DEVELOPMENT OF SOMATIC CELL GENETICS SYSTEMS IN *VIGNA RADIATA* AND *PHASEOLUS VULGARIS*

By

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ABSTRACT

DEVELOPMENT OF SOMATIC CELL GENETICS SYSTEMS IN *VIGNA RADIATA* AND *PHASEOLUS VULGARIS*

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Limited genetic diversity within the food legume genera *Vigna* and *Phaseolus* prompted us to develop a somatic cell genetic system by which these sexually incompatible genera might ultimately be mutually introgressed. Grain legume cell lines were subjected to three positive selections in an attempt to generate a system of complementing biochemical markers for protoplast fusion hybridization.

In developing a mutagenesis protocol for *Vigna radiata* cells *in vitro*, photoreactivation of UV induced growth inhibition was demonstrated. A *V. radiata* cell line was UV mutagenized and selected for resistance to the folic acid analog, methotrexate (MTX). Among many surviving colonies, several clones showed a stable low level of drug resistance. Variant frequency was enhanced by mutagenesis. To better design further selections for higher level methotrexate resistance, the drug's effects on five cell lines were studied. Natural interspecific variation in MTX tolerance spanned a two hundred fold concentration range and drug sensitivity was directly proportional to intrinsic growth rate. Folinic acid reversed methotrexate toxicity wherever tested. Stoichiometric inhibition of dihydrofolate reductase (DHFR) by MTX was

demonstrated in crude extracts of cultured V. radiata and Nicotiana tabacum. DHFR's from these cell lines were shown to be kinetically indistinguishable although their pH optima were 7.6 and 6.8 respectively. DHFR exhibits exponential phase induction in batch cell cultures of tomato, tobacco and V. angularis. The level of peak DHFR specific activity is proportional to a cell line's intrinsic growth rate. The enzyme could not be induced with folic acid, MTX or folinic acid. The implications of these findings for in vitro uses of methotrexate in plant cell systems are discussed.

Cultured Phaseolus vulgaris cell populations were screened for ethionine resistant and galactose utilizing mutants. Although no variants were recovered from these selections, characterization of the selective systems revealed some relevant aspects of amino acid and carbohydrate metabolism.

An anthocyanin pigmented variant cell line of a complex Vigna species hybrid was fortuitously recovered. Synthesis of the pigment, a vacuolar anthocyanin tentatively identified as a malvidin derivative, is enhanced by media sucrose supplementation, but not by light.

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Finally, I owe my greatest debt of gratitude to my family and especially my wife, Gracia, who always kept the faith.

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ABBREVIATIONS AND TERMINOLOGY

A_x	Spectrophotometric absorbance at wavelength x (nm)
ACES	N-(2-acetamido)-2-aminoethanesulfonic acid
ANOVA	Analysis of variance
BTP	1,3-bis [tris(hydroxymethyl-methylamino-propane)]
C_1	Single carbon moiety, associated with THF in this context
cAMP	3',5'-cyclic adenosine monophosphate
CHO	Chinese hamster ovary
CRD	Completely randomized design
D_{37}	Dose resulting in a 37 percent reduction in measured parameter
d/d	Doublings (generations) per day
DHF	Dihydrofolic acid
DHFR	Dihydrofolate Reductase
EMS	Ethyl methane sulfonate
FACS	Fluorescence activated cell sorter
FnA	Folinic acid
I_{50}, I_{100}	Dose resulting in a 50 or 100 percent viability reduction, respectively
J/m^2	Joules per square meter
K_i,K_D	Dissociation constant
K_m	Michaelis constant, substrate concentration at which velocity is half-maximal
k_p	Turnover number, molecular activity, catalytic center activity
Lawn	The confluence of colonies resulting from high density slosh inoculation of petri dish cultures

LSD	Least significant difference
MES	2(N-morpholino) ethane sulfonic acid
$\mu Em^{-2}s^{-1}$	Microeinsteins per square meter per second
MTX	Methotrexate, amethopterin
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NTG	Nitrosoguanidine
OD	Optical density
pCMB	Para-mercuribenzoate
PCV_{20}	Packed cell volume after 20 minutes at 1 x g
PCV_{20-1}	Packed cell volume after 20 minutes at 1 x g on first day of experiment
PR	Photoreactivation
r	Correlation coefficient
r^2	Coefficient of determination
S.A.	Specific activity (units per milligram protein)
se	Standard error
Slosh	Cell plating technique whereby a suspension culture is poured onto solid medium in a petri dish. The excess suspension is then serially poured onto succeeding dishes.
t_d	Doubling (generation) time
TES	N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid
THF	Tetrahydrofolic acid
TRIS	Tris(hydroxymethyl)aminomethane
2,4-D	2,4-dichlorophenoxyacetic acid
UV, UVL	Ultraviolet, ultraviolet light
V_T	Total volume

