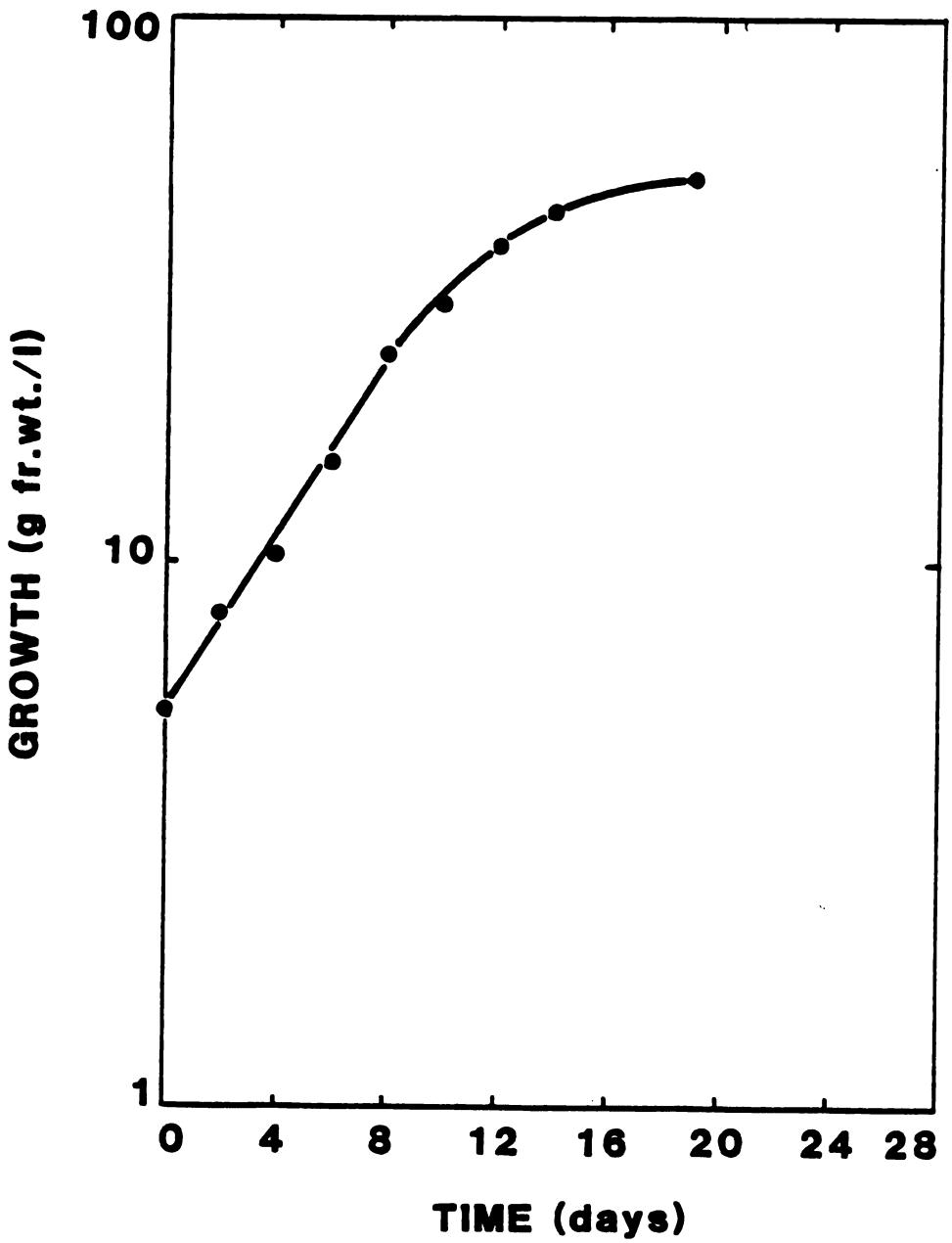

Figure 50: Growth of *L. esculentum* cv. VF-36 batch suspension cultured cells in urea-M1D medium.

ammonium succinate-T_{12} medium from a 2 l suspension culture were transfered to 500 ml flasks containing 200 ml of ammonium succinate-T_{12} medium and $KClO_3$ at concentrations ranging from 0 to 100 mM. Every three days 2 flasks per treatment were harvested and fresh weight determined. Under these conditions, the chlorate I_{50} (= the concentration resulting in a 50% reduction in growth rate with respect to the control) was 10 mM (Figures 51 and 52). This value is lower than that obtained for 14U tobacco cells grown in urea-M1D (I_{50} = 40 mM, See Figure 43), indicating that, at the cellular level, tomato and tobacco do not differ significantly in their sensitivity to chlorate.

Chlorate Toxicity in Tomato Seedlings.

Chlorate toxicity in *L. esculentum* cv VF36 plants was measured by transfering 2 day old seedlings into test tubes containing 20 ml of Hoagland's solution (7.5 mM KNO_3) and $KClO_3$ concentrations ranging from 0 to 100 mM. Seedlings were harvested and their fresh and dry weight determined 1, 2 and 3 weeks after transfer to the growth tubes. The I_{50} for the effect of chlorate on seedling growth was 620 μM (Figure 53). This value was very low compared to the concentration required to elicit the same response in tomato cells (I_{50} = 10.5 mM, Figure 52) and tobacco cells (I_{50} = 13 mM, Figure 43).

In a preliminary experiment, the response of tomato seedlings to chlorate was also measured in Hoagland's solution in which KNO_3 had been substituted with 7.5 mM NH_4Cl. $KClO_3$ was supplied at 1 mM. Seedlings growing in chlorate-free Hoagland's solution (7.5 mM nitrate) were used as controls. The data in Table 16-A indicate that: 1)ammonia-grown plants developed poorly compared to those grown on nitrate and 2) chlorate toxicity was greater, not less, in ammonia grown plants than in those grown on

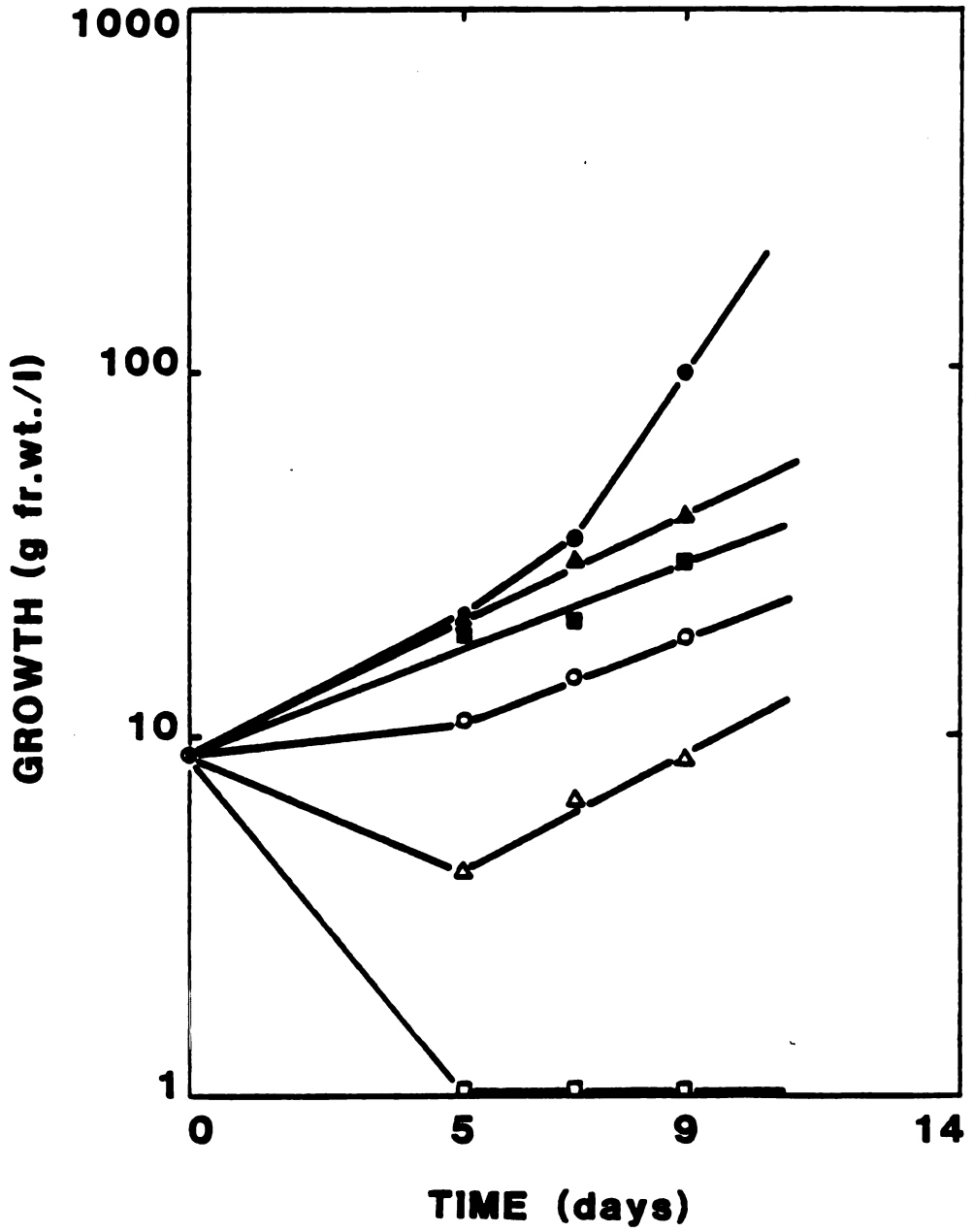

Figure 51: Chlorate toxicity in batch suspension cultures of *L. esculentum* cv. VF-36. Cultures were grown in ammonium succinate T_{12} medium supplemented with 0 (●), 1 (▲), 3 (■), 10 (○), 30 (△), or 100 (□) mM $KClO_3$, and harvested by vacuum filtration for fresh weight determinations on indicated days.

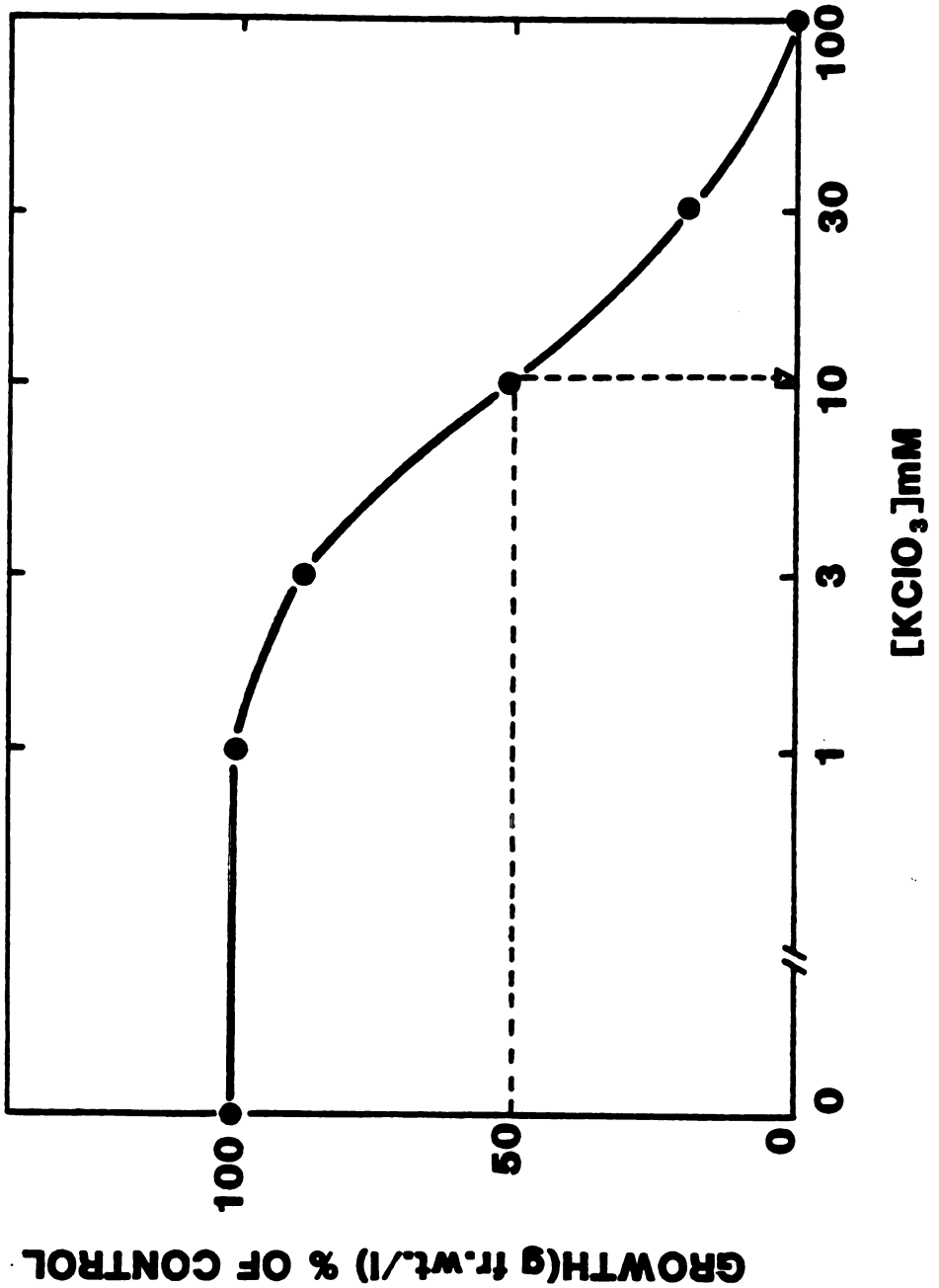

Figure 52: Growth response of tomato cell cultures to chlorate. Five day growth data from Figure 51 are plotted as a function of chlorate concentration, expressed as a percent of the 0-chlorate control.

