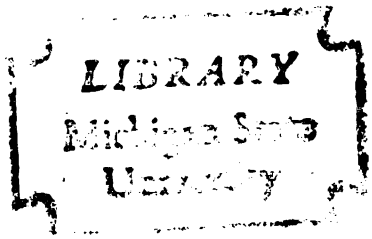
THESIS



This is to certify that the

thesis entitled

Development of Selection Strategies for the Isolation of Methionine Accumulating Cell Lines in Solanum Tuberosum L.

presented by

John Paul Hunsperger

has been accepted towards fulfillment
of the requirements for

Doctor of Philosophy degree in Crop and Soil Sciences

and

Genetics Program

Major professor

for P.S. Carlson

Date 7/12/82

O-7639

DEVELOPMENT OF SELECTION STRATEGIES FOR THE ISOLATION OF METHIONINE ACCUMULATING CELL LINES IN *SOLANUM TUBEROSUM* L.

By

John Paul Hunsperger

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Crop and Soil Science
and
Program of Genetics

1982

ABSTRACT

DEVELOPMENT OF SELECTION STRATEGIES FOR THE ISOLATION OF METHIONINE ACCUMULATING CELL LINES IN SOLANUM TUBEROSUM L.

By

John Paul Hunsperger

In vitro selection strategies were defined which are capable of identifying cell lines that accumulate methionine, the first nutritionally limiting amino acid in potato. Inhibitory levels of cysteine plus threonine, feedback regulators of homoserine dehydrogenase, were not overcome by methionine, homoserine, or homocysteine. Methionine relieved growth inhibition due to lysine plus threonine, end product inhibitors of aspartate kinase and homoserine dehydrogenase. Selenomethionine toxicity was not overcome by equimolar concentrations of methionine or precursors. Ethionine toxicity was relieved by low levels of methionine and homocysteine. Kinetic analysis of methionine-ethionine antagonism indicated competitive interaction between these compounds. Glutamyl- γ- methyl ester was established as a higher plant methionine analog by virtue of methionine- mediated reversal of growth depression. The presence of 6.8 mM glutamine conferred tolerance to ten fold higher concentrations of growth inhibiting amino acids and analogs.

Stable ethionine-resistant cell lines were isolated from a dihaploid clone at a frequency of 9×10^{-10}. Among ten ethionine tolerant clones, resistance level correlated negatively with molar percent free methionine, but positively with total free pool amino acids. However, one isolate expressed 44 % greater free methionine than its progenitor line. Stability of ethionine resistance was monitored for 450 days in a second isolate continuously cultured in the presence or absence of ethionine. Resistance decreased from high to moderate, and high level resistance was not reacquired upon reselection. Spontaneous resistance to selenomethionine in cultures of tetraploid cultivar 'Superior' occured at a frequency of 2×10^{-8}. One variant which was cross resistant to ethionine contained 2.29 times as much free pool methionine and 1.37 times as much total methionine as unselected cells.

Analog resistant clones could not be regenerated from callus cultures; however, a protocol for regenerating potato shoots from true roots was designed which involves zeatin, gibberellic acid, and abscisic acid.

ACKNOWLEDGMENT

The writer gratefully acknowledges past and current members of his guidance committee for time spent in planning an academic program, for helpful suggestions concerning the thesis project, and for thoughtful evaluation of the resulting dissertation. Special gratitude is extended to Peter Carlson for maintaining a stimulating environment for research and discussion and for encouraging exploration of research areas beyond the thesis project.

Dr. Werner Bergen is acknowledged for providing access to ion exchange facilities used in amino acid determinations.

Acknowledgment is also made to fellow students and post-doctoral fellows for thoughtful discussion and laboratory interactions and to Brenda Floyd for unfailing attention to laboratory needs.

Finally, the author expresses his sincere appreciation to Mary for her love, understanding, encouragment, and assistance during the course of these studies.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. THE POTATO AS AN EXPERIMENTAL SYSTEM FOR *IN VITRO* SELECTION OF ENHANCED METHIONINE PRODUCTION

Introduction

The potential for crop improvement through *in vitro* selection of specific traits has been much heralded in the literature (56, 80). The prospect of generating *de novo* a useful character in an agricultural crop for which an appropriate gene does not exist is very appealing. Such a character might overcome a specific defect in a crop or adapt the crop to a new environmental situation. In recent years interest in somatic cell genetics has accelerated as the number of realistically manipulatable plant species has expanded, as new applications and the means for achieving those applications are recognized, and as examples of successful *in vitro* genetic modifications accumulate.

Plant Cell *in vitro* Selection Systems

For plant cells as well as for microorganisms successful selection systems require that rare variants be easily and unambiguously distinguished from a wild type background. While physiological advantage is the usual basis for selection, other distinguishing properties such as morphology, fluorescence, or production of a colored product might be used. Most selection systems require the creation of a stress to which resistant genotypes are sought. Growth advantage is the usual criterion for assessing resistance; however,

for selection strategies based upon lethal synthesis, it is the absence of a function or a state of metabolic quiescence which confers resistance.

It is axiomatic that resistance to any stress can be selected without prior knowledge of the mechanism underlying such resistance. Selection for resistance to stresses such as sustained high or low temperature, high osmoticum, anoxia, heavy metals, pathotoxins, and many herbicides and antibiotics is performed with only a rudimentary knowledge of how a resistant cell might cope with the stress. Alternatively, an understanding of the biochemistry involved in a metabolic process can provide a basis for devising a straightforward strategy capable of selecting a particular phenotype directly. The latter approach is especially well suited to selecting for resistance to analogs of normal metabolites as well as to the products of lethal syntheses. In these instances the target molecule is frequently an enzyme. Tests for altered regulatory properties or catalytic function are direct and can often indicate the nature of a mutational change.

Manipulation of *Solanum* species *in vitro*

Until recent years the potato has not been a particularly strong candidate for tissue culture manipulations. Early reported difficulties with potato tissue cultures are primarily attributable to investigators' restricted use of cultivated varieties. Once the focus was broadened to include wild germplasm and hybrid derivatives between *S. tuberosum* and wild or less developed species, almost all of the *in vitro* techniques established for other species could be performed with one or more tuber-bearing *Solanum* clones. This technology has since been

extended to material which is taxonomically *S. tuberosum* and in certain instances to agronomically important cultivars as well.

In vitro propagation through axillary shoot proliferation is now a routine procedure for all potato cultivars (103). A refinement of this procedure has proved useful for virus elimination (93). Shoot regeneration from unorganized tissues, however, is somewhat more difficult. Shoot regeneration from tuber explants has been reported for several cultivars (47,54), and regeneration from callus cultures has been achieved with certain clones (5, 55). Experiments leading to shoot regeneration from true roots will be described in a subsequent chapter.

Considerable effort has been invested in defining conditions conducive to protoplast production and culture. Upadhya (99) first described procedures for reproducible protoplast release and proliferation. Grun (38) later succeeded in regenerating an entire plant from protoplast-derived cultures from a *S. phureja X S. chacoense* hybrid. Subsequently, shoot regeneration was described for dihaploid *S. tuberosum* clones (7, 101).

The elaborate protocols described by Shepard (82-84) now permit high frequency regeneration from protoplasts originating from commercial tetraploid cultivars. Concomitant with advances in protoplast technology, systems have been developed for somatic cell fusion. Butenko has utilized fusion between protoplasts produced from regenerative leaf mesophyll from *S. chacoense* and nonregenerative callus cell protoplasts from *S. tuberosum* as a means for rescuing the *S. tuberosum* cell line from culture (14). Potato protoplasts have also been fused with tomato protoplasts to produce plants exhibiting features of each progenitor species (64).

Interest in breeding systems utilizing dihaploid germplasm extracted from tetraploid clones has encouraged the development of anther culture procedures suitable for potatoes (29, 44, 92). Many species, including *S. tuberosum*, have been demonstrated to be capable of producing dihaploids and/or monohaploids through anther culture but response is highly genotype dependent. Indeed, heritable components conferring androgenic response in anther culture have been recombined among dihaploid *S. tuberosum* clones to produce clones with exceptionally high rates of haploid embryoid production (46).

As a consequence of the *in vitro* techniques developed for potato, selection of variant cell types has already been performed. A cell line of *S. stenotomum* which is resistant to 5-methyltryptophan, an analog of tryptophan, has been described (45). Callus and regenerated roots from this line exhibited diminished feedback sensitivity for anthranilate synthetase. A second 5-methyltryptophan cell line derived from 'Merrimac' which accumulates free tryptophan to nearly 50 times wild type levels has also been described (15). Recently, potato variants have been selected which are resistant to semi-purified pathotoxin filtrates. Matern et al. (6) report differential sensitivity among protoplast regenerates of 'Russet Burbank' to *Alternaria solani* toxins. Resistant clones have remained resistant through two tuber generations. Several 'Russet Burbank' clones of similar origin express differential sensitivity to inoculation with *Phytophthora infestans* (83). Behnke (6) has succeeded in selecting dihaploid *S. tuberosum* clones *in vitro* which are resistant to culture filtrates of *Phytophthora infestans* and *Fusarium oxysporum*. These examples demonstrate the utility of potato cultures as subjects for *in vitro* selection.

The Nutritional Value of Potato Tubers : a Problem and a Proposal

Among world food crops the potato ranks fourth in total production (96). Its principal contribution to human nutrition lies in its value as a source of calories and high quality protein. Despite widely held beliefs, the protein value of potatoes exceeds its caloric value. The recommended National Academy of Science's daily caloric requirement for adult males can be satisfied by approximately 3.3 kg of potatoes, a quantity which also supplies 115% of the daily amino acid requirement (3).

Various measures of protein quality support the potato as the highest quality source of all major food crops (96). Schuphan (79) has reported a biological value of 80 for potato. Rexen (72) has calculated essential amino acid indices, EAAI, ranging from 55 to 84 for European potato varieties. Surprisingly, clones cultivated for starch production tended to rank higher in EAAI than did clones raised for human consumption. Kaldy (48) has calculated protein scores for several potato clones. These varieties ranged in value from 60 to 78.

Amino acid analyses of whole potato tubers reveal methionine as the first limiting amino acid (48,72,75). Kaldy (48) has determined an average chemical score of 69 for potato tubers. While a score of this magnitude is exceptionally high for a food of plant origin, improvement of tuber methionine levels can well be expected to enhance utilization of the remaining amino acids. Evidence that more efficient amino acid utilization is possible derives from feeding studies with small animals. Voles fed methionine-supplemented potato diets exhibited markedly improved growth relative to nonsupplemented controls (73).

Calculated PER values of methionine supplemented diets were as much as twice those of unsupplemented controls.

Partitioning the tuber into nitrogenous fractions is useful in identifying sources of nutritional inadequacy. Kapoor et al.(50) have separated tuber proteins into albumin, globulin, prolamin, glutelin, and residual fractions and have performed amino acid analysis on each. The EAAI for all fractions except prolamin were equivalent at a value of 83. The prolamin fraction with an EAAI of 53 was identified as limiting. To focus upon improvement within the prolamin fraction is nonetheless unjustified since it represents no more than four percent of the total tuber protein. The relative contributions of each protein fraction to total protein have been assessed in *S. andigena* and *S. phureja X S. tuberosum* progeny (90). Despite a greater than two fold difference in total protein among the clones examined, the proportions of each fraction were constant. Selection based upon total protein content will therefore not improve protein quality.

The nonprotein nitrogen fraction contains a very substantial proportion of the total amino acids within a potato tuber. The free amino acid pool has been determined to contribute from 23% (24) to over 50%(13) of the total amino acids present in the tuber. It is noteworthy that the nutritional value of the nonprotein fraction is inferior to that of the protein fraction (24). The proportion of nonprotein nitrogen to protein nitrogen is relatively constant for both high and low protein clones cultured under similar conditions (89, 91).

Fertilization practice strongly influences the proportion of free to protein bound amino acids. Hoff (42) nearly doubled the free pool by increasing nitrogen application from 40 to 376 kg/ha. Under the same

conditions of fertilization, total protein increased by 36% (107). Among free amino acids the amides increased most dramatically. Free methionine was observed to increase by 40%; however, its relative proportion declined. Rexon (72) also observed amino acid increases in whole tuber digests as a result of increased nitrogen fertilization. Very significantly, he noted that not all amino acids increased in response to higher nitrogen levels. Among the stable amino acids was methionine. As a consequence, the EAAI of tubers harvested from plots receiving more fertilizer was diminished.

Variability of methionine levels among potato clones is high. Free pool methionine levels vary as much as two fold among commonly cultivated British clones (21). Protein bound methionine among progeny of several *S. phureja X S. tuberosum* families has ranged from 0.19 to 2.69 mg/g dry tuber weight, an almost 14 fold difference (24). Luescher has shown that tuber methionine concentration is a selectable trait (58). His heritability estimates of 79% to 94% indicate strong potential for a breeding approach as a means for enhancing methionine levels in potatoes.

The preceding discussion demonstrates that from a nutritional perspective, methionine limits the value of potatoes as an amino acid source. Selection for increased total protein will certainly increase the abundance of methionine, but can have only a modest effect on the relative proportion of methionine. Overall quality will therefore not be improved. It is presently not feasible to identify particular polypeptides which are high in methionine content with the objective of selecting for enhanced expression of such a gene product. Plant breeding techniques, however, can be used to concentrate genes which,

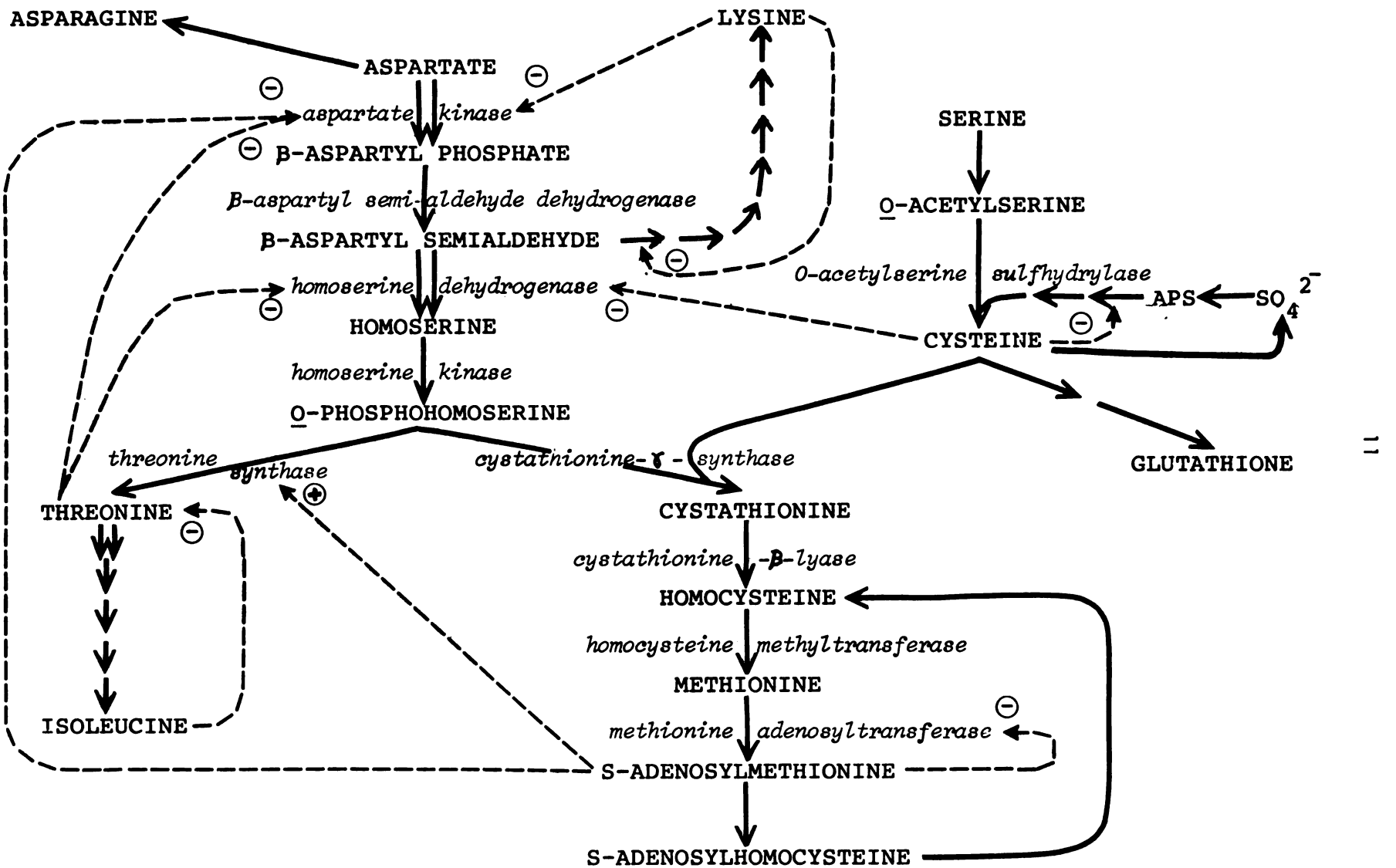
by as yet unrecognized mechanisms, enhance methionine content in some clones. Despite real improvement that might be realized, this approach is constrained by the natural occurrence of genes which contribute to methionine accumulation. These limitations have prompted an alternative proposal to directly alter cellular metabolism in such a way as to favor methionine accumulation. The structural and regulatory features of the metabolic pathways leading to methionine synthesis are now sufficiently well understood as to permit the design of *in vitro* selection systems capable of conferring a growth advantage to methionine accumulating cells. The potato tuber is already highly adapted to storing much of its amino acid content in the free pool; consequently, it should not be necessary to require processing of additional methionine into a polypeptide product. Finally, the techniques necessary to transform a selected cell into a whole plant have now been worked out for several *S. tuberosum* clones. For these reasons a program with the objective of selecting potato cells which accumulate methionine by a deregulated synthetic mechanism was initiated.

II. REGULATION OF METHIONINE SYNTHESIS IN HIGHER PLANTS

The regulation of aspartate family amino acid synthesis has received considerable attention in recent years. The sulfur amino acids in particular have been intensively investigated since it is partly through the aspartate pathway that net assimilatory reduction of sulfur occurs. While much has been learned of the mechanisms by which inorganic sulfur becomes incorporated into cysteine and methionine, neither the structural pathways nor the regulation of these pathways are as yet fully understood. Yet despite occasional inconsistencies in what is known of sulfur amino acid synthesis in higher plant species, a fairly cohesive model of the pathway can be constructed.

Figure 1 encompasses much of what is generally recognized concerning the synthesis and regulation of the protein bound sulfur amino acids, cysteine and methionine. Cysteine is produced by the reaction of activated serine with a sulfhydryl donor originating from reductively assimilated sulfur (108). The convergence of cysteine with an activated derivative of aspartate, O-phosphohomoserine, is mediated by cystathionine-γ-synthase. With cleavage of cystathionine by cystathionine-β-lyase, the transfer of a sulfur atom from cysteine to an aspartate derived carbon backbone is achieved. Methylation of homocysteine completes the synthesis of methionine. Unlike bacteria and many fungi in which direct sulfhydrylation from S^- to activated

Figure 1. Regulation of aspartate family amino acid synthesis in higher plants.


ASPARAGINE
ASPARTATE
LYSINE
aspartate kinase
β-ASPARTYL PHOSPHATE
β-aspartyl semi-aldehyde dehydrogenase
β-ASPARTYL SEMIALDEHYDE
homoserine dehydrogenase
HOMOSERINE
homoserine kinase
O-PHOSPHOHOMOSERINE
threonine synthase
THREONINE
ISOLEUCINE
SERINE
O-ACETYLSERINE
O-acetylserine sulfhydrylase
APS
SO4 2-
CYSTEINE
GLUTATHIONE
cystathionine-γ-synthase
CYSTATHIONINE
cystathionine-β-lyase
HOMOCYSTEINE
homocysteine methyltransferase
METHIONINE
methionine adenosyltransferase
S-ADENOSYLMETHIONINE
S-ADENOSYLHOMOCYSTEINE

homoserine is possible, transsulfuration from cysteine to O-phosphohomoserine is the predominant, if not sole, route by which methionine acquires its sulfur atom in higher plants. This course of methionine biosynthesis is corroborated by $^{35}SO_4^{2-}$ tracer studies (35, 36, 59) and by relief of inhibition at different points along the pathway with specific intermediates (19, 96).

Although the structural aspects of sulfur amino acid synthesis are reasonably well characterized, regulation is less clearly understood. Feedback inhibition of several key enzymes serves to coordinate sulfur amino acid synthesis with the production of lysine, threonine, and isoleucine. Features of aspartate family feedback inhibition which are recognized by most researchers are depicted in Figure 1, and regulated enzymes on the course to methionine will be discussed below.

Aspartate kinase, the first enzyme on the path to methionine, exhibits an elaborate feedback mechanism. In most species aspartate kinase activity is expressed by two distinct isozymes. In carrot one isozymic species is inhibited by high concentrations of lysine while the other loses activity in response to threonine (22, 75). Concurrent inhibition of both activities results in a strictly additive reduction of activity. In pea as well, lysine sensitive (74) and threonine sensitive (2) aspartate kinase enzymes exist. Barley also contains both isozymes although the threonine sensitive form is not strongly expressed (74). Maize is characterized by a lysine sensitive aspartate kinase (32). The lysine sensitive aspartate kinases of pea, broad bean, maize, barley, and melon have been shown to be sensitive to a product of methionine, S-adenosylmethionine,

which interacts synergistically with lysine to inhibit activity (74). The consequence of these interactions with aspartate kinase isozymes is that lysine, threonine, and methionine jointly direct the flow of carbon through their own and through each other's synthetic pathways.

Homoserine dehydrogenase also comes under regulatory control. All homoserine dehydrogenase activity purified from pea or barley is sensitive to cysteine inhibition; however, forms which are either sensitive or insensitive to threonine inhibition can be separated (1, 2). Sensitivity to threonine occurs in maize (25, 32) and soybean (106). In these species several bands of activity which include threonine-sensitive low molecular weight forms and threonine-insensitive high molecular weight forms can be resolved. These bands appear to be related as an oligomeric series of the same basic unit. Inhibition of homoserine dehydrogenase by threonine in concert with lysine inhibition of lysine-sensitive aspartate kinase is a potent technique for inducing methionine deprivation.

The positioning of O-phosphohomoserine at a metabolic branch point implies a regulatory property for at least one of the enzymes which utilizes this substance as substrate. It is therefore not surprising that threonine synthase is highly sensitive to the methionine derivative, S-adenosylmethionine. Yet regulation of threonine synthase by S-adenosylmethionine is not by inhibition. S-adenosylmethionine acts as a strong promotor of threonine synthase activity (60). Since O-phosphohomoserine occurs at a cellular concentration approximately two orders of magnitude below the K_m of either threonine synthase or cystathionine-γ-synthase, promotion of threonine synthase by S-adenosylmethionine sharply enhances threonine synthesis at the

expense of methionine (36). In many species enhancement of threonine synthase activity by S-adenosylmethionine is inhibited by cysteine, thereby providing further modulation of regulatory control (35).

The regulatory controls described for aspartate kinase, homoserine dehydrogenase, and threonine synthase are generally recognized features of the aspartate amino acid family in higher plants. However, observations from other studies suggest that additional or alternative controls exist. Soluble O-acetylserine sulfhydrylase from wheat is competitively inhibited by methionine (4) while pea and clover chloroplast O-acetylserine sulfhydrylase can be competitively inhibited by cystathionine (70). In maize suspension cultures or in partially organized maize callus cultures β-aspartylsemialdehyde dehydrogenase is suppressed by methionine (32). In *Pisum* isoleucine enhances activity of threonine-sensitive aspartate kinase (2) but strongly inhibits homoserine kinase (36). It is particularly interesting that O-phosphohomoserine dependent cystathionine-γ-synthase activity in *Lemna* increases several fold in response to lysine plus threonine treatment (96). This observation is consistent with enzyme induction.

Much of the information concerning regulatory properties in the aspartate amino acid pathway is based upon *in vitro* characterization of partially purified enzymes. While information of this nature is useful, it does not necessarily reflect the situation *in vivo*. One of the first assessments of *in vivo* aspartate family regulation was performed with *Mimulus* seedlings (39). In this system lysine or threonine alone decreased ^{14}C-leucine incorporation into protein while their combination led to the rapid cessation of all protein synthesis. Addition of methionine, however, totally negated inhibition of protein

synthesis. By feeding with ^{14}C-aspartate and analysing radioactive products, it could be shown that lysine plus threonine had suppressed methionine synthesis.

Barley and wheat embryos as well are subject to growth inhibition by lysine plus threonine (10). That intermediates leading to methionine production are depleted is substantiated by reversal of growth inhibition when homoserine or homocysteine are provided. Similarly, growth inhibition in maize embryos (37) and *Lemna* (19) induced by lysine plus threonine is relieved by methionine or methionine precursors. Growth inhibition in *Lemna* induced by propargylglycine, an inactivator cystathionine-β-synthase, or by aminoethoxyvinylglycine, an inhibitor of cystathionine-γ-lyase, are also relieved by methionine or the appropriate methionine precursors (19).

Intracellular regulation can be complicated by compartmentation of substrate pools or of isozymes with distinct regulatory properties. In actively photosynthesizing chloroplasts, aspartate family amino acid synthesis proceeds rapidly, indicating light stimulation of the process (65). Isolated chloroplasts appear to contain the requisite enzymes for synthesis of all aspartate family amino acids as well as for cysteine. Many of these enzymes are subject to feedback inhibition. While few reports concerning isozyme localization are available, the conjunction of specific isozymes with particular substrate pools can influence synthetic rates. Homoserine dehydrogenase occurs in both chloroplasts and cytosol (100). Differential regulation is indicated by threonine inhibition of the chloroplast enzyme while the cytosol enzymes remains insensitive. In contrast cytoplasmic homoserine dehydrogenase is subject to cysteine inhibition while chloroplast

activity is relatively insensitive. A second enzyme, O-acetylserine sulfhydrylase, has been isolated from the chloroplast stroma of some species and from the cytoplasm of others (70). Finally, homocysteine methyltransferase has been identified in both mitochondria and chloroplast preparations (36). Multiple substrate pools also occur. Free cysteine has been identified in the chloroplast, the cytosol, and the vacuole (59). The pool which most likely contributes to net methionine synthesis occurs in the chloroplast. Additionally, S-adenosylmethionine is found in the vacuole where it may act as a methionine reserve (36).