PROLOGUE

Origins of the Present Work. Grain legumes of the genera Vigna and Phaseolus are major staple foods for the burgeoning peasant classes of South Asia and Latin America, respectively. In these Third World regions, where animal protein is a luxury obtainable by only the affluent few, the broad masses of people rely on a diet of legume and cereal mixtures for adequate protein and caloric nutrition. A proper balance of legume and cereal consumption must be maintained to assure adequate protein nutrition in a diet based primarily on vegetable protein (Lappe, 1971). The Green Revolution of the 1960s boosted cereal production in these regions dramatically. However, increases in grain legume yields have not been as impressive. Therefore, the "protein gap" has widened in countries where the enhanced profitability of cereal cultivation has reduced both the production acreage of grain legumes and, consequently, the availability of legume protein to the consumer (Borlaug, 1975; Swaminathan and Jain, 1975; Collins and Lappe, 1977). Radical measures may be necessary in the coming decades to maintain even a minimum supply of balanced protein in the Third World. It has been toward the ultimate goal of genetic improvement of the food legumes that the present study has been directed.

Bressani (1975) has stressed that breeding advances in grain legumes might best be brought about by 1) Devising better methods of population improvement; 2) Broadening the genetic base; and, 3) Applying advanced genetic manipulative techniques. Population improvement can, in fact, be served by the latter two approaches. There is disagreement as to whether the germplasm bases of

Vigna spp. and Phaseolus spp. are in fact narrow (Chaven et al., 1965; Smart, 1971) or merely underexploited (Froussios, 1970; Frankel, 1975; Swaminathan and Jain, 1975; Evans and Davis, 1976; Imbrie et al., 1981). Adams (1977) and others (National Academy of Sciences, 1972) concluded that the genetic base of cultivated P. vulgaris is narrow enough to warrent concern over its genetic vulnerability. That valuable additions to legume breeders' populations cannot be made by further building and probing of germplasm banks has yet to be proved. The merits of such a traditional approach notwithstanding, plant breeders are frequently frustrated by the rare, weakly expressed or unfavorably linked occurrence of desireable agronomic characters in their populations. The interspecific transfer of such characters, blatantly expressed in a taxonomically proximal, yet sexually isolated, species represents a desideratum worthy of rational pursuit.

Disease and pest resistance is considered to be the most important breeding objective for short term mung bean yield improvement (Poehlman, 1978; Yang, 1978, Imbrie et al., 1981). The crop is acutely susceptible to rust, powdery mildew, Cercospora leaf spot, Rhizoctonium root rots and beanfly infestations (Yang, 1978). In addition, harvest efficiency suffers from pod shattering, the crop's decumbent habit and non-uniformity of maturation (Park and Yang, 1978). Vigorous research efforts in the U.S. have resulted in the characterization of both major sources of genetic resistance to the former diseases (Zaumeyer and Meiners, 1975) and genetic controls over the latter characters in Phaseolus vulgaris (Adams, 1973). Likewise, the American Phaseolus vulgaris crop suffers from predation by the Mexican bean beetle (Smartt, 1976), yield losses due to pod and flower drop and limited sources of resistance to its major diseases (Zaumeyer and Meiners, 1975). Genetic sources of amelioration of these yield-limiting factors can be found in some Vigna

species (Strand, 1943; Yang, 1978). Therefore, it appears that intergeneric hybridization would serve breeders on both sides of the Pacific. Interspecific sexual hybridization within the genera Vigna and Phaseolus have thus far produced some promising (Chen, 1980) and valuable (Honma, 1956) results. However, attempts to achieve intergeneric sexual hybridization within the Papilionoideae have been, at best, unsuccessful (McComb, 1975; Smart, 1979) and at worst, "ill-advised" (Smart, 1981).

Interspecific hybridization has been achieved by protoplast fusion in a number of model systems (Chaleff, 1981). Successful recovery of intergeneric hybrids by somatic cell hybridization has been reported in the families Umbelliferae (Dudits et al., 1980), Solanaceae (Melchers et al., 1978; Krumbeigel and Schieder, 1979) and Cruciferae (Gleba and Hoffman, 1978). In each of these studies, the fusion partners seem to have been chosen so as to maximize not the practical utility of the hybrid, but the probability of recovering a hybrid at all. In spite of the fact that a number of forage legume plants can now be regenerated from cultured cells (Saunders and Bingham, 1972; Scowcroft and Adamson, 1976; Oswald et al., 1977; Moktarzedeh and Constantin, 1978; Bharal and Rashid, 1979; Beach and Smith, 1979; Phillips and Collins, 1979; Gharyal and Maheshwari, 1980; and Meijer and Broughton, 1981), a somatic hybrid with a legume in its pedigree has yet to be reported. The only large seeded grain legume with which cell-to-plant regeneration can be routinely accomplished is the winged bean, Psophocarpus tetragonologus (Venketeswaran, 1978; Bottino et al., 1979; Gregory et al., 1980; and Mehta and Mohan Ram, 1981). A reproducible method for whole plant regeneration from cultured cells of Phaseolus vulgaris or Vigna radiata has not been reported. In the present work, it is assumed that, eventually, this technical obstacle will be surmounted, so that the products of this and similar endeavors can be put to practical use.