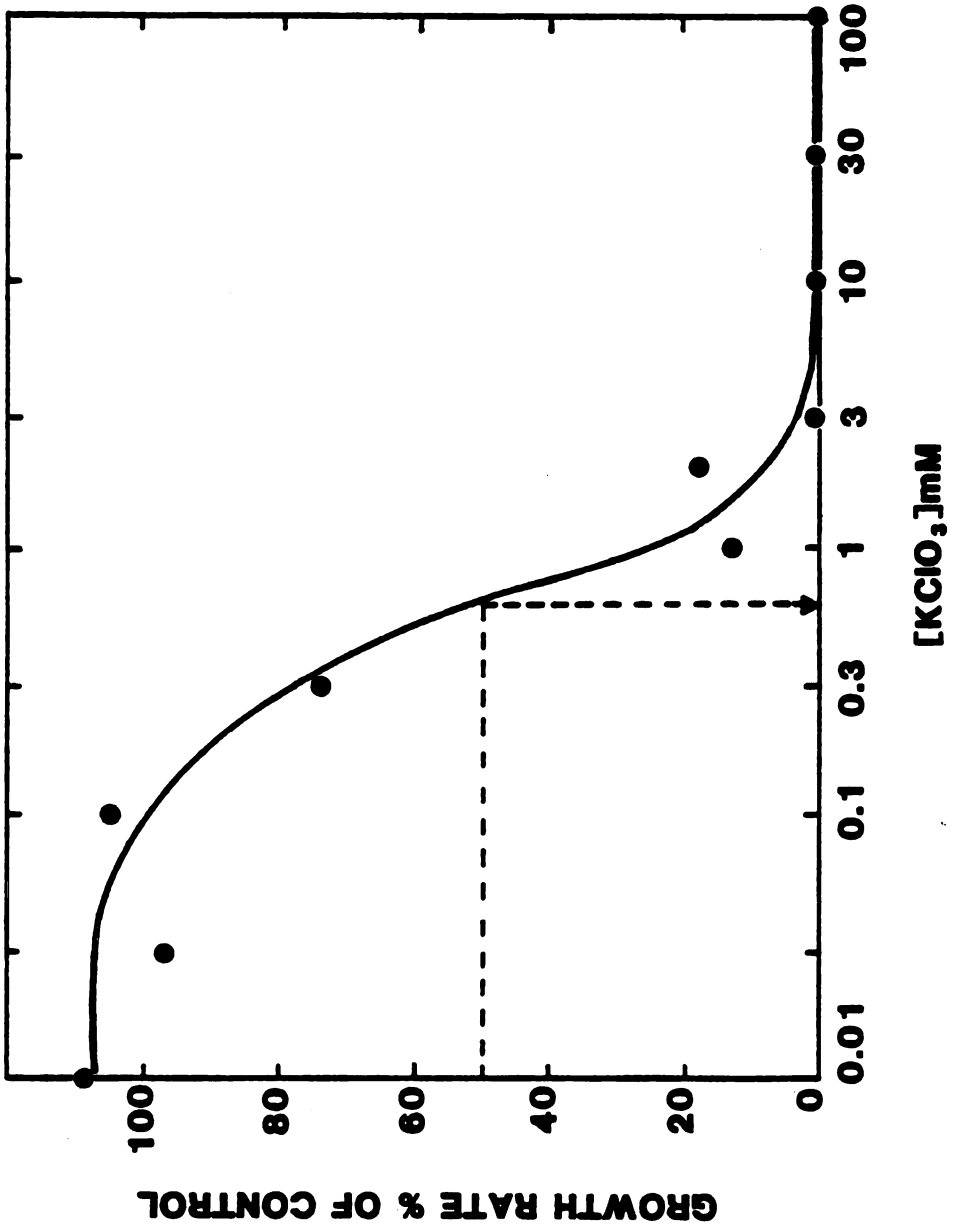

Figure 53: Effect of chlorate on tomato seedling growth. Each point represents the log-linear growth rate of 3 replicate seedlings over a 3 week period, expressed as a percent of control.

Table 16A: Nitrogen source dependence of chlorate sensitivity in tomato seedlings grown in unbuffered solutions. Seedlings were harvested and weighed after 3 weeks' growth on the N-source/$KClO_3$ combination indicated. Data are means of three replicate measurements.

	Fresh Weights (% Control)	
	KNO_3 (7.5 mM)	NH_4Cl (7.5 mM)
-$KClO_3$	100	21
+$KClO_3$(1mM)	22	2

Table 16B: Effect of nitrogen source and chlorate on pH of tomato seedling nutrient solution. The pH of seedling nutrient solution was determined after 7 days growth in the N-Source/$KClO_3$ combination indicated. Data are means of 3 replicate samples.

	pH	
	KNO_3 (7.5 mM)	NH_4Cl (7.5 mM)
-$KClO_3$	6.29	3.79
+$KClO_3$(1mM)	6.07	5.29

nitrate. Since the growth of the tomato seedlings was reduced dramatically when grown in ammonia, it was possible that their high sensitivity to chlorate was a consequence of their weak physiological state. Poor growth on ammonia may result from the media's lowered pH which developed as a consequence of ammonium metabolism in growing seedlings. Table 16-B summarizes the final pH values of the media in which the seedlings were grown for the above experiment. The necessity of a buffered medium was obvious.

The buffering capacity of MES and its effect on seedlings was examined by its addition at 5 and 20 mM to Hoagland and ammonim-Hoagland nutrient solutions. Fresh and dry weights of the seedlings as well as the pH changes of the media were recorded at weekly intervals. 20 mM MES maintained the pH of the Hoagland's solution; however, its buffering capacity is insufficient for the ammonium-Hoagland solution. These results are summarized in Table 17 and correspond to the seedlings harvested after three weeks. As in previous experiments, the ammonium grown plants showed greater sensitivity to chlorate than those grown on nitrate. Even though MES (20 mM) maintained the pH for the first 2 weeks (Table 17) we cannot rule out the possibility that the difference in response was due to a difference in pH created by nitrate and ammonia assimilation. Supplementation of ammonium-Hoagland's with succinic acid also achieved a measure of pH stabilization (Table 18). Still, a pH drop from 5.4 to 5.0 was observed with 15 mM succinic acid. Furthermore, plants grown in 15 mM succinic acid buffered ammonium-Hoagland's did not attain fresh or dry weight comparable to growth on nitrate.

One possibility for the poor performance of seedlings under these conditions was that 7.5 mM ammonium was toxic. Therefore combinations of MES and succinic acid and decreasing amounts of ammonium were tested. The results of these experiments (Tables 19 and 20) suggested that 4 mM

Table 17: Effect of MES, ammonium and chlorate on growth and nutrient solution pH of tomato seedlings. Fresh and dry weights of tomato seedlings were determined after 3 weeks of growth under the nutrient conditions indicated. Seedlings were harvested weekly for nutrient pH determinations. Each datum represents mean and standard error of 3 replicate determinations. H = Hoagland's solution. N-less H = nitrogen-free Hoagland's solution. Nutrient solutions were titrated to pH 5.4 prior to planting.

Table 17: Effect of MES, ammonium and chlorate on growth and nutrient solution pH of tomato seedlings.

Treatment	Fresh Weight (mg)	Dry Weight (mg)	pH First Week	pH Second Week	pH Third Week
H	687 ± 34	25 ± 1	6.44 ± .11	7.05 ± .08	7.46 ± .07
H + 5 mM MES	745 ± 33	28 ± 2	5.53 ± .01	5.71 ± .01	5.86 ± .05
H + 20 mM MES	692 ± 3	29 ± 1	5.37 ± .00	5.42 ± .01	5.47 ± .02
H + 1 mM $KClO_3$	159 ± 8	12 ± 1	5.87 ± .18	6.32 ± .14	6.66 ± .13
H + 5 mM MES + 1 mM $KClO_3$	110 ± 38	9 ± 2	5.39 ± .04	5.41 ± .04	5.43 ± .03
H + 20 mM MES + 1 mM $KClO_3$	136 ± 42	11 ± 3	5.34 ± .01	5.33 ± .00	5.34 ± .01
N-less H + 7.5 mM NH_4Cl	90 ± 4	12 ± 1	3.38 ± .05	3.42 ± .03	3.55 ± .04
N-less H + 7.5 mM + 5 mM MES	120 ± 21	15 ± 1	4.63 ± .15	3.27 ± .04	3.33 ± .07
N-less H + 7.5 mM + 20 mM MES	355 ± 14	21 ± 0	5.20 ± .02	4.76 ± .13	3.33 ± .06
N-less H + 7.5 mM NH_4Cl + 1 mM $KClO_3$	15 ± 5	2 ± 0	5.48 ± .09	5.49 ± .07	5.51 ± .09
N-less H + 7.5 mM NH_4Cl + 5 mM MES + 1 mM $KClO_3$	15 ± 6	2 ± 0	5.35 ± .01	5.38 ± .01	5.32 ± .01
N-less H + 7.5 mM NH_4Cl + 20 mM MES + 1 mM $KClO_3$	14 ± 3	2 ± 1	15.34 ± .01	5.35 ± .00	5.32 ± .01

Table 18: Effect of MES, ammonium and succinic acid on growth and nutrient solution pH of tomato seedlings. Fresh weights, dry weights and nutrient solution pHs were determined after 3 weeks growth under treatment conditions indicated. Each datum represents mean and standard error of 3 replicate determinations. SA = succinic acid. N-less H = Nitrogen-free Hoagland's solution. H = Hoagland's solution.

Treatment	Fresh Weight (mg)	Dry Weight (mg)	pH
N-less H + 7.5 mM NH_4Cl + O mM SA	96 ± 10	14 ± 0	3.46 ± 0.5
N-less H + 7.5 mM NH_4Cl + 7.5 mM SA	546 ± 43	27 ± 2	4.41 ± .14
N-less H + 7.5 mM NH_4Cl + 15 mM SA	503 ± 6	25 ± 1	5.00 ± .03
N-less H + 7.5 mM NH_4Cl + 20 mM MES	354 ± 11	22 ± 2	3.39 ± 0.5
H + 20 mM MES	678 ± 41	30 ± 2	5.57 ± .00

Table 19: Effect of MES, ammonium and succinic acid on growth and nutrient solution pH of tomato seedlings. Fresh weights, dry weights and nutrient solution pHs were determined after 3 weeks growth under treatment conditions indicated. Each datum represents mean and standard error of 3 replicate determinations. SA = succinic acid. N-less H = nitrogen-free Hoagland's solution. H = Hoagland's solution.

Treatment	Fresh Weight (mg)	Dry Weight (mg)	pH
H + 20 mM MES	587 ± 24	27 ± 1	5.49 ± .03
N-less H + 7.5 mM NH_4Cl	118 ± 22	14 ± 1	3.08 ± .04
N-less H + 7.5 mM NH_4Cl + 15 mM SA	392 ± 48	21 ± 1	4.56 ± .04
N-less H + 7.5 mM NH_4Cl + 20 mM SA	413 ± 8	22 ± 1	4.75 ± .03
N-less H + 7.5 mM NH_4Cl + 25 mM SA	402 ± 17	22 ± 1	4.87 ± .03
N-less H + 7.5 mM NH_4Cl + 15 mM SA + 15 mM MES	394 ± 20	21 ± 2	4.95 ± .02
N-less H + 7.5 mM NH_4Cl + 20 mM SA + 20 mM MES	389 ± 42	22 ± 2	5.05 ± .04

Table 20: Effect of MES, ammonium and succinic acid on growth and nutrient solution pH of tomato seedlings. Fresh weights, dry weights and nutrient solution pHs were determined weekly under treatment conditions indicated. Each datum represents mean and standard error of 3 replicate determinations. SA = succinic acid. N-less H = nitrogen-free Hoagland's solution. H = Hoagland's solution.