A further complication to aspartate family regulation relates to regulatory differences associated with different developmental states of tissues and organs. The relative susceptibility of carrot aspartate kinase to lysine or threonine inhibition is a direct consequence of the proportions of threonine-sensitive and lysine-sensitive isozymes present (22, 75). These levels vary among fresh root tissues, cultured root discs, and embryogenic suspension cultures at different times after subculture. Aspartate kinase activities in soybean cotyledons and suspension cultures also differ in their sensitivity to lysine inhibition (106). Differences among shoots, roots, kernels, callus and suspension cell cultures of maize to methionine inhibition of β-aspartylsemialdehyde dehydrogenase have also been described (32). Threonine sensitivity of homoserine dehydrogenase is dependent upon developmental stage in wheat (100) and maize (25) such that progressive desensitization occurs shortly after germination.

A particularly poignant example of the dependency of enzyme activity upon physiological state is given by anthranilate synthetase,

an enzyme in the tryptophan synthetic pathway. A tobacco cell line isolated as resistant to 5-methyltryptophan accumulated tryptophan to levels 25 times those occurring in wild type cells. Whole plants regenerated from this line fail to express the tryptophan accumulator phenotype, yet cultures reinitiated from leaf explants from these plants resume tryptophan overproduction (106).

The regulatory features of the aspartate amino acid family discussed in this section point to a highly integrated system of controls which coordinately buffer the cell against wide fluctuations in the synthesis of any particular end product. Resistance to fluctuation is expressed by control redundancy in certain branches of the pathway. Further complicationg the situation are higher level controls which compartmentalize substrate pools, localize differentially regulated isozymes to separate regions of the cell, and alter the production or sensitivity of particular enzyme activities as the cell varies in its physiological mode.

Despite the intricate regulation of end product synthetic rates in the aspartate family pathway, the system is nonetheless vulnerable to genetic manipulation. With respect to methionine synthesis, enhanced production might result by altering sites of allosteric interactions between enzymes and their feedback inhibitors or promotors. Pertinent targets might be aspartate kinase isozymes, homoserine dehydrogenase, or threonine synthase. Alternatively, the activity of methionine adenosyltransferase might be reduced so as to concurrently alleviate S-adenosylmethionine enhanced inhibition of lysine sensitive aspartatekinase and its promotion of threonine synthase. Each of these mechanisms should facilitate the flow of

carbon to methionine and lead to expression of a methionine accumulator phenotype.

III. DEVELOPMENT OF IN VITRO PROCEDURES FOR SELECTION OF METHIONINE-ACCUMULATING POTATO CELL LINES

Introduction

The determination of optimal *in vitro* growth conditions is a necessary prerequisite to any study which relies upon cell growth rate as a measure of the effectiveness of treatments designed to repress growth or to relieve growth inhibition. For potato cell cultures requirements for optimal growth vary from clone to clone and must be empirically determined for each clone investigated. The development of conditions promoting rapid growth rate in two potato clones follows.

Optimization of Cell Growth in Dihaploid Potato Clone 'W-973'

Several dihaploid (2x = 24) potato selections recovered as parthenogenic offspring from tetraploid *S. tuberosum* clones were provided by the USDA, ARS, North Central Region Potato Introduction Station at Sturgeon Bay, Wisconsin. Potato plants were raised in the greenhouse, and newly mature leaves were harvested for callus initiation. As a first approximation of an appropriate culture medium, surface disinfested tuber cortex, petiole, and lamina sections were placed on media prepared with Murashige and Skoog inorganic components (68), Nitsch and Nitsch vitamin components (69), 30 g/l sucrose, and either 15 mg/l indoleacetic acid (IAA) or 2 mg/l 2,4-dichlorophenoxy-

acetic acid (2,4-D). Cultures were placed in the dark at 25° C and evaluated after ten weeks of growth. Among six dihaploid clones examined, two clones derived from tetraploid seedling clone 'Wis AG-231' proliferated as uniformly soft, cream-colored callus. One of these clones,'US-W 973', hereafter referred to as 'W-973', proliferated especially well on medium containing 2,4-D. This clone was used in all subsequent work involving dihaploid potato cultures.

Growth factor requirements were optimized by examining growth rate in a factorial design composed of benzylaminopurine (BAP) at 0, 0.02, 0.1, and 0.5 mg/l and 2,4-D at 0, 0.5, 1, 2, 3, 4, and 5 mg/l. Growth assessment was performed as described in a later section titled 'Growth Assay'. The generation time varied from 8.7 days to 14.4 days. The growth rate deemed optimal was provided by 3 mg/l 2,4-D without cytokinin. This hormone level resulted in a 9.4 day generation time, equivalent to a growth rate of 0.106 doublings per day. Callus on this medium was soft, bright, cream-colored, and free from large cell aggregates. Although treatments containing BAP as well as 2,4-D grew somewhat more rapidly, the resultant callus morphology was hard, compact, and therefore inappropriate for growth rate assessments. Results of further growth optimization experiments fixed sucrose at 30 g/l and nitrogen provided as ammonium and nitrate at concentrations recommended by Murashige and Skoog. A complete amino acid mixture equivalent to 0.75 times that found in the free amino acid pool of potato tubers further accelerated growth; however, 1000 mg/l glutamine proved a simpler substitute and yielded a similar response.

The optimized basal medium on which all 'W-973' callus cultures

were maintained consisted of Murashige and Skoog inorganic salts, Nitsch and Nitsch vitamins, 30 g/l sucrose, 3 mg/l 2,4-D, and 1000 mg/l glutamine and was designated D_3 + Gln_{1000} medium. Growth on this medium proceeded at a rate of 0.17 doublings per day for a generation time of 5.8 days. While a generation time of this magnitude appears slow compared to 24 hours for well established soybean suspension cultures, the rate is quite rapid for potato cultures. Over the course of three years the growth rate of 'W-973' callus on D_3 + Gln_{1000} medium gradually declined. Within this period the ploidy status of these cells had also changed. By two years of culture the chromosome number of the majority of cells had increased to approximately the tetraploid level with aneuploids predominating.

Optimization of Cell Growth in Tetraploid Potato Clone 'Superior'

The 'Superior' clone of *S. tuberosum* was selected because of its culturability and its reported regenerability following up to one year in culture (54,55). This feature was deemed critical if whole plants were later to be established from selected variant cell lines.

Cultures initiated from 'Superior' originated from greenhouse grown plants or tubers derived from premier certified stock 'seed'. Explants from either tuber cortex or stem produced the callus used for growth optimization. As an initial approximation of satisfactory culture conditions, the complex medium described by Lam was examined (54,55). This medium contained 0.5 mg/l benzylaminopurine (BAP), 0.01 mg/l kinetin, 0.2 mg/l gibberellic acid, and 10 mg/l naphthalene acetic acid (NAA). Under our conditions more rapid growth rates were consistently observed when cytokinins and gibberellic acid were

omitted and when 2 mg/l 2,4-D was included in the culture medium. Under the latter growth factor regime callus was softer and more uniform in texture as well. The inclusion of 1000 mg/l glutamine and 0.3 mM adenine sulfate further enhanced growth rate. The standard medium on which 'Superior' callus was routinely subcultured consisted of Murashige and Skoog inorganic salts (68), Nitsch and Nitsch vitamins (69), 30 g/l sucrose, 10 mg/l NAA, 2 mg/l 2,4-D, and 1000 mg/l glutamine. This medium was designated N_{10} D_2 + Gln_{1000}. Glutamine was frequently reduced to 500 mg/l during culture maintenance to decrease subculture frequency. Cultures were routinely maintained at ambient room temperature and illuminated with 40 - 80 foot-candles of fluorescent light.

Growth Assay

Accurate assessment of growth is essential to understanding the mechanisms by which growth processes are affected. Without a universally recognized method of reporting growth in a form which is compatible with all experimental conditions, comparisons of the effects of different growth promotors or inhibitors among unrelated tissues cultured under dissimilar conditions is not possible. Unfortunately, almost all reports in the plant literature which describe the effects of growth promotors or inhibitors on cell cultures are meaningful only within the rigid confines of the specific experimental conditions defined by the investigator. This dilemma is due to the common practice of reporting growth promotion or inhibition as percent increase or reduction of control growth at an arbitrary point in time following initiation of the experiment. The

flaw in this approach is to have compared two points from an exponential growth curve in linear terms without having first transformed to linearity. With this approach an apparent 50% growth reduction attributed to a particular concentration of inhibitor can just as easily be reduced to an apparent 20% growth reduction by shortening the duration of the experiment. Alternatively, it can be increased to an apparent 90% growth reduction by extending the duration of the experiment. Figure 2, a plot of fresh weight increase over time as affected by various concentrations of ethionine, illustrates this situation. The apparent percent reduction in fresh weight increase attributable to any level of ethionine is wholly dependent upon the time at which the comparison is made. Data reported in this manner is highly subject to manipulation and lacks real meaning.

Among the few investigators who legitimately report their results on a linearized basis such as change in growth rate (doublings per unit of time) or change in generation time, almost all assume constant growth velocity from initiation to completion of an experiment. However, when growth is monitored over frequent time intervals, growth rate can often be shown to vary over the course of an experiment. Figure 3, a linear transformation of Figure 2, demonstrates that growth rate was in fact *not* constant for all treatments throughout the experimental growth period. The initially steep slope of all treatments receiving ethionine diminished as ethionine toxicity appeared. Following an equilibration period of approximately five days, the growth rate became constant and accurately reflected the level of inhibitor present. For other systems an initial lag phase followed

Figure 2. Time course of the effect of ethionine concentration on fresh weight increase in 'W-973' potato cell cultures.

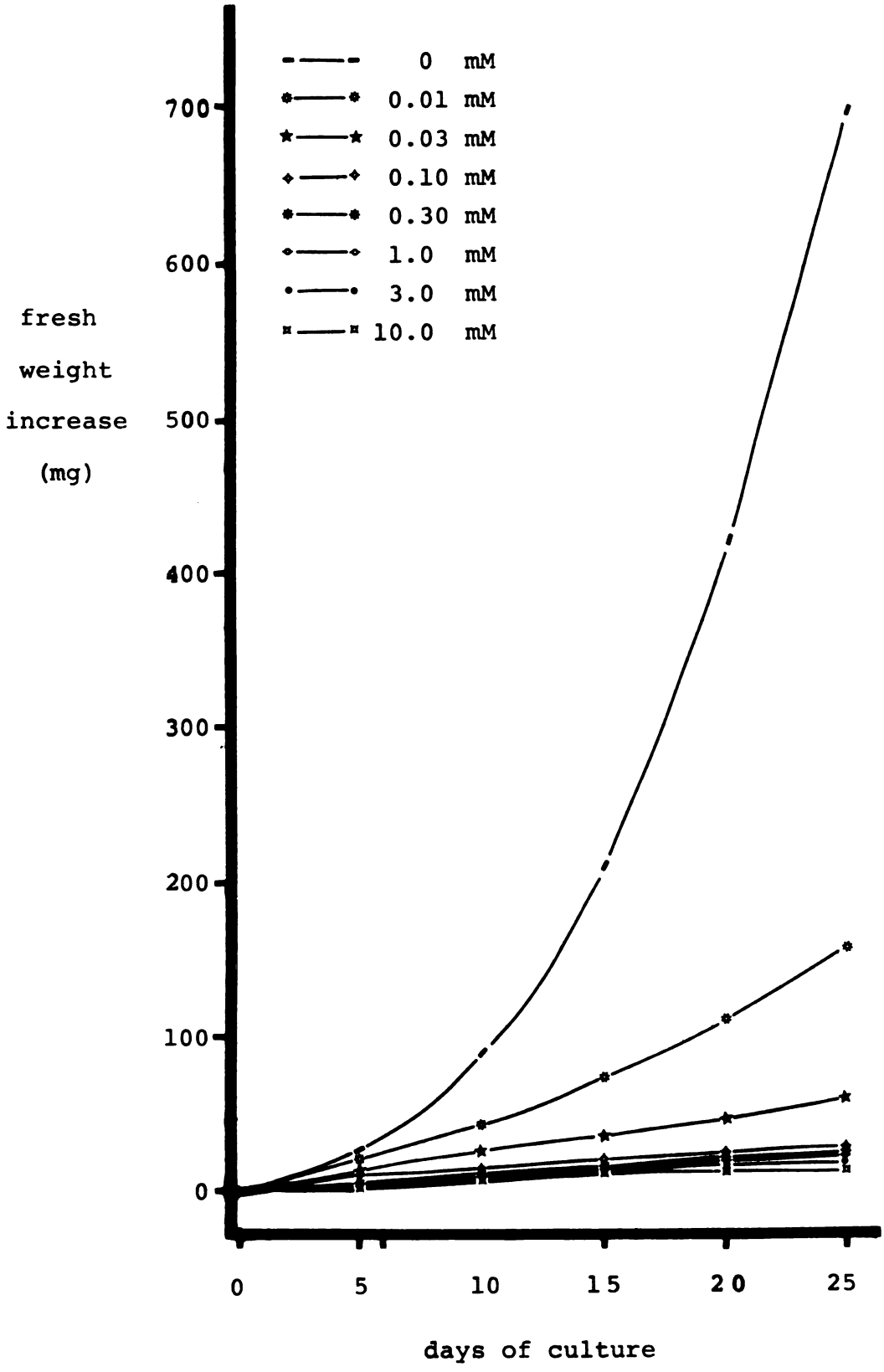
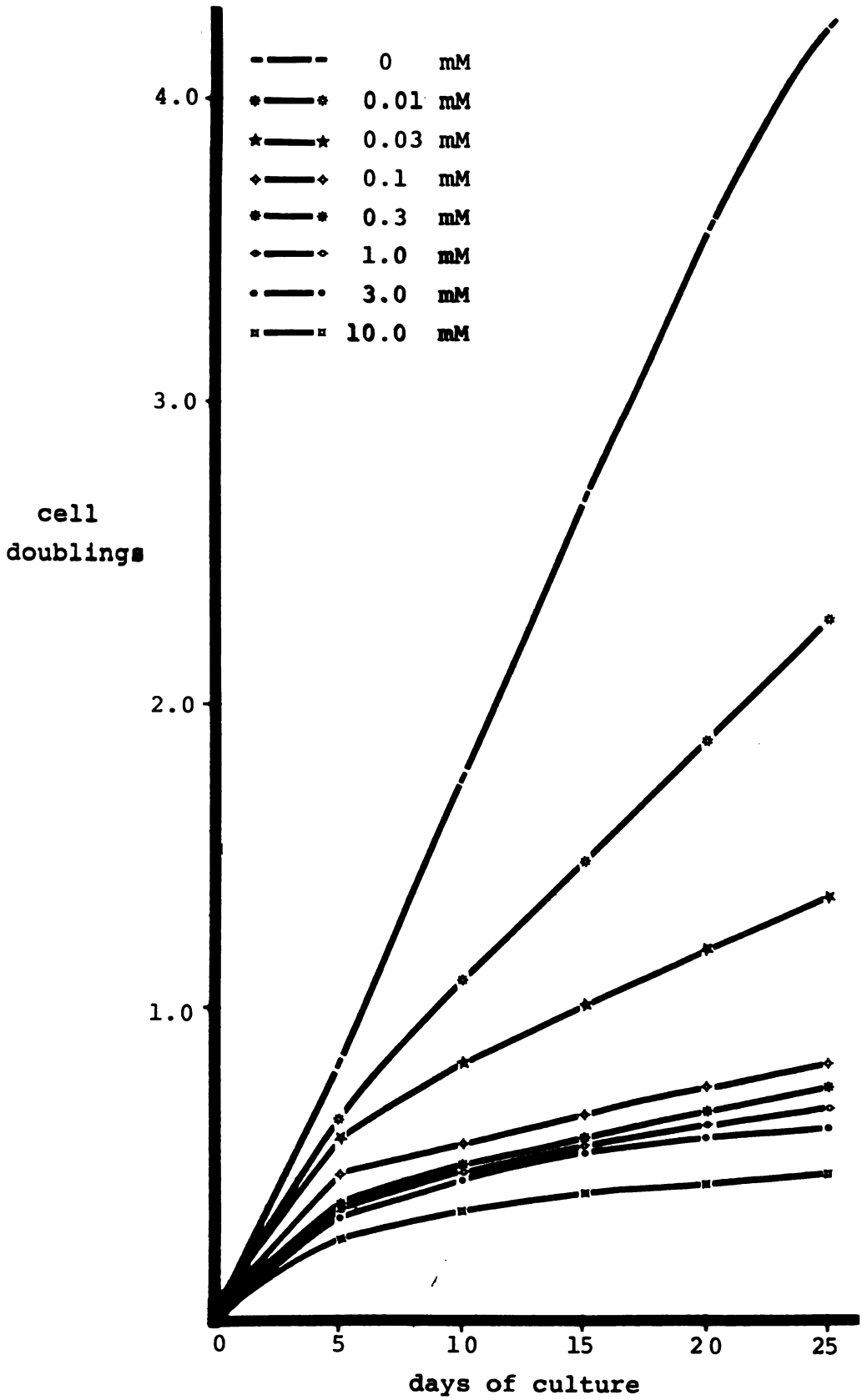

0 mM
0.01 mM
0.03 mM
0.10 mM
0.30 mM
1.0 mM
3.0 mM
10.0 mM
fresh weight increase (mg)
700
600
500
400
300
200
100
0
0
5
10
15
20
25
days of culture

Figure 3. Time course of cell doubling in 'W-973' potato cell cultures as affected by ethionine concentration.


0 mM
0.01 mM
0.03 mM
0.1 mM
0.3 mM
1.0 mM
3.0 mM
10.0 mM
4.0
3.0
2.0
1.0
cell doublings
0
5
10
15
20
25
days of culture

by a period of constant exponential growth and a later growth rate decrease may be noted. For some of the inhibitors investigated in this project, high concentrations induced an initial growth increase followed by an apparent negative growth rate as cells died and released their contents into the medium. Such negative growth was later followed by growth stasis. From these examples it is apparent that an accurate growth rate can be determined only within a time frame during which growth proceeds at a constant rate.

To assess growth rate in potato cells a nondestructive method was devised in which growth of all cells within an individual callus could be followed without disturbing the orientation of cells within the callus or of cells at the callus-culture medium interface. This was accomplished by culturing cells on a nitrocellulose filter which could be aseptically weighed at convenient time intervals. Nitrocellulose filters are rigid enough to be easily transported to a sterile petri dish for weighing. Because they absorb a fixed amount of liquid from the agar medium, the weight of filter plus liquid, recorded prior to transfer of cells to the filter, can be subtracted from the measurement of callus plus filter weight.

Cultures were routinely prepared by transferring approximately 40 mg of cells to a filter which had previously been saturated with liquid, lightly blotted, and weighed. These cultures were then weighed in a tared petri dish on an analytical balance in a laminar flow hood. Subsequent weighings were performed at carefully monitored time intervals. When weights were recorded to the nearest tenth milligram, the method proved to be extremely sensitive to very slight growth increments. Because of this, a growth *rate* as low as

two orders of magnitude below that of uninhibited cells could be detected within a 20 - 30 day assay period.

Cell growth proceeding at a constant rate can be accurately described as $y = a2^N$ where y is the fresh weight increase from time $t = 0$, a is the initial fresh weight, and N is the number of mass doublings per unit of time. Growth rates for individual callus cultures in all experiments were calculated by a least squares exponential regression procedure. The regression equation was

$$b = \left[\frac{\Sigma\, t \cdot \ln y \;-\; \frac{\Sigma\, t \;\Sigma \ln y}{n}}{\Sigma\, t^2 \;-\; \frac{(\Sigma\, t)^2}{n}} \right] \Big/ \ln 2$$

where growth rate, b, equals N/t, the number of doublings per unit of time and n is the number of time points sampled within the exponential phase of growth. The unit of time was standardized to days; b is therefore reported as doublings per day.

In preliminary experiments designed to characterize normal and inhibited growth of W-973 cells, frequent time intervals of 4 or 5 days were sampled throughout a period of up to six weeks. These experiments indicated that constant growth rates occurred within the period of approximately eight to thirty-five or forty days following culture initiation. Therefore culture weighings were routinely performed between days 10 and 30. A minimum of three sampling points was used for all regressions calculated. The coefficient of determination served as a useful check that growth rate was indeed constant. Rarely did this indicator fall below a value of 0.98. Experimental treatments were typically replicated at least four times while control

treatments were replicated a minimum of eight times.

The physical properties of cells used in growth assays were critical to the accuracy of growth determinations. Only exponential phase callus which was soft, finely dispersive, and brightly cream-colored was used. To overcome difficulties due to somaclonal variation, callus for an entire experiment or for an entire replication was thoroughly mixed to assure uniform response.

Preparation of culture medium was accomplished by autoclaving a pH adjusted,concentrated version of the final medium devoid of all amino acids and/or growth inhibitors. These were prepared as pH adjusted, concentrated stocks and were filter-sterilized before their addition to the basal medium. All cultures on which growth assays were performed were placed in a dark incubator maintained at 24° - 25° C.

Effects of Aspartate Family Amino Acids on Growth of Potato Cultures

Methionine

A selection strategy dependent upon methionine accumulation should be designed so as to permit growth of variant clones which synthesize product at levels that do not adversely affect normal growth processes. This requires an estimate of methionine levels which are toxic to cell growth. While growth inhibition by exogenously supplied methionine can provide a minimal estimate of the concentration at which toxicity occurs, information provided by a methionine growth inhibition curve is useful in studies designed to determine whether methionine can overcome growth inhibition due to competition with methionine analogs or to feedback-induced methionine

deficiency.

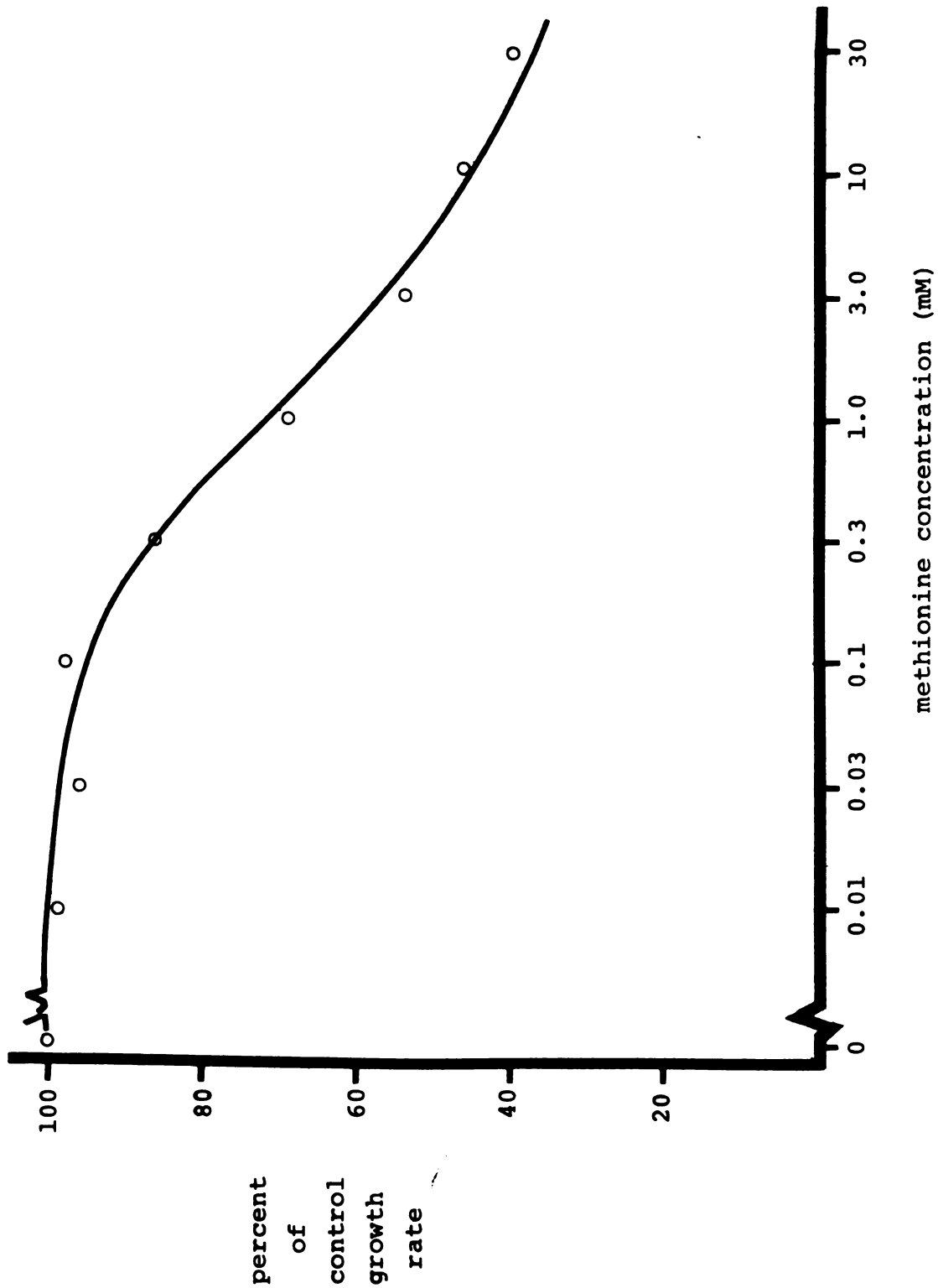
The effect of methionine on growth rate in 'W-973' cells was determined by culturing cells on D_3 + Gln_{1000} medium containing methionine at concentrations ranging from 0 to 30 mM. Cultures were prepared, maintained, and assayed as described under 'Growth Assay'.

Figure 4 depicts the response of potato cell cultures to supplemental methionine. Concentrations between 0.01 and 0.1 could not be distinguished from the control ($\alpha = 0.05$) while higher concentrations resulted in growth depression.