Grain Legume Cell Cultures: Physiological Studies. Despite their intractability toward regeneration, cell cultures of large seeded grain legumes, particularly soybean (Glycine max) and P. vulgaris, have served as experimental material in some classic investigations. The standard bioassay for cytokinin activity employs soybean callus and is sensitive to 20 nM kinetin (Miller, 1963). Fosket's group has extended the pioneering studies of Blaydes (1966) on the effect of cytokinin withdrawal and replenishment on the cell cycle and the translational control of protein synthesis in soybean cell cultures (Fosket et al., 1977; Muren and Fosket, 1977; Fosket and Tepfer, 1978). Other hormonal studies with soybean cells have focused on the effects and production of ethylene (LaRue and Gamborg, 1971; Constabel et al., 1977) and the intracellular target of the auxin indole-3-acetic acid (Matthysee and Phillips, 1969). Cell cultures of G. max were partners in the first diazotrophic association in vitro (Holsten et al., 1971; Child and LaRue, 1974), and have continued to play a role in the dissection of symbiotic nitrogen fixation (Ludden and Carlson, 1980). Gamborg's (1975) SB-1 soybean cell line has become a veritable E. coli for the study of DNA replication and the cell cycle by Lark's group (Chu and Lark, 1976; Cress et al., 1978; Roman et al., 1980), cell wall synthesis (Klein, 1981) and virus infection (Jarvis, 1979). Cultured soybean cells have also been the subjects of studies on nitrogen assimilation (Bayley et al., 1972; King and Hirji, 1975; King, 1976; Polacco, 1976, 1977), enzyme localization and regulation (Hahlbrock et al., 1971; Postius and Kindl, 1978; Polacco and Havir, 1979), the physiological effects of ultraviolet irradiation (Ohyama et al., 1974; Reilly and Klarman, 1980) and mRNA complexity and metabolism (Silflow and Key, 1979, Silflow et al., 1979).

The first long term batch suspension culture of plant cells was composed of hypocotyl derived tissue of Phaseolus vulgaris (Nickell, 1956). In a classic paper, Bergmann (1960) described what has become a widely adapted method for plating

suspension cultured cells of P. vulgaris in soft agar. Through the continued efforts of Nickell and others, the cellular morphology, nutritional requirements and growth kinetics of suspension cultured P. vulgaris have been thoroughly characterized (Tulecke and Nickell, 1959; Nickell and Tulecke, 1960; Mehta et al., 1967; Liau and Boll, 1971, 1972). Lamport (1964) rejected P. vulgaris in favor of sycamore cells for studies on growth energetics, due to the "variable (and therefore) inferior pipettability" of the former. However, Dougall (1964) found Phaseolus suitable for such studies. Veliky and Martin (1970) solved the aggregation problem encountered by Lamport (1964) by growing P. vulgaris cells in "semi-continuous" culture, whereby the larger aggregates were periodically drawn from the bottom of the fermentor. Bertola and Klis (1979) found that P. vulgaris suspensions could be entrained to generate "mainly small aggregates of spherical cells" by growing them under glucose limitation in a modified bacterial fermentor.

In contrast, their prolific elaboration of extracellular polysaccharide, no doubt causally linked to cellular aggregation (Lamport, 1964), has made P. vulgaris cell cultures the systems of choice for studies of cellular (though not tissue) differentiation. Boll's group has characterized these compounds and the conditions required for the induction of their synthesis (Mante and Boll, 1975, 1976, 1978). The finding that subtle shifts in culture conditions could effect differentiation events led this group to investigate exogenous signals for gene expression at the isozyme level in Phaseolus callus and suspension cultures (Arnison and Boll, 1974, 1976, 1978). Their surprising revelation of multiple, discrete clonal differences in allozyme patterns was perhaps a foreshadowing of later studies of variability in vitro by Carlson (1978) and Secor and Shepard (1981). In a similar vein, Northcote's group has used the P. vulgaris cell culture system to study vascular differentiation at the histochemical (Jeffs and

Northcote, 1966), physiological (Jeffs and Northcote, 1967), enzymatic (Rubery and Northcote, 1968; Bevan and Northcote, 1979; Dudley and Northcote, 1979) and mRNA levels (Dudley and Northcote, 1978).