Treatment	Fresh Weight (mg) 1 week	2 weeks	3 weeks
H + 20 mM MES	120 ± 10	322 ± 6	516 ± 60
4 mM NH_4Cl + 4 mM SA + 20 mM MES	92 ± 5	238 ± 10	345 ± 5
4 mM NH_4Cl + 4 mM SA + 40 mM MES	91 ± 13	229 ± 12	341 ± 38
4 mM NH_4Cl + 10 mM SA + 20 mM MES	99 ± 5	207 ± 9	330 ± 26
7.5 mM NH_4Cl + 20 mM SA + 40 mM MES	82 ± 7	235 ± 11	270 ± 20

Treatment	Dry Weight (mg) 1 week	2 weeks	3 weeks
H + 20 mM MES	7 ± 0	16 ± 1	24 ± 2
4 mM NH_4Cl + 4 mM SA + 20 mM MES	5 ± 0	14 ± 1	17 ± 0
4 mM NH_4Cl + 4 mM SA + 40 mM MES	5 ± 1	13 ± 1	18 ± 1
4 mM NH_4Cl + 10 mM SA + 20 mM MES	6 ± 0	13 ± 1	19 ± 1
7.5 mM NH_4Cl + 20 mM SA + 40 mM MES	5 ± 0	12 ± 1	16 ± 1

Treatment	pH 1 week	2 weeks	3 weeks
H + 20 mM MES	5.56 ± 0.00	5.59 ± 0.01	5.59 ± 0.01
4 mM NH_4Cl + 4 mM SA + 20 mM MES	5.47 ± 0.02	5.32 ± 0.00	4.96 ± 0.04
4 mM NH_4Cl + 4 mM SA + 40 mM MES	5,51 ± 0.01	5.36 ± 0.00	5.13 ± 0.02
4 mM NH_4Cl + 10 mM SA + 20 mM MES	5.49 ± 0.00	5.41 ± 0.00	5.19 ± 0.01
7.5 mM NH_4Cl + 20 mM SA + 40 mM MES	5.50 ± 0.00	5.26 ± 0.03	5.31 ± 0.00

NH_4Cl was optimal for both growth and pH maintenance. Therefore, for a reevaluation of the effect of chlorate on ammonia-grown plants the following conditions were adopted: 4 mM NH_4Cl + 4 mM succinic acid + 20 mM MES.

L. esculentum cv. VF-36 seedlings were 7 times more sensitive to chlorate when grown on medium containing ammonia than when they grew on nitrate (Table 21). The weak physiological state of the ammonium grown plants and the pH involvement in this increased sensitivity to chlorate can now be ruled out.

These results contradict those of Hofstra (1977) and do not support the mechanism of chlorate toxicity proposed by Åberg (1947). Could the seedlings contain enough nitrate reductase activity (constitutive or remnant from seed storate) to account for the reduction and toxicity of chlorate? The levels of nitrate reductase acitivity in these young seedlings were measured. Whole seedlings (a total of 2.27 g fr. wt) were homogenized in a morter and pestle with 11.35 ml phosphate buffer (0.1 M K_2HPO_4, 1 mM EGTA, 1 μM FAD and 1 mM L-cysteine free base) pH 7.5. The enzyme extract obtained as described in MATERIALS AND METHODS of PART II was assayed for nitrate reductase activity as described in that same section. Nitrate reductase activity of 0.39 nmoles / min / g fr. wt was recovered from the seedlings, approximately two orders of magnitude less than the fully induced activity of cultured XD cells (see PART II). Two possibilities exist to explain the data. The first is that the seedlings' low NR activity is sufficient to generate a lethal level of chlorite, and that the nitrate present in the $KN0_3$ treatment competes with and therefore protects the seedlings from chlorate. The second is that ammonia acts in an unexplained synergistic fashion to render seedlings more sensitive to chlorate.

The effect of tungstate on chlorate toxicity was also evaluated. In the

Table 21: Nitrogen source dependence of chlorate sensitivity in tomato seedlings grown in buffered solutions. Seedlings were harvested and weighed after 14 day's growth on the N-source/$KClO_3$ combination indicated. Data represent means of three replicate measurements. Buffers = MES (20 mM) + succinic acid (4 mM). Initial pH = 5.4. Final pH = 5.5 (KNO_3), 5.3 (NH_4Cl).

	Fresh Weight (% of control)	
	KNO_3 (7.5 mM)	NH_4Cl (4mM)
-$KClO_3$	100	96
+$KClO_3$	59	8

presence of tungstate, an analog of molybdate, a tungsto-analog of the molybdoenzyme (Heimer and Filner, 1971) nitrate reductase is formed. The tungsto-enzyme cannot catalyze reduction of nitrate. Consequently, if tungstate-treated seedlings develop toxicity symptoms in the presence of chlorate to the same extent as untreated ones (no tungstate), it would indicate that chlorate toxicity results from either 1) reduction of chlorate by preexisting embryonic or long-lived constitutive NR, or 2) a mechanism of toxicity, not involving nitrate reductase, which is either enhanced by ammonium or manifested when nitrate is not present to compete. The first explanation seems unlikely, because molybdate can replace tungstate in the absence of protein synthesis. Therefore, tungstate should be able to replace molybdate in the same way

L. esculentum cv. VF-36 seedlings were transfered to test tubes containing GABA as the nitrogen source and 100 μM tungstate. $KClO_3$ was diluted into the nutrient solution of two separate groups of test tubes to give final concentrations of 0.5 or 1 mM. Parallel test tubes were prepared to be used as controls in which 1) $KClO_3$, 2) tungstate or 3) both $KClO_3$ and tungstate were lacking.

Tungstate alone slightly inhibited seedling development (Figure 54). Chlorate toxicity symptoms of seedlings treated with tungstate were comparable to those of seedlings grown in the absence of tungstate (Figure 54).

Antidote Effect of Nitrate on Chlorate Toxicity.

In 1939 Crafts reported experiments with Kanota oats and Hubbard squash using single-salt water cultures which suggested that chlorate in the presence of high nitrate concentrations was not taken up by plant roots, whereas in low-nitrate or nitrate-free cultures, it was taken up and

Figure 54: Effect of tungstate on chlorate toxicity in tomato seedlings. GABA = 3 mM γ-amino butyric acid; TUNG. = 0.1 mM tungstate; chl. = 0.5 mM $KClO_3$; γ = GABA.

concentrated above the level of the external culture solution. That observation supports the hypothesized interaction between chlorate and nitrate at the uptake and/or reduction level in whole plants. We proceeded to reexamine this finding using our culture system, free of bacterial and fungal contamination.

Three mM $KClO_3$ was the minimum lethal concentration for stopping seedling growth in Hoagland's solution containing 7.5 mM $KClO_3$ (see Figure 53). That chlorate concentration was chosen for this experiment. Symptoms displayed by seedlings in 3 mM $KClO_3$ served as the standard of comparison for those to which KNO_3 was added at 7.5 to 37.5 mM. No buffer was supplied in this experiment. The seedlings were harvested 1, 2 and 3 weeks after transfer to test tubes. Fresh and dry weights were determined at each harvest.

The plot of the fresh weight (% of control) of 21 day old seedlings *versus* the $[KNO_3]/[KClO_3]$ ratio shows a linear response, proportional to KNO_3 concentration (Figure 55). These results support Crafts' report of the protection afforded by nitrate against chlorate toxicity. However, this protection is not as complete as one would expect if nitrate and chlorate were taken up by the same carrier and reduced by the same nitrate reductase which has an affinity 18 times higher for nitrate than for chlorate (see PART II). The 70% growth inhibition resulting from 3 mM chlorate + 37.5 mM nitrate may indicate: 1) the existence of another chlorate transport system not shared by nitrate, 2) toxicity of high nitrate concentrations or 3) that seedlings are extremely sensitive to even minute amounts of chlorite.

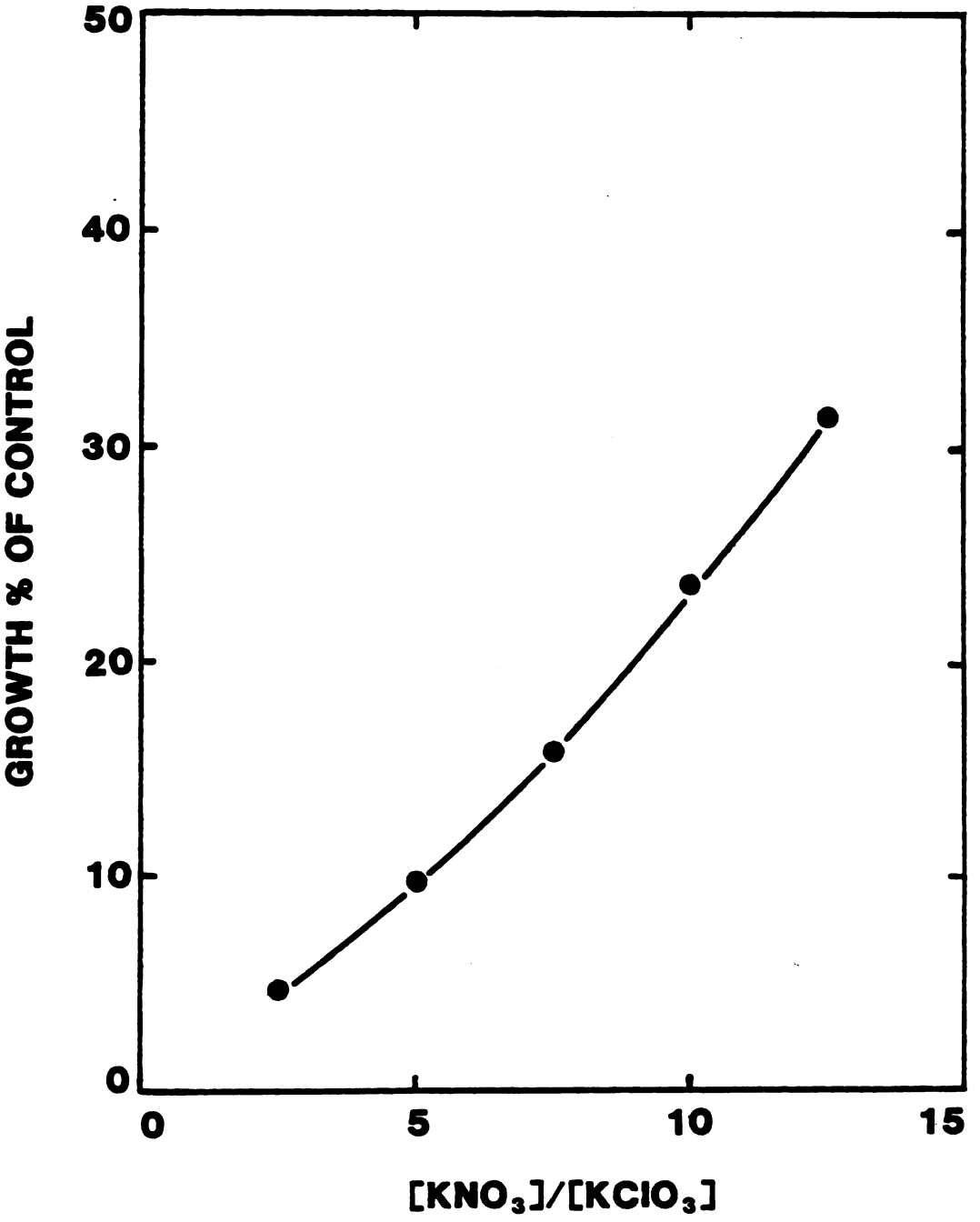

Figure 55: Nitrate protection of tomato seedlings from chlorate toxicity. Tomato seedlings were grown in 3 mM $KClO_3$ to which 7.5 to 37.5 mM KNO_3 was added. Fresh weights of 3 replicate seedlings were taken after 21 days.

Effect of Nitrogen Compounds on Tomato Seedling Development..

As with the tomato cell cultures, we again sought nitrogen compounds to replace nitrate and ammonia that could sustain tomato seedling's growth without interacting with nitrate-chlorate uptake mechanisms. Among the compounds tested in the first trial were: urea, glutamine, arginine and GABA all at 3 mM. Hoagland's solution containing 7.5 mM nitrate was the control. In the same experiment the effect of chlorate on plants growing on these compounds was also evaluated. An NH_4Cl treatment was incorporated into the study to confirm the ammonium-chlorate toxicity synergy described above. In all cases, the nutrient solutions were buffered with 20 mM MES and the pH adjusted to 5.5. Fresh and dry weights of the tomato seedlings were obtained 1, 2 and 3 weeks after transfering two day old seedlings to the test tubes. The pH's of the solutions were recorded after harvest.

The results obtained after three weeks of culture (Table 22) indicate that nitrate grown plants were less sensitive to chlorate than those given alternative treatments (Figure 56). Once again, it appeared that nitrate protected the seedlings from the toxic effect of chlorate, presumably by competing with chlorate for uptake and/or reduction. Arginine was not a good nitrogen source, but the response to glutamine was similar to that of the KNO_3 control. Seedling development was retarded in ammonium succinate, urea, and GABA, the latter giving the slowest response. These nitrogen sources can be arranged in order of decreasing ability to support growth of *L. esculentum* cv. VF-36 seedlings:

KNO_3 > Glutamine > NH_4Cl > GABA > Urea > Arginine

Table 22: Effect of nitrogen source on chlorate toxicity in tomato seedlings. Seedlings were weighed after 3 weeks' growth under the conditions indicated. pH measurements of nutrient solutions were taken at harvesting. All nutrient treatments contained 20 mM MES. Each datum represents mean and standard error of 3 replicate determinations. H = Hoagland's solution (7.5 mM KNO_3). SA = succinic acid.