Since the cells were not assayed for methionine content, the degree to which they concentrated methionine from the culture medium cannot be discerned. Other amino acids have been shown to be transported by energy dependent, saturable permease systems which function at amino acid concentrations of up to approximately 5 mM (9, 63). These systems can concentrate an amino acid from 10 to nearly 100 fold, depending upon the uptake phase of the permease. At still higher amino acid concentrations an energy independent, unsaturable uptake system is noted; this system may be primarily diffusional. Therefore at very high levels of supplemental methionine, the cell's endogenous methionine level may more closely relate to the concentration provided to the culture medium.

A lower limit estimate of the highest intracellular methionine concentration which fails to evoke toxicity can be inferred from free pool concentrations in cells in which methionine occurs in greatest abundance, i.e. tuber cells. Assuming potato tubers to be 80% water, the free methionine pool in 'W-973' tubers can be estimated at 1.14 mM (see Appendix B). A comparison of this estimate with the highest

Figure 4. Effect of methionine concentration on growth rate in 'W-973' potato cell cultures.

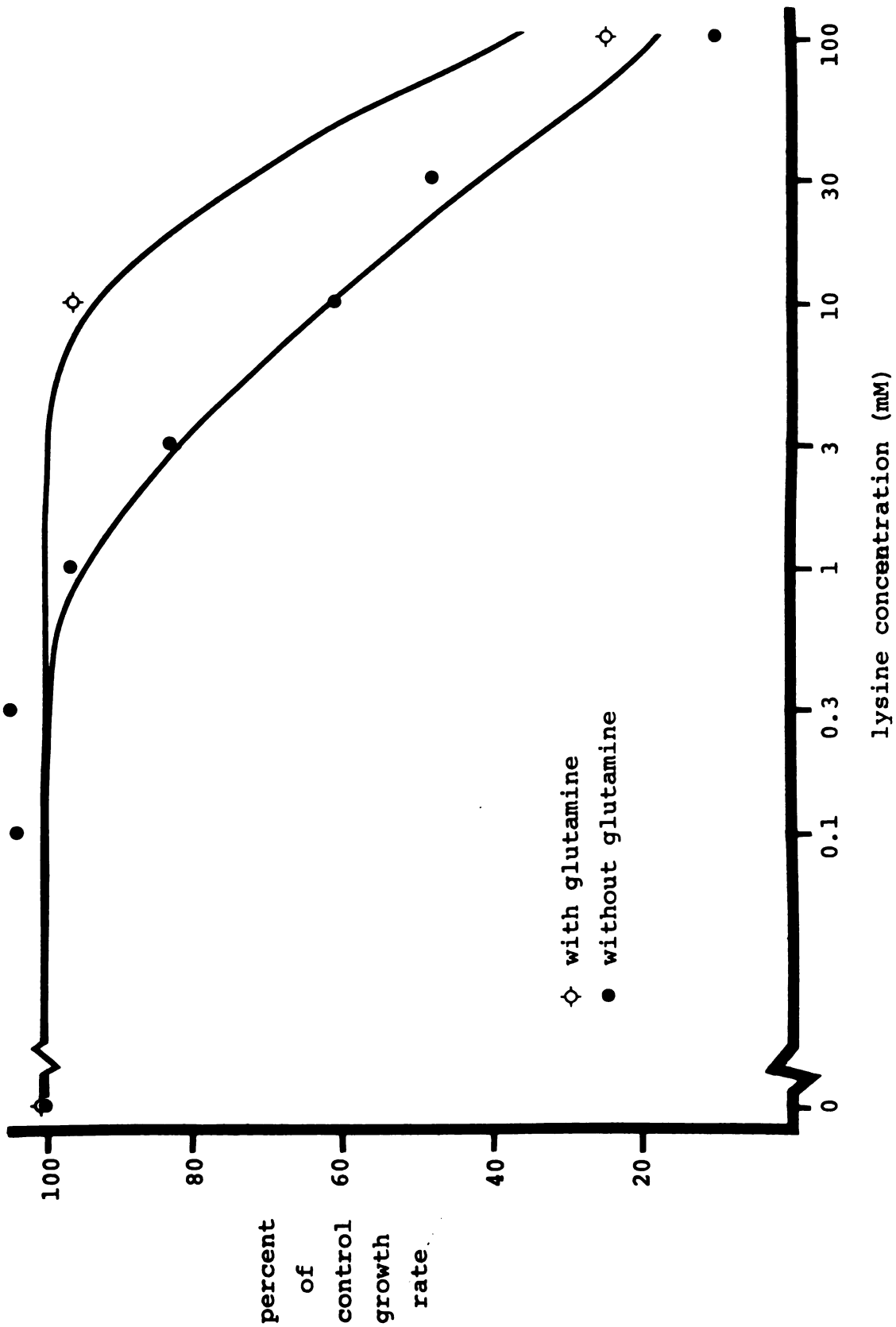

percent of control growth rate
100
80
60
40
20
0
0.01
0.03
0.1
0.3
1.0
3.0
10
30
methionine concentration (mM)

extracellular level of methionine which does not elicit toxicity leads to the conclusion that potato cells are capable of concentrating methionine by a factor at least ten times that provided in the culture medium. This estimate, of course, is valid only for methionine levels for which transport is permease dependent. As will later become apparent, the limit of potato cell tolerance to supplemental methionine is less than for lysine, threonine, or cysteine.

Lysine

Lysine-induced growth inhibition was examined by assaying growth in 'W-973' callus cultured as described under 'Growth Assay' on $D_3 \pm Gln_{1000}$ medium supplemented with lysine from 0 to 100 mM. Inhibition curves relativized against control growth rates appear in Figure 5. It is clearly evident that glutamine relieves lysine-induced growth inhibition, particularly at weakly toxic lysine levels. A 50% growth rate reduction was noted at 25 mM in glutamine-free medium while in the presence of glutamine, this level of growth rate depression occurred at 60 mM. The phenomenon of glutamine-conferred protection against growth inhibition will later be discussed in relation to other amino acids and analogs. While the basis for protection is unknown, glutamine is known to competitively affect uptake of other amino acids in cells cultured in its presence (18). It is therefore conceivable that in cells cultured in the presence of glutamine, the internal pool of competing amino acid is effectively reduced. Nevertheless, glutamine was included in most culture media because of its effect in accelerating growth rate. While not apparent in Figure 5, control growth rate in medium containing glutamine was 20% greater

Figure 5. Lysine-induced growth inhibition in 'W-973' potato cell cultures.


percent of control growth rate
100
80
60
40
20
0
0.1
0.3
1
3
10
30
100
lysine concentration (mM)
with glutamine
without glutamine

than that in glutamine-free medium.

Threonine

The influence of threonine on growth rate in 'W-973' potato cell cultures was examined over a range of 0 to 100 mM on $D_3 \pm Gln_{1000}$ medium. Growth rates relativized against glutamine-supplemented or glutamine-free controls are shown in Figure 6. As with lysine, glutamine conferred a nearly 100 fold protection against threonine-induced toxicity. The 50% level of growth inhibition in the absence of glutamine was 4 mM while in its presence, 40 mM threonine was required. Glutamine enhanced control growth rate by 19%.

Cysteine

Cysteine-induced growth depression in 'W-973' cells was examined in cultures grown on $D_3 \pm Gln_{1000}$ medium. Over the range of 0.15 to 100 mM cysteine, provided as cysteine HCl, toxicity was most prominent in glutamine-free medium (Figure 7). At approximately 6 mM cysteine, glutamine rapidly lost its capacity to protect against toxicity. In glutamine-free medium 50% growth inhibition occurred at 0.8 mM; in glutamine-supplemented medium the half maximal growth rate occurred at 10 mM. Cell stasis or death was elicited by 4 and 15 mM cysteine in D_3 and $D_3 + Gln_{1000}$ medium, respectively.

Consistent growth responses were not always observed when cysteine was included in the medium. Unanticipated differences in toxicity between cysteine HCl and the free base were noted; the latter was much more toxic. Since a time dependent pH decrease in cysteine-supplemented medium was suspected, medium prepared with the free base

Figure 6. Threonine-induced growth inhibition in 'W-973' potato cell cultures.

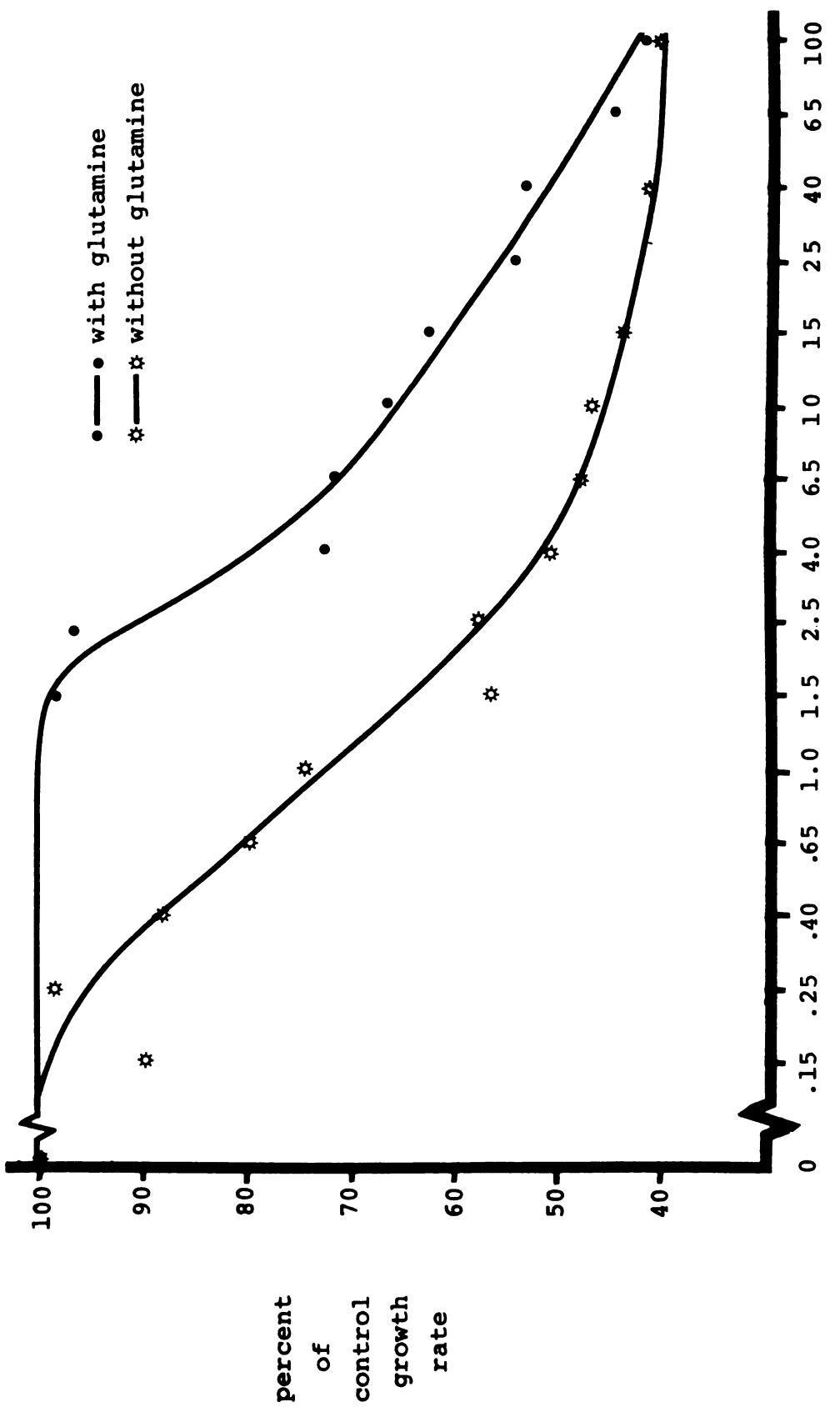
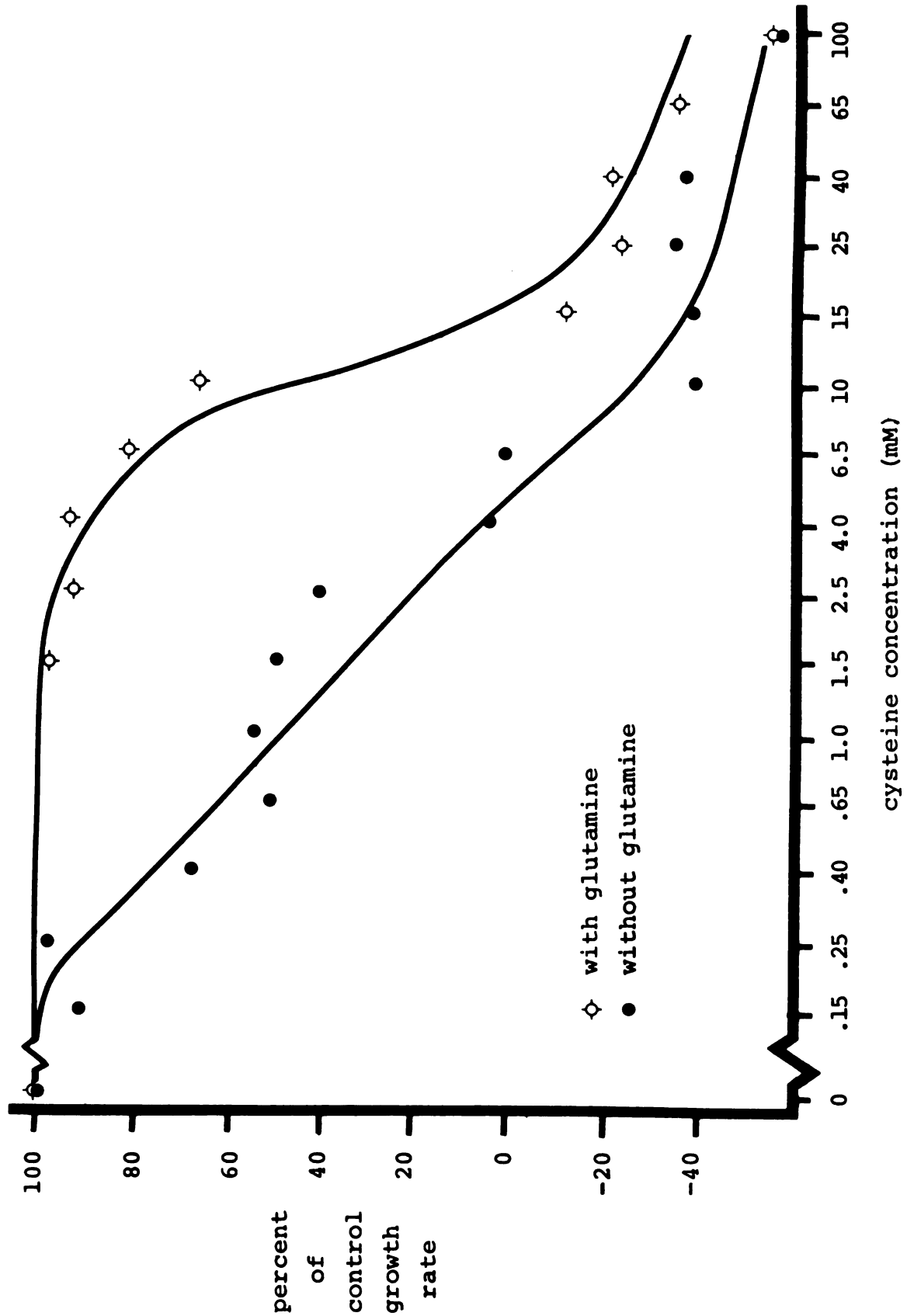

• with glutamine
✡ without glutamine
percent of control growth rate
100
90
80
70
60
50
40
0
.15
.25
.40
.65
1.0
1.5
2.5
4.0
6.5
10
15
25
40
65
100
threonine concentration (mMoles/l)

Figure 7. Effect of cysteine on growth rate in 'W-973' potato cell cultures.


100
80
60
40
20
0
-20
-40
percent of control growth rate
with glutamine
without glutamine
0
.15
.25
.40
.65
1.0
1.5
2.5
4.0
6.5
10
15
25
40
65
100
cysteine concentration (mM)

form of cysteine was buffered with MES at concentrations ranging from 0 to 20 mM. After adjusting the pH of filter sterilized cysteine and MES to 5.7, D_3 + Gln_{1000} medium was prepared and the pH monitored over several days. In unbuffered medium the pH had spontaneously dropped to 3.95 by 115 hours while at 20 mM MES the pH had only decreased to 5.42. The inclusion of 20 mM MES in culture medium supplemented with cysteine only slightly reduced cysteine toxicity. Despite this weak relief MES was provided in several experiments involving cysteine-induced growth inhibition.

A further difficulty encountered with cysteine was the development of a dense precipitate at high cysteine concentrations. Cysteine is known to be catalytically oxidized by ferrous ion; indeed, deletion of iron and microelements from the culture medium diminished the rate at which precipitation occurred. As a consequence of cysteine precipitation, the quantity of cysteine that was available to the cells could not be accurately known.

Selective Systems Based upon Methionine Analogs

Preliminary Considerations

An understanding of the regulatory controls discussed in the preceeding chapter allows one to construct selection schemes in which the overproduction of methionine might confer growth advantage. One useful approach is to provide structural analogs of methionine to a cell population. Presumably the analogs will be transported into the cell where they will be perceived as methionine. As the analogs interact with the cell's metabolic machinery, they may inhibit

certain metabolic processes and/or they may be used to produce defective products, often with the expenditure of considerable metabolic energy. With regard to analogs, any of a number of mutations which might increase the quantity of intracellular free methionine,and thereby lead to effective methionine-analog competition, could be expected to enhance cell proliferation of a variant clone. Alternatively, a scheme based upon artificially induced methionine starvation through concerted feedback inhibition might result in selection of a mutant with relaxed feedback control. Resumption of synthesis of the precursors required for methionine production could permit such a cell to grow while wild type cells would die. Selection based upon relaxed feedback inhibition would be expected to involve fewer mutational classes than selection for analog resistance because desirable mutations downstream from the target feedback genes would be unable to be expressed.

Although one can develop selective schemes which favor growth of methionine overproducers, other mutational classes might be represented among variant cell lines capable of resisting the selection pressure. Permease mutants in which the uptake of feedback inhibitors or methionine analogs is impaired could escape growth inhibition. Improved enzyme specificities capable of distinguishing between methionine and its analog(s) would also confer resistance. Increased relative specificity of methionyl-tRNA synthetase for methionine would result in reduced analog incorporation into protein. Similarly, improved specificity of methionine adenosyltransferase or other methyl transferases would prevent an analog such as ethionine from propagating

its alkylating effects to other metabolic pathways. Finally, an epigenetic (or even possibly stable) resistance might be conferred by the acquisition of a mode in which synthesis of inhibited enzymes is elevated or by induction of a feedback insensitive isozyme not normally produced by the cell type under selection. Assumption of a dormancy mechanism in which overall metabolism during the period of selection is reduced could also result in an apparently resistant variant.

Selenomethionine

Selenomethionine serves as an analog of methionine by virtue of a selenium substitution for sulfur. Its incorporation into protein has been examined in order to determine whether translational processes are affected. In *Vigna radiata*, a plant species sensitive to selenium, selenomethionine is efficiently charged onto methionyl-tRNA,and ribosome binding is normal (26,27). Peptide formation, however, is reduced. This reduction is especially pronounced when selenomethionine is charged to methionyl-tRNA-1, the polypeptide initiator species, with the result that polypeptide initiation is frequently aborted. Despite this effect, selenomethionine is freely incorporated into protein. *E. coli* proteins in which selenomethionine has largely replaced methionine function at near normal levels. In hepatoma cells selenomethionine increases the heat lability of enzymes into which it has been incorporated (52). Methionine adenosyltransferase utilizes selenomethionine to produce Se-adenosylselenomethionine. Methyl transferases can transfer the methyl group of this species to methyl acceptors.

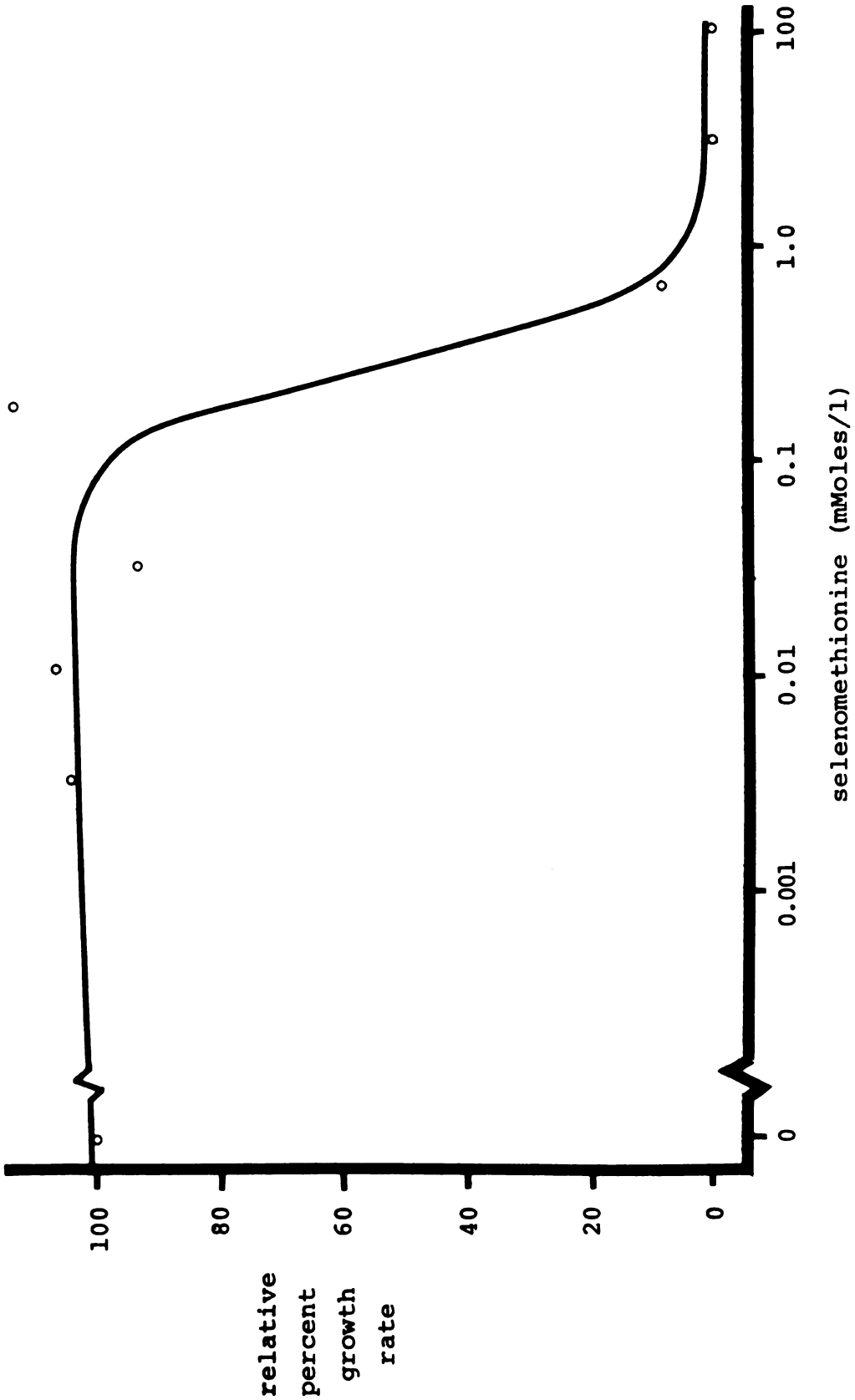
Resistance to selenoamino acids has been described for tobacco cultures (28). Two mutants resistant to selenocystine alone and one mutant resistant to selenocystine and selenomethionine in combination were isolated from cultures treated with both analogs. The mechanism of resistance in these mutants has not been determined.

To assess conditions under which cell selection for resistance to selenomethionine might be performed, the toxicity of this compound to 'W-973' cells was determined. Cultures which had been prepared as described under 'Growth Assay' were transferred to $D_3 + Gln_{1000}$ medium supplemented with selenomethionine at concentrations ranging from 0 to 10 mM. Growth rates expressed as a percentage of control growth rate are presented in Figure 8. The selenomethionine inhibition curve displays an unusually sharp transition between innocuous and highly toxic concentrations. A response of this nature leads to difficulty in accurately assessing the degree to which growth rate is reduced within the transition portion of the curve. Fifty percent growth rate inhibition occurs near 0.3mM selenomethionine while ninety percent depression is caused by approximately 0.7 mM analog.