As in the case of soybean, cell cultures of P. vulgaris normally exhibit auxin and cytokinin dependence. The Mok group has employed this system to explore the genotypic contribution to hormonal response in vitro (Mok and Mok, 1977; Mok et al., 1980), as well as structure-function relationships of cytokinins (Mok et al., 1978) and the exogenous control of cytokinin autonomy (Mok et al., 1979). Finally, the Phaseolus system has been exploited in comparative studies of whole plant and in vitro reponses to mineral stress (Christianson, 1979) and pathotoxin symptomology (Rudolff and Warick, 1968; Bajaj and Saettler, 1970; Russell, 1970).

Very few reports have appeared wherein cultures of mung bean, Vigna radiata (usually called by its former name, Phaseolus aureus) have been used. Gamborg (1966b) found mung bean cell suspensions to be rich sources of eight enzymes of aromatic metabolism. The first plant quinate dehydrogenase activity was detected in these cultures. Barz's group has employed cultured mung bean cells for their studies of the synthesis and degradation of plant phenolics (Barz and Berlin, 1970; Dewick et al., 1970; Berlin and Barz, 1971). It is interesting to note that neither of these groups reported a cytokinin requirement for optimal mung bean culture growth.

Grain Legume Cell Cultures: Genetic Studies. Despite their impressive service in these physiological and biochemical areas, the Glycine and Phaseolus cell culture systems have not been adopted widely for genetic studies. The reasons for this are threefold: 1) Whole plant regeneration from callus or suspension cultures has not been achieved in either system. Therefore, any variants selected in vitro are doomed to remain there, with a classical genetic

characterization impossible. 2) Haploids have not been recovered by in vitro techniques in either species; although, sexually generated soybean haploids have been reported (Beversdorf and Bingham, 1977), and are beginning to be exploited in vitro (Weber and Lark, 1980; Zhou, personal communication). Confinement of the systems, to date, to diplontic selections has narrowed the scope of variants obtainable. 3) Aggregation, slow growth and high density requirements have made microbial methodologies less readily adaptable to those species than to the model solanaceous and carrot systems. Nonetheless, some important results have been obtained with the soybean cell system.

The Saskatoon group used soybean protoplasts in their pioneering studies of the feasilibity of interfamilial hybridization by protoplast fusion (Kao et al., 1971). Cell divisions were reported in heterokaryocytes of barley-soybean, corn-soybean, pea-soybean and Vicia-soybean fusions (Kao et al., 1974). Although never regenerated into interfamilial hybrid plants, Kao's tobacco-soybean hybrid clones retained cytological (Kao, 1977) and isozymic (Wetter, 1977) evidence of hybridity for six months (100 generations) following fusion. The preferential loss of the larger Nicotiana glauca chromosomes was accompanied by the loss of tobacco isozyme markers.

Somatic cell selections have yielded two different auxotrophic soybean cell lines. Polacco (1979) recovered a deficiency variant by using arsenate as a negative selection agent. The variant cell line, M36-2, displayed a growth dependence on bovine serum albumin, casein hydrolysate or conditioned medium. Its presumed amino acid auxotrophy could not, however, be tested, since the line was unstable and lost its apparent deficiency after nine months in culture. Zhou (personal communication) has isolated Na^+ dependent cell lines using UV mutagenesis and a non-selective isolation procedure. Among several sodium resistant clones recovered, two were also sodium dependent. The deficiency

appeared to result from decreased Na^+ transport capacity, as judged by the variants' impaired ability to take up $^{22}Na^+$. Revertants could only be obtained with further mutagenesis.

Three groups have reported attempts to select soybean cells with new metabolic capabilities. Ikeda et al. (1979) transferred soybean calluses from complete medium to one in which thiamine had been substituted with a precursor. Three surviving calluses survived transfer to a medium containing an upstream precursor. The authors suggested that the acquired autotrophy was perhaps epigenetic, by comparing the frequency of variant recovery with that of cytokinin habituation. Limberg et al. (1979) and Weber and Lark (1980) have selected a large number of soybean cell mutants, in haploid and diploid cultures, for their ability to assimilate maltose as a sole carbon-energy source. In the former study, a multi-step selection protocol was used to recover variants of decreasing generation time on maltose. No biochemical differences could be detected between the wild type and the maltose-adapted cells. However, the latter were larger and transported maltose more efficiently than the wild type. The variants were stable in the absence of selection (100 generations on sucrose) and their frequency of occurrence could be enhanced with mutagenesis (see below). Polacco et al. (1979) have reported preliminary explorations into the conditions for selection of high urease soybean cells, although no variants have yet been reported.