Treatment	Fresh Weight (mg)	Dry Weight (mg)	pH
H	398 ± 135	18 ± 6	5.56 ± .02
H + 1 mM $KClO_3$	161 ± 1	11 ± 0	5.64 ± .00
N-less H + 3 mM Arginine	39 ± 2	6 ± 0	5.48 ± .01
N-less H + 3 mM Arginine + 1 mM $KClO_3$	13 ± 1	2 ± 0	5.48 ± .00
N-less H + 4 mM NH_4Cl + 4 mM SA	309 ± 6	17 ± 0	4.85 ± .02
N-less H + 4 mM NH_4Cl + 4 mM SA + 1 mM $KClO_3$	21 ± 4	3 ± 0	5.51 ± .01
N-less H + 3 mM Urea	220 ± 33	15 ± 2	5.40 ± .03
N-less H + 3 mM Urea + 1 mM $KClO_3$	18 ± 8	3 ± 1	5.47 ± .01
N-less H + 3 mM GABA	234 ± 22	15 ± 1	5.42 ± .01
N-less H + 3 mM GABA + 1 mM $KClO_3$	20 ± 3	3 ± 0	5.49 ± .01

Figure 56: Effect of nitrogen source on chlorate toxicity in tomato seedlings. Seedlings shown are 3 weeks old.

These compounds can also be ordered according to their ability to maintain the pH of the culture:

$$\text{Glutamine} > \text{GABA} > \text{Urea} > KNO_3 > NH_4Cl$$

Since glutamine was the overall most efficient in sustaining growth and stabilizing pH, it was chosen as the nitrogen source for the uptake studies.

In a subsequent experiment, the growth of *L. esculentum* cv. VF-36 seedlings in nitrate-free Hoagland's solution supplemented with asparagine and several degradation products of purines (hypoxanthine, xanthine,uric acid and allantion) was evaluated. The pH of the stock solutions of these compounds was adjusted to 5.5 before they were diluted into nitrate-free Hoagland's solution to a final concentration of 3 mM . MES (20 mM) was used to buffer the nutrient solutions. Some difficulty was enountered in adjusting the pHs of the uric acid, xanthine and hypoxanthine solutions. Consequently, the pH of the final solutions was higher than planned (see Table 23). GABA and glutamine treatments were incorporated into this experiment to confirm previous observations. Plants grown on standard Hoagland's solution (KNO_3) were used as controls.

The effect that these nitrogen compounds had on the growth of *L. esculentum* cv. VF-36 seedlings is summarized in Table 23, where the fresh and dry weights of 12 day old seedlings are tabulated. As in the previous experiment, seedling growth and development were extremely sensitive to the source of nitrogen . Even though the size (Table 23) and timing of primary leaf expansion was affected by these compounds, more dramatic changes were observed in root morphology and development (Figures 57 and 58, Table 24).

Table 23: Effect of ureide and amide nitrogen sources on tomato seedling growth. All data represent mean and standard error of 3 replicate determinations on 12 day old seedlings. Stem length represents distance from cotyledonary node to tip of most distal leaflet. pHs of nutrient solutions were taken at harvesting.

Treatment	Fresh Weight (mg)	Dry Weight (mg)	pH Harvest	Stem Length (cm)
KNO_3	237 ± 24	10 ± 1	5.60	7.5
Allantoin	157 ± 4	6 ± 0	5.53	5.2
Uric Acid	88 ± 2	6 ± 0	7.22	2.6
Hypoxanthine	160 ± -	7 ± -	5.98	5.2
Xanthine	129 ± 12	6 ± 1	6.57	4.3
Asparagine	200 ± -	11 ± -	5.55	5.6

Figure 57: Effect of ureide nitrogen on tomato seedling root morphology. Xerographs were made of 2 week old tomato seedling roots which had been grown with the indicated compound as the sole nitrogen source.

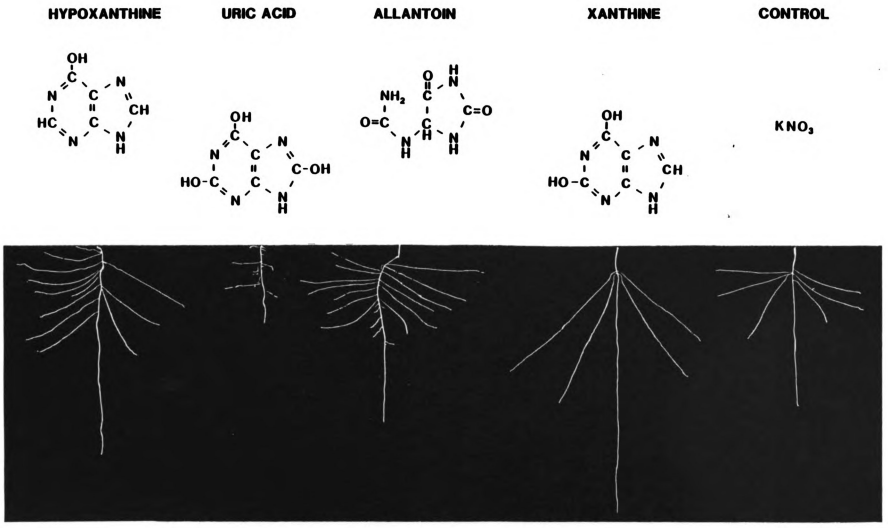

Figure 57: Effect of ureide nitrogen on tomato seedling root morphology.

Figure 58: Effect of amide nitrogen and GABA on tomato root morphology. Seedling roots were xerographed after two weeks' growth on the indicated compound as a sole source of nitrogen. One-sidedness of lateral root growth on glutamine treatment is an artifact of arranging roots for photography.

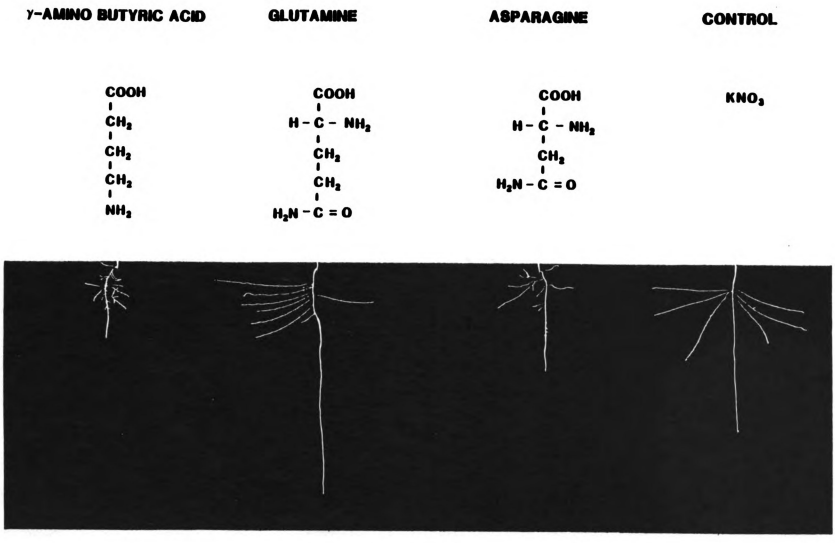

Figure 58: Effect of amide nitrogen and GABA on tomato root morphology.

Figure 24: Effect of ureide and amide nitrogen on the pH of the nutrient solution and on growth of roots of tomato plants. All data were taken after growth of seedlings for 12 days. All treatments contained 20 mM MES.

Treatment	pH at Harvest	Main Root Length (cm)	Number of Lateral Roots	Average Length Laterals (cm)	Total Root Length (cm)
KNO_3	5.60	14.9	8	5.3	47.6
Allantoin	5.53	16.2	19	4.6	103.0
Uric Acid	7.22	7.3	11	1.1	12.9
Hypoxanthine	5.98	20.2	12	6.7	100.0
Xanthine	6.57	19.0	4	10.5	65.0
Asparagine	5.55	8.7	16	1.0	26.4

Treatments that resulted in larger root systems [e.g., allantion and hypoxanthine (Table 24)] had the opposite effect on stem and leaf size and development (Table 23).

In a third experiment, the effect on seedling growth of some of the nitrogen compounds previously tested (GABA, allantoin, xanthine and glutamine) was re-examined. The nutrient solutions were prepared so as to allow accurate pH adjustment, particularly in the case of xanthine. The nitrate treatment was modified to make the final KNO_3 concentration 3 mM. The growth response of the seedlings was compared to that of two new controls: 1) Deionized water + 20 mM MES and 2) Nitrate-free Hoagland's solution + 20 mM MES.

Parameters portraying response in root morphology after two weeks of culture are summarized in Table 25. They suggest that, among other responses, *L. esculentum* cv. VF-36 seedlings develop extensive root systems in the absense of a nitrogen source. However, the aerial plant parts appeared more sensitive to the lack of nitrogen. Nitrate-grown seedlings possessed 5 true leaves after 14 days of culture while seedlings maintained in nitrate-free Hoagland's solution only developed one diminutive true leaf.

γ-Amino Butyric Acid Effect on Root Development

γ-Amino butyric acid (GABA) had a striking effect on root morphology and root hair development (Figure 59 and 60). The main and lateral roots of seedlings grown on GABA were shorter than those of seedlings grown on nitrate. Both main and lateral roots of GABA-treated plants developed root hairs in a periodic pattern in which portions of the epidermal layer had no root hairs while the intervening regions developed long and numerous root hairs. Also, the regions of the root with hairs had a greater diameter than the

Table 25: Effect of nitrogen source on tomato seedling root growth. Data were taken after 14 days' growth. All treatments contained 20 mM MES.

Treatment	pH at Harvest	Main Root Length (cm)	Number of Lateral Roots	Average Length Laterals (cm)	Total Root Length (cm)
Water	5.57	8.0	16	3.7	68.4
N-less H	5.55	17.2	16	5.2	100.2
KNO_3	5.61	15.0	16	4.4	72.5
GABA	5.53	6.2	19	1.2	29.8
Allantoin	5.60	18.6	10	8.7	108.9
Xanthine	5.55	23.1	14	4.4	85.7
Glutamine	5.51	19.6	14	5.6	101.1

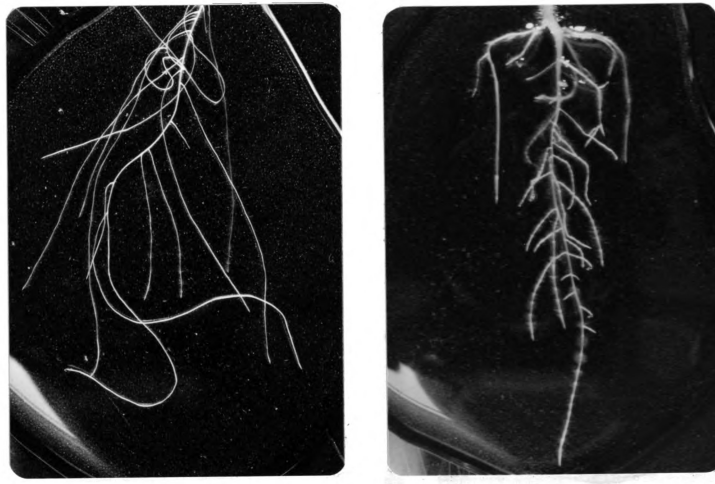

Figure 59: Effect of nitrate (left) and GABA (right) on tomato root morphology.

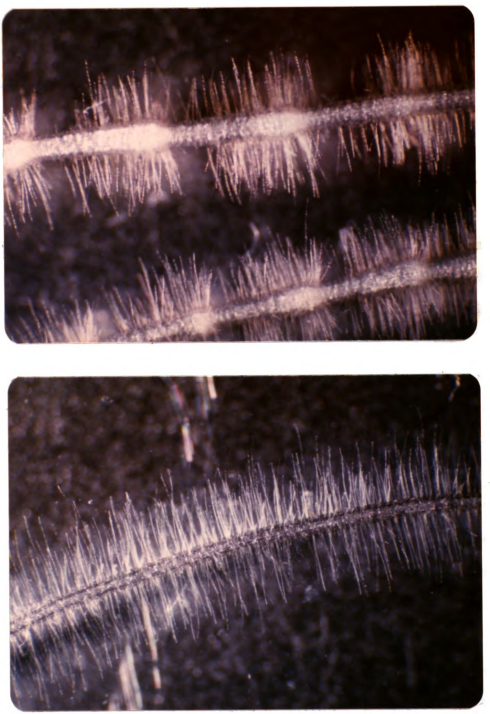

Figure 60: Effect of GABA (above) and nitrate (below) on tomato seedling root hair growth.

portions without them. In contrast, the roots of seedlings grown on nitrate had root hairs uniformily distributed along the main and lateral roots and the root diameter was smaller and uniform all along the main and lateral roots.

These observations prompted us to characterize further the nature of the GABA effect on root development. To examine whether this GABA effect is "dominant", *L. esculentum* cv. VF-36 seedlings were grown in nitrogen-free Hoagland's solution containing 3 mM GABA plus 3 mM of each of the following nitrogen compounds, singly: nitrate, glutamine, allantoin, xanthine and asparagine. In all combinations with the exception of asparagine, the GABA effect (specifically root hair periodicity) was evident (data not shown). Several amino compounds similar in structure to GABA (6-amino caproic acid, 5-amino valeric acid, β-alanine, γ-amino levulinic acid, 3-amino propane sulfonic acid, and β-amino butyric acid) were evaluated for their ability to elicit the GABA effect. None of these compounds elicited GABA's effect of root hair periodicity. All inhibited growth to differing degrees except for 3-amino propane sulfonic acid (3-APSA), which seemed to stimulate root growth compared to the nitrate control (Figure 61). This response in root development was more prominent early in seedling growth. Figure 62 shows the difference in root size and morphology between KNO_3, GABA and 3-APSA treatments in 7 day-old seedlings. The roots shown in Figure 61 correspond to 12 day-old seedlings.