Ethionine

Ethionine is a structural analog of methionine in which an ethyl group is substituted for the methyl moity of methionine. This analog is effectively incorporated into protein, but little is known concerning its effects on enzyme activity (30). Perhaps the most pervasive effects attributable to ethionine derive from its activity as an ethyl donor in reactions which normally accept methyl groups. S-adenosylethionine is readily synthesized by yeast and *Coprinus*

Figure 8. Selenomethionine toxicity in 'W-973' potato cell cultures.


relative percent growth rate
100
80
60
40
20
0
0
0.001
0.01
0.1
1.0
100
selenomethionine (mMoles/l)

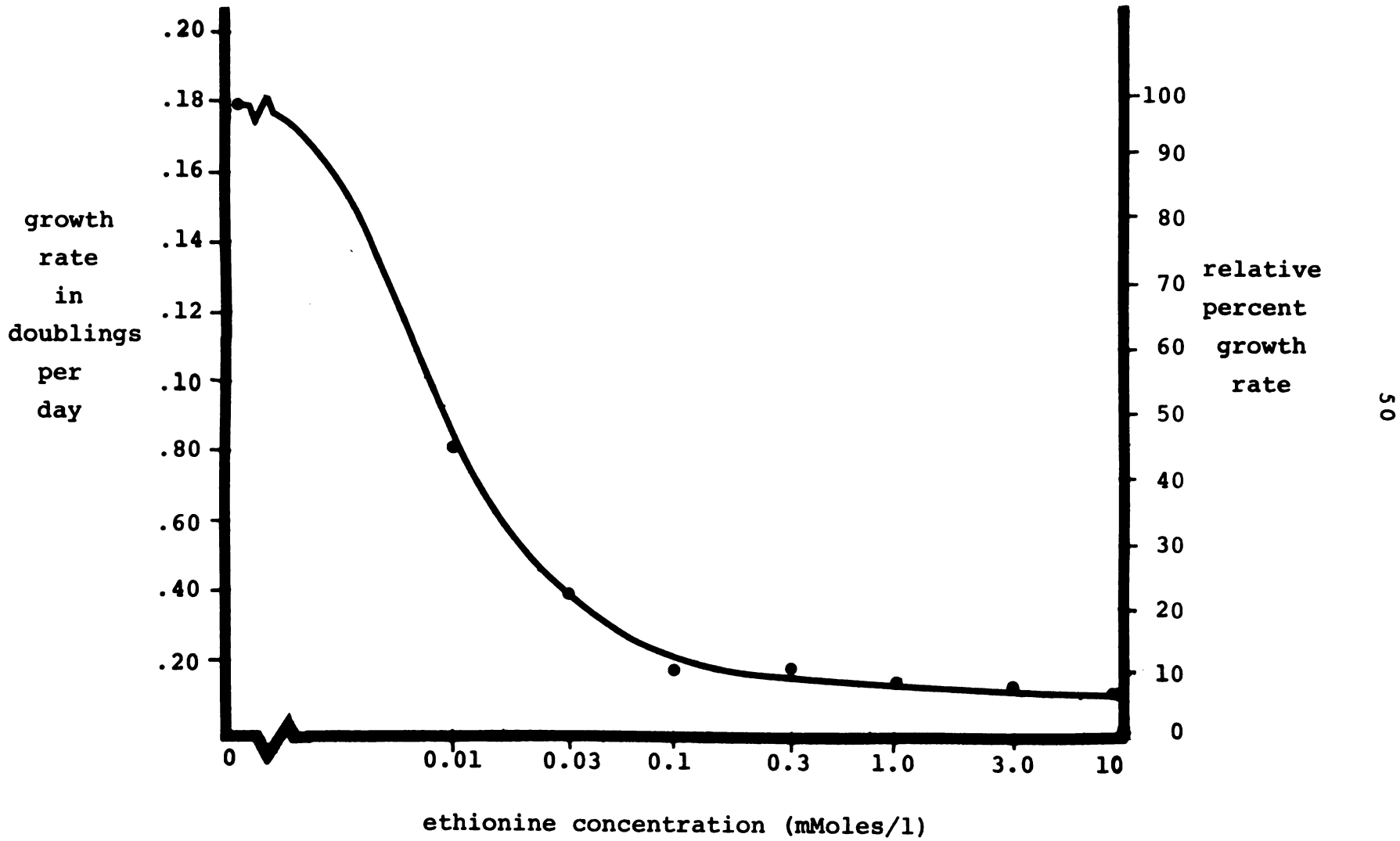
methionine adenosyltransferase, thereby setting the stage for extensive ethylation. Ethyl groups originating from S-adenosylethionine have been identified in choline and in rat liver DNA and RNA. Ethionine is recognized as a mutagen and displays carcinogenic activity as well.

Resistance to ethionine can occur by a number of mechanisms (30). A permease mutant which excludes ethionine and concurrently exhibits resistance to a phenylalanine analog has been described in *Saccharomyces*. A *Neurospora* mutant is resistant by virtue of methionine overproduction. Enhanced discrimination in methionyl-tRNA charging has been reported in a *Coprinus* mutant. Other mutants also exist for which the resistance mechanism is not understood.

As a guide to identifying a concentration of ethionine appropriate for variant selection, an ethionine growth inhibition curve was established. The response of 'W-973' cell cultures prepared as described under 'Growth Assay' was examined over the range of 0 to 10 mM ethionine. Fresh weight increase, cumulative cell doublings, and percent growth rate relative to a control are presented in Figures 2, 3, and 9, respectively. In Figure 9 the concentration producing 50% growth inhibition appears as 0.01 mM; 90% growth rate depression occurred near 0.1 mM ethionine.

It is noteworthy that during the course of this experiment an occasional callus sector appeared which grew distinctively more rapidly than background cells on medium containing 0.1 mM ethionine. One such variant which continued to express resistance was propagated and designated clone E1. These observations indicated that selection in the vicinity of 0.1 mM ethionine might allow relatively rapid growth of

Figure 9. Growth rate inhibition in 'W-973' potato cell cultures as a function of ethionine concentration.


growth rate in doublings per day
.20
.18
.16
.14
.12
.10
.80
.60
.40
.20
100
90
80
70
60
50
40
30
20
10
0
relative percent growth rate
0
0.01
0.03
0.1
0.3
1.0
3.0
10
ethionine concentration (mMoles/l)

resistant cells which could later be analyzed under more stringent selection pressure.

The kinetics of methionine-ethionine antagonism were assessed to determine whether these compounds act in a competitive or noncompetitive manner. Even though the biochemical process most disturbed by ethionine is not known, the effect of ethionine on any rate limiting step should be reflected in cellular growth rate. Therefore the extent of antagonism can be measured. The nature of methionine-ethionine antagonism is important because a noncompetitive interaction will not necessarily permit selection for resistance based upon methionine overproduction. It was also of interest to determine the level of supplemental methionine required to achieve a particular level of relief from inhibition at various ethionine concentrations. Since the extent to which ethionine and methionine are transported and concentrated within the cell was not known, the influence of the endogenous free methionine pool (0.24 mM in 'W-973' cells) upon antagonism could not be determined.

From Table 1 it is evident that methionine can negate ethionine-induced growth inhibition. An analysis of variance performed on growth rates corrected for methionine-induced toxicity indicated strong interaction between methionine and ethionine ($F < 0.0005$). That the nature of interaction is competitive is demonstrated by the double reciprocal plot in Figure 10. As required of competitive interactions, all curves converge at the reciprocal value of the uninhibited growth rate. Growth depression was relieved to the extent of fifty percent for 0.01, 0.03, and 0.1 mM ethionine by 0.023, 0.046, and 0.057 mM methionine, respectively (Figure 11).

Table 1. Relief of ethionine-induced growth inhibition by methionine in 'W-973' callus cultures.[z]

ethionine concentration (mM)	methionine concentration (mM)						
	0	0.003	0.01	0.03	0.1	0.3	1.0
0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
0.003	73.6	76.7	90.2	86.4	91.5	96.1	95.1
0.01	25.7	28.4	38.0	54.4	73.2	95.3	100.4
0.03	8.6	8.8	18.3	39.6	63.5	95.4	95.6
0.1	0.4	-1.8	1.8	30.7	64.5	98.2	98.3

z- growth rates are expressed as percentage of growth rate in ethionine-free controls and are corrected for inhibition due to methionine. The experiment-wise standard error is 2.6 percent.

Figure 10. Competitive interactions between ethionine and methionine in 'W-973' potato cell cultures.

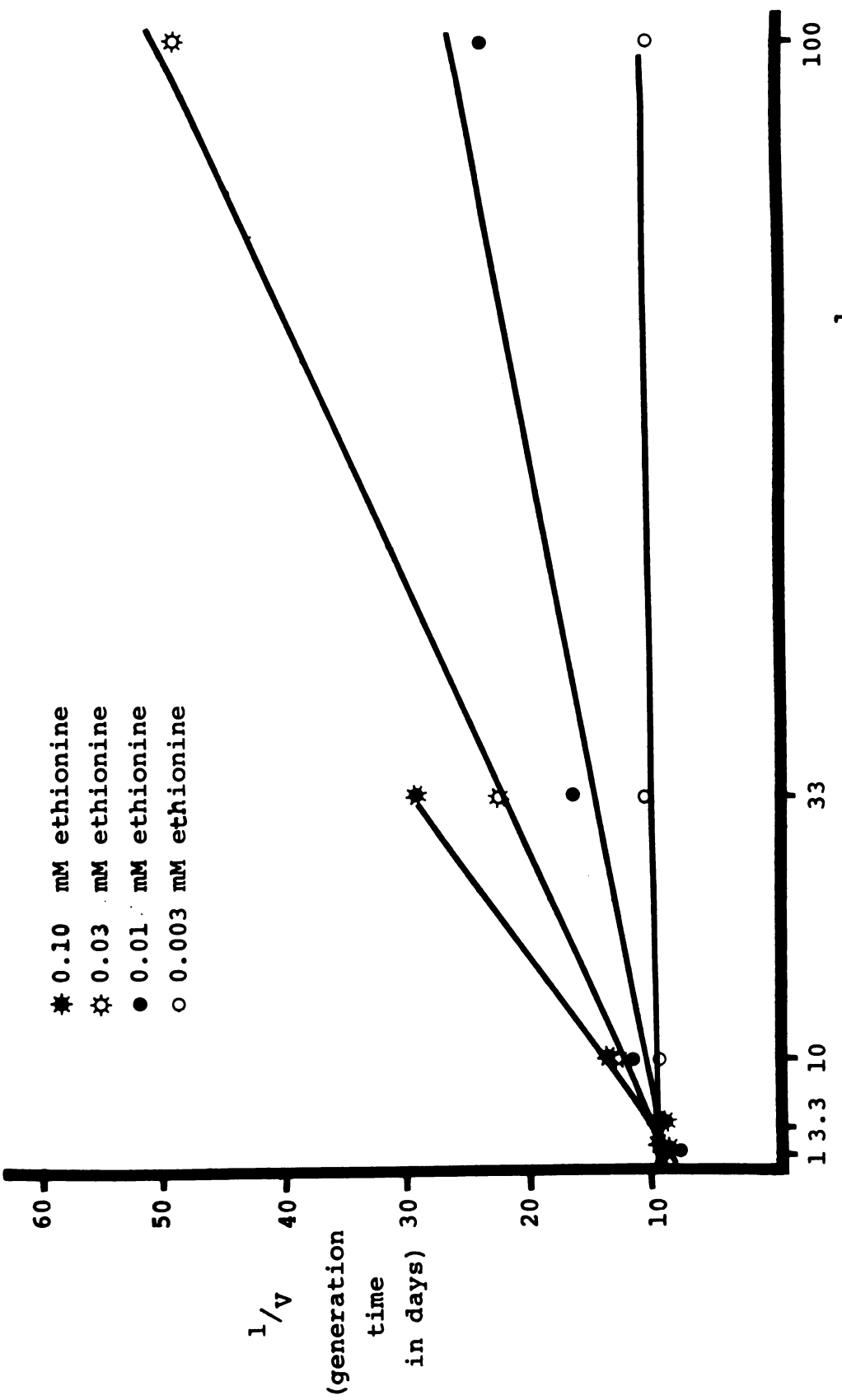
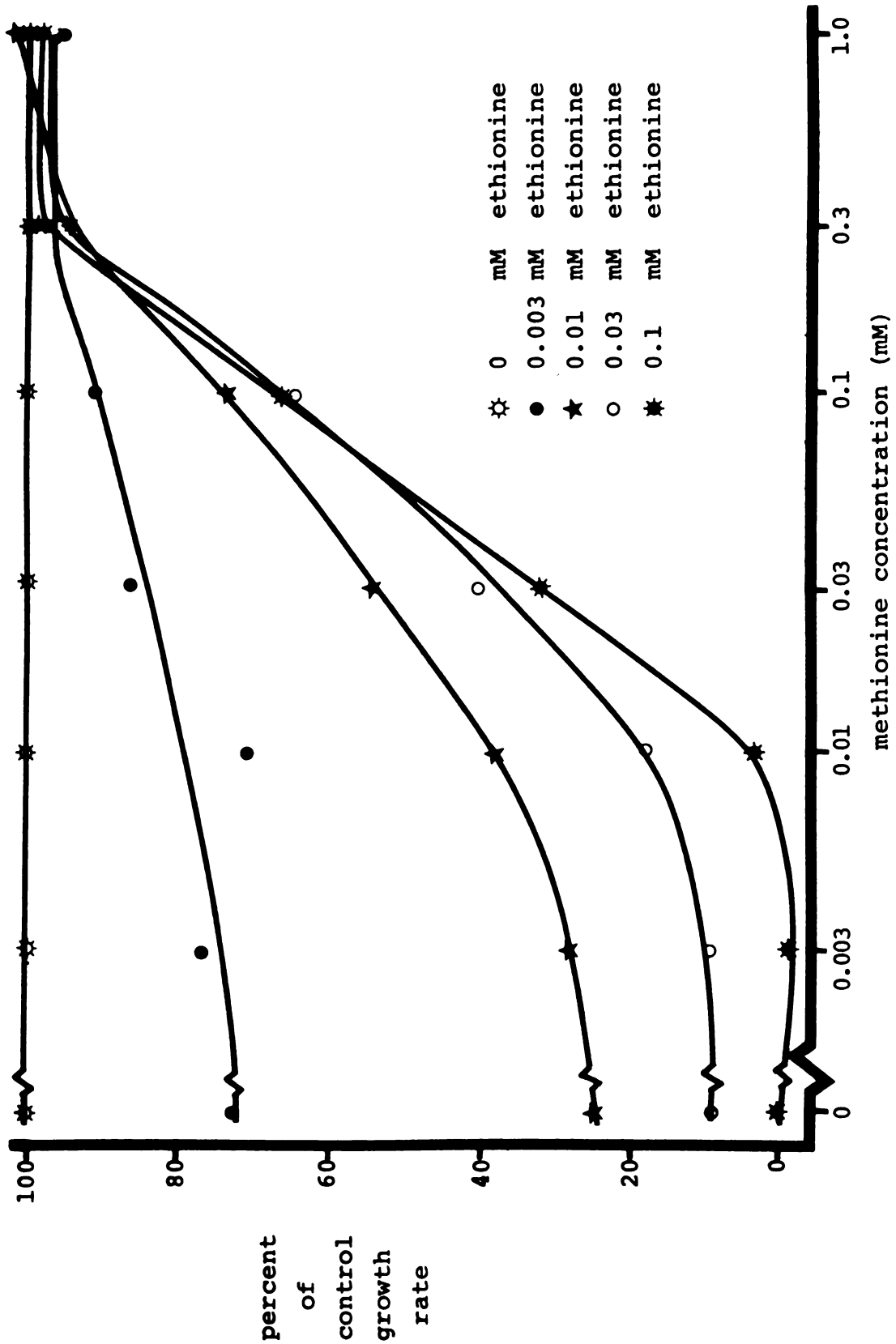

0.10 mM ethionine
0.03 mM ethionine
0.01 mM ethionine
0.003 mM ethionine
1/v (generation time in days)
60
50
40
30
20
10
1
3.3
10
33
100
1/S (mM methionine concentration)-1

Figure 11. Reversal of ethionine-induced growth inhibition in 'W-973' potato cell cultures.


percent of control growth rate
100
80
60
40
20
0
0
0.003
0.01
0.03
0.1
0.3
1.0
methionine concentration (mM)
0 mM ethionine
0.003 mM ethionine
0.01 mM ethionine
0.03 mM ethionine
0.1 mM ethionine

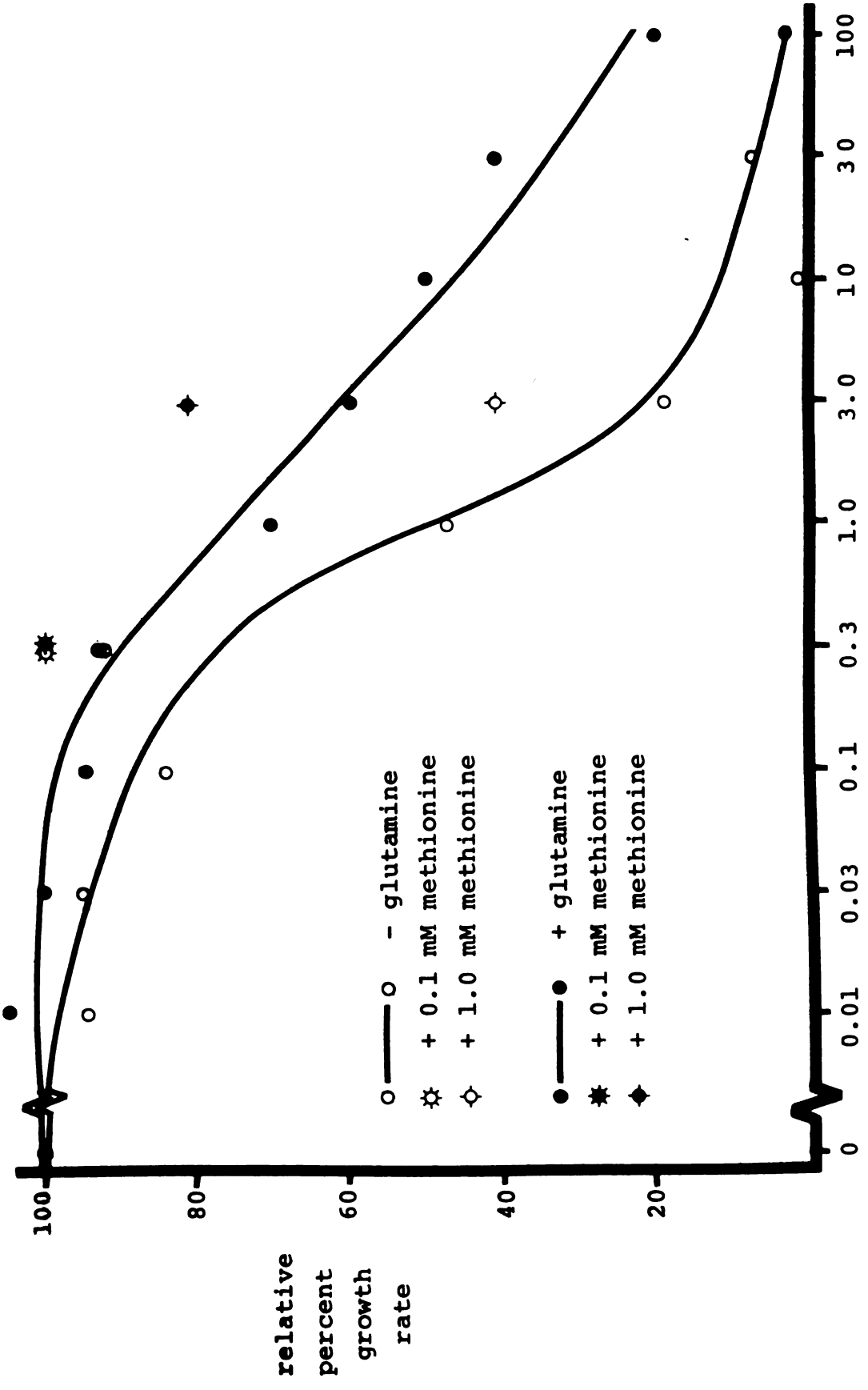
Glutamyl-γ-Methyl Ester

Glutamyl-γ-methyl ester (GluOMe) is an amino acid analog known to antagonize glutamate through its activity with glutamyl-tRNA synthetase in *E. coli*. In the course of screening *E. coli* for resistance to GluOMe, mutants were isolated which mapped to genes associated with the methionine pathway (53). These mutants also exhibited resistance to other methionine analogs though not to analogs of other amino acids. The cross resistance patterns to other methionine analogs proved compatible with those of mutants known to reside in the methionine biochemical pathway. Since GluOMe had not previously been recognized as an analog of methionine, it was of interest to determine whether GluOMe might exhibit methionine analog activity in a higher plant.

An inhibition curve for GluOMe within the range of 0 - 100 mM was determined both in the presence and absence of supplemental glutamine. Reversal of GluOMe by methionine was also examined. As is evident in Figure 12, the inclusion of glutamine in the culture medium confers resistance to the analog. Whether this indicates that GluOMe also acts as an analog of either glutamine or an immediate product of glutamine is not clear. It is possible that activity as a dual analog may not even be cause for great concern. Another methionine analog, methionine sulfoximine, is also rescued by methionine in *E. coli* and has been successfully used for the selection of methionine overproducers in tobacco (16), yet this analog also acts as an analog of glutamine.

Figure 12 shows that GluOMe toxicity is partially reversed by

Figure 12. Inhibition of growth rate in 'W-973' potato cell cultures by glutamyl-γ-methyl ester.


relative percent growth rate
100
80
60
40
20
0
0.01
0.03
0.1
0.3
1.0
3.0
10
30
100
glutamic acid-γ-methyl ester (mMoles/l)
- glutamine
+ 0.1 mM methionine
+ 1.0 mM methionine
+ glutamine
+ 0.1 mM methionine
+ 1.0 mM methionine

nontoxic levels of methionine. This observation suggests that GluOMe may well be useful for selecting methionine overproducers in plant cells. Selections might most effectively be performed in the absence of added glutamine since glutamine-free medium provided a greater growth differential between GluOMe toxicity and methionine rescue. Selection on glutamine-free medium might also identify variants which need not produce self-inhibitory quantities of methionine.

Selection Strategies Utilizing Feedback Inhibition

Preliminary Considerations

The extensive system of feedback controls operating within the aspartate family of amino acids has led to the development of selection schemes capable of favoring growth in cells expressing a reduced sensitivity to feedback regulation. Selection for concomitant resistance to lysine and threonine has been performed in maize and barley with the objective of isolating mutants in which lysine content may be enhanced. Selection in partially organized maize tissue cultures has revealed a mutant with diminished sensitivity to the lysine sensitive isozyme of aspartate kinase (40). This mutant expresses an approximately 1.3 fold increase in free lysine and a four fold increase in free methionine in homozygous seed but is most remarkable for a 75 fold increase of free threonine (C.E. Green, personal communication). It is very likely that threonine enhancement in this mutant is due to stimulation of threonine synthase activity by elevated levels of S-adenosylmethionine (refer to Figure 1). In experiments similar to those with maize, selections performed with M_2 embryos from mutagenized barley seeds resulted in the

identification of adominant gene responsible for increased concentrations of lysine, threonine, and methionine (10).

Cysteine-Threonine Selection

Reports concerning selections based upon the combined effects of cysteine and threonine do not exist. However, feedback regulatory controls described for homoserine dehydrogenase suggest that cysteine-threonine selection might be useful in identifying variants with deregulated carbon flow to homoserine. Since elevated methionine levels might overcome cysteine-threonine inhibition, the effect of cysteine and threonine on growth of potato cell cultures was investigated.

Cysteine-threonine studies were performed with 'W-973' cultures. Selection of cysteine and threonine levels was based upon previous determinations of growth inhibition individually induced by these amino acids. Cysteine (free base) levels of 3, 11, 21, and 38 mM and threonine concentrations of 1, 2.1, 3.3, and 7.2 mM had previously been determined to result in 0, 10, 25, and 50 percent growth rate depression, respectively. These levels of inhibition were selected so as to provide evidence for synergism. Cysteine-threonine combinations containing cysteine at 11 mM or less reduced growth rate no more than 43% while at cysteine levels of 21 mM or more all cultures were killed.

A subsequent experiment was performed to identify cysteine-threonine combinations which might result in growth rate reductions greater than the moderate levels observed yet less stringent than the most severe. In this second experiment cysteine HCl was substituted

for the free base. Table 2 reveals a very different pattern of response than that observed in the preliminary experiment. The pattern of growth inhibition closely paralleled the response elicited by threonine alone with little effect attributable to cysteine. This result indicates that selection for resistance to cysteine HCl-threonine may be of no more value than selecting for resistance to threonine alone.

The basis for discrepancy in these two experiments is not known. While it is tempting to attribute differences to the form or batch of cysteine used, the results might just as easily be attributed to differences in inherent cysteine sensitivities of 'wild type' cells. It is well known that cell cultures maintained for long periods of time can be separated into subclones which are distinguishable from the parental population (56). The potato cultures designated as 'wild type' were under continuous selection for characters associated with good culturability. It is very possible that subclones with altered sensitivity to amino acid inhibition were inadvertantly selected. Even in the absence of selection for culturability characteristics, unselected differences in amino acid sensitivity could occur. This possibility is supported by the frequent observation that apparently identical subclones isolated from apparently homogeneous regions from a single callus mass and propagated under identical conditions for a period as brief as four or five cell generations can respond very dissimilarly when treated as replicates in the same experiment. Such instability in wild type populations required frequent standardization of subclone response to individual amino acids and analogs. Of all the amino acids and analogs

Table 2. Influence of various concentrations of cysteine and threonine on growth rate in 'W-973' potato cell cultures.[z]

cysteine (mM)	threonine (mM) 0	3	6	15	50
0	100	62	51	37	25
5	100	78	62	45	34
8	86	73	48	39	33
10	74	68	50	38	35
12	82	68	57	47	35
20	81	44	42	36	29

z- values are growth rate means relativized against the growth rate of cells cultured on medium devoid of cysteine and threonine.

which have been restandardized over time, wild type cell sensitivity to cysteine shifted most dramatically.

Lysine-Threonine Selection

End product regulation of aspartate kinase activity by lysine and threonine results in growth inhibition due to methionine starvation. Mutations which desensitize an aspartate kinase isozyme to feedback inhibition should result in the resumption of carbon flow through the aspartate pathway and thereby relieve methione deficiency. In the absence of artificially maintained levels of lysine and threonine, cells with altered aspartate kinase feedback sensitivity should synthesize aspartate amino acids at rates greater than in regulated cells. Selection for resistance to the joint effects of lysine and threonine should therefore provide a means for enhancing free methionine levels.

To determine the effects of lysine and threonine on growth of

potato cell cultures various combinations of these amino acids were added to D_3 + Gln_{1000} medium. 'W-973'cultures were prepared as described under 'Growth Assay'. Figure 13 depicts the response of potato cells to lysine-threonine induced growth inhibition. Unexpectedly, the surface topography is very irregular and displays frequent departures from what might be anticipated in a model based upon concerted feedback inhibition. Nevertheless, the establishment of a topographical feature by several contiguous points was deemed as evidence for a real effect. Of particular interest is the relief of lysine inhibition in the 85 to 140 mM range conferred by low levels of threonine. From this observation it might be inferred that lysine sensitive aspartate kinase represents the bulk of the total aspartate kinase activity. Its inhibition by excess lysine would be expected to lead to cell deprivation of both threonine and methionine. Threonine supplementation of lysine inhibited cells may then restore growth rate to the level observed in cells inhibited by threonine alone. At high levels of both lysine and threonine, cell death occurs.