Soybean cells have been selected for resistance to a number of cytotoxic agents. Weber and Lark (1979) demonstrated the efficacy of their nurse culture selection technique by selecting a variant resistant to 8-azaguanine. The cell line did not lack the enzyme hypoxanthine-guanine phosphoribosyl transferase, which is commonly defective in or missing from mammalian cell mutants resistant to this purine analog. A soybean cell line resistant to 6-thioguanine

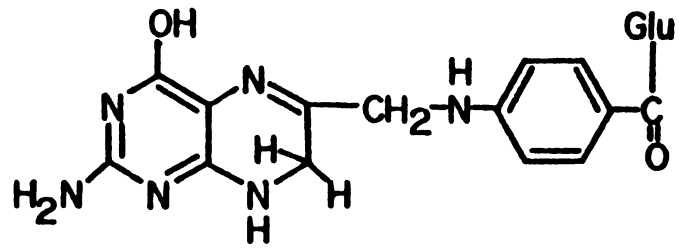
(another purine analog), reported in the same communication, was selected by stepwise adaptation to the analog over a 6 month period of suspension culture incubation. The biochemical or physiological basis of neither of these variants has been reported. Preliminary communications of soybean cells resistant to ethionine, paraquat and diquat have also appeared (Chaleff, 1981).

The most thoroughly characterized variant legume cell lines thus far selected were BU-5 and BU-54, Ohyama's (1974, 1976) soybean cell lines exhibiting resistance to the thymidine analog, 5-bromodeoxyuridine (BrdU). BU-5 was one among twenty putative mutant clones selected at a frequency of 4×10^{-5} by plating regenerated cells from SB-1 soybean protoplasts, mutagenized with N-methyl-N-nitro-N-nitrosoguanidine, onto BrdU-containing medium. The variant displayed resistance BrdU 250 fold greater than that of wild type, provided that the medium was supplemented with uridine. Changes in thymidine uptake and thymidine kinase activities could not account for the BrdU resistance. DNA bouyant density analysis revealed that BU-5 nuclear DNA accumulated BrdU to levels normally expected to be mutagenic, if not lethal. The basis of BU-5's resistance was never established, and the line is no longer available (K. Ohyama, personal communication). A second BrdU resistant cell line, BU-54, was subsequently recovered by subjecting BU-5 to another round of selection for resistance to higher levels of BrdU and fluorescent light (Ohyama, 1976). The phenotype of BU-54 was stable and exhibited both 1000 fold greater BrdU and aminopterin resistance than wild type SB-1, in addition to uridine independence. Resistance to 5-fluorodeoxyuridine (FdU) was also demonstrted in Bu-54. The activity of thymidylate synthetase (TS) was slightly higher in BU-54 than in SB-1 when the former was grown non-selectively. However, TS activity doubled when BU-54 (but not SB-1) was grown in BrdU. Dihydrofolate reductase activity of BU-54 was 50 fold higher than that of wild type, whether the variant

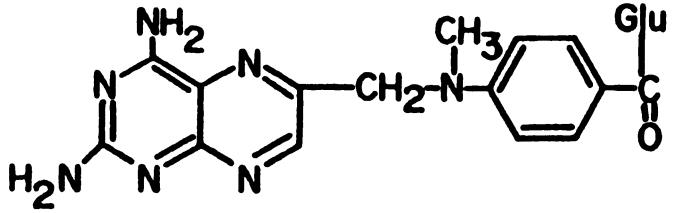
was maintained selectively or non-selectively. BrdU uptake was normal in BU-54, but incorporation of the analog into DNA was half or twice that in wild type, depending on the absence or presence of FdU, respectively. Ohyama concluded that increased synthesis of dTMP resulted in reduced BrdU incorporation into DNA in BU-54. The basis for the amplified enzyme activities in BU-54 was not clarified. In the light of the present study and subsequent revelations about the origins of increased enzyme activities in variant mammalian cell lines (especially dihydrofolate reductase, see below), further analysis of this cell line would be valuable.

In the present study, three selections are described: resistance to methotrexate and ethionine, and ability to assimilate galactose. No published precedents for these selections exist in the realm of grain legume cell culture, although experience has been gained in other plant and animal systems.

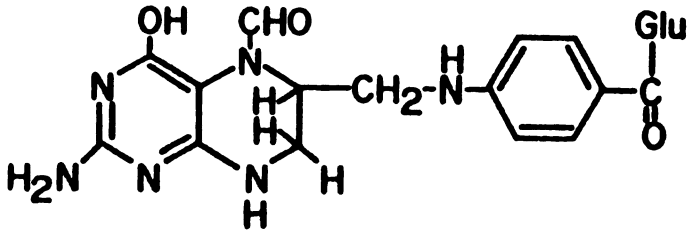
Dihydrofolate Reductase: Inhibition by Methotrexate. The potent toxicities of aminopterin (N-[4-{[(1,4-Diamino-6-pteridyl)-methyl]-amino}-benzolyl]-glutamic acid) and its N^{10}-methyl homologue, methotrexate (Figure 1), were acknowledged in the same communication in which their syntheses were first reported (Seeger et al., 1949). However, it was not until the simultaneous reports of Futterman (1957), Osborn et al. (1958) and Zakrzewski and Nichol (1958) that the identity of their cellular target, dihydrofolate reductase (DHFR), was clearly established. These early workers used standard enzyme inhibition kinetics techniques to conclude that methotrexate (MTX) inhibition of DHFR was competitive with respect to the enzyme's normal substrate, dihydrofolate (DHF, Figure 1). However, Werkheiser (1961) would seem to have established a different mechanism. He showed that, under appropriate (and approximately physiological) conditions, inhibition was "stoichiometric". That is, inhibition of DHFR was linearly proportional to the amount of MTX present, suggesting that