The GABA effect on root hair development is light dependent. The roots of etiolated seedlings did not develop lateral roots and no periodicity was evident on the main root. The inhibition of root development was proportional to GABA concentration. High levels of GABA so inhibited root growth that the root hair periodicity was substantially obscured.

The root system of *L. esculentum* cv. Floridade and *Solanum pennellii*

Figure 61: Effect of GABA analogues on tomato seedling root morphology. Seedlings were grown for two weeks in the indicated compound, after which roots were spread and xerographed.

Figure 61: Effect of GABA analogues on tomato seedling root morphology.

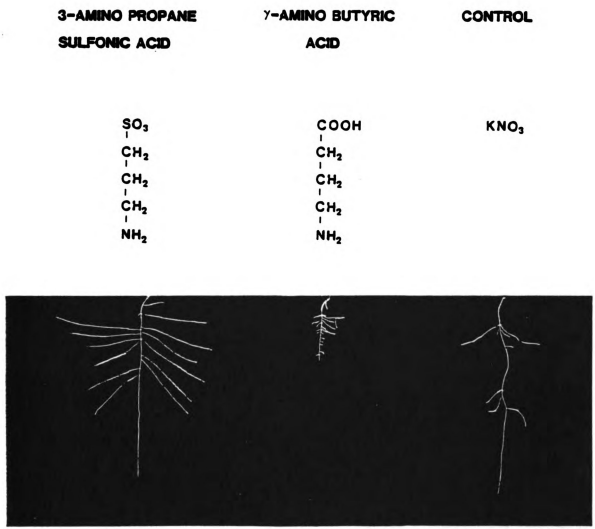

Figure 62: Comparison of GABA and 3-APSA effects on tomato seedling root morphology. Tomato seedlings were grown for one week in the indicated compound, after which their roots were spread and xerographed.

seedlings grown in GABA-Hoagland's solution responded to GABA in the same fashion as *L. esculentum* cv. VF-36 did. Therefore, the effect of GABA is not species or cultivar specific.

Compartmentalization of Labelled Chlorate in Tomato Roots.

Prior to measuring chlorate uptake by tomato seedlings, it was necessary to determine the time required to remove the radioactive label present in the apoplast. This was carried out by means of a compartmentalization analysis as follows.

The amount of radioactivity remaining in the root tissues of *L. esculentum* cv. VF-36 after each wash, obtained from ^{36}Cl-chlorate efflux data during the "unloading" period (see MATERIALS AND METHODS) was plotted as a function of time (Figure 63). The initial loss of radioactivity from tissues was rapid, but declined to a slower, constant rate after approximately one hour. This loss is presumed to be from the vacuolar compartment (Hodges, 1973) and by extrapolation to $t=0$, the amount of radioisotope in the vacuole at the beginning of the "unloading" period was estimated (52.3 nmoles / g f wt.). The half-time ($t_{1/2}=0.693/k$) for loss of radioisotope from the vacuolar compartment was determined from the slope of the linear component [slope = $-K/2.303$ where K = first-order rate constant in the equation $\log A = (-K/2.303)t + \log A_0$. A_0 = initial amount of the radioisotope]. Eight hours is required to replace half of the $^{36}ClO_3^-$ ions in the vacuoles of these tomato root cells.

The amount of radioisotope remaining in the vacuoles at any time was directly determined from the extrapolated line. These values were then subtracted from the total radioactivity in the tissue at the various times. These remainders were plotted as a function of time. The loss of radioisotope

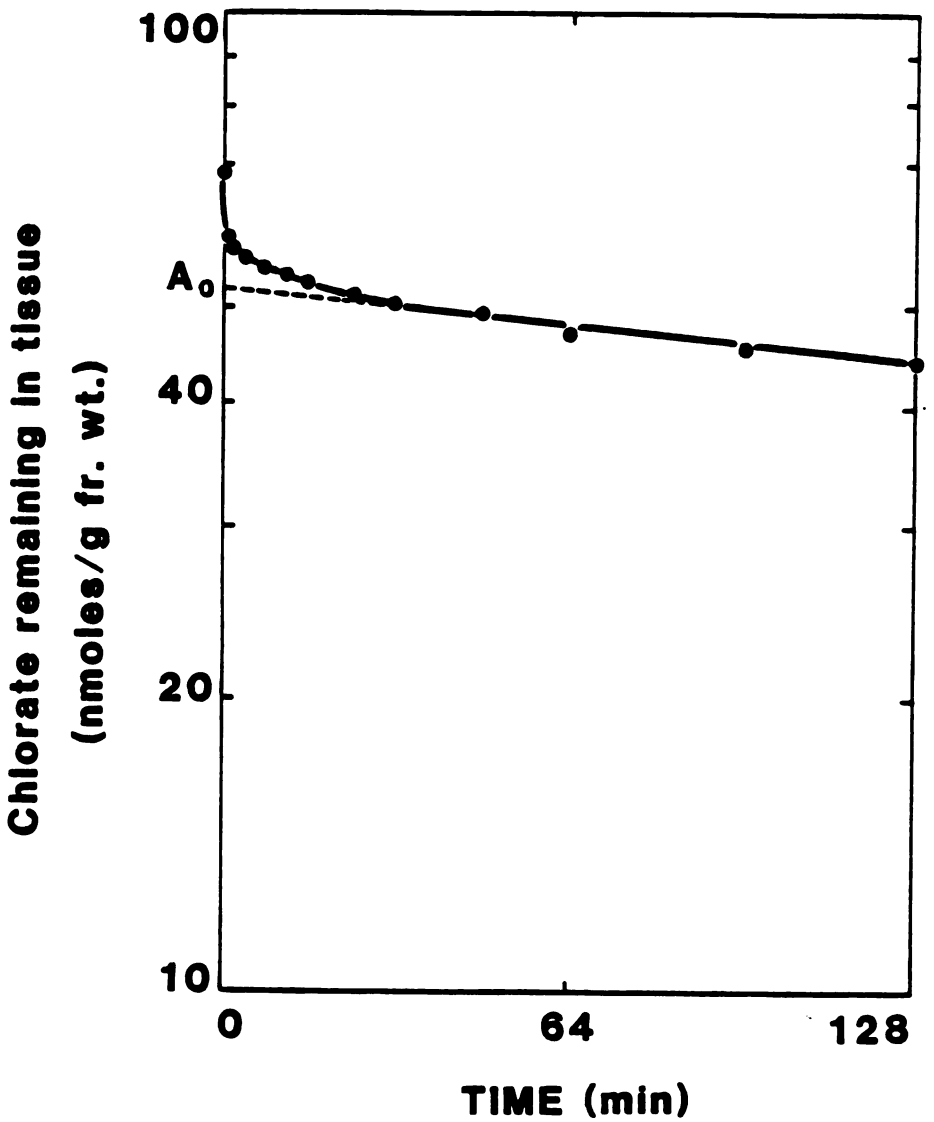

Figure 63: Compartmentalization analysis of chlorate in tomato seedling roots: vacuole compartment. Intersection of back-extrapolation of curve to ordinate (dashed line) yields chlorate content of vacuolar compartment at end of "loading" period.

from the tissue was at first rapid, then declined to a slower, constant rate, as in vacuolar unloading, described above(Figure 64). This linear component represents the loss from the second largest cell compartment, the cytoplasm (Hodges, 1973). The rate constant for chlorate loss from the cytoplasm was determined from the slope. The half-time for radioisotope loss from the cytoplasm was calculated as described above. 9.4 minutes was required to replace half of the ions in the cytoplasm of these tomato root cells.

By subtracting the radioactivity lost from the cytoplasm from the radioactivity remaining in the tissue, the loss of radioisotope from two additional compartments was distinguished, these being the cell walls and surface film (Figure 65). In this way, the time required to remove the chlorate ions from the cell wall interstitial spaces was estimatedto be 12 minutes. Consequently, a rinsing period of 12 minutes was chosen to remove the extracellular ions from the tomato seedlings used for the chlorate uptake experiments described below.

Chlorate Uptake by Tomato Roots. Effect of Nitrate.

Chlorate uptake by roots of *L. esculentum* cv. VF-36 seedlings growing on glutamine-Hoagland's solution was measured in the absence and in the presence of nitrate. Glutamine was chosen as the nitrogen source for the growth of the seedlings prior to and during the experimental incubation time. However, since interactions of glutamine with nitrate metabolism have been reported (Stewart and Rhodes, 1977), it is possible that glutamine also interferes with nitrate and/or chlorate uptake into root cells. For that reason, chlorate uptake was also measured in *L. esculentum* cv. VF-36 seedlings grown on GABA-Hoagland's solution to make possible an estimate of the extent to which glutamine influences the uptake of chlorate.

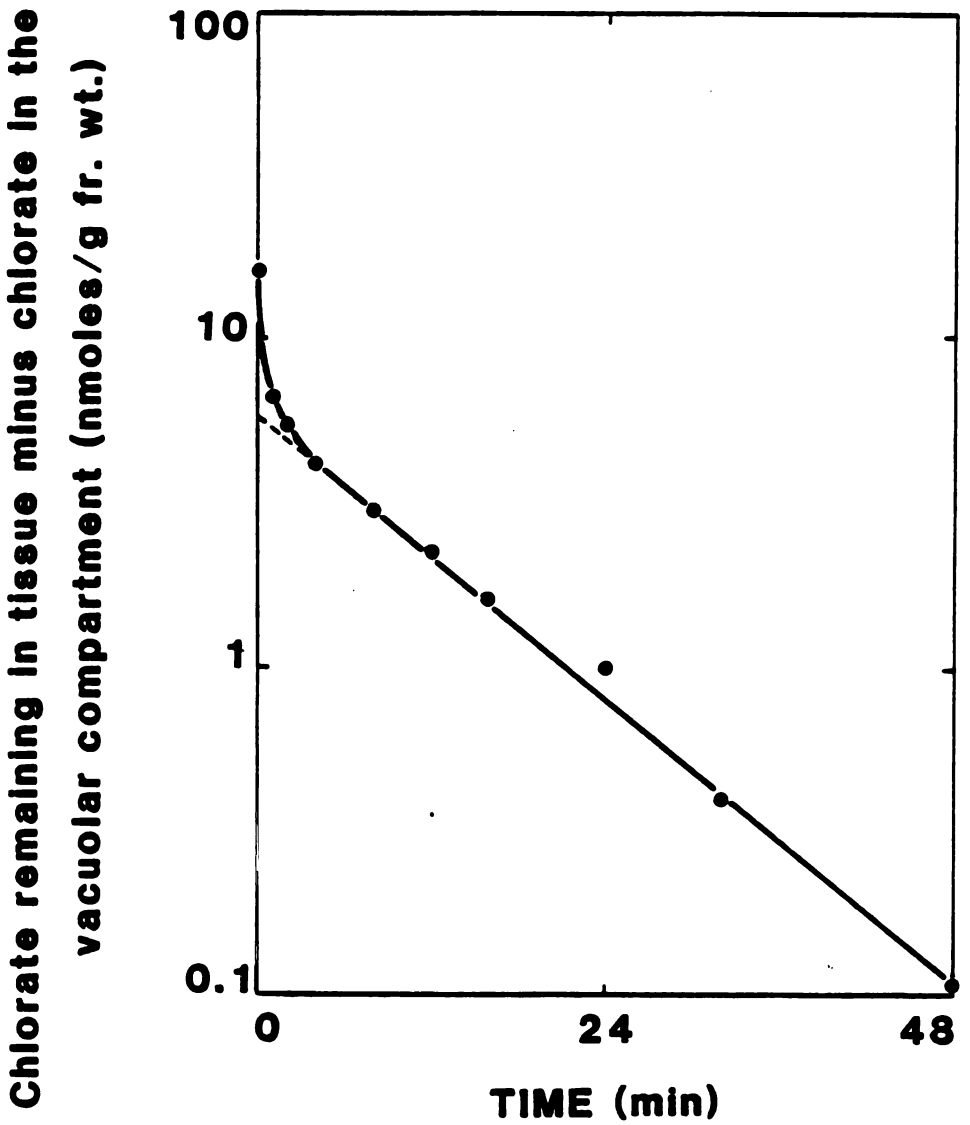

Figure 64: Compartmentalization analysis of chlorate in tomato seedling roots: cytoplasmic compartment. Curve was generated from data plotted in Figure 63, as described in the text. Intersection of back extrapolation of curve to ordinate (dashed line) yields chlorate content of cytoplasmic compartment at end of "unloading" period.