It has recently been reported that high concentrations of lysine antagonize arginine dependent processes (17). In this context, growth inhibition observed in lysine-threonine treated cells may not be attributed wholly to methionine starvation.

Figure 13. Response surface topography of growth inhibition in 'W-973' potato cell cultures induced by lysine plus threonine.

Interactions Among Lysine, Threonine, Cysteine, and Methionine

The effects of lysine plus threonine and cysteine plus threonine on growth of potato cell cultures have already been described. It was of interest as well to determine whether lysine, threonine, and cysteine might depress growth rate to an even greater extent when acting in concert. Additionally, it was necessary to determine the degree to which supplemental methionine could alleviate growth inhibition due to lysine, threonine, and cysteine individually and in various combinations. A factorial experiment was designed to assess these interactions.

'W-973' potato cultures were transferred to D_3 + Gln_{1000} medium containing 2.5 mM MES (pH 5.7) and various combinations of lysine, threonine, cysteine, and methionine. Distinct growth inhibition was observed over the range of each individual amino acid except cysteine (refer to Table 3). Methionine relieved growth inhibition due to threonine but was not effective in reversing lysine inhibition. Growth depression due to lysine plus threonine was not consistently observed, nor was it regularly relieved by methionine. Cysteine generally exerted little effect on lysine-induced growth inhibition. However, cysteine partially reversed growth reduction due to threonine. Interactions among lysine, threonine and cysteine were weak. Had arginine been included in the culture medium, methionine reversal of lysine-threonine inhibition may well have been more pronounced (17).

Table 3. The influence of lysine, threonine, cysteine, and methionine and their interactions on growth of 'W-973' potato cell cultures.[z]

Lysine (mM)		0				10				50				120			
Threonine (mM)		0	1.5	5	50	0	1.5	5	50	0	1.5	5	50	0	1.5	5	50
Cysteine (mM)	Methionine (mM)																
0	0	100	93	61	29	96	97	60	37	92	74	70	31	34	34	48	28
	0.3	105	112	97	66	94	108	87	70	90	103	90	72	17	32	43	37
	3	76	85	79	76	82	87	84	79	79	86	59	86	41	40	58	61
12	0	93	92	92	43	100	91	100	56	92	86	80	64	19	56	62	44
	0.3	95	82	78	61	93	97	92	63	103	87	87	65	20	29	26	45
	3	56	53	64	61	65	58	70	62	72	58	60	66	33	34	32	38
16	0	105	92	77	47	107	93	70	44	118	87	61	54	11	22	23	51
	0.3	101	82	81	49	86	89	92	66	86	94	77	68	24	23	29	41
	3	60	62	48	65	60	68	64	58	66	63	57	65	25	29	26	34

[z] Values are growth rate means relativized against a control lacking lysine, threonine, cysteine, and methionine in the culture medium. Each treatment was replicated a minimum of four times. The relativized experiment-wise standard error is 4.3.

Reversal of Analog and Feedback Inhibitor-Induced Growth Rate Depression by Methionine and Aspartate Pathway Intermediates

Specific reversal of end product induced growth depression by methionine precursors synthesized downstream of the target block in methionine synthesis indicates that, (1) no other enzymes occurring downstream of the feedback inhibited enzyme are also subject to feedback inhibition by end product(s) effective against the target enzyme and, (2) a variant which overcomes end product growth inhibition by virtue of relaxed feedback regulation will likely synthesize methionine at elevated rates. Similarly, reversal of growth inhibition due to methionine analogs by methionine and methionine precursors indicates, (1) the capacity of methionine to overcome analog-induced growth depression and, (2) the noninterference of analog with upstream processes in methionine synthesis. Each of these conditions is required for analog selection of a methionine overproducer.

To test these criteria, methionine and the methionine precursors, homoserine and homocysteine, were challenged with methionine analogs and with proposed feedback inhibitor selection systems. Table 4 demonstrates complete reversal of ethionine-induced growth inhibition by a two fold excess of methionine. Very weak relief of growth inhibition was conferred by homocysteine at ten fold molar excess. Equimolar concentrations of either homoserine or homocysteine were without effect. Nevertheless, higher concentrations of these compounds might be expected to overcome ethionine inhibition. It was unanticipated that neither methionine nor its precursors would reverse

Table 4. Relief of analog and feedback inhibitor-induced growth depression in 'W-973' potato cell cultures by methionine and methionine precursors. [z]

	compensating metabolites absent	homoserine		homocysteine		methionine	
		0.1 mM	1.0 mM	0.1 mM	1.0 mM	0.1 mM	1.0 mM
inhibitor-free	100 ± *4*	101 ± *4*	97 ± *4*	101 ± *5*	95 ± *4*	94 ± *5*	79 ± *3*
0.1 mM ethionine	1 ± *3*	5 ± *5*	—	1 ± *3*	16 ± *3*	58 ± *3*	99 ± *7*
1.0 mM selenomethionine	0 ± *0*	0 ± *0*	0 ± *0*	0 ± *0*	0 ± *0*	0 ± *0*	0 ± *0*
80 mM lys + 12 mM thr	52 ± *4*	54 ± *4*	43 ± *8*	60 ± *2*	46 ± *6*	65 ± *2*	71 ± *6*
21 mM cys + 2 mM thr	0 ± *0*	0 ± *0*	0 ± *0*	0 ± *0*	0 ± *0*	0 ± *0*	0 ± *0*

z- values are relative growth rates ± standard error. Treatments were replicated a minimum of four times.

growth inhibition due to selenomethionine. This suggests that selenomethionine may act noncompetitively with methionine, that toxicity may reach into pathways outside those directly related to methionine synthesis, or that the levels of methionine and its precursors tested were inadequate to overcome growth inhibition. In any event, selenomethionine is decidedly more toxic to the cell than ethionine.

Relief of growth inhibition induced by glutamyl-γ-methyl ester was not examined in this experiment since methionine at concentrations well below those of the inhibitor were shown to provide substantial reversal of growth inhibition.

End product growth depression due to 80 mM lysine plus 12 mM threonine was clearly relieved by 1 mM methionine. Homoserine and homocysteine, however, appeared to have no statistically discernible effect on growth.

Medium containing 2 mM threonine and 21 mM cysteine (provided as free base) was highly toxic and could not be reversed by methionine or methionine precursors.

The choice of an appropriate selection system useful in identifying methionine accumulators is somewhat simplified by the information provided in Table 4. Cysteine-threonine selection is not tenable, primarily because of the failure of methionine to rescue inhibited cells. With selenomethionine rescue by methionine was not demonstrated; however very high methionine levels relative to selenomethionine have negated toxicity in tobacco cells (28). Selenomethionine may still be useful, although less stringent selective conditions may be necessary. Selections based upon lysine-threonine growth inhibition may well prove useful in revealing methionine

overproducers, particularly if arginine supplementation can be shown to increase the ability of methionine to promote growth. In this vein it is particularly noteworthy that methionine failed to relieve lysine-threonine inhibition in callus cultures of *Arabidopsis* and barley (but not in barley seedlings) until arginine was supplied to the culture medium (17).

Ethionine selection may also prove useful in identifying methionine accumulators. Growth inhibition is strongly relieved by methionine and less strongly so by homocysteine, and mutations at sites in addition to the gene for aspartate kinase can result in oversynthesis. Variants of alfalfa (71) and carrot (104,105) in which free methionine pools appear to be elevated have been revealed by ethionine selection.

The usefulness of glutamyl- γ -methyl ester as a selective agent in plant cells also appears promising. Successful reversal of growth inhibition by relatively low methionine supplements suggests that mutants in which methionine need be only moderately elevated can be selected.

IV. SELECTIONS AND RESISTANCE PROPERTIES OF RECOVERED VARIANT CELL LINES

Introduction

Cell selections were performed with three of the selection strategies described earlier. Of these strategies ethionine and selenomethionine were anticipated to produce useful variant cell lines. Selection for resistance to cysteine-threonine was deemed less promising but nevertheless was performed to see what types of variants might appear.

Ethionine Selection and Variant Characterization

A spontaneous ethionine resistant variant from 'W-973' identified among callus cultures growing on 0.1 mM ethionine has already been mentioned. This variant, clone E1, appeared as one of several healthy proliferating nodules on a background of deteriorating cells. Further variants were sought using a more intense selective pressure of 0.5 mM ethionine.

Suspension cultures of clone 'W-973' were prepared by adding six grams (5.4×10^7 cells) of friable agar-cultured callus to 30 ml of liquid $D_3 + Gln_{1000}$ medium in 125 ml erlenmeyer flasks. These suspension cultures were placed on a gyrotory shaker operating at 125 rpm at ambient room temperature in darkness. Following six days of acclimatization, filter sterilized ethionine was added to provide

a final dilution of 0.5 mM. Seventy days later the cultures were decanted. The cells were spread onto fine mesh nylon microfilament discs and transferred to nonselective solid medium for a three week growth period. A second cycle of selection was then initiated by transferring the nylon screens to solid medium containing 0.1 mM ethionine.

Among 15 original ethionine-treated suspension cultures, 13 produced from three to several hundred colonies on nonselective medium. Resistance was retained in colonies from 10 flasks following a second cycle of selection. From each of the latter 10 flasks a single, healthy colony was isolated; these were designated clones E3 - E12. Thereafter these variant clones were maintained on D_3 + Gln_{1000} medium. The frequency of resistance was calculated to be 9.4×10^{-10} on the basis of the number of flasks retaining ethionine resistance after two cycles of selection.

Although ethionine resistant variants E3 - E12 successfully survived two cycles of ethionine selection, it was of interest to determine whether these clones might retain their resistance following long term growth in the absence of selection pressure. Accordingly, wild type potato cell clone 'W-973' and ethionine selected clones E3 - E12 were cultured on D_3 + Gln_{1000} medium containing 0, 0.01, and 0.1 mM ethionine following 260 days of continuous growth on ethionine-free medium.

Table 5 demonstrates that all variants expressed high level resistance to 0.01 mM ethionine after greater than eight months' unchallenged growth. At 0.1 mM ethionine, 'W-973' and E9 were totally inhibited while the remaining variants continued to grow. The

Table 5. Long term resistance to growth inhibition by ethionine among ethionine-resistant potato clones.

clone	generations of growth on ethionine-free medium	unselected growth rate relative to W-973 [z]	percent of normal growth rate on 0.01 mM ethionine [y]	percent of normal growth rate on 0.1 mM ethionine [y]
W-973	—	100.0 ± *2.0*	34.5 ± *2.2*	-6.1 ± *1.0*
E3	23	98.7 ± *7.2*	80.9 ± *7.2*	19.9 ± *10.6*
E5	28	121.4 ± *3.4*	83.1 ± *2.6*	36.5 ± *2.1*
E6	16	70.2 ± *0.9*	98.3 ± *4.6*	8.2 ± *3.0*
E7	22	93.5 ± *5.6*	72.4 ± *5.0*	18.9 ± *2.2*
E8	19	80.3 ± *1.9*	85.2 ± *2.5*	5.3 ± *4.6*
E9	15	65.8 ± *2.3*	74.8 ± *7.1*	-15.6 ± *1.7*
E10	35	150.7 ± *6.5*	96.2 ± *1.4*	36.8 ± *5.1*
E11	25	109.6 ± *2.2*	87.2 ± *1.8*	10.4 ± *2.1*
E12	17	71.2 ± *4.0*	104.8 ± *3.1*	25.0 ± *8.4*

z- calculated as (variant growth rate ÷ W-973 growth rate) x 100 ± standard error on D_3+GlN_{1000} medium. Uninhibited growth rate of W-973 was 0.0899 doublings/day.

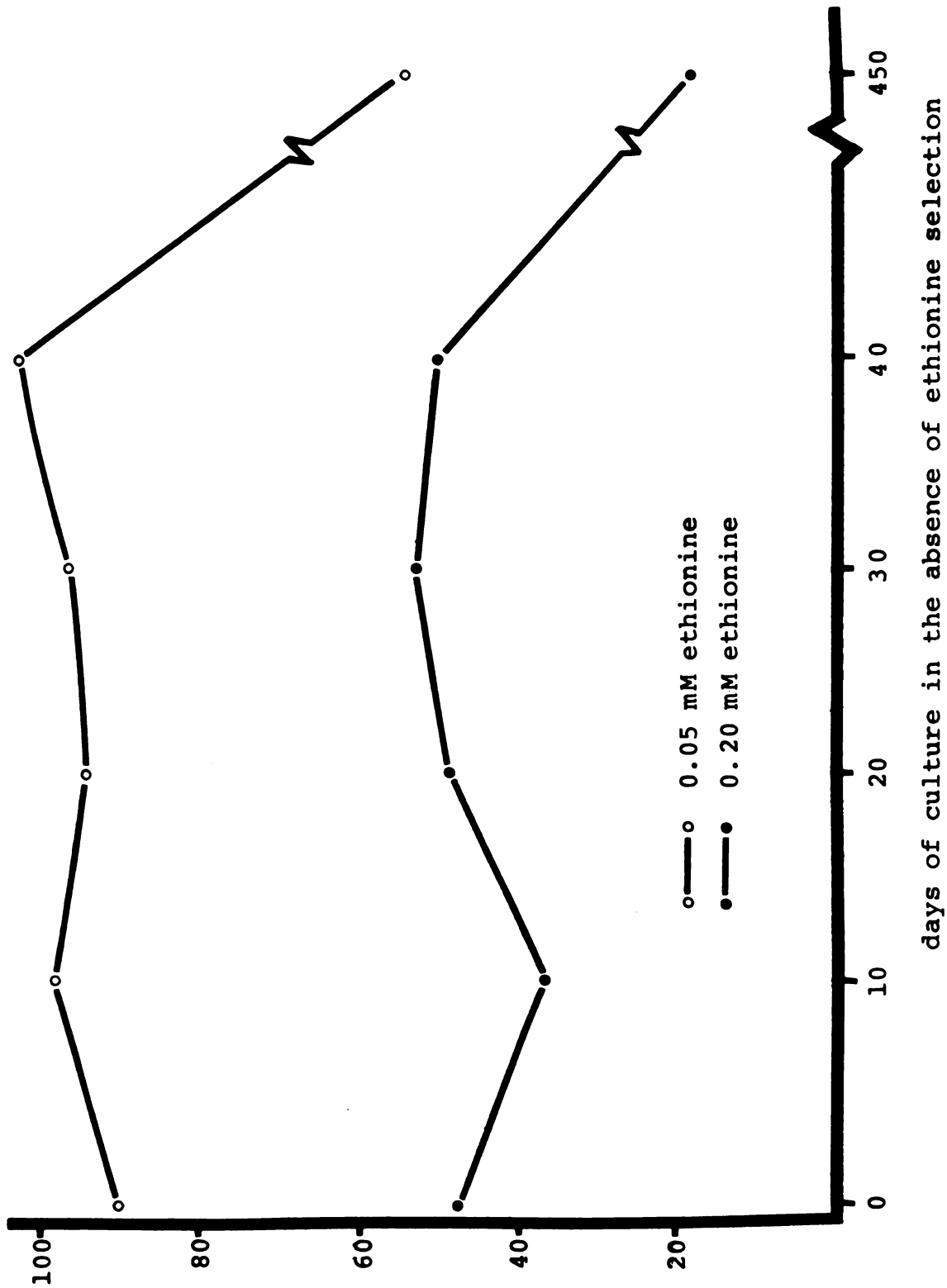
y- calculated as (growth rate on ethionine ÷ growth rate on ethionine-free medium) x 100.

resistant clones fluctuated widely in their inherent, unchallenged growth rates. Clones E5 and E10 exhibited the strongest growth rates on ethionine-free medium and also proliferated to the greatest extent on 0.1 mM ethionine. Further characterization of these two clones revealed a complete absence of cross resistance to 1 mM seleno-methionine. When cultured on medium containing 160 mM lysine and 24 mM threonine, E5, E10, and 'W-973' grew at 20.2 $\pm$ 2, 35.6 $\pm$ 1, and 22.2 $\pm$ 3 percent of their normal growth rates.

Further characterization of clonal stability to ethionine resistance was performed with clone E1, the earliest isolated ethionine resistant potato clone. Shortly following its isolation clone E1 was divided into two subclones. One subclone, E1-0 was continuously subcultured on D_3 + Gln_{1000} medium plus 0.1 mM ethionine while its sister subclone, E1-1, was maintained on nonselective D_3 + Gln_{1000} medium. At various time intervals secondary subclones from E1-0 were transferred to ethionine-free medium.

The resistance of these subclones to ethionine challenge following various periods of time in the absence of ethionine is depicted in Figure 14. Since the unstressed growth rate of each subclone varied, the resistance of each subclone is expressed as a percent growth rate relative to growth on ethionine-free medium. Growth rates of wild type cells were reduced to 16 and 4 percent on 0.05 and 0.2 mM ethionine, respectively. From 0 to 40 days following culture on ethionine-free medium, relative growth rates of rechallenged cells were not substantially altered. In contrast, subclone E1-1, which had been maintained free of selection pressure for 450 days, exhibited a sharp growth reduction when again challenged

Figure 14. Stability of resistance to ethionine among clone E1 derivatives as a function of duration and intensity of selection.


percent nonselected growth rate
100
80
60
40
20
0
10
20
30
40
450
days of culture in the absence of ethionine selection
0.05 mM ethionine
0.20 mM ethionine

with ethionine. Despite this growth reduction its phenotype was still resistant in comparison to wild type cells.

That long term culture in the absence of selective pressure should be associated with partial loss of ethionine resistance might be explained in several ways. Had resistance been due to the maintenance of multiple copies of a major resistance gene by multiplication of the chromosome bearing such a gene, then loss of one or more of these chromosomes umder nonselective conditions would have reduced the level of resistance expressed upon resumption of selective conditions. Alternatively, the partial resistance expressed by subclone E1-1 might indicate an epigenetic component to the high level state of resistance expressed by subclone E1-0. Loss of the epigenetic component could be expected to lead to moderate level resistance.

In a parallel experiment subclone E1-1 was cultured without ethionine and rechallenged 11 cell generations before growth rates were measured. This was done to determine whether E1-1 was composed of two phenotypically, and perhaps genetically, distinct subpopulations, one highly resistant to ethionine and the other moderately resistant. The prechallenge period was sufficiently long to permit a subpopulation of highly resistant cells occuring at a frequency of 2% to accrue to greater than 30% of the total population at the time of growth assessment. When growth rates of prechallenged and nonprechallenged E1-1 cells on ethionine medium were measured, no substantial differences were noted. This indicates that the cells comprising E1-1 are relatively homogeneous with respect to their tolerance to ethionine.

Selenomethionine Selection and Variant Characterization

Selection for resistance to selenomethionine was performed with 'Superior' callus at an analog concentration capable of inducing growth stasis in 'W-973' cultures. Photosynthetic 'Superior' callus cultured under 40 foot-candles of fluorescent light was plated thinly on fine mesh nylon microfilament discs at a rate of 2 g (1.8×10^7 cells) per 30 ml of $N_{10}\ D_2 + Gln_{1000}$ medium containing 1 mM selenomethionine. Thirty plates were selected in the light; fifteen were cultured in darkness. After 80 days of culture the cells were transferred to fresh selection medium. Following two additional 45 day passages on selective medium, four slowly growing resistant clones were isolated and transferred to $N_{10}\ D_2 + Gln_{1000}$ medium. Three of these clones, SEM1, SEM2, and SEM3, originated from cultures selected in darkness. SEM4 was isolated under light selection. The overall frequency of resistant variants was calculated directly as 2×10^{-8}.

Selenomethionine resistant variants were subsequently cultured for 160 days on selenomethionine-free medium. They were then retested for resistance to growth inhibition by the seleno-analog as well as to ethionine and lysine plus threonine. A feature common to all selenomethionine selections was their sharply reduced unstressed growth rates relative to wild type 'Superior' callus. This may indicate a greater than necessary stringency of the original selection procedures since clones with diminished metabolic rates would tend to be favored in an inhibitor-laden environment. The wild type clone proved sensitive to selenomethionine while SEM1

Table 6. Resistance of selenomethionine-selected potato clones to growth inhibition by selenomethionine, ethionine, and lysine plus threonine.

clone	generations of growth on selenomethionine-free medium	unselected growth rate relative to Superior [z]	percent growth rate on 1 mM selenomethionine [y]	percent growth rate on 0.1 mM ethionine [y]	percent growth rate on 160 mM lys + 24 mM thr [y]
Superior	—	100.0 ± *2.6*	18.7 ± *0.5*	-5.8 ± *0.7*	-6.3 ± *1.2*
SEM 1	15	40.9 ± *1.4*	76.3 ± *2.4*	47.6 ± *4.1*	—
SEM 2	21	55.8 ± *1.2*	-6.8 ± *3.6*	3.9 ± *4.2*	—
SEM 3	21	56.8 ± *1.3*	-20.4 ± *3.6*	76.1 ± *9.3*	—
SEM 4	13	34.4 ± *0.7*	43.1 ± *3.4*	0.4 ± *8.1*	-6.4 ± *1.9*

z- calculated as (variant growth rate ÷ Superior growth rate) x 100 ± standard error on N_{10} D_2 + GlN_{1000} medium. Uninhibited growth rate of Superior callus was 0.2345 doublings per day.

y- calculated as (growth rate on medium containing inhibitor ÷ growth rate on inhibitor-free medium) x 100 ± standard error.

and SEM4 exhibited strong and moderate resistance, respectively (see Table 6). Clones SEM2 and SEM3 had lost all their earlier expressed resistance. Cross resistance to a first time exposure to 0.1 mM ethionine was noted for SEM1 and SEM3. SEM2 and SEM4 proved sensitive to ethionine. One clone, SEM4, was examined for tolerance to lysine plus threonine, and like wild type callus, proved sensitive.

Among the four selenomethionine selected variants all possible combinations of resistance and sensitivity to selenomethionine and ethionine appeared. The dual resistance expressed by SEM1 may conceivably be due to analog competition due to enhanced methionine production or to mutual discrimination by an altered permease. Selenomethionine resistance in SEM4 appeared to be due to a mechanism specific to this analog since ethionine completely suppressed growth. SEM3 is an anomaly. This variant proved highly resistant to 0.1 mM ethionine but lost all earlier expressed resistance to selenomethionine during growth away from this analog. SEM2 was also no longer resistant to selenomethionine and proved sensitive to ethionine as well.

Cysteine-Threonine Selection

Selection for concurrent resistance to cysteine and threonine was performed as a test to determine whether such a selection might result in methionine overproduction. On the basis of the regulatory controls operating in the aspartate amino acid family, this selection would appear to be directed toward homoserine dehydrogenase activity. Desensitization of homoserine dehydrogenase might be expected to increase carbon flow through the pathway. The inability of

supplemental methionine to reverse cysteine-threonine induced growth rate depression was described earlier. Nevertheless it was considered of interest to isolate resistant variants since such selections had not previously been described.

For selection six grams of 'W-973' cells were dispersed per 125 ml erlenmeyer flask containing 35 ml D_3 + Gln_{1000} liquid medium with 21 mM cysteine (free base) and 2 mM threonine. Flasks were dark incubated on gyrotory shakers rotating 125 rpm. Selection continued over a 90 day period during which the culture medium was once decanted and replenished. Following selection only three of thirty-two flasks contained viable cells. One isolate from each was maintained on D_3 + Gln_{1000} medium. The frequency with which resistant variants appeared was calculated as 3.9×10^{-11}.

The three surviving clones, designated CT1, CT2, and CT3, were maintained for 360 days in the absence of selection. Upon retesting these clones for resistance to 21 mM cysteine and 2 mM threonine, all were killed. Resistance in these clones may therefore have been epigenetic.

V. AMINO ACID ANALYSIS OF WILD TYPE AND VARIANT CLONAL SELECTIONS

Methionine-Specific Assays

To assess whether endogenous methionine levels in variant clonal selections correlate with the degree of resistance to methionine analogs, it was necessary to select a methionine assay procedure sufficiently sensitive to discriminate between real and trivial differences in both free and protein-bound methionine levels.

Turbidometric microbial bioassays utilizing *Streptococcus zymogenes* or *Leuconostoc mesenteroides* have frequently been used to estimate methionine levels in biological material including potato (57). Such tests detect an *available* fraction of the *total* methionine present.

Several assays dependent upon specific chemical properties of methionine have been described. Gehrke (33) devised an automated method for determining methionine levels in hydrolyzed seed meals. This assay is based upon a methionine-nitroprusside color reaction. A second assay depends upon the selective oxidation of methionine by chloramine T (98). Due to its simplicity and promised specificity, considerable effort was invested to adapt this procedure for use with potato tissue.

The chloramine T assay originated from the observation that under mildly alkaline conditions methionine alone among the common amino

acids is oxidized (81). Upon oxidation of methionine to methionine sulfoxide with known excess of chloramine T, the remaining chloramine T can be assayed in a second reaction. Unreacted chloramine T oxidizes the highly colored reagent, 2-nitro-5-thiobenzoic acid (NTB) to a colorless species, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). The quantity of chloramine T remaining after the methionine oxidation step can be determined spectrophotometrically at 412 nm. Absorbance at 412 nm is therefore directly related to the quantity of methionine originally present. Positive interference due to sulfhydryl or amino groups can be removed by prior acylation with diethylpyrocarbonate (DEPC). Although DEPC also reacts with chloramine T, small quantities of DEPC hydrolyze to CO_2 and ethanol within several hours.

In a preliminary examination of the chloramine T method of methionine estimation, the procedures suggested by Trout (98) were tested. By this method the relation between methionine concentration and absorbance at 412 nm was linear for methionine within the range of 0.12 and 1.88 µg/ml.

To more closely simulate true sampling conditions the effectiveness of DEPC as a remedy for amino and sulfhydryl interferences was examined. Methionine standards were assayed in the presence of glutamine and cysteine at levels anticipated to exceed those which might interfere in potato tuber samples. DEPC was provided at the level recommended by Trout, and acylation accompanied by DEPC decay proceeded for 24 hours.