DIHYDROFOLIC ACID



METHOTREXATE



5-formyl THF (Folinic Acid)

Figure 1: Structures of folate derivatives used in this study.

the enzyme was being titrated by the drug. That inhibition by other 4-amino folate analogs exhibited precisely the same concentration dependence as that by MTX lent support to Werkheiser's stoichiometric or "pseudo-irreversible" hypothetical mechanism. Still, other workers (Mathews and Huennekens, 1963; Burchall and Hitchings, 1965) could not unequivocally corroborate the model until it was demonstrated (Williams et al., 1973) that the exact assay conditions dictated the form MTX inhibition would take. It has since been repeatedly demonstrated that the following conditions must be controlled for "stoichiometric" inhibition of DHFR by MTX to be observed: 1) The enzyme must be preincubated with the inhibitor, and in most cases, with the cofactor NADPH (Williams et al., 1973). 2) The assay should be carried out at the lowest possible pH (DHFR frequently exhibits multiple pH optima, see below and Bertino et al., 1964). 3) The DHF concentration must be well above the K_m, but not so high as to effectively compete with the inhibotor (Werkheiser, 1961). 4) The ionic strength of the reaction mixture must be kept to a minimum (Williams et al., 1973). Under condition (1), above, binding of the inhibitor to the enzyme is completed before the normal substrate is added. No competition is possible. NADPH is included in the preincubation mixture because the ternary complex (DHFR-MTX-NADPH) is usually more stable (Perkins and Bertino, 1966) than the binary (DHFR-MTX) complex. However, the binary complex of soybean DHFR and MTX is apparently no less stable that the ternary complex (Reddy and Rao, 1977). Stabilization of the complex by NADPH in other systems has been attributed to the cofactor's blocking access of solvent to DHFR's hydrophobic cavity where hydrogen bonds join the enzyme and inhibitor (Baker, 1959).

A proper method for the quantification of the tightness of MTX binding to DHFRs is not generally recognized. In applying classical Lineweaver-Burk (1934) inhibition analysis, it is assumed that at any time, essentially all of the inhibitor

is free (Segal, 1975). This steady state condition does not obtain in the case of a very tight binding inhibitor such as methotrexate. Thus, Werkheiser (1961) slightly modified Lineweaver-Burk procedures, after Goldstein (1961), to account for the depletion of free inhibitor by enzyme titration, and arrived at a value of 3×10^{-11} M MTX as a maximum K_i for rat liver DHFR. Bertino et al. (1964) took advantage of the (operationally) competetive inhibition by MTX of Erlich ascites tumor DHFR which obtains at its higher pH optimum. Using Dixon's (1953) method, these workers calculated a maximum K_i of 6.7×10^{-10} M MTX. Domin et al. (1979) used Cha (1975) and Cha et al.'s (1975) adaptation of the Ackermann-Potter (1949) procedure to calculate K_i's of 4×10^{-11} and 1.4×10^{-10} M MTX for human and mouse S180/AT3000 DHFR's respectively. In all of these reports, different algebraic adaptations of classical inhibition analysis were employed. Greco and Hakala (1979) evaluated these and 8 other classically-derived methods of extimating the K_i of tight-binding enzyme inhibitors by a computerized Monte Carlo simulation. The methods of Ackermnn and Potter (1949) and Morrison (1969) (actually a modification of the former) were judged to be the most precise, given the accessibility of computer software for non-linear regression. Flintoff and Essani (1980) determiend K_i's for DHFRs from several MTX resistant CHO cell lines (and wild type) by a [^{3}H]-methotrexate binding assay using gel filtration to separate bound from free drug (Lo and Sanwal, 1975). Their apparent K_i determinations ranged from 5×10^{-10} to 3.2×10^{-9} M MTX. However, the theoretical support for their technique is questionable (C. Suelter, personal communication). More recently, Haber et al. (1981) reported K_i's of 2×10^{-10} and 5.4×10^{-8} M MTX for wild type and mutant mouse DHFR's, respectively, determined by standard equilibrium dialysis techniques and Scatchard (1949) plots. The assays were performed at a relatively high pH (7.5) to weaken MTX-DHFR binding (otherwise this procedure

might not have been appropriate). Consequently, their values are, once again, maximum estimates. In the end, it appears that an ideal method has not yet been established for determinations of such low K_i's. At best, comparisons of K_i's within a single system (e.g., mutant vs. wild type) and laboratory (Jackson et al., 1979) are valid, but the many estimates (Greco and Hakala, 1979) are not comparable *inter se*. No such values have been reported for plant DHFRs, although Crosti (1981) has reported meaningless I_{50}'s for MTX inhibition of the maize and pea enzymes.