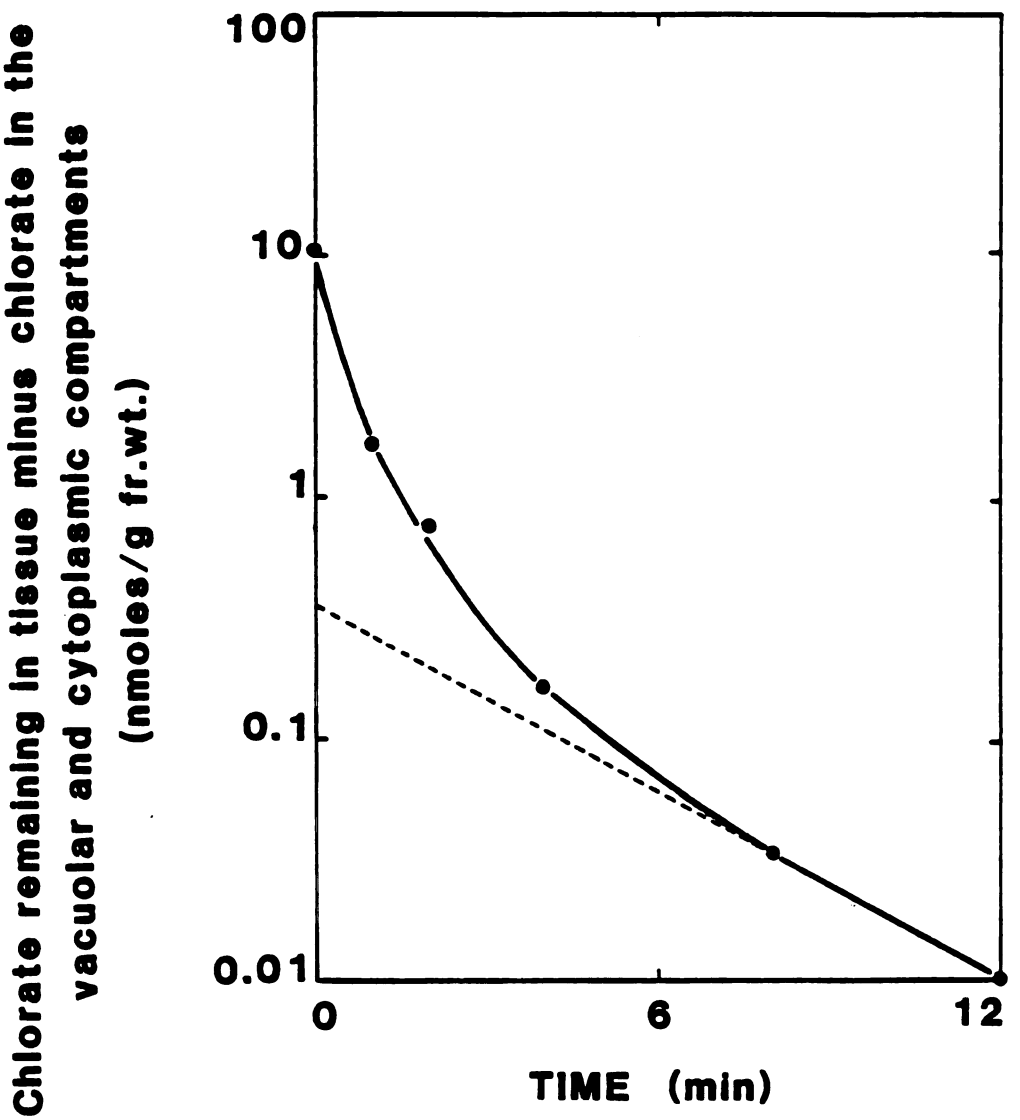

Figure 65: Compartmentalization analysis of chlorate in tomato seedling roots: apoplast compartment. Curve was generated from data plotted in Figure 64, as described in the text. Intersection of back extrapolation of curve to ordinate (dashed line) yields chlorate content of apoplast compartment at end of "unloading" period.

$KClO_3$ and tracer amounts of ^{36}Cl-chlorate were added to nutrient solutions to give concentrations and specific activities ranging from 0.2 to 6.0 mM and 176,860 to 8958 cpm/μmole, respectively. To study the effect of nitrate on chlorate uptake, two sets of parallel cultures were supplied with KNO_3, to give final concentrations of 5 and 15 mM.

The results shown in Figures 66 and 67 suggest that chlorate uptake from solutions containg glutamine was slightly less than that from solutions containing GABA, although the difference could be attributed to experimental error. The kinetics of chlorate uptake, i.e., an unsaturable rate of uptake at increasing chlorate concentrations, resemble those obtained with the tobacco cells (see Figure 17). Nitrate inhibition of chlorate uptake into the root cells was similar to that observed with the tobacco cells, especially at 15 mM KNO_3 (see Figure 19).

Figure 66: Chlorate uptake by tomato seedlings in the presence of GABA or glutamine. GABA (■) and glutamine (●) were supplied at 3 mM for 3 weeks prior to addition of $K^{36}ClO_3$. Total ^{36}Cl accumulated by the seedlings 24 hours later was determined by scintillation counting.

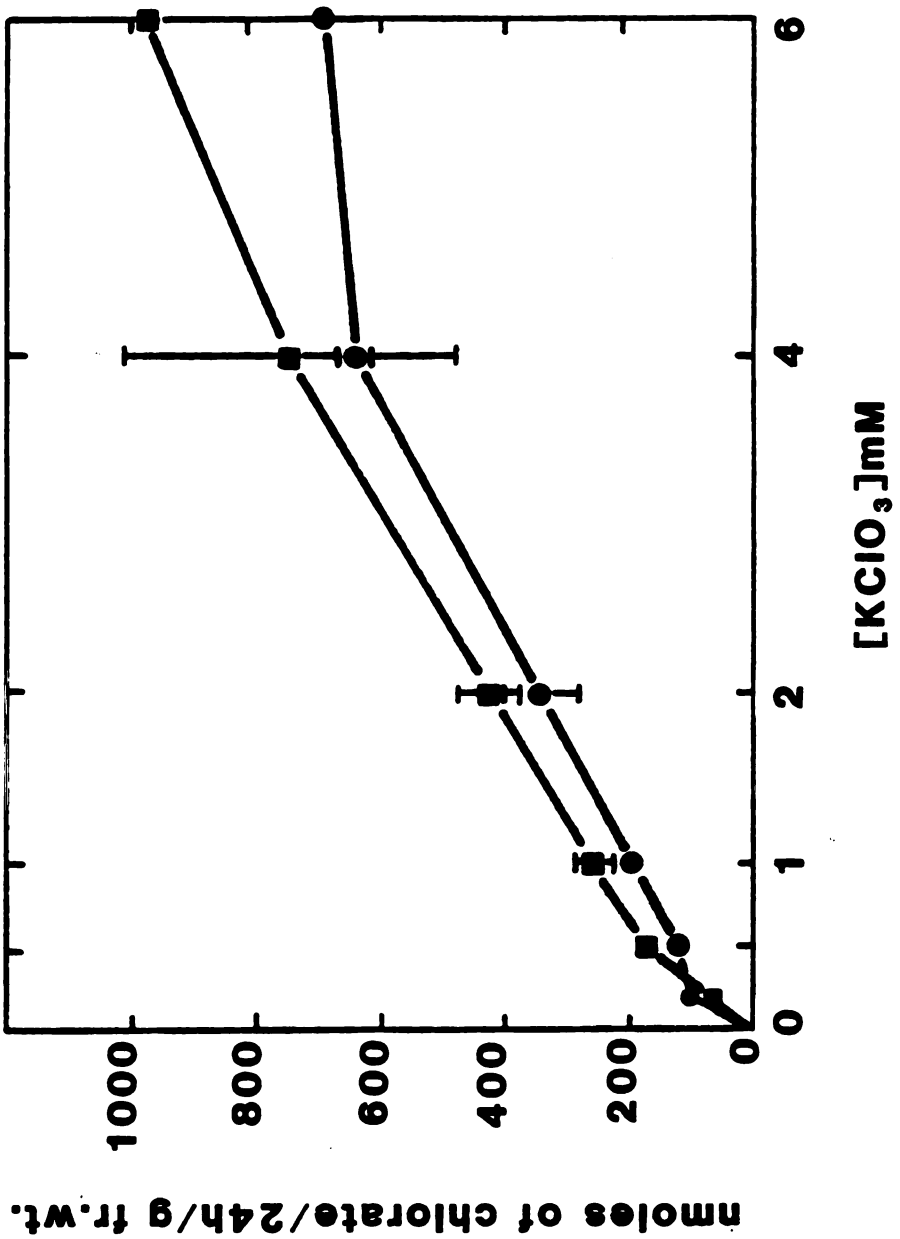

Figure 66: Chlorate uptake by tomato seedlings in the presence of GABA or glutamine.

Figure 67: Effect of nitrate on chlorate uptake in tomato seedlings. Three week old tomato seedlings, grown in 0 (●), 5 (■) and 10 (▲) mM KNO_3, were treated with the indicated $^{36}ClO_3^-$ concentrations for 24 hours. Total accumulation of ^{36}Cl was determined by scintillation counting.

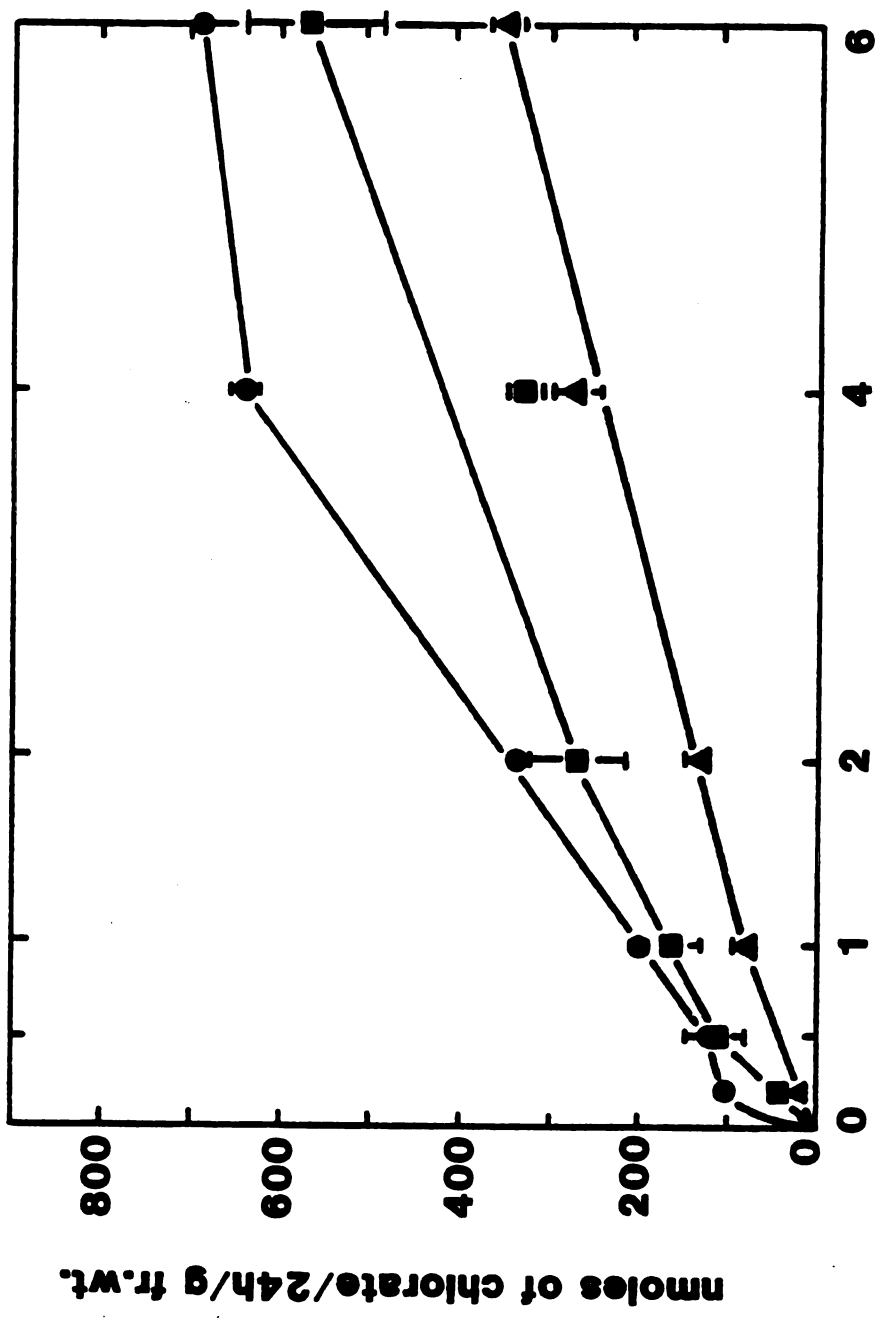

Figure 67: Effect of nitrate on chlorate uptake in tomato seedlings.

DISCUSSION

A necessary prerequisite to any attempt at improving plants' ability to scavenge nitrate from soils is an understanding of the nature and regulation of nitrate uptake. These processes in both prokaryotes and eukaryotes, are at present incompletely understood. In addition to the inherent complexity of nitrate absorption, the absence of a relatively long-lived radioisotope of nitrogen is the major reason for the lack of understanding of such an important process.