Positive interferences proved not to be completely suppressed. In subsequent tests DEPC levels up to 10 fold that recommended by Trout were used, and the decay period was extended to 48 hours. At

the ten fold DEPC level a very pronounced negative interference was observed due to NTB oxidation. Reducing the DEPC to six fold the recommended level eliminated both DEPC-induced negative interference and the positive interference contributed by amino and sulfhydryl groups. A test could now be performed on potato tissue samples.

A whole, sound tuber of the cultivar 'Superior' was cubed, frozen in liquid nitrogen, lyophilized, and milled to pass a 60 mesh sieve. Free amino acids were extracted by agitation in distilled water or 80% ethanol for periods of 0.5 to 24 hours. Samples clarified by two centrifugations at 12000 X g were than assayed. Samples extracted in 80% ethanol appeared to yield twice the free methionine level that had previously been reported for 'Superior' tubers (42). The apparent methionine levels measured in aqueous extracts were approximately four times greater than the published value. The association of unduly high apparent methionine levels with the development of phenolic substances in these extracts suggested that enzyme activities might well be causing positive interference in the assay. Accordingly, DEPC, which also acts as a potent enzyme inactivator, was included during the extraction step. This adjustment resulted in apparent free methionine levels more comparable to published results. The concentration of DEPC required to reduce positive interferences was so high that residual, undecomposed DEPC interfered with subsequent steps in the assay. Methods to accelerate DEPC decomposition were therefore investigated.

To eliminate DEPC interference,a compound capable of reducing DEPC as well as being easily removed from the assay system was sought. Sodium borohydride is a very potent reducing agent which is reported to

be very reactive with methanol and labile to heat (77). Post extraction reaction of $NaBH_4$ with DEPC eliminated negative interference attributable to DEPC; however, excess $NaBH_4$ could not in turn be purged from the system by heating in the presence of methanol. Therefore attempts fo measure methionine with the chloramine T technique were discontinued in favor of ion exchange chromatography.

Ion Exchange Chromatography

High level resolution and quantitative determination of amino acids is readily provided by ion exchange chromotography (8). With reference to selection systems for methionine overproduction which might be expected to alter synthetic rates of amino acids other than methionine, ion exchange chromatography is useful in comparing amino acid profiles among variant and wild type clones. Recognition of altered amino acid spectra coupled with the regulatory phenomena associated with amino acid synthesis can reveal the genetic lesion underlying a novel variant phenotype. For this reason alone the additional effort and expense involved in generating the complete amino acid profile for a variant clone is justified.

One of the more critical steps in automated amino acid analysis relates to sample preparation. For carbohydrate-containing samples, the conditions under which acid hydrolysis is performed become especially crucial (78). The sulfur amino acids as well as tryptophan, serine, threonine, and tyrosine are highly susceptible to degradation and require special treatment (51, 62, 76, 87). Performic acid oxidation of cysteine to cysteic acid and methionine to methionine sulfone (41, 66) or derivatization of cysteine to acid stable compounds

(31, 76) prior to hydrolysis permit quantitative determination of the sulfur amino acids. Alternatively, providing mercaptoacetic acid or mercaptoethanol during hydrolysis enhances methionine recovery (62, 87). Tyrosine and serine recoveries are improved by addition of phenol (51, 76). Preparation of free amino acid samples requires that all free amino acids are extracted under conditions that do not degrade polypeptides. Furthermore, soluble proteins must be excluded from the sample (49).

Amino acid analyses were performed on potato tissues representing variable levels of morphogenetic organization, physiological state, and resistance to *in vitro* selection procedures. Callus cultures of wild type and variant 'W-973' clones were dark grown at 24° C on D_3 + Gln_{1000} medium for 26 days (late exponential phase) before harvest. Wild type callus cultured on medium lacking glutamine was included as a control to assess the effects of supplemental glutamine on the resulting amino acid profile. Callus cultures of wild type and variant 'Superior' clones were raised at 24° C under 40 foot-candles of fluorescent light. Cells were harvested following 16 days of culture (mid-exponential phase) on N_{10} D_2 + Gln_{500} medium. Young, fully expanded leaves were collected from greenhouse grown plants. Field grown tubers from 'Superior' were stored two months at 4° C prior to sampling. Tubers from 'W-973' were freshly harvested from greenhouse grown plants. All tissues were frozen in liquid nitrogen, lyophilized, milled to pass a 60 mesh screen, and stored dessicated and frozen at -20° C before use.

For free amino acid extractions a carefully weighed tissue sample of approximately 50 mg was vortexed in 1 ml of 1% sulfosalicylic acid

containing 1 µmol norleucine and 10 µmols dithiothreitol. This mixture was then agitated for one hour on a gyrotory shaker adjusted to 125 rpm. The sample was twice centrifuged for 15 minutes at 12000 X g, the pH was adjusted to 2.2 with crystalline lithium citrate, and the volume was doubled with lithium citrate buffer. A white flocculent, precipitate was removed by a final centrifugation, and the sample was filtered through a 0.2 µm nitrocellulose filter before ion exchange chromatography.

For total amino acid digests tissue samples of approximately 6 mg were very carefully weighed in pretared 40 ml ignition tubes. To each tube was delivered 6 ml of 6 N HCl containing 0.5% phenol, 0.67 µmols norleucine, and 300 µmols dithiothreitol. After degassing the samples the tubes were flushed with nitrogen and tightly sealed with teflon lined caps. Acid hydrolysis proceeded for 16 hours in an autoclave at 121° C. Digested samples were filtered, ether extracted, and evaporated under vacuum on a rotory evaporator at 55° C. After two cycles of dissolving the residue in 10 ml water followed by rotory evaporation, the final residue was taken up in 1 ml of lithium citrate buffer plus 1 ml of 0.01 N HCl, and the sample was twice centrifuged for 15 minutes at 12000 X g. Dithiothreitol was added to provide a 50 mM concentration and the sample was then stored in darkness at 35° C for 72 hours to reduce methionine sulfoxide to methionine (43). After filtration through a 0.2 µm nitrocellulose filter the sample was analyzed. In all sample preparations great care was taken to assure cleanliness of glassware and reagent purity. Double-distilled deionized water was used for all reagent stock solutions and sample dilutions.

Ion exchange chromatography was performed on a 27 cm X 3.2 cm single column instrument utilizing Dionex DC-4 resin in conjunction with a Pico IV lithium buffer and ninhydrin detection system. The flow rate was 0.1 ml per minute. Chromatography proceeded at 37° C to glycine elution whereupon the temperature was increased at a rate of 1° per minute to 60° C. A single amino acid profile was determined for each tissue sample. Traces were hand integrated for all amino acids, and actual concentrations were determined by relation to a norleucine internal standard after compensation for absorbance factors specific to each amino acid.

The capabilities of this system in large measure determined the methods of sample preparation. Since protected sulfur amino acid derivatives such as methionine sulfone and cysteic acid are not well resolved from other amino acids in this system, it was necessary that unoxidized methionine and cysteine be measured. These compounds are especially subject to degradation so a new means for their protection was assessed. The use of mercaptoacetic acid and mercaptoethanol as methionine protectants had been reported (62, 87), yet because of their noxious nature dithiothreitol was used instead. It was found that 50 mM dithiothreitol during a 16 hour hydrolysis period allowed 39% greater recovery of methionine compared to a control lacking dithiothreitol. Post hydrolysis treatment with 50 mM dithiothreitol of a sample hydrolyzed in the absence of dithiothreitol resulted in 16% greater recovery of methionine. As a result of these observations dithiothreitol was routinely used both to recover methionine from methionine sulfoxide and to prevent methionine destruction during acid hydrolysis.

Cysteine is also recognized as being labile to oxidation and degradation (8). Its oxidation was investigated with dithiothreitol protected and nonprotected digests of insulin. In the absence of dithiothreitol cysteine was completely converted to cystine. Cysteine recovered from samples hydrolysed in the presence of dithiothreitol occurred wholly in the reduced state. In potato tissue cysteine occurs at very low concentrations. Its precise measurement is therefore difficult and subject to error.

Additional limitations imposed by the available chromatography system relate to its inability to resolve several amino acids. Asparagine in free amino acid extracts was not resolved from glutamate. Cystathionine cochromatographed with isoleucine. Homoserine eluted in the tail of glutamate. Glutathione was confounded with the aspartate peak. Cystathionine, homoserine, and glutathione were of particular interest as intermediates and end products of sulfur metabolism in plant cells. Although cystathionine occurs in very low quantities in higher plants, glutathione can be present at greater than 10 fold the level of methionine (20). In this regard it is remarkable that levels of a compound interpreted as cystathionine in wild type and ethionine resistant alfalfa clones ranged from two to ten times the level of recovered methionine (71).

Total Amino Acid Profiles

The primary objective of this project has been to devise *in vitro* selection strategies capable of revealing cell lines whose synthetic capacities for methionine have been enhanced. Realization of this objective requires that stable mutational events leading to an

overproducer phenotype be distinguishable from transitory events which might lead to an apparent overproduction only. It has become a tenet in plant cell culture work that the process of growing cells in culture generates variability. The greater the temporal displacement of two cell lines from their progenitor, the more likely their phenotypes will have diverged. Cell culture conditions tend not to select against aberrant cytogenetic changes which would quickly be suppressed in a whole plant growing in a natural environment. The resulting collage of different euploid, aneuploid, and chromosomally rearranged cell types, with their attendant perturbations in gene dosage, cell cycle time, and gene regulation, provides a highly heterogeneous background against which a phenotype which represents a truly novel genetic element must be distinguished from a phenotype which is merely the consequence of a new pattern of expression for essentially unaltered genetic material. For this reason the patterns of amino acid variability expressed in different variant clones must be compared in different ways and on different bases.

The amino acid profiles presented in Appendices A and B are cataloged so as to allow direct comparisons of individual and total amino acid distributions within and among clones on a reference index related to wild type callus cultures. All data presented are on a molar basis. The 'μmoles per gram' category provides the molar amino acid concentration per gram dry weight as determined by ion exchange chromatography. The 'molar %' category indicates the distribution of any amino acid as a percent of the total amino acid concentration. 'PDI' or percent distribution index is a relative measure comparing the molar % distribution of an amino acid with the molar % distribution

of that amino acid in wild type callus tissue. The 'AAR' or amino acid ratio measures the total amino acid content of a tissue relative to the content of all amino acids in wild type callus. Tables 7 and 8 summarize these indices for methionine alone and also introduce a 'methionine ratio' for comparisons of relative methionine levels among variant lines.

Upon examination of Appendices A and B it quickly becomes apparent that substantial variation in amino acid expression exists among the tissue samples analyzed. Tubers of 'W-973' and 'Superior' possess a much greater total free amino acid pool than wild type callus, yet total amino acids from hydrolysates (AAR) are sharply depressed, indicating much lower relative levels of tuber protein. In contrast leaf tissue contains a much depressed total free amino acid level relative to wild type callus while amino nitrogen in leaf hydrolysates is enhanced. It is noteworthy that total amino nitrogen in leaf tissues is two to three times that observed in the tuber. In tubers of both 'W-973' and 'Superior' the free amino acid pool represented 40% of the total amino nitrogen. Since one third of the methionine in 'W-973' and one half of the methionine in 'Superior' were found in free form, it becomes clearly apparent that methionine accumulation in tubers is not dependent upon its incorporation into protein. It is also notable that free methionine in the tuber represents a much higher proportion of total free amino acids than does free methionine in cell cultures. A small increase in methionine synthetic capacity might therefore translate to substantial quantities in the tuber. These observations strongly support the thesis that potato cell clones selected for enhanced methionine production

might well express this phenotype in the tuber.

Supplemental glutamine increased total free amino acids by more than 50%. Despite this increase the proportion of methionine in glutamine-supplemented cells was half that in cells cultured without glutamine. This observation suggests that the inclusion of glutamine in selective medium may actually increase the stringency of selection.

As an aid in determining whether the various indices of methionine content calculated for each cell line are associated with either total amino acid levels or growth kinetics of the cell lines, correlation coefficients among these variables were computed for ethionine-resistant variants of clone 'W-973' (Table 7). Of critical interest is the association of ethionine resistance with methionine concentration in variant tissue samples. In neither the free pool nor in total digests is such a correlation apparent. This observation suggests that in the majority of ethionine-resistant variants the mechanism of resistance may not be via methionine overproduction.

A paradoxical but explainable correlation is the observation that as the proportion of methionine in the free pool increases, the level of resistance to ethionine decreases. This apparent anomaly can be resolved by noting the high correlation of ethionine resistance with total free pool amino acids. Ethionine resistance then is more likely to be associated with cell lines in which the synthetic capacity for amino acids *other* than methionine is generally enhanced. An inspection of the PDI values for all free pool amino acids of ethionine-selected cell lines exhibiting a total AAR greater than 1.00 reveals that while the proportion of methionine generally decreases, the relative contribution of aspartate to the free pool increases. Glutamine and/or

Table 7. Correlations among ethionine stressed or nonstressed growth rates, methionine concentrations, and total amino acid levels for wild type and ethionine resistant variant cell lines derived from cultures of clone 'W-973'.[z]

	nonstressed growth rate[y]	ethionine stressed growth rate[x]	µmoles/g methionine[w]	molar % methionine[v]	AAR[u]
nonstressed growth rate[y]		*+.674** s*	*-.219*	*-.114*	*-.292*
ethionine stressed growth rate[x]	+.674** t		*+.116*	*+.266*	*-.096*
µmoles/g methionine[w]	+.272	+.077		*+.823***	*+.865***
molar % methionine[v]	-.292	-.650*	+.367		*+.382*
AAR[u]	+.451	+.723**	+.492	-.686**	

z -correlation coefficients have been adjusted for small sample size. significance tests distinguish between $\rho = 0$ vs $\rho < 0$ or $\rho > 0$.

y -correlations calculated from growth rates in doublings per day for wt and variant clones E1-E12 cultured on medium D3.

x -correlations based on relativized growth rates calculated as (doublings per day on medium D3 + $\text{Ethionine}_{0.1}$ ÷ doublings per day on medium D3) x 100 for each of wt and variant clones E3-E12.

w -µg methionine per g dry weight of samples harvested from late exponential phase callus cultures for wt and variant clones E1-E12. Correlations using 'µg/g' are equivalent to correlations based upon 'MR'.

v -molar percent methionine = (moles methionine ÷ total moles of all amino acids) x 100. Correlations using 'molar percent methionine' are equivalent to correlations based on 'PDI'.

u -AAR or amino acid ratio is calculated as total moles of all amino acids in variant samples ÷ total moles of all amino acids in wild type samples.

t -correlations in elite type are for free amino acid pools.

s -correlations in *italic* type are for total sample hydrolysates and include free plus protein bound amino acids.

* - $\rho < 0$ or $\rho > 0$ at $\alpha = 0.05$.

**- $\rho < 0$ or $\rho > 0$ at $\alpha = 0.01$.

glutamate tend to increase as well. This suggests that these amino acids somehow interfere with ethionine.

The indices of methionine concentration presented in Tables 8 and 9 allow an assessment of whether methionine has in fact been increased in any variant cell lines. Among variant derivatives of clone 'W-973' few exhibit elevated methionine concentrations. Clone E3 contains approximately 20% more methionine in both the free pool and in total digests. This clone is also characterized by an exceptionally large total free amino acid pool. Clone E5, a selection which is highly resistant to ethionine, is similarly distinguished by a large free amino acid pool. Its methionine ratio in total hydrolysates is 1.44. Since its amino acid ratio for the total hydrolysate is not greatly increased compared to 'W-973', methionine may be accumulated by incorporation into protein(s) which are overexpressed relative to other proteins. A very high free methionine pool exists in clone E11; however, the high concentration of the free pool is not reflected in the levels found in total tissue digests.

Among selenomethionine-selected clones SEM1 and SEM3 are clearly distinguished from wild type in their methionine content. SEM1 was earlier shown to be highly resistant to selenomethionine and ethionine while SEM3 is resistant to ethionine alone. For SEM1 free methionine is elevated 2.29 times that of wild type cells. This increase is the result of both a 30% increase in total free amino acids and a 76% greater proportion methionine in the free pool. SEM3 also expresses a greater proportion of its total free amino acid pool as methionine yet accumulates free amino acids to a lesser extent than wild type cells. The increase in free methionine concentration in

Table 8. Measures of free and total methionine in 'W-973' cell line derivatives and whole plant organs.

	free pool					total hydrolysate				
sample	µmoles per g[z]	molar %[y]	PDI[x]	MR[w]	AAR[v]	µmoles per g[z]	molar %[y]	PDI[x]	MR[w]	AAR[v]
wt control	1.18	0.49	1.00	1.00	1.00	28.8	1.69	1.00	1.00	1.00
wt w/o GlN	1.46	0.96	1.96	1.25	0.64	28.9	2.05	1.21	1.00	0.83
E1	0.92	0.41	0.84	0.80	0.95	25.4	1.84	1.09	0.88	0.81
E3	1.37	0.37	0.75	1.18	1.55	34.2	1.85	1.09	1.20	1.09
E5	1.29	0.36	0.73	1.10	1.51	41.1	2.24	1.32	1.44	1.08
E6	1.30	0.54	1.10	1.11	1.01	29.6	1.69	1.00	1.03	1.03
E7	1.33	0.45	0.92	1.15	1.25	24.3	1.73	1.02	0.84	0.82
E8	0.98	0.34	0.69	0.83	1.20	36.1	1.82	1.08	1.26	1.17
E9	1.06	0.47	0.96	0.90	0.94	34.3	1.88	1.11	1.19	1.07
E10	1.07	0.33	0.67	0.92	1.37	26.9	1.59	0.94	0.93	0.99
E11	1.63	0.41	0.84	1.41	1.68	25.6	1.67	0.99	0.89	0.90
E12	0.94	0.25	0.51	0.81	1.58	34.4	1.90	1.12	1.20	1.07
CT1	1.27	0.50	1.02	1.09	1.07	26.6	1.80	1.06	0.93	0.87
CT2	0.57	0.21	0.43	0.50	1.16	22.6	1.25	0.74	0.78	1.06
CT3	0.93	0.35	0.71	0.79	1.11	29.4	1.99	1.18	1.03	0.87
tuber	5.69	1.27	2.59	4.87	1.88	17.0	1.67	0.99	0.59	0.60
leaf	0.68	0.76	1.55	0.57	0.37	41.9	2.06	1.22	1.45	1.19

z - µmoles/g is the quantity of methionine per gram (dry weight) of sample
y - molar % is the percent of total amino acid content represented by methionine
x - PDI or 'percent distribution index' is the ratio of variant molar % methionine ÷ wt molar % methionine
w - MR or 'methionine ratio' is the ratio of variant µmoles methionine/g sample ÷ wt µmoles methionine/g sample.
v - AAR or 'amino acid ratio' is the total quantity of amino acids/g variant sample ÷ the total quantity of amino acids/g wt sample.

Table 9. Measures of free and total methionine in various 'Superior' cell line derivatives and whole plant organs.

	free pool					total hydrolysate				
sample	µmoles per g[z]	molar % [y]	PDI[x]	MR[w]	AAR[v]	µmoles per g[z]	molar % [y]	PDI[x]	MR[w]	AAR[v]
wt control	0.72	0.41	1.00	1.00	1.00	19.5	1.59	1.00	1.00	1.00
SEM 1	1.61	0.72	1.76	2.29	1.30	26.7	2.01	1.26	1.37	1.09
SEM 2	0.53	0.34	0.83	0.73	0.88	21.3	1.79	1.13	1.10	0.97
SEM 3	1.15	0.76	1.85	1.61	0.87	26.8	1.90	1.19	1.38	1.15
SEM 4	1.34	0.65	1.58	1.89	1.19	19.3	1.66	1.04	0.99	0.95
tuber	7.99	3.26	7.95	11.21	1.41	15.1	2.44	1.45	0.72	0.50
leaf	0.89	1.09	2.66	1.25	0.47	35.9	2.03	1.28	1.84	1.44

z - µmoles/g is the quantity of methionine per gram (dry weight) of sample
y - molar % is the per cent of total amino acid content represented by methionine
x - PDI or 'percent distribution index' is the ratio of variant molar % methionine ÷ wt molar % methionine
w - MR or 'methionine ratio' is the ratio of variant µmoles methionine / g sample ÷ wt µmoles methionine / g sample
v - AAR or 'amino acid ratio' is the total quantity of amino acids / g variant sample ÷ the total quantity of amino acids / g wt sample.

SEM3 was 61% greater than that of wild type tissue. In total cell digests methionine increased 38%.

In both these variants the enhancement of methionine levels can be attributed to free methionine rather than to protein bound methionine. Since the tuber contains more than eleven times the free methionine contained in callus tissue, it is reasonable to expect that tuber methionine levels will be sharply enhanced in these variants. If the percent increase in methionine concentration observed in callus cultures of SEM1 and SEM3 prevail in the tuber as well, the potato will have become a wholly adequate source of dietary methionine. This prediction, however, must await regeneration of these variants to intact plants.

VI. SHOOT AND ROOT MORPHOGENESIS IN POTATO TISSUE CULTURES

Until such time that a mutant population of *in vitro* selected cells can be regenerated into an entire, healthy, plant capable of expressing its newly acquired character at the whole plant level, the value of such a mutant will be extremely limited. For this reason the process of shoot regeneration in potato cultures was examined.

Initial experiments were designed to define conditions promotive of shoot regeneration in primary explants, i.e., tuber, petiole, stem, and lamina segments. For cultivar 'Superior', conditions previously defined by Jarret (47) and Lam (54) proved satisfactory. Cool temperatures (18^{o} C) and a light intensity of 500 foot-candles substantially enhanced regeneration frequency and plant quality. Clone 'W-973' and twelve commercially important cultivars produced shoots on media modified from Behnke (7).

Secondary callus, i.e., callus no longer in association with primary explants was also examined for shoot regenerability. A number of modifications of the conditions suggested by Behnke (5), Binding (7), Lam (55), and Shepard (85) were examined as a possible means for regenerating shoots from long term callus of 'W-973', 'Superior', and 'Russet Burbank'. No treatment resulted in shoot regeneration. It is noteworthy that among the several protocols published for potato shoot regeneration, very dissimilar culture

media and growth conditions are employed. Inorganic salts, vitamins, carbon sources, osmotic stabilizers, organic supplements, and hormones are highly variable. Protocols effective with some cultivars are frequently ineffective with others. Typically, culture age is a critical factor in shoot regeneration from unorganized tissues. As cultures age, their resistance to shoot induction increases. Since selection for altered cell phenotypes is a long term process which cannot easily be hastened, the selection process impedes the final objective of regenerating whole plants.

In the course of identifying media which promote growth of potato callus, several were found which induced root regeneration. Specifically, combinations of benzylaminopurine from 0.1 to 1.0 mg/l and naphthalene acetic acid from 0.1 to 3.0 mg/l promoted root formation while 2,4-D suppressed the process. Shoot regeneration from primary explants appears to involve the vascular regions of these explants. Since roots also possess well developed vascular tissues, it was inferred that regenerated roots might serve as a transitional organ from which shoots might later be regenerated. Very few attempts to regenerate plants from root tissues have been described (95). However, observations that certain solanaceous plants infrequently produce shoots from true roots under field conditions suggested that *in vitro* attempts to regenerate shoots from roots in potato might be well worthwhile.

Aseptic shoot cultures from the cultivars 'Superior' and 'Michibonne' were from disinfested tuber buds and were maintained as lighted shaker cultures in 125 ml erlenmeyer flasks supplied with

30 ml of hormone-free Murashige and Skoog basal medium. Healthy, true roots proliferated and were harvested for subsequent culture on solid medium. Explants consisting of 5 to 8 mm root segments were placed on medium composed of Murashige and Skoog FeEDTA and macro salts with the exception of NH_4NO_3 which was replaced by 8.3 mM NH_4Cl, half strength micro salts, Nitsch and Nitsch vitamins, 500 gm/l low salt acid hydrolysed casein, 0.3 mM adenine sulfate, 0.2 M mannitol, and 5 g/l sucrose at pH 5.7. This basal medium was modified from Shepard (82). Plant hormones included zeatin, the phenylalanine conjugate of indole acetic acid (IAA-phe), abscisic acid, and gibberellin A_3. These were examined in various combinations over a range of concentrations. All cultures were maintained at ambient room temperature and were illuminated with 40 foot-candles of fluorescent light.

Of all the hormones examined, zeatin proved most critical for shoot initiation. For 'Superior' 0.1 to 0.3 µM zeatin elicited the greatest response; for 'Michibonne' 0.3 to 1.0 µM zeatin was more effective. In the absence of zeatin or at concentrations of 3 µM or more, shoots never appeared. IAA-phe contributed very little to shoot initiation. Responses at 0, 0.1, 1.0, and 5.0 µM IAA-phe were all very similar. In later experiments auxin was entirely omitted from the culture medium. Gibberellic acid at 0.65 or 1.3 µM decisively promoted both the frequency of responsive roots and the numbers of emergent shoots per root. Additionally, it reduced the period to shoot emergence from three months to three weeks. In the absence of gibberellin brachytic shoots or buds originated directly from the distal surface of the root segment with no intervening

callus development. Proliferation of soft callus composed of shoots and buds in various stages of development occurred when gibberellin was present. By promoting shoot elongation gibberellin also led to the formation of morphologically normal shoots which readily produced roots upon transfer to hormone-free medium. Abscisic acid had previously been reported to enhance shoot organization in protoplast derived cultures (82). Its influence at 0.3 μM on shoot production from root segments was very modest and not always reproducible. Higher levels suppressed shoot organization.

The physiological state of the root segment also influenced shoot regenerability. Young white roots less than 10 days old were more responsive than were older, thicker roots in which chlorophyll had developed. Mother plant culture conditions also influenced shoot initiation. Roots from plants cultured with 30 g/l sucrose were several times more responsive than roots from plants cultured with 5 g/l sucrose.

These results indicated that a transition from callus to root to shoot might be useful in overcoming the recalcitrance of long term callus cultures toward shoot regeneration. To date this concept has not been confirmed because of newly arisen difficulties in regenerating roots from cultures which a year earlier had produced roots freely. Efforts to identify conditions under which rhizogenesis might again occur are currently in progress. The success of this approach will be critical to the determination of whether selected genotypes in which methionine is modestly elevated in cell cultures express this phenotype at the whole plant level as well.