Methotrexate I_{50}'s for growth inhibition in plant and mammalian systems have been frequently reported. Cultured mammalian cells, which are folate-requiring and assimilate folates by active transport (Huennekens and Henderson, 1975) display MTX I_{50}'s of 2-5 x 10^{-8} M (Eagle and Foley, 1956; Hakala et al., 1961). Nielsen et al. (1979) reported values of 2 x 10^{-9} to 3 x 10^{-6} M MTX for several suspension cultured plant cell lines, as judged by fresh weight increase. Cocking (1976) reported minimum aminopterin concentrations of 4 x 10^{-7} and 5 x 10^{-5} M for complete growth inhibition of "small colonies" of Petunia hybrida and Nicotiana tabacum cells, respectively. Mastrangelo and Smith (1977) found that 5 x 10^{-8} M aminopterin reduced by fifty percent the colony forming ability of small aggregates and single cells of Datura innoxia. Somewhat higher concentrations (in the micromolar range) of anti-folates were required to inhibit the growth of Brassica sp. (Ruddenberg et al., 1955), pea (Suzuki and Iwai, 1970) and soybean (Reddy and Rao, 1975) seedlings. It is perhaps worth noting that plant cell cultures, by virtue of their relatively high inoculum density requirements, represent the only in vitro systems for MTX inhibition studies where the volume occupied by the cells themselves is a significant proportion of the total culture volume, even at the beginning of a growth experiment. This consideration renders I_{50}'s expressed as molarities less than optimally

comparable. Consequently, in the data presented in Part V, MTX levels are expressed as moles of drug per milliliter of packed cell volume on the first day of the experiment. This compromise is not ideal, but represents one approach to a problem unique to plant cell systems and worthy of further investigation (Parke and Carlson, 1979).

Cellular Mechanisms of Resistance to Methotrexate. The immediate recognition of the cytotoxicity of the 4-amino folate analogues prompted early researchers to evaluate their chemotheraputic capacity (Farber et al., 1948). Aminopterin and methotrexate have since become "magic bullet" anti-tumor drugs, exhibiting remarkable effectiveness in the control and frequent apparent cure of choriocarcinoma, Burkitt's lymphoma and acute leukemia (Blakley and Morrison, 1970). These successes have inspired intensive activity in the synthesis and clinical evaluation of a galaxy of folate analogs (Baker, 1967). However, two problems accompany so-called "high-dose methotrexate chemotherapy" (MTX having been the most widely adopted in chemotherapy). First, the drug is toxic to normal, rapidly proliferating cells, particularly those of the intestinal mucosa and bone marrow. Second, tumor cell populations, whether treated in situ or in vitro with anti-folates, frequently develop drug resistant clones. In chemotherapy, this latter event necessitates either increasing the drug dosage or abandoning the therapy.

The obstacle of methotrexate toxicity has been greatly reduced by the discovery that the simultaneous administration of either actinomycin D (Ross et al., 1965) or 5-formyl tetrahydrofolate (5-formyl THF, folinic acid, Figure 1) selectively rescues normal cells (Hyrniuk et al., 1967). The physiological mechanism by which these antidotes apparently protect normal cells but not tumor cells is still at issue (Djerassi, 1975). The popular current model for folinic acid "rescue" is as follows: Tumor cells have an impaired active transport