Chlorate has been shown to behave as an analogue of nitrate in the nitrate reductase reaction (Hofstra, 1979; Pichinoty, 1969; Rhodes and Filner, 1979; Solomonson and Vennesland, 1972). However, conclusive data is lacking concerning the role of chlorate as a competitor with nitrate at the level of absorption. Were it demonstrable that chlorate functions as an analogue of nitrate in the uptake process, radioactive ^{36}Cl-chlorate could be used for more precise and extensive studies of nitrate transport than were heretofore possible. Furthermore, if nitrate uptake were rate-limiting for growth, then it might be possible to use chlorate to screen plants for genetic material with superior nitrate transport properties.

^{36}Cl-chlorate is not commercially available, but was synthesized by electrolysis of $H^{36}Cl$ under alkaline conditions following the procedure used by Trombolla (1970). Approximately 30% of the $H^{36}Cl$ was converted to chlorate. The product had a purity of better than 99%. A report has since appeared which indicates that recovery could have been doubled with an alternate electrode design and lower electrolysis temperature (Deane-

Drummond, 1981). We achieved greatest purity of the synthesized ^{36}Cl-chlorate when thin layer chromatograms, on which the products of electrolysis had been separated, were visualized by autoradiography. Chlorate and perchlorate have Rfs of 0.73 and 0.54, respectively. Irregularities in the migration of these bands would have made it very difficult to clearly separate the chlorate from the perchlorate fractions simply on the basis of Rf.

I examined the characteristics of chlorate uptake and the interactions of nitrate and chlorate uptake in tobacco cells. Chlorate uptake from urea-M1D medium had a sharp pH optimum at 5.5, the rate at pH 6.5 being one-fourth that at pH 5.5 (Figure 16). This ratio changed slightly with chlorate concentration (Figure 17). Tobacco cells concentrated chlorate against a gradient at pH 5.5 but not at pH 6.5. After 20 hours of uptake, cells incubated at pH 5.5 accumulated 5 times more chlorate (48 nmoles / g fr. wt.) than those incubated at pH 6.5 (9.4 nmoles / g fr. wt.), when the chlorate concentration of the external medium was 15 nmoles / ml (Figure 16). Measurements of chlorate accumulation after 20 hours could be misleading since ^{36}Cl-radioactivity reflects both chlorate accumulation and metabolism (chlorate is reduced to $^{36}Cl^-$ *in vivo* as discussed below). However, chromatography of cell homogenates showed that only 20% of the chlorate taken up was metabolized (M. Guy, personal communication). Accumulation of ^{36}Cl radioactivity is therefore a useful way to measure chlorate transport. Another indication that active transport of chlorate takes place only near pH 5.5 comes from the observation that the accumulation ratio (chlorate accumulated / amount of chlorate in the medium at different chlorate concentrations) at pH 5.5 was greater than 1, but remained below 1 at pH 6.5 (Figure 18). This suggests that protons could conceiveably play a role in the active process or cotransport of chlorate at pH 5.5. Ullrich and Novasky (1981), from data obtained with

nitrate-induced *Lemna gibba* cells, suggested that an H^+ / NO_3^- cotransport mechanism for nitrate uptake. Also, Thibond and Grignon (1981) suggested that the mechanism of nitrate uptake in corn roots is a $2NO_3^- / 1OH^-$ antiport. Further evidence that chlorate is taken up by an active transport system comes from the inhibitory effect of inhibitors of ATP synthesis, ATPase activity and proton conductors such as arsenate, diethylstilbestrol, azide and CCCP (M. Guy, personal communication).

Uptake of chlorate by tobacco cells (14U) from urea-M1D medium was not induced or activated by the substrate chlorate (Figure 16). However, when chlorate accumulation was measured in XD cells preincubated for 30 minutes in N-less M1D medium, a lag phase of 2 to 3 hours was observed (Figures 5, 8, and 15). This pattern initially was thought to indicate that the chlorate uptake system is inducible. However, the lag in chlorate absorption during the first 2 hours was later found to be a consequence of the vigorous harvesting procedure employed (vacuum filtration). Thom *et al.* (1981), in studies of amino acid transport into XD cells, reported that arginine uptake displayed a 2-hour lag after harvesting by vacuum filtration, whereas gentle (gravity) filtration resulted in no appreciable lag. Indeed, the lag in chlorate uptake was absent in cells harvested by gentle gravity filtration. This was shown with 14U cells rather than XD cells because the 14U variant of the XD cell line was used in the later stages of this study. 14U cells had the advantage that they grew equally well in nitrate or urea, whereas XD cells grew about 20% as rapidly on urea as on nitrate. In all other respects, the XD and 14U cells are virtually indistinguishable.

Heimer and Filner (1971) showed that, in contrast to the non-inducibility of chlorate uptake by chlorate, nitrate induces or activates an active nitrate uptake system in XD cells. Induction was characterized by a 3

hour lag when nitrate was added to XD cells growing in urea. Induction by nitrate of the nitrate uptake system has been demonstrated in many other plants (Jackson, 1978).

The rate of chlorate uptake by the 14U cells increased with increasing chlorate concentrations (Figures 17, 19, 20, 21 and 22). The complex uptake kinetics indicated the existence of two systems of chlorate uptake: a saturable component functioning at concentrations below 1 mM and a linear one which accounts for rate increases at concentrations above 1 mM. The linear component of the curve could be the result of either diffusion of chlorate into the cells such that the rate would be proportional to the external chlorate concentration, or a second potentially saturable transport system with low affinity for chlorate.

The kinetics of chlorate uptake measured in the presence of nitrate allowed us to distinguish between these two kinetic components. The saturable components of the curves shown in Figure 17 were absent when the experiment was repeated with the addition of 15 mM nitrate (Figures 19 and 20). This result suggests that 15 mM nitrate completely inhibited the saturable component of chlorate transport at chlorate concentrations below 1 mM (the high affinity system) but did not inhibit transport at high chlorate concentrations (low affinity system or diffusion) as evidenced by the parallel lines above 1 mM chlorate (Figures 19 and 20). Subtraction of the low affinity or diffusion component from the v *versus* [S] curves in Figures 19 and 20 revealed the saturation kinetics of the high affinity system (insets in Figures 19 and 20).

The nature of the linear component of chlorate uptake was examined (Figure 21). Chloride inhibited chlorate uptake at high chlorate concentrations and this inhibition cannot be accounted for by a diffusion

process. Therefore, the linear component of chlorate uptake appears to be an uptake system with low affinity for chlorate.

The apparent Km values for chlorate uptake by the high affinity system were 0.33 mM at pH 5.5 and 0.29 at pH 6.5. The apparent Km values for chlorate uptake by the low affinity system were greater than 5 mM at both pH 5.5 and 6.5 (Table 4).

Heimer and Filner (1971) reported that nitrate uptake by the XD cells is also concentration dependent but with saturation kinetics in a nitrate concentration range from 0 to 30 mM. The apparent Km value was 0.4 mM and the Vmax was 2 to 5 μmoles nitrate / h / g fr. wt. at pH 6.2. In agreement with our data, no linear component of nitrate uptake was observed at high substrate concentrations.

The high affinity chlorate uptake system was subject to nitrate inhibition at pHs 5.5 and 6.5 in 14U cells, and at pH 6.2 in XD cells (Figures 8 and 9). The kinetics of the latter inhibition were of the competitive type (Figure 9) with a K_i value of 0.1 mM (Figure 10). However, when the effect of chlorate on nitrate transport was evaluated in XD cells, only slight inhibition was apparent (Figures 11 and 12). This suggests that nitrate is taken up by a different system than the high and low affinity uptake systems described above for chlorate transport. Also, if this particular nitrate uptake system were the inducible one (Heimer and Filner, 1971), the discrepancy between the inducibility and non-inducibility of nitrate and chlorate uptake, respectively, could be resolved.

Evidence for the existence of two nitrate uptake systems, one inducible and chlorate insensitive, the other constitutive and chlorate sensitive, was gained in the following experiment. During the first two hours following transfer of urea-grown cells to a medium containing nitrate and chlorate, the

activity of the inducible nitrate uptake system is low enough to unmask the effect of chlorate on a constitutive nitrate uptake system. Indeed, chlorate inhibition of nitrate uptake was observed during this period (Figure 13). One interpretation of this result is the existence of a constitutive chlorate-sensitive nitrate uptake system. Heimer and Filner (1971) were unable to detect a constitutive nitrate uptake system in XD cells grown in urea-M1D. However, the low sensitivity of the mass spectrometric methods used for their measurements may have precluded detection of the low uptake levels indicated by the present data.

KCl inhibited the low affinity chlorate transport system (Figures 21,22,23). This inhibition was due to the chloride ion rather than to the counter ion, K^+, since no difference was observed in the degree of inhibition caused by the K^+ and Na^+ salts of chloride at either pH 5.5 or 6.5 (Figure 24).

The characteristics of chloride uptake were examined to define better the relationship between the chlorate and chloride uptake systems. Chloride transport was relatively insensitive to pH (Figure 28). This has also been observed in *Chara* cells by Keiger *et al.* (1982). In contrast to that of chlorate, chloride transport at pHs 5.5 and 6.5 displayed saturation kinetics (Figure 28). However, the double reciprocal plots (Figure 29) for chloride transport suggest the existence of both high and low affinity uptake systems. Chlorate inhibited only the low affinity chloride uptake system (Figure 29). The apparent Km values for chloride uptake by the high affinity system were 0.15 mM at pH 5.5 and 0.10 mM at pH 6.5. The apparent Km values for chloride uptake by the low affinity system were 0.27 mM at pH 5.5 and 0.21 mM at pH 6.5.

The effect of perchlorate on chlorate uptake was also evaluated and the results suggested that perchlorate, like nitrate, interacts with the high affinity component of chlorate uptake (Figure 25).

Ambient ammonium has been shown to inhibit the absorption of nitrate in many systems (Jackson, 1978), including XD tobacco cells (Heimer and Filner, 1971). However, ammonium does not inhibit chlorate accumulation by the XD cells, but rather seems to stimulate chlorate uptake (Figure 15). It is well documented (Ohta *et al.*, 1981; Raven and Smith, 1976) that ammonium uptake by cultured plant cells or roots brings about an increase in the acidity of the medium. As discussed above, the optimum pH for chlorate uptake is 5.5. The medium (M1D) used for evaluating the effect of ammonium on chlorate uptake was initially titrated to pH 6.2. Therefore, a decrease in the pH of the medium brought about by assimilation of ammonium into the cells could explain the enhancement of chlorate uptake. However, succinic acid was always supplied to the experimental solutions along with ammonium. This anion is a hydrogen ion buffer in this pH range and should have tended to maintain pH despite the production of protons by ammonium metabolism (Ohta *et al.*, 1981). It remains possible that the buffering capacity of 1 mM succinic acid was insufficient to maintain a pH of 6.2.

Cycloheximide inhibited chlorate uptake very rapidly upon its addition to the experimental solution (Figure 15). This high cycloheximide sensitivity indicates that the carrier for chlorate may have a high turnover rate. This is unexpected since the chlorate uptake system is constitutive. However, cycloheximide has been shown to have other effects besides inhibition of protein synthesis and could have inhibited the uptake system directly. Cycloheximide also very rapidly inhibited nitrate uptake into XD cells (Heimer and Filner 1971).

These accumulated data on chlorate transport suggest the model shown in Figure 68. This model indicates that the hypothesis of chlorate functioning as an analogue of nitrate in the process of uptake is only partially correct for

tobacco cells grown under the conditions used in this study. The uptake of these anions was found to be more complex than expected. It should be noted that the data used in the kinetic studies were the result of cellular chlorate accumulation by the end of a 24 hour period. These data do not give initial rates of uptake, but rather the net result of ion movement from the external medium into the cytoplasm and from this compartment both into the vacuole and out of the cell. Because of this compartmental complexity it remains to be determined whether or not systems I, II, and III (Figure 68) are independent proteins or carriers located at the plasmalemma, or elsewhere.

Nitrate uptake rates have been reported to be higher in nitrogen-starved than in nitrogen-fed plants (Jackson *et al.*, 1976a,b). I examined the effect of preexisting nitrogen nutritional status on the sensitivity of the XD cells' chlorate uptake system. The apparent Km for chlorate uptake decreased from 2.25 mM to 0.65 mM following 4 hours of N-starvation (Table 2). This apparent increase in affinity of the chlorate uptake system for chlorate with time in N-less medium suggests that nitrogen starvation may derepress or activate the chlorate transport system. Alternatively, the greater affinity demonstrated by the transport system after 2 or 4 hours may simply reflect recovery from harvesting injury as discussed above. Finally, nitrate leaking out of the cells may compete with chlorate for a shared uptake system, resulting in the initially higher apparent Km values.

Parallel experiments carried out to determine the effect of nitrogen starvation on nitrate uptake revealed no changes in the affinity of the nitrate uptake system for nitrate, the apparent Km for nitrate uptake remaining constant, around 0.1 mM (Figure 7 and Table 3). This result could reflect a true difference between the chlorate and nitrate uptake systems.