APPENDICES

Appendix A

Patterns of amino acid distribution in wild type tubers, leaves, and callus cultures and in variant callus cultures of tetraploid S. tuberosum cv 'Superior'.

The amino acid ratio, AAR, measures the total amino acid content of a particular callus selection or tissue source relative to the total amino acid content found in wild type 'Superior' callus. The PDI or percent distribution index measures the molar percent frequency of any amino acid in a callus selection or tissue source divided by the molar percent frequency of that amino acid in wild type 'Superior' callus.

Appendix A.

Amino acid	Wild type 'Superior' callus				SEM 1					
	free		hydrolysate		free			hydrolysate		
	μmoles per g	molar %	μmoles per g	molar %	μmoles per g	molar %	PDI	μmoles per g	molar %	PDI
Asp	5.27	3.02	121.30	9.90	7.72	3.41	1.13	114.00	8.56	0.86
Thr	3.01	1.73	58.82	4.80	6.48	2.87	1.66	48.66	3.66	0.76
Ser	5.58	3.20	82.72	6.75	9.64	4.26	1.33	62.17	4.67	0.69
Glx	72.59	41.64	166.70	13.60	66.23	29.29	0.70	207.43	15.59	1.15
Gly	3.02	1.73	107.90	8.80	9.40	4.16	2.40	115.68	8.69	0.99
Ala	23.00	13.20	103.10	8.41	33.23	14.70	1.11	131.88	9.91	1.18
Val	5.74	3.29	95.63	7.80	17.43	7.71	2.34	97.76	7.34	0.94
Met	0.72	0.41	19.46	1.59	1.62	0.72	1.76	26.71	2.01	1.26
Ile	1.24	0.71	54.27	4.43	2.48	1.10	1.55	55.88	4.20	0.95
Leu	2.20	1.26	96.75	7.89	4.49	1.99	1.58	107.30	8.06	1.02
Tyr	2.31	1.33	42.95	3.50	3.56	1.58	1.18	40.25	3.03	0.87
Phe	2.77	1.59	42.17	3.44	2.33	1.03	0.65	45.47	3.42	0.99
GABA	35.90	20.60	49.25	4.02	48.05	21.30	1.03	120.50	9.06	2.25
Lys	0.76	0.44	83.62	6.82	2.11	0.93	2.11	76.94	5.78	0.85
His	6.71	3.84	38.93	3.18	3.26	1.44	0.38	29.11	2.19	0.69
Arg	1.86	1.07	48.78	3.98	2.28	1.01	0.94	50.72	3.81	0.96
Cys	1.62	0.93	13.50	1.10	5.80	2.56	2.75	0.00	—	—
Total	174.30	100.00	1225.85	100.00	226.12	100.00		1330.46	100.00	
AAR						1.30			1.09	

Appendix A (cont'd).

Amino acid	Sem 2 free µmoles per g	Sem 2 free molar %	Sem 2 free PDI	Sem 2 hydrolysate µmoles per g	Sem 2 hydrolysate molar %	Sem 2 hydrolysate PDI	Sem 3 free µmoles per g	Sem 3 free molar %	Sem 3 free PDI	Sem 3 hydrolysate µmoles per g	Sem 3 hydrolysate molar %	Sem 3 hydrolysate PDI
Asp	6.08	3.96	1.31	117.50	9.83	0.99	7.08	4.67	1.55	130.80	9.29	0.94
Thr	2.85	1.86	1.08	57.35	4.80	1.00	6.17	4.07	2.35	63.35	4.50	0.94
Ser	5.01	3.27	1.02	68.55	5.74	0.85	6.83	4.51	1.41	77.32	5.49	0.81
Glx	58.71	38.27	0.92	191.00	15.60	1.15	41.43	27.34	0.66	175.20	12.44	0.91
Gly	4.48	2.92	1.69	110.85	9.28	1.05	7.60	5.01	2.90	130.10	9.24	1.05
Ala	14.14	9.22	0.70	89.78	7.51	0.89	16.44	10.85	0.82	121.10	8.60	1.02
Val	4.52	2.95	0.90	81.72	6.84	0.88	7.03	4.64	1.41	106.70	7.68	0.97
Met	0.53	0.34	0.83	21.34	1.79	1.13	1.15	0.76	1.85	26.77	1.90	1.85
Ile	1.09	0.71	1.00	55.66	4.66	1.05	2.66	1.76	2.48	65.03	4.62	1.04
Leu	1.60	1.05	0.83	89.44	7.48	0.95	3.20	2.11	1.68	115.90	8.23	1.07
Tyr	1.04	0.68	0.51	35.80	0.30	0.86	1.15	0.76	0.57	41.15	2.92	0.83
Phe	1.70	1.11	0.70	41.59	3.48	1.01	2.01	1.33	0.84	51.46	3.65	1.06
GABA	40.04	26.10	1.27	65.44	5.48	1.36	34.78	22.95	1.11	96.00	6.82	1.70
Lys	1.29	0.84	1.91	79.18	6.63	0.97	1.89	1.25	2.84	95.67	6.79	1.00
His	3.69	2.41	0.63	31.10	2.60	0.82	3.10	2.04	0.53	32.12	2.28	0.72
Arg	1.93	1.26	1.18	47.36	3.96	1.00	3.75	2.47	2.31	66.67	4.73	1.19
Cys	4.72	3.08	3.31	11.33	0.95	0.86	5.28	3.48	3.74	12.88	0.91	0.83
Total	153.42	100.00		1194.99	100.00		151.54	100.00		1408.22	100.00	
AAR		0.88			0.97			0.87			1.15	

Appendix A (cont'd).

	SEM 4						'Superior' tuber					
	free			hydrolysate			free			hydrolysate		
Amino acid	µmoles per g	molar %	PDI	µmoles per g	molar %	PDI	µmoles per g	molar %	PDI	µmoles per g	molar %	PDI
Asp	12.86	6.18	2.05	149.30	12.82	1.29	15.50	6.31	2.09	148.58	24.04	2.43
Thr	4.41	2.12	1.23	55.44	4.76	0.99	5.88	2.39	1.38	25.73	4.16	0.87
Ser	9.42	4.53	1.42	72.47	6.22	0.92	5.34	2.17	0.68	29.13	4.71	0.70
Glx	113.86	54.69	1.31	181.46	15.58	1.15	134.28	54.70	1.31	102.72	16.62	1.22
Gly	3.91	1.88	1.09	94.73	8.13	0.92	1.92	0.78	0.45	41.52	6.72	0.72
Ala	9.48	4.55	0.34	76.74	6.59	0.78	2.86	1.17	0.89	30.63	4.95	0.56
Val	5.02	2.41	0.73	87.89	7.55	0.97	17.11	6.97	2.12	46.07	7.45	0.91
Met	1.34	0.65	1.59	19.28	1.66	1.04	7.99	3.26	7.95	15.07	2.44	1.45
Ile	1.17	0.56	0.79	50.84	4.37	0.99	4.89	1.99	2.80	28.42	4.60	0.98
Leu	2.01	0.96	0.76	82.68	7.10	0.90	1.73	0.70	0.56	42.33	6.85	0.82
Tyr	0.83	0.40	0.30	40.76	3.50	1.00	5.25	2.14	1.61	20.27	3.28	0.89
Phe	1.20	0.57	0.36	37.02	3.18	0.92	6.98	2.84	1.79	24.09	3.90	1.07
GABA	30.09	14.46	0.70	40.05	3.44	0.76	12.43	5.06	0.25	19.27	3.12	0.74
Lys	1.65	0.79	1.80	78.00	6.70	0.98	8.72	3.55	8.07	32.82	5.31	0.74
His	7.49	3.60	0.99	39.74	3.41	1.07	13.36	1.37	0.36	11.37	1.84	0.55
Arg	2.99	1.44	1.35	43.14	3.70	0.93	11.23	4.57	4.27	nd*	nd*	nd*
Cys	0.45	0.22	0.24	15.15	1.30	1.18	0.00	—	—	0.00	—	—
Total	208.16	100.00		1164.69	100.00		245.47	100.00		618.22	100.00	
AAR		1.19			0.95			1.41			0.50	

*not determined

Appendix A (cont'd).

	'Superior' leaf					
	free			hydrolysate		
Amino acid	µmoles per g	molar %	PDI	µmoles per g	molar %	PDI
Asp	14.68	17.99	5.96	188.86	10.67	1.08
Thr	1.81	2.22	1.28	92.91	5.25	1.09
Ser	2.96	3.62	1.13	91.98	5.20	0.77
Glx	30.84	37.78	0.91	192.24	10.86	0.80
Gly	1.06	1.29	0.75	167.94	9.49	1.08
Ala	2.63	3.22	0.24	153.99	8.70	1.03
Val	1.01	1.24	0.38	142.15	8.03	1.03
Met	0.89	1.09	2.66	35.87	2.03	1.28
Ile	0.73	0.90	1.27	95.46	5.39	1.22
Leu	0.57	0.69	0.55	173.21	9.79	1.24
Tyr	0.65	0.80	0.60	64.65	3.65	1.04
Phe	1.27	1.56	0.98	82.16	4.64	1.35
GABA	20.42	25.02	1.21	30.57	1.73	0.43
Lys	0.76	0.93	2.11	110.95	6.27	0.92
His	0.47	0.58	0.15	39.40	2.23	0.71
Arg	0.77	0.94	0.88	89.38	5.05	1.27
Cys	0.11	0.13	0.14	17.80	1.01	0.92
Total	81.63	100.00		1769.52	100.00	
AAR		0.47			1.44	

Appendix B

Patterns of amino acid distribution in wild type tubers, leaves, and callus cultures and in variant callus cultures of dihaploid S. tuberosum clone 'Wis AG-231 US-W973'.

The amino acid ratio, AAR, measures the total amino acid content of a particular callus selection or tissue source relative to the total amino acid content found in wild type 'W-973' callus. The PDI or percent distribution index measures the molar percent frequency of any amino acid in a callus selection or tissue source divided by the molar percent frequency of that amino acid in wild type 'W-973' callus.

Appendix B.

Amino acid	Wild Type 'W-973' callus				'W-973' callus cultured w/o Gln					
	free		hydrolysate		free			hydrolysate		
	µmoles per g	molar %	µmoles per g	molar %	µmoles per g	molar %	PDI	µmoles per g	molar %	PDI
Asp	3.86	1.54	173.40	10.19	3.59	2.36	1.53	130.20	9.23	0.91
Thr	4.25	1.78	74.10	4.35	3.69	2.42	1.36	61.59	4.37	1.00
Ser	6.65	2.78	87.90	5.16	5.20	3.42	1.23	76.16	5.40	1.05
Glx	67.14	28.11	224.30	13.18	43.84	28.80	1.02	142.77	10.12	0.77
Gly	6.45	2.70	131.80	7.74	6.09	4.00	1.48	115.60	8.20	1.06
Ala	36.37	15.23	177.10	10.40	27.36	17.97	1.18	135.40	9.60	0.92
Val	9.96	4.13	113.20	6.64	6.11	4.01	0.97	103.00	7.30	1.10
Met	1.18	0.49	28.74	1.69	1.46	0.96	1.96	28.88	2.05	1.21
Ile	2.70	1.13	97.24	5.71	2.66	1.75	1.55	66.81	4.74	0.83
Leu	4.02	1.68	130.49	7.66	4.10	2.69	1.60	123.70	8.77	1.15
Tyr	2.47	1.03	41.60	2.44	2.84	1.87	1.82	37.84	2.68	1.10
Phe	2.57	1.07	60.01	3.52	2.78	1.83	1.71	50.89	3.61	1.03
Gaba	75.44	31.59	130.19	7.64	29.52	19.39	0.61	120.43	8.54	1.12
Lys	2.90	1.21	114.90	6.74	2.71	1.78	1.47	105.20	7.46	1.11
His	2.47	1.04	34.40	2.02	1.68	1.10	1.06	29.57	2.10	1.04
Arg	4.89	2.15	72.79	4.28	3.71	2.44	1.19	64.45	4.57	1.07
Cys	5.63	2.36	10.27	0.60	4.90	3.22	1.36	17.94	1.27	2.12
Total	238.84	100.00	1702.43	100.00	152.24	100.00		1410.35	100.00	
AAR						0.64			0.83	

Appendix B (cont'd).

	E 1						E 3					
	free			hydrolysate			free			hydrolysate		
Amino acid	µmoles per g	molar %	PDI	µmoles per g	molar %	PDI	µmoles per g	molar %	PDI	µmoles per g	molar %	PDI
Asp	6.16	2.73	1.77	134.70	9.77	0.96	15.75	4.26	2.77	191.90	10.37	1.02
Thr	8.91	3.95	2.22	57.80	4.19	0.96	10.90	2.95	1.66	74.56	4.03	0.93
Ser	7.02	3.11	1.12	73.24	5.31	1.03	16.85	4.56	1.64	117.20	6.33	1.23
Glx	77.25	34.21	1.22	230.90	16.74	1.27	109.34	29.57	1.05	246.02	13.30	1.01
Gly	6.62	2.93	1.09	119.40	8.66	1.12	17.32	4.68	1.73	145.07	7.84	1.01
Ala	17.54	7.69	0.50	97.94	7.10	0.68	45.09	12.19	0.80	188.21	10.17	0.98
Val	9.56	4.23	1.02	96.28	6.98	1.05	11.74	3.17	0.77	117.23	6.34	0.95
Met	0.92	0.41	0.84	25.41	1.84	1.09	1.37	0.37	0.76	34.23	1.95	1.10
Ile	3.58	1.58	1.40	70.81	5.13	0.90	3.81	1.03	0.91	79.95	4.32	0.76
Leu	5.95	2.65	1.57	97.93	7.10	0.93	5.48	1.48	0.88	128.14	6.93	0.91
Tyr	5.73	2.54	2.47	42.03	3.05	1.25	6.14	1.66	1.61	45.20	2.44	1.00
Phe	2.65	1.17	1.09	47.54	3.45	0.98	1.80	0.49	0.46	59.60	3.22	0.92
GABA	50.22	22.23	0.70	85.11	6.17	0.81	97.75	26.43	0.84	173.60	9.98	1.23
Lys	3.31	1.47	1.22	93.41	6.77	1.00	8.54	2.31	1.91	113.27	6.12	0.91
His	4.03	1.79	1.72	31.52	2.29	1.13	1.06	0.29	0.28	33.30	1.80	0.89
Arg	3.98	1.76	0.86	50.28	3.64	0.85	8.95	2.42	1.18	76.93	4.76	0.97
Cys	12.41	5.49	2.33	24.81	1.80	3.00	7.92	2.14	0.91	25.96	1.40	2.33
Total	225.84	100.00		1379.21	100.00		369.79	100.00		1850.37	100.00	
AAR		0.95			0.81			1.55			1.09	

Appendix B (cont'd).

	E 5						E 6					
	free			hydrolysate			free			hydrolysate		
Amino acid	µmoles per g	molar %	PDI	µmoles per g	molar %	PDI	µmoles per g	molar %	PDI	µmoles per g	molar %	PDI
Asp	27.81	7.73	5.02	181.80	9.91	0.97	6.74	2.80	1.82	172.96	9.90	0.97
Thr.	9.71	2.70	1.52	75.49	4.11	0.95	5.79	2.41	1.35	72.27	4.13	0.95
Ser	11.53	3.20	1.15	80.75	4.40	0.85	7.29	3.03	1.09	82.49	4.72	0.91
Glx	133.60	37.12	1.32	287.40	15.67	1.19	50.16	20.85	0.74	220.00	12.70	0.96
Gly	9.85	2.74	1.01	144.30	7.87	1.02	13.55	5.63	2.09	193.90	11.09	1.43
Ala	40.30	11.20	0.74	172.90	9.42	0.91	38.26	15.90	1.04	159.40	9.12	0.88
Val	10.12	2.81	0.68	126.70	6.91	1.04	6.13	2.55	0.62	112.50	6.44	0.97
Met	1.29	0.36	0.73	41.09	2.24	1.33	1.30	0.54	1.10	29.59	1.69	1.00
Ile	3.76	1.05	0.93	74.68	4.07	0.71	2.36	0.98	0.87	75.96	4.35	0.76
Leu	6.36	1.77	1.05	137.80	7.51	0.98	3.83	1.59	0.95	130.42	7.46	0.97
Tyr	3.76	1.04	1.01	49.57	2.70	1.11	2.40	1.00	0.97	45.02	2.58	1.06
Phe	1.75	0.49	0.46	58.91	3.21	0.91	1.22	0.51	0.48	59.27	3.39	0.96
GABA	66.64	18.52	0.59	156.90	8.55	1.12	85.22	35.43	1.12	152.60	8.73	1.14
Lys	6.14	1.71	1.41	116.50	6.35	0.94	3.67	1.52	1.26	110.40	6.32	0.94
His	3.07	0.85	0.82	36.56	1.99	0.99	2.44	1.01	0.97	32.99	1.89	0.94
Arg	8.28	2.30	1.12	76.94	4.19	0.98	5.57	2.31	1.13	78.62	4.50	1.05
Cys	15.95	4.43	1.88	16.27	0.89	1.48	4.63	1.92	0.81	17.46	1.00	1.67
Total	359.91	100.00		1834.56	100.00		240.55	100.00		1747.85	100.00	
AAR		1.51			1.08			1.01			1.03	

Appendix B (cont'd).

Amino acid	E 7 free µmoles per g	E 7 free molar %	E 7 free PDI	E 7 hydrolysate µmoles per g	E 7 hydrolysate molar %	E 7 hydrolysate PDI	E 8 free µmoles per g	E 8 free molar %	E 8 free PDI	E 8 hydrolysate µmoles per g	E 8 hydrolysate molar %	E 8 hydrolysate PDI
Asp	3.73	1.25	0.81	115.78	8.25	0.81	12.64	4.42	2.87	204.80	10.32	1.01
Thr	5.20	1.74	0.98	50.36	3.59	0.83	7.36	2.57	1.44	75.63	3.81	0.88
Ser	11.07	3.71	1.34	68.72	4.90	0.95	8.93	3.12	1.12	83.25	4.19	0.82
Glx	61.43	2 58	0.73	176.70	12.59	0.96	58.10	20.30	0.72	202.40	10.19	0.77
Gly	9.15	3.07	1.14	104.59	7.45	0.96	9.93	3.47	1.29	180.60	9.10	1.18
Ala	45.34	15.18	1.00	157.30	11.21	1.08	38.81	13.56	0.89	160.60	8.09	0.78
Val	7.53	2.52	0.61	84.90	6.05	0.91	9.99	3.49	0.85	125.25	6.32	0.95
Met	1.33	0.45	0.92	24.34	1.73	1.02	0.98	0.34	0.69	36.12	1.82	1.08
Ile	3.99	1.34	1.19	63.32	4.51	0.79	3.55	1.24	1.10	85.41	4.30	0.75
Leu	6.52	2.18	1.30	96.08	6.84	0.89	5.68	1.99	1.18	142.10	7.16	0.94
Tyr	4.86	1.63	1.58	35.84	2.55	1.05	3.49	1.22	1.18	47.36	2.39	0.98
Phe	3.28	1.10	1.03	44.77	3.19	0.91	1.96	0.68	0.64	63.61	3.20	0.91
GABA	109.12	36.55	1.16	207.14	14.76	1.93	97.33	34.01	1.08	302.14	15.22	1.99
Lys	6.06	2.03	1.68	82.43	5.87	0.87	4.02	1.41	1.17	127.70	6.43	0.95
His	3.55	1.19	1.14	26.76	1.91	0.95	3.63	1.27	1.22	35.95	1.81	0.90
Arg	6.47	2.17	1.06	54.99	3.92	0.92	8.97	3.13	1.53	84.10	4.24	0.90
Cys	9.96	3.33	1.41	9.46	0.67	1.12	10.79	3.77	1.60	28.04	1.41	2.35
Total	298.57	100.00		1403.48	100.00		286.16	100.00		1985.31	100.00	
AAR		1.25			0.82			1.20			1.17	

Appendix B (cont'd).

	E 9						E 10					
	free			hydrolysate			free			hydrolysate		
Amino acid	µmoles per g	molar %	PDI	µmoles per g	molar %	PDI	µmoles per g	molar %	PDI	µmoles per g	molar %	PDI
Asp	2.97	1.32	0.86	170.50	9.33	0.92	12.61	3.86	2.51	164.70	9.75	0.96
Thr	6.36	2.83	1.59	72.77	3.98	0.91	6.84	2.10	1.18	64.75	3.83	0.88
Ser	6.40	2.85	1.03	88.46	4.84	0.94	6.52	2.00	0.72	73.15	4.33	0.84
Glx	43.59	19.41	0.69	223.70	12.24	0.93	120.40	36.91	1.31	230.80	13.66	1.04
Gly	8.26	3.68	1.36	171.80	9.40	1.21	15.01	4.60	1.70	211.30	12.51	1.62
Ala	26.38	11.74	0.77	163.10	8.92	0.86	22.79	6.99	0.46	123.80	7.33	0.71
Val	7.06	3.14	0.76	127.50	6.97	1.05	8.50	2.60	0.63	115.20	6.82	1.03
Met	1.06	0.47	0.96	34.30	1.88	1.11	1.07	0.33	0.67	26.86	1.59	0.94
Ile	4.16	1.85	1.64	86.44	4.93	0.83	3.48	1.07	0.95	76.10	4.50	0.79
Leu	5.51	2.45	1.46	154.80	8.47	1.11	3.97	1.22	0.73	129.30	7.65	1.00
Tyr	2.42	1.08	1.05	47.70	2.61	1.07	2.94	0.90	0.87	43.80	2.59	1.06
Phe	3.38	1.50	1.40	72.08	3.94	1.12	2.19	0.67	0.63	53.42	3.16	0.90
GABA	81.05	36.08	1.14	144.50	7.90	1.04	94.84	29.07	0.92	161.00	9.53	1.25
Lys	4.94	2.20	1.82	125.80	6.88	1.02	4.32	1.32	1.09	105.10	6.22	0.92
His	3.04	1.35	1.30	37.60	2.06	1.02	2.23	0.68	0.65	31.04	1.84	0.91
Arg	7.17	3.19	1.56	86.80	4.75	1.11	5.74	1.76	0.86	63.52	3.76	0.88
Cys	10.88	4.84	2.05	20.44	1.12	1.87	12.78	3.92	1.66	15.74	0.93	1.55
Total	224.62	100.00		1828.25	100.00		326.21	100.00		1689.58	100.00	
AAR		0.94			1.07			1.37			0.99	

Appendix B (cont'd).

	E 11						E 12					
	free			hydrolysate			free			hydrolysate		
Amino acid	µmoles per g	molar %	PDI	µmoles per g	molar %	PDI	µmoles per g	molar %	PDI	µmoles per g	molar %	PDI
Asp	17.75	4.42	2.87	176.98	11.58	1.14	15.57	4.13	2.68	175.35	9.65	0.95
Thr	5.62	1.40	0.79	58.33	3.82	0.88	8.10	2.14	1.20	73.13	4.03	0.93
Ser	7.61	1.89	0.68	68.59	4.49	0.87	8.18	2.17	0.78	80.21	4.42	0.86
Glx	105.80	26.33	0.94	221.40	14.48	1.10	142.14	37.73	1.34	267.10	14.70	1.12
Gly	13.46	3.35	1.24	125.37	8.20	1.06	9.92	2.63	0.97	144.90	7.98	1.03
Ala	40.32	10.03	0.66	143.44	9.38	0.90	39.16	10.39	0.68	149.00	8.20	0.79
Val	10.21	2.54	0.62	96.34	6.30	0.95	8.01	2.13	0.52	116.10	6.39	0.96
Met	1.63	0.41	0.84	25.59	1.67	0.99	0.94	0.25	0.51	34.44	1.90	1.12
Ile	3.31	0.82	0.73	76.11	4.98	0.87	3.19	0.85	0.75	83.28	4.58	0.80
Leu	4.27	1.06	0.63	109.61	7.17	0.94	4.65	1.23	0.73	136.60	7.52	0.98
Tyr	2.27	0.57	0.55	36.17	2.37	0.97	2.59	0.69	0.67	47.96	2.64	1.08
Phe	1.68	0.42	0.39	50.26	3.29	0.93	1.53	0.41	0.38	58.66	3.23	0.92
GABA	171.30	42.64	1.35	142.60	9.33	1.22	104.03	27.62	0.87	191.30	10.53	1.38
Lys	3.70	0.92	0.76	95.09	6.22	0.92	3.45	0.91	0.75	117.88	6.49	0.96
His	2.16	0.54	0.52	28.14	1.84	0.91	3.64	0.97	0.93	35.44	1.95	0.97
Arg	4.86	1.21	0.59	60.27	3.94	0.92	9.19	2.44	1.22	78.13	4.30	1.01
Cys	5.81	1.45	0.61	14.48	0.95	1.58	12.45	3.30	1.40	27.19	1.50	2.50
Total	401.76	100.00		1528.77	100.00		376.72	100.00		1816.67	100.00	
AAR		1.68			0.90			1.58			1.07	

Appendix B (cont'd).