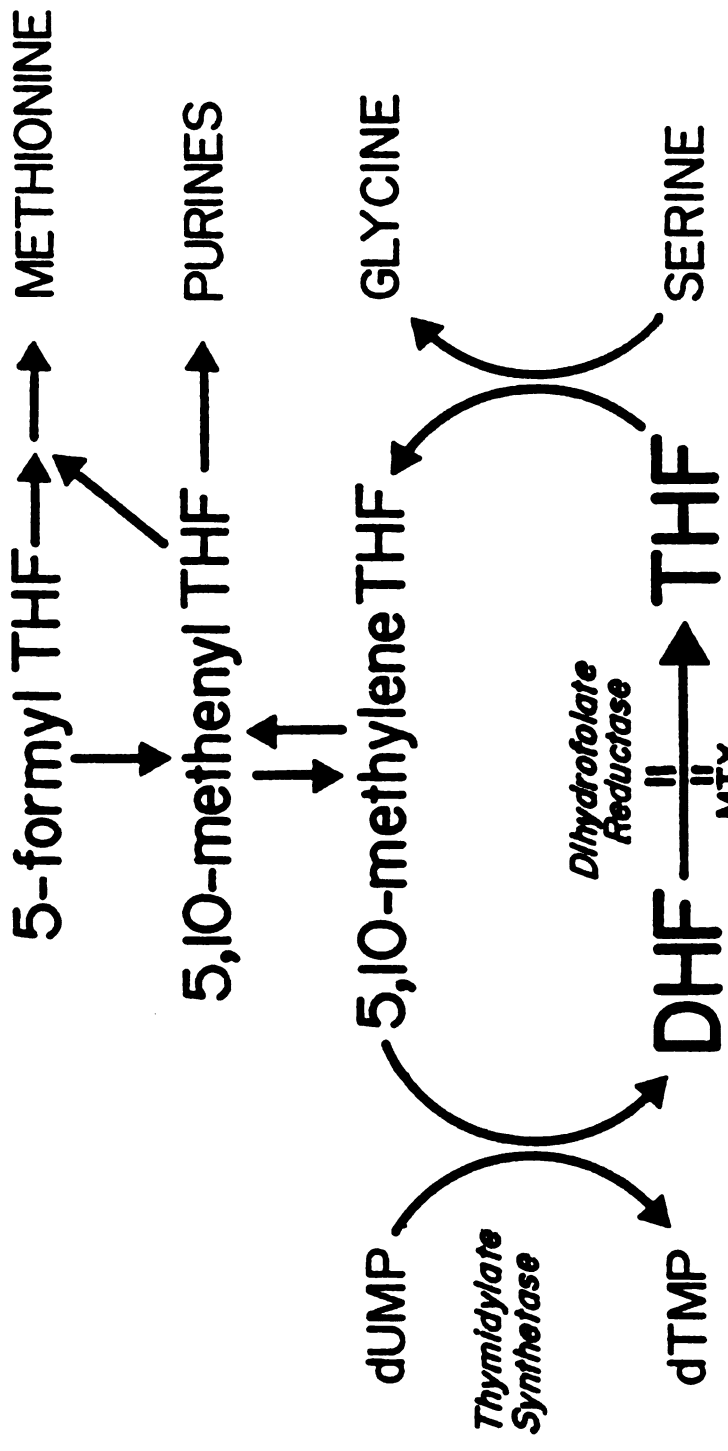


Figure 2: Pathways of folic acid metabolism.

system for folates. At high serum concentration, MTX enters tumor and normal cells by diffusion. When low concentrations of 5-formyl THF are administered, normal cells accumulate the antidote and thereby sufvive, whereas transformed cells do not, and die (Saucer et al, 1979). Once 5-formyl THF enters a MTX arrested cell, the former compound supplies reduced folates directly for DNA and protein synthesis, obviating the cell's normal dependence on DHFR (Figure 2). 5-formyl THF rescue of MTX inhibited Brassica sp. and pea seedling growth has been demonstrated (Ruddenberg et al., 1955; Suzuki and Iwai, 1970).

The problem of acquired resistance to MTX in tumorous tissue has long been recognized (Law, 1956). A sizable literature has accumulated concerning the question of how such resistance is attained. Three modes of resistance have been observed in microorganisms and eukaryotic cells in culture. These include impaired transport of the drug, altered affinity of the intracellular target (DHFR) for the drug, of increased levels of the target enzyme in the cell. The first two mechanisms appear most frequently in mutants recovered in single step selections, especially with mutagenesis (Flintoff et al., 1976a). The latter mechanism usually requires multistep selections (but see below). Sirotnak et al. (1967) isolated six mutants of Diplococcus pneumoniae which exhibited MTX resistance 10 to 100 fold greater than wild type. The transport Km for labeled drug was 2.5 to 10 fold higher in the mutants, though the V_{max} was unaffected. Flintoff et al. (1976a) isolated four mutants of cultured CHO cells, by EMS mutagensis, which displayed high MTX resistance and drastically reduced transport of the drug. In a subsequent study, it was shown that this class of mutant arose at a spontaneous rate of less than or equal to 2×10^{-9} per locus per generation. Somatic cell fusion studies suggested that the impaired transport character was a recessive trait (Flintoff et al., 1976b).

Biedler et al. (1972) reported the isolation of several CHO mutant cell lines, highly resistant to methotrexate. Some displayed high levels of DHFR, whereas others turned out to have DHFRs with altered affinity for the drug (Albrecht et al., 1972). This historic report was the first good indication that structural gene mutations could be isolated in mammalian cell cultures (Wright et al., 1980). One of Albrecht et al.'s mutants displayed reversible (as opposed to "stoichiometric") inhibition of DHFR by MTX. In vitro translation of mRNAs encoded by this mutant and a wild type