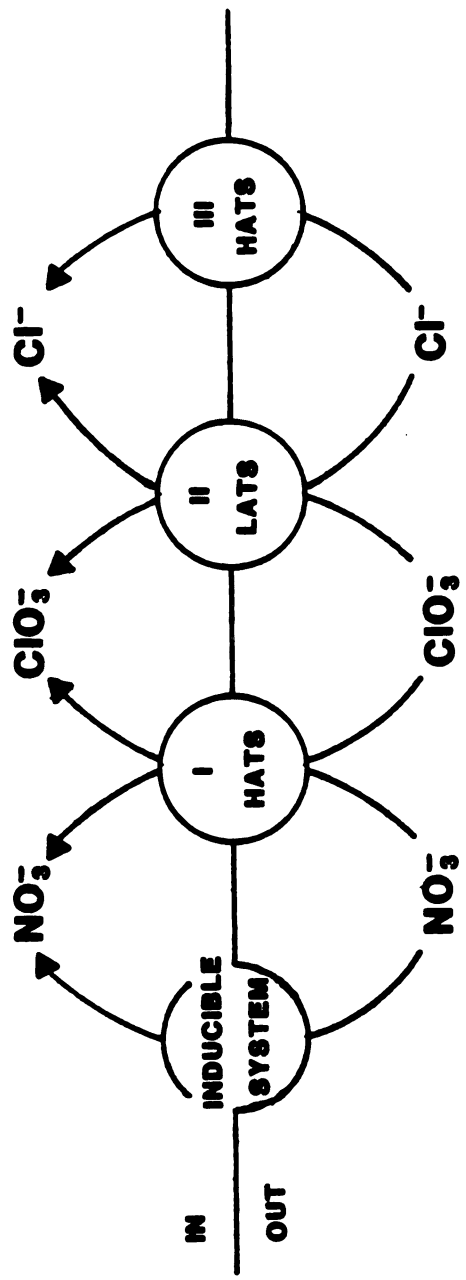

Figure 68: Model for interactions between ClO_3^-, NO_3^- and Cl^- transport systems.

According to Åberg's hypothesis (1947), chlorate is rendered toxic upon its reduction to chlorite by the enzyme nitrate reductase (NR). While competition between chlorate and nitrate in the nitrate reductase reaction has been demonstrated in many organisms, the actual mechanism of chlorate action has not been completely established.

The XD and 14U tobacco cell lines provided a suitable system in which to examine details of the mechanism of chlorate toxicity proposed by Åberg. According to the hypothesis, XD cells growing on a medium with nitrate (M1D) as the sole nitrogen source and containing high (induced) levels of nitrate reductase, should be more sensitive to chlorate than the 14U cells growing on a medium with urea as the sole nitrogen source and containing much lower levels of nitrate reductase. The effect of chlorate on the growth response of XD and 14U cells grown in M1D and urea-M1D, respectively, was examined. The dose-response curves yielded I_{50} values of 13 mM and 42 mM for the XD and 14U cell lines, respectively, supporting the proposed hypothesis (Figure 43).

Using ^{36}Cl-chlorate, we examined chlorate reduction and the kinetics of nitrate and chlorate competition at the enzyme nitrate reductase in tobacco cells. Chlorate was reduced by NR from XD cells' crude enzyme preparations (Figure 32). However, no ^{36}Cl-chlorite was found as a product of the reaction. All of the radioactivity was recovered as $^{36}Cl^-$ (Table 6). This explains the failure of previous attempts to apply a colorimetric assay for chlorite formation to the XD tobacco system. Chlorite formation upon reduction of chlorate by another source of NR (soybean nodule bacteroids) was detectable (Table 8). This suggests that the amount of chlorite accumulated upon reduction of chlorate by XD cells' crude NR enzyme was too small to be detected in this assay. Presumably, XD cells have a very active enzyme which

reduces ClO_2^- to Cl^-. Such an activity would both prevent ClO_2^- from accumulating and perhaps thereby render ClO_3^- less toxic. Indeed, XD cells can grow in the presence of 30 mM chlorate (Figure 31).

Evidence that NR is directly involved in the reduction of chlorate came from assays of desalted enzyme extracts of XD cells grown on urea-M1D. These cells had only barely detectable NR activity (46 pmoles / min / g fr. wt.) and undetectable "chlorate reductase" activity (Table 9).

The kinetics of chlorate and nitrate reduction with increasing chlorate or nitrate concentrations indicated that tobacco nitrate reductase has a higher affinity for nitrate (Km values ranged from 0.18 to 0.28 mM) than for chlorate (Km values ranged from 2.3 to 6 mM) (Table 11). These values are similar to those reported for tomato plants, green algae and several bacteria (Table 12).

Chlorate was found to inhibit nitrate reduction competitively, with a K_i of 5 to 8.9 mM (Table 11, Figure 36). These values are comparable to the Km values (2.3 to 6 mM) obtained for chlorate reduction, as expected for a competitive inhibitor. Nitrate competitively inhibited chlorate reduction. The K_i values obtained for inhibition of chlorate reduction by nitrate were between 0.13 and 0.20 mM (Table 11, Figure 37), once again very close to the Km values for reduction of nitrate (Table 11).

Chlorate is not a gratuitous inducer of nitrate reductase in tobacco cells. The barely detectable NR actitvity of 14U cells grown in urea did not increase when ClO_3^- was added to the growth medium for a period of 8 hours (Table 13) or several days (Table 14). Addition of nitrate increased NR activity in both cases (Tables 13 and 14). These results are congruent with those of Hofstra (1977) and Hackenthal *et al.* (1965). In their studies, chlorate did not induce NR activity in tomato leaf discs or in *Bacillus cereus*, respectively.

Addition of nitrate to the urea-M1D medium results in an induction of

NR activity in 14U cells. According to Åberg's hypothesis, the cells' chlorate sensitivity should increase concomitantly. Chlorate toxicity was therefore evaluated in 14U cells grown with and without nitrate. Unexpectedly, there was little difference between the chlorate sensitivities of 14U cells grown under these two conditions (Figures 41 and 42). However, in light of the finding that no ClO_2^- could be detected in extracts or cells with NR exposed to ClO_3^-. perhaps a difference in toxicity with or without NO_3^- should not be expected.

Several miscellaneous aspects of the cellular chlorate response observed during the course of this study are worth noting. Cells could eventually recover from initial growth inhibition at high chlorate concentrations (30 to 100 mM) (Figures 30A and 30B). The larger the initial inoculum size the less sensitive the cells were to chlorate (Figures 31 and 43). Low $KClO_3$ concentrations seemed to stimulate culture growth in several instances (Figures 41 and 43). This growth stimulation by chlorate has also been reported for cultures of *Datura innoxia* (King and Khann, 1980). Doddema and Telkamp (1979) reported enhanced uptake of nitrate by *Arabidopsis* plants in the presence of very low concentrations of chlorate.

Tomato plants were used to evaluate the nitrate-chlorate interactions at the whole plant level. To avoid unwanted interactions with the nitrate and chlorate uptake systems, alternative nitrogen sources to NO_3^- and NH_4^+ were initially sought. We found that cells could eventually adapt to growth on urea or arginine, though relatively poor growth was observed on the latter (Table 15). No developmental differences were found in tomato's growth response to nitrate, ammonium, urea or arginine (Tables 15 and 22). Both seedlings and cultured cells showed the following descending order of preference for nitrogen

source:

Nitrate > Ammonium Succinate > Urea > Arginine

I proceeded to evaluate the growth response of tomato seedlings to several other nitrogen sources including glutamine, γ-amino butyric acid (GABA), asparagine, hypoxanthine, xanthine, uric acid and allantoin. Seedling growth and development was extremely sensitive to the source of nitrogen. The size (Table 23) and timing of primary leaf expansion was somewhat affected by these nitrogen sources, but more dramatic changes were observed in root morphology and development (Figures 57, 58 and Table 24). Treatments which resulted in larger root systems (e.g., allantoin and hypoxanthine (Table 24)) had the opposite effect on stem and leaf size and development (Table 23). It was also observed that tomato seedlings developed extensive root systems in the absence of a nitrogen source, while growth of the aerial plant parts was retarded by the lack of nitrogen (Table 25). This finding concurs with the observation by Jackson *et al.* (1976b) that root surface area increases with suboptimal nitrate nutritional conditions. Such a response would enable a plant to scavenge nitrogen more efficiently from a nitrogen-poor soil.

GABA had a striking effect on root morphology and root hair development (Figures 59 and 60). The growth of the main and lateral roots was inhibited when tomato seedlings were grown on GABA. Both main and lateral roots of GABA-treated plants developed root hairs in a periodic pattern in which portions of the epidermis had no root hairs whereas the intervening regions developed long and numerous root hairs. The regions of the GABA-treated roots where root hairs appeared had a greater diameter than the adjacent hairless portion. In contrast, the distribution of root hairs of seedlings grown on nitrate was uniform, with a progressively smaller root diameter toward the apices of the main and lateral roots.

This effect was specific for GABA, since none of the other nitrogen sources tested elicited such a response. The GABA effect was "dominant", in that seedlings grown on solutions containing mixtures of GABA and other nitrogen compounds showed root hair growth periodicity. No GABA analogue tested elicited the GABA effect (Figure 61). All inhibited growth to differing degrees, with the exception of 3-amino propane sulfonic acid, which stimulated root growth when compared with the nitrate control (Figures 61 and 62). The GABA effect was light dependent. Roots of etiolated GABA-treated seedlings did not develop lateral roots and no periodicity was evident in the main root. Finally, the GABA effect was not species or cultivar specific. The root system of *L. esculentum* cv. Floridale, *L. esculentum* cv. VF-36 and *L. pennellii* seedlings all responded to GABA with the characteristic root hair periodicity.

The response of the tomato seedlings to GABA is of interest because little is known about the role or function of this compound in higher plants. It has been reported that GABA accumulates in plant roots under anaerobiosis (Wickremasinghe and Swain, 1965). Selman and Cooper (1978) found GABA to be distributed throughout tomato plant parts and postulated that its role was that of a nitrogen storage compound.

The results obtained with tobacco cells in the first part of this study indicated that chlorate is not an ideal analogue of nitrate for transport studies. In contrast, Deane-Drummond and Glass (1982) have reported that chlorate is an excellent analogue of nitrate for studying nitrate uptake by intact barley roots. I used tomato plants to determine whether whole plants show nitrate-chlorate responses similar to those of cultured cells. The I_{50} for chlorate inhibition of tomato seedling growth was 0.62 mM. Seedlings treated with 3 mM ClO_3^- showed a antidotal response to NO_3^- in the 7.5 to 37.5 mM NO_3^-

range. At the highest NO_3^- concentration tested, 3 mM ClO_3^- still inhibited growth by 17%.

The uptake of chlorate by roots of *L. esculentum* cv. VF-36 seedlings growing on glutamine-Hoagland's solution was measured in the presence and absence of nitrate. The kinetics of chlorate uptake (Figures 66 and 67) resembled those obtained with tobacco cells (Figure 15). Nitrate inhibited chlorate uptake into roots in a fashion similar to that observed with tobacco cells *in vitro*, in that 15 mM KNO_3 reduced chlorate uptake to a linear, non-saturable response (Figure 67). However, further experiments would be required to characterize this interaction to the extent described above in the *in vitro* tobacco cell system.

Chlorate was much more toxic to seedlings of *L. esculentum* cv. VF-36 grown in Hoagland's solution (I_{50} = 620 μM, Figure 53) than to cells of the same cultivar, grown *in vitro* on ammonium succinate T_{12} (I_{50} = 10.5 mM, Figure 51) or to tobacco cells cultured on M1D (I_{50} = 13 mM, Figure 41) or the latter grown on urea-M1D (I_{50} = 40 mM, Figure 43). Seedlings may concentrate chlorate in their aerial portions via transpiration, thus accounting for their enhanced sensitivity.

Hofstra (1977) reported that tomato plants were insensitive to chlorate when grown on ammonium as the sole source of nitrogen. This finding agrees with the predictions of Åberg's hypothesis of chlorate becoming toxic only after its reduction to chlorite by the enzyme nitrate reductase. However, we found that alternative nitrogen sources such as ammonium succinate, urea, GABA, glutamine, and arginine increased the toxicity of chlorate over that observed when seedlings were grown on nitrate (Tables 20 and 22, Figure 56). Doddema and Telkamp (1979) also reported this unexpected phenomenon in studies using *Arabidopsis* plants.

In addition to Cove's (1976) alternative mechanism of chlorate toxicity (see INTRODUCTION), yet another explanation for these results can be proposed, based on Åberg's hypothesis: If these young seedlings had a constitutive nitrate reductase, this activity could reduce chlorate to toxic chlorite without competition from medium nitrate. Tomato seedlings at this stage had a level of nitrate reductase activity (0.39 nmoles / min / g fr. wt.) two orders of magnitude below the full induced activity of cultured XD tobacco cells (see PART II). It is unlikely that such a low activity could generate cytotoxic levels of chlorite, considering the low affinity of NR for chlorate (Table 12). Furthermore, ammonium inhibits both nitrate reductase and nitrate transport in plants (see INTRODUCTION).

Seedlings treated with tungstate, in order to block insertion of molybdenum into NR, developed toxicity symptoms in the presence of chlorate to the same extent as untreated ones. This, and the results discussed immediately above, suggest that chlorate toxicity results from either (i) reduction of chlorate by (not readily detectable) preexisting constitutive NR, or (ii) a mechanism not involving nitrate reductase. Any model based on this second alternative would have to account for the apparent enhancement of chlorate toxicity by ammonium, GABA, urea and glutamine in tomato seedlings.

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