Amino acid	CT 1 free µmoles per g	CT 1 free molar %	CT 1 free PDI	CT 1 hydrolysate µmoles per g	CT 1 hydrolysate molar %	CT 1 hydrolysate PDI	CT 2 free µmoles per g	CT 2 free molar %	CT 2 free PDI	CT 2 hydrolysate µmoles per g	CT 2 hydrolysate molar %	CT 2 hydrolysate PDI
Asp	4.68	1.83	1.18	144.90	9.81	0.96	12.00	4.43	2.81	208.24	11.49	1.13
Thr	4.36	1.70	0.96	57.62	3.90	0.90	3.98	1.44	0.81	50.55	2.79	0.64
Ser	5.44	2.11	0.76	71.23	4.82	0.93	4.94	1.79	0.64	64.17	3.54	0.69
Glx	63.60	24.83	0.88	199.50	13.51	1.02	127.97	46.23	1.64	373.20	2.58	1.56
Gly	13.21	5.16	1.91	124.27	8.41	1.09	9.42	3.40	1.26	116.43	6.42	0.83
Ala	29.09	11.35	0.75	120.60	8.17	0.79	20.33	7.34	0.48	112.74	6.22	0.60
Val	6.69	2.61	0.63	96.12	6.51	0.98	4.59	1.66	0.40	84.63	4.67	0.70
Met	1.27	0.50	1.02	26.64	1.80	1.07	0.57	0.21	0.43	22.63	1.25	0.74
Ile	3.14	1.23	1.09	65.86	4.46	0.78	1.56	0.56	0.50	57.25	3.16	0.55
Leu	5.42	2.12	1.26	108.20	7.63	0.96	2.50	0.90	0.54	96.64	5.22	0.68
Tyr	3.22	1.26	1.22	37.03	2.51	1.03	1.61	0.58	0.57	38.26	2.11	0.87
Phe	2.30	0.90	0.84	49.15	3.33	0.95	0.94	0.34	0.32	42.83	2.36	0.67
GABA	95.89	37.43	1.19	175.43	11.88	1.56	68.92	24.90	0.79	151.33	8.35	1.09
Lys	4.59	1.79	1.48	100.95	6.83	1.01	2.07	0.75	0.62	293.20	16.17	2.40
His	2.34	0.91	0.88	27.25	1.84	0.91	2.33	0.84	0.81	28.97	1.60	0.79
Arg	6.91	2.70	1.32	64.10	4.34	1.01	2.60	0.94	0.46	54.38	3.00	0.70
Cys	4.00	1.56	0.66	8.25	0.56	0.66	10.47	3.78	1.60	19.60	1.08	1.80
Total	256.15	100.00		1477.10	100.00		276.79	100.00		1813.05	100.00	
AAR		1.07			0.87			1.16			1.06	

Appendix B (cont'd).

	CT 3					
	free			hydrolysate		
Amino acid	µmoles per g	molar %	PDI	µmoles per g	molar %	PDI
Asp	9.50	3.57	2.32	169.80	11.52	1.13
Thr	5.28	1.99	1.12	60.00	4.07	0.94
Ser	5.76	2.17	0.78	79.11	5.37	1.04
Glx	94.74	35.65	1.27	212.00	14.38	1.09
Gly	13.47	5.07	1.88	112.00	7.60	0.98
Ala	16.99	6.39	0.42	110.00	7.46	0.72
Val	7.54	2.83	0.69	105.70	7.17	1.08
Met	0.93	0.35	0.71	29.37	1.99	1.18
Ile	2.90	1.09	0.96	67.26	4.56	0.80
Leu	4.93	1.86	1.11	116.80	7.92	1.03
Tyr	3.00	1.13	1.10	45.46	3.08	1.26
Phe	1.87	0.70	0.65	52.44	3.56	1.01
GABA	63.80	24.01	0.76	93.05	6.31	0.83
Lys	4.81	1.81	1.50	110.00	7.46	1.11
His	2.20	0.83	0.80	32.02	2.17	1.07
Arg	5.49	2.07	1.01	60.39	4.10	0.96
Cys	22.55	8.49	3.60	18.87	1.28	2.13
Total	265.75	100.00		1474.27	100.00	
AAR		1.11			0.87	

Appendix B (cont'd).

	'W-973'tuber						'W-973'leaf					
	free			hydrolysate			free			hydrolysate		
Amino acid	µmoles per g	molar %	PDI	µmoles per g	molar %	PDI	µmoles per g	molar %	PDI	µmoles per g	molar %	PDI
Asp	13.87	3.09	2.01	295.20	29.01	2.85	21.98	24.80	16.10	206.37	10.17	1.00
Thr	6.73	1.50	0.84	38.60	3.79	0.87	3.05	3.44	1.93	134.73	6.64	1.53
Ser	9.29	2.07	0.74	45.07	4.43	0.86	5.39	6.08	2.19	114.39	5.36	1.09
Glx	237.40	52.92	1.88	142.30	13.99	1.06	13.89	15.67	0.56	193.80	9.55	0.72
Gly	1.55	0.34	0.13	61.05	6.00	0.78	0.82	0.92	0.34	177.90	8.76	1.13
Ala	6.69	1.49	0.10	43.50	4.28	0.41	2.71	3.06	0.20	172.30	8.49	0.82
Val	14.36	3.20	0.78	60.23	5.92	0.89	1.32	1.49	0.36	162.46	8.00	1.20
Met	5.69	1.27	2.59	16.98	1.67	0.99	0.68	0.76	1.55	41.92	2.06	1.22
Ile	13.81	3.08	2.73	42.77	4.20	0.74	0.99	1.12	0.99	118.26	5.83	1.02
Leu	5.19	1.16	0.69	57.46	5.65	0.74	0.98	1.10	0.66	205.30	10.11	1.32
Tyr	2.85	0.63	0.61	25.42	2.50	1.02	1.23	1.39	1.35	75.70	3.73	1.53
Phe	1.56	0.35	0.33	29.64	2.91	0.83	1.48	1.67	1.56	86.42	4.26	1.21
GABA	16.95	3.78	0.12	24.95	2.45	0.32	28.33	31.98	1.01	39.82	1.96	0.26
Lys	9.62	2.15	1.78	48.40	4.76	0.71	0.83	0.93	0.77	135.50	6.67	0.99
His	13.62	3.04	2.92	23.29	2.29	1.13	1.06	1.19	1.14	45.54	2.24	1.11
Arg	40.04	8.93	4.36	52.47	5.16	1.21	0.93	1.05	0.51	105.33	5.19	1.21
Cys	1.29	0.29	0.12	10.12	0.99	1.65	2.94	3.32	1.41	14.62	0.72	1.20
Total	448.56	100.00		1017.45	100.00		88.60	100.00		2030.20	100.00	
AAR		1.88			0.60			0.37			1.19	

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Aarnes, H. 1977. A lysine-sensitive aspartate kinase and two molecular forms of homoserine dehydrogenase from barley seedlings. Plant Sci. Letters 9:137-145.

2. Aarnes, H. and Rognes, S.E. 1974. Threonine-sensitive aspartate kinase and homoserine dehydrogenase from Pisum sativum. Phytochemistry 13:2717-2724.

3. Anonymous. 1968. Recommended Dietary Allowances. NAS Food and Nutritional Board. Publ. No. 1694. 100pp.

4. Ascano, A. and Nicholas, D.J.D. 1977. Purification and properties of O-acetyl-L-serine sulfhydrolyase from wheat leaves. Phytochemistry 16:889-893.

5. Behnke, M. 1975. Regeneration in Gewegekulturen einiger dihaploider Solanum tuberosum-klone. Z. Pflanzenzuchtg 75:262-265.

6. Behnke, M. 1979. Selection of potato callus for resistance to culture filtrates of Phytophthora infestans and regeneration of resistant plants. Theor. Appl. Genet. 55:69-71.

7. Binding, H., Nehls, R., Schieder, O., Sopory, S.K., and Wenzel, G. 1978. Regeneration of mesophyll protoplasts isolated from dihaploid clones of Solanum tuberosum. Physiol. Plant. 43:52-54.

8. Blackburn, S. 1978. Amino Acid Determination- Methods and Techniques. Second edition. Marcel Dekker, Inc. 367 pp.

9. Blackman, M.S. and McDaniel C.N. 1980. Amino acid transport in suspension-cultured plant cells.II Characterization of L-leucine uptake. Plant Physiol. 66:261-266.

10. Bright, S.W.J., Norbury, P.B. and Miflin, B.J. 1980. Isolation and characterization of barley mutants resistant to aminoethylcysteine and lysine plus threonine. in Plant Cell Cultures: Results and Perspectives. Sala, F., Parisi, B., Cella, R., and Ciferri, O. eds. Elsevier. p.179-182.

11. Bright, S.W.J., Wood, E.A., and Miflin, B.J. 1978. The effect of aspartate derived amino acids (lysine, threonine, methionine) on the growth of excised embryos of wheat and barley. Planta 139:113-117.

12. Bryan, J.K. 1976. Amino acid biosynthesis and its regulation. in Plant Biochemistry. Third edition. Bonner, J. and Varner, J.E. eds. Academic Press. p 525-560.

13. Burton, W.G. 1966. The Potato: a survey of its history and of factors influencing its yield, nutritive value, quality, and storage. Second edition. H. Veenman & Zonen.

14. Butenko, R.G. and Kuchko, A.A. 1979. Physiological aspects of procurement, cultivation, and hybridization of isolated potato protoplasts. Fiziol. Rast. 26:1110-1119.

15. Carlson, J.E. and Widholm, J.M. 1978. Separation of two forms of anthranilate synthetase from 5-methyltryptophan susceptible and resistant cultured Solanum tuberosum cells. Physiol. Plant. 44:251-255.

16. Carlson, P.S. 1973. Methionine sulfoximine-resistant mutants of tobacco. Science 180:1366-1368.

17. Cattoir-Reynaerts, A., Degryse, E., Negrutris, I., Aerts M., and Jacobs, M. 1981. Effects of aspartate-derived amino acids on growth of barley and Arabidopsis plants and callus. Z. Pflanzenphysiol. 101:67-74.

18. Cheruel, J., Jullien, M., and Surdin-Kerjan, Y. 1979. Amino acid uptake into cultivated mesophyll cells from Asparagus officinalis L. Plant Physiol. 63:621-626.

19. Datko, A.H. and Mudd, S.H. 1980. Methionine biosynthesis in Lemna: Inhibitor studies. Plant Physiol. v 65 supplement:16.

20. Datko, A.H., Mudd, S.H., Giovanelli, J., and Macnird, P.K. 1978. Sulfur containing compounds in Lemna perpusilla 6746 grown at a range of sulfite concentrations. Plant Physiol. 62:629-635.

21. Davies, A.M.C. 1977. The free amino acids of tubers ofpotato varieties grown in England and Ireland. Potato Res. 20:9-21.

22. Davies, H.M. and Miflin, B.J. 1978. Regulatory isozymes of aspartate kinase and the control of lysine and threonine biosynthesis in carrot cell suspension culture. Plant Physiol. 62:536-541.

23. Desborough, S. and Lauer, F. 1977. Improvement of potato protein II. Selection for protein and yield. Amer. Potato J. 54;371-376.

24. Desborough, S.L. and Weiser, C.J. 1974. Improving potato protein I. Evaluation of selection techniques. Amer. Potato J. 51:185-196.

25. Di Camelli, C.A. and Bryan, J.K. 1980. Comparison of sensitive and desensitized forms of maize homoserine dehydrogenase. Plant Physiol. 65:176-183.

26. Eustice, D.C., Foster, I., Kull, F.J., and Shrift, A. 1980. In vitro incorporation of selenomethionine into protein by Vigna radiata polysomes. Plant Physiol. 66:182-186.

27. Eustice, D.C., Kull, F.J., and Shrift, A. 1981. Selenium toxicity: amino acylation and peptide bond formation with selenomethionine. Plant Physiol. 67:1054-1058.

28. Flashman, S.M. and Filner, P. 1978. Selection of tobacco cell lines resistant to seleno amino acids. Plant Sci. Letters 13:219-229.

29. Foroughi-Wehr B., Wilson, H.M., Mix, G., and Gaul, H. 1977. Monohaploid plants from anthers of a dihaploid genotype of Solanum tuberosum L. Euphytica 26:361-367.

30. Fowden, L., Lewis, D. and Tristram, H. 1967. Toxic amino acids: their action as antimetabolites. in Advances in Enzymology. Volume 29. Nord, F.F. ed. Wiley.

31. Friedman, M., Krull, L.H., and Cavins, J.F. 1970. The chromatographic determination of cystine and cysteine residues in proteins as S-β-(4-pyridylethyl) cysteine. J. Biol. Chem. 245:3868-3871.

32. Gengenbach, B.G., Walter, T.J., Green, C.E., and Hibberd, K.A. 1978. Feedback regulation of lysine, threonine, and methionine biosynthetic enzymes in corn. Crop Science 18:472-476.

33. Gehrke, C.W. and Neuner, T.E. 1974. Automated chemical determination of methionine. J. Assoc. Offic. Anal. Chem. 57:682-688.

34. Gill, J. 1978. Design and Analysis of Experiments in the Animal and Medical Sciences. Volumes 1-3. Iowa State Univ. Press.

35. Giovanelli, J., Mudd, S.H., and Datko, A. 1980. Homocysteine biosynthesis in plants. in Natural Sulfur Compounds: novel biochemical and structural aspects. Cavallini, D., Gaull, G.E., and Zappia, V. eds. Plenum.

36. Giovanelli, J., Mudd, S.H., and Datko, A. 1980. Sulfur amino acids in plants. in Biochemistry of Plants: a comprehensive treatise. Volume 5, ch 12. Stumpf, P.K. and Conn, E.E. eds. Academic Press.

37. Green, C.E. and Donovan, C.M. 1980. Effect of aspartate-derived amino acids and aminoethyl cysteine on growth of excised mature embryos of maize. Crop Science 20:358-362.

38. Grun , P. and Chu,L-J. 1978. Development of plants from protoplasts of Solanum (Solanaceae). Amer. J. Bot. 65:538-543.

39. Henke, R.R. and Wilson, K.G. 1974. In-vivo evidence for metabolic control of amino acid and protein synthesis by exogenous lysine and threonine in Mimulus cardinalis. Planta 121:155-166.

40. Hibberd, K.A., Walter, T., Green, C.E., and Gengenbach, B.G. 1980. Selection and characterization of a feedback-insensitive tissue culture of maize. Planta 148:183-187.

41. Hirs, C.H.W. 1956. The oxidation of ribonuclease with performic acid. J. Biol. Chem. 219:611-621.

42. Hoff, J.E., Jones, C.M., Wilcox, G.E., and Castro, M.D. 1971. The effect of nitrogen fertilization on the composition of the free amino acid pool of potato tubers. Am. Pot J. 48:390-391.

43. Houghton, R.A. and Li, C.H. 1979. Reduction of sulfoxides in peptides and proteins. Anal. Biochem. 98:36-46.

44. Irikura, Y. 1975. Induction of haploid plants by anther culture in tuber-bearing species and interspecific hybrids of Solanum. Potato Res. 18:133-140.

45. Isaacs, C.E. 1977. An in vitro system for culture of Solanum cells: selection of an in vitro marker. PhD dissertation. Rutgers University. Department of Biological Sciences.

46. Jacobsen, E. and Sopory, S.K. 1978. The influence and possible recombination of genotypes on production of microspore embryoids in anther cultures of Solanum tuberosum and dihaploid hybrids. Theor. Appl. Genet. 52:119-123.

47. Jarret, R.L., and Hasegawa, P.M., and Erickson, H.T. 1980. Factors affecting shoot initiation from tuber discs of potato (Solanum tuberosum). Physiol. Plant. 48:177-184.

48. Kaldy, M.S. and Markakis, P. 1972. Amino acid composition of selected potato varieties. J. Food Sci. 37:375-377.

49. Kapoor, A.C., Desborough, S.L., and Li, P.H. 1975. Extraction of nonprotein nitrogen from potato tuber and its amino acid composition. Potato Res. 18:582-587.

50. Kapoor, A.C., Desborough, S.L., and Li, P.H. 1975. Potato tuber proteins and their nutritional quality. Potato Res. 18:469-478.

51. Kistler, W.S., Noyes, C., Hsu, R., and Heinrikson, R.L. 1973. The amino acid sequences of a testis-specific basic protein that is associated with spermatogenesis. J. Biol. Chem. 250:1847-1853.

52. Knowles, S.E. and Ballard, F.J. 1978. Effects of amino acid analogues on protein synthesis and degredation in isolated cells. Br. J. Nutr. 40:275-287.

53. Kraus, J., Soll, D. and Low, K.B. 1979. Glutamyl-δ- methyl ester acts as a methionine analogue in Escherichia coli: analogue resistant mutants map at the met J and met K loci. Genet. Res., Camb. 33:49-55.

54. Lam, S.L. 1977. Plantlet formation from potato tuber discs in vitro. Amer. Potato J. 54:465-468.

55. Lam, S.L. 1977. Regeneration of plantlets from single cells in potatoes. Amer. Potato J. 54:575-580.

56. Larkin, P.J. and Scowcroft, W.R. 1981. Somaclonal variation- a novel source of variability from cell culture for plant improvement. Theor. Appl. Genet. 60:197-214.

57. Luescher, R. 1971. Evaluation of methods to determine the sulfur containing amino acids in potatoes. Master of Science thesis. Michigan State Univer.

58. Luescher, R. 1972. Genetic variability of 'available' methionine, total protein, specific gravity, and other traits in tetraploid potatoes. PhD thesis. Michigan State Univer.

59. Macnicol, P.K., Datko, A.H., Giovanelli, J., and Mudd, S.H. 1981. Homocysteinebiosynthesis in green plants:Physiological importance of the transsulfuration pathway in Lemna paucicostata. Plant Physiol. 68:619-625.

60. Madison, J.T. and Thompson, J.F. 1976. Threonine synthetase from higher plants: stimulation by S- adenosylmethionine and inhibition by cysteine. Biochem. Biophys. Res. Commun. 71:684-691.

61. Matern, U., Strobel, G., and Shepard, J. 1978. Reactions to phytotoxins in a potato population derived from mesophyll protoplasts. Proc. Nat. Acad. Sci. (USA) 75:4935-4939.

62. Matsubara, H. and Sasaki, R.M. 1969. High recovery of tryptophan from acid hydrolysates of proteins. Biochem. Biophys. Res. Commun. 35:175-181.

63. McDaniel, C.N.,Lyons,R.A., and Blockman, M.S. 1981. Amino acid transport in suspension cultured plant cells. IV biphasic saturable uptake kinetics of L-leucine in isolates from six Nicotiana tabacum plants. Pl. Sci. Letters 23:17-23.

64. Melchers, G., Sacristan, M.D., and Holder, A.A. 1978. Somatic hybrid plants of potato and tomato regenerated from fused protoplasts. Carlsberg Res. Commun. 43:203-218.

65. Mills, W.R., Lea P.G., and Miflin, B.J. 1980. Photosynthetic formation of the aspartate family of amino acids in isolated chloroplasts. Plant Physiol. 65:1166-1172.

66. Moore, S. 1963. On the determination of cysteine as cysteic acid. J. Biol. Chem. 238:235-237.

67. Muhitch, M.J. and Wilson, K.G. 1980. Regulation of aspartate kinase (AK) and homoserine dehydrogenase (HSD) in Mimulus cardinalis

(5031). Plant Physiol. 65: supplement: 126.

68. Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

69. Nitsch, J.P. and Nitsch,C. 1969. Haploid plants from pollen grains. Science 163:85-87.

70. Ng, B.H. and Anderson, J.W. 1978. Chloroplast cysteine synthases of Trifolium repens and Pisum sativum. Phytochemistry 17:879-885.

71. Reisch, B., Duke, S.H., and Bingham, E.T. 1981. Selection and characterization of ethionine-resistant alfalfa (Medicago sativa L.) cell lines. Theor. Appl. Genet. 59:89-94.

72. Rexen, B. 1976. Studies of proteins of potatoes. Potato Res. 19:189-202.

73. Rios-Iriarte, B.J., Thompson, N.R., and Bedford, C.C. 1972. Protein in potato flakes: evaluation by the meadow vole (Microtus pennsylvanicus). Amer. Potato J. 49:255-260.

74. Rognes, S.E., Lea, P.J., and Miflin, B.J. 1980. S-adenosylmethionine- a novel regulator of aspartate kinase. Nature 287:357-359.

75. Sakano, K. and Komamine, A. 1978. Change in the proportion of two aspartokinases in carrot root tissue in response to in vitro culture. Plant Physiol. 61:115-118.

76. Salnikow, J., Liao, T.A., Moore, S., and Stein, W.H. 1973. Bovine pancreatic deoxyribonuclease A. Isolation, composition, and amino acid sequences of the tryptic and chymotryptic peptides. J. Biol. Chem. 248:1480-1488.

77. Schlesinger, H.I., Brown, H.C., Hockstra, H.R., and Rapp, L.R. 1953. Reactions of diborane with alkali metal hydrides and their addition compounds. New syntheses of borohydride. Sodium and potassium borohydrides. J. Amer. Chem. Soc. 75:199-204.

78. Schram, E., Dustin, J.P., Moore, S., and Bigwood, E.J. 1953. Application de la chromatographie sur échangeur d'ions à l'étude de la composition des aliments acides aminés. Anal. Chim. Acta 9:149-162.

79. Schuphan, W. 1959. Studien über essentielle Aminosaüren in Kartoffeln. II. Mitteilung Die Biologische Eiweisswertigheit der Kartoffel (Solanum tuberosum L.) in Ernahrungsversuche und im Spiegel der essentiellen Aminosäuren. Qual. Pl. Mat. veg 6:16-38.

80. Scowcroft, W.R. 1977. Somatic cell genetics and plant improvement. Advan. Agron. 29:39-81.

81. Shechter, Y., Burstein, Y., and Patchornik, A. 1975. Selective oxidation of methionine residues in proteins. Biochem. 14:4497-4503.

82. Shepard, J.F. 1980. Abscissic acid-enhanced shoot initiation in protoplast-derived calli of potato. Plant Sci. Letters 18:327-333.

83. Shepard, J.F. 1980. Mutant selection and plant regeneration from potato mesophyll protoplasts. in Emergent Techniques for the Genetic Improvement of Crops. Rubenstein, S., Gengenbach, B., and Green, C.E.(eds.) Univer. Minnesota Press.

84. Shepard, J.F., Bidney, D., and Shahin, E. 1980. Potato protoplasts in crop improvement. Science 208:17-24.

85. Shepard, J.F. and Totten, R.E. 1977. Mesophyll cell protoplasts of potato. Isolation, proliferation, and plant regeneration. Plant Physiol. 60:313-316.

86. Skirvin, R.M. 1978. Natural and induced variations in tissue culture. Euphytica 27:241-266.

87. Sletten, K., Dus, K., de Klerk, H., and Kamen, M.D. 1968. Cytochrome C_2 of Rhodospirillum rubrum. I Molecular properties of the protein and amino acid sequences of its peptides derived by the action of trypsin and thermolysin. J. Biol. Chem. 243:5492.

88. Smith, I.K. 1981. Compartmentation of sulfur metabolites in tobacco cells. Use of efflux analysis. Plant Physiol. 68:937-940.

89. Snyder, J. and Desborough, S. 1978. Protein, starch, and nonprotein nitrogen accumulation in high protein hybrids and low protein cultivars. Amer. Potato J. 55:453-465.

90. Snyder, J.C. and Desborough, S.L. 1980. Total protein and protein fractions in tubers of group andigena and phureja-tuberosum hybrids. Qual. Plant. 30:123-134.

91. Snyder, J., Desborough, S., amd Holm, D. 1977. Accumulation of protein, non-protein nitrogen, and starch during tuber growth of three potato cultivars. Amer Potato J. 54:545-555.

92. Sopory, S.K., Jacobsen, E., and Wenzel, G. 1978. Production of monohaploid embryoids and plantlets in cultured anthers of Solanum tuberosum. Plant Science Letters 12:47-54.

93. Stace-Smith, F.C. and Mellos, R. 1968. Eradication of potato viruses X and S by thermo therapy and axillary bud culture. Phytopathology 58:199-203.

94. Synge, R.L.M. 1977. Free amino acids of potato tubers: a survey of published results set out according to potato variety. Potato Res. 20:1-7.

95. Thomas, E. and Street, H.E. 1972. Factors influencing morphogenesis in excised roots and suspension cultures of Atropa belladonna. Am. Bot. 36:239-247.

96. Thompson, G.A., Mudd, S.H., Datko, A., and Giovanelli, J. 1980. Regulation of cystathionine-γ- synthase and O-phosphohomoserine sulfhydrolyase in Lemna. Plant Physiol. Vol. 65 supplement:16.

97. Thompson, N.R. 1978. Potatoes. in Protein Resources and Technology. Milner, Scrimshaw, and Wang. (eds.) pp485-501.

98. Trout, G.E. 1979. The estimation of microgram amounts of methionine by reaction with chloramine-T. Anal. Biochem. 93:419-422.

99. Upadhya, M.D. 1975. Isolation and culture of mesophyll protoplasts of potato (Solanum tuberosum L.). Potato Res. 18:438-445.

100. Wallsgrove, R.M., Sainis, J.K., Lea, P.J., and Miflin, B.J. 1980. The regulation and subcellular localization of homoserine dehydrogenase (HSDH) in the leaves of Hordeum vulgare. Plant Physiol. Vol. 65 supplement:27.

101. Wenzel, G., Schieder, O., Przewozny, T., Sopory, S.K., and Melchers, G. 1979. Comparison of single cell culture derived Solanum tuberosum L. plants and a model for their application in breeding programs. Theor. Appl. Genet. 55:49-55.

102. Werner, G., Hossli, R., and Neukom. 1969. Nachweis von S-methyl-methionin und seinen Zersetzprodukten in Kartoffeln. Lebensmittel-Wissenschaft- Technologie 2:145.

103. Wescott, R.J., Henshaw, G.G., and Roca, W.M. 1977. Tissue culture storage of potato germplasm: culture initiation and plant regeneration. Plant Science Letters 9:309-315.

104. Widholm, J.M. 1976. Selection and characterization of cultured carrot and tobacco cells resistant to lysine, methionine, and proline analogs. Can. J. Bot. 54:1523-1529.

105. Widholm, J. 1977. Selection and characterization of amino acid analog resistant plant cell cultures. Crop Science 17:597-600.

106. Widholm, J. 1980. Differential expression of amino acid biosynthetic control of isoenzymes in plants and cultured cells. in Plant Cell Cultures: Results and Perspectives. Sala, F., Parisi, B., Cella, R., and Ciferri, O. (eds.) Elsevier. p 157-159.

107. Wilcox, G.E. and Hoff, J.G. 1970. Nitrogen fertilization of potatoes for early summer harvest. Am. Potato J. 47:99-102.

108. Wilson, L.G. and Reuveny, Z. 1976. Sulfate Reduction in Plant Biochemistry. Third edition. Bonner, J. and Varner, J.E. (eds.) Academic Press. pp 599-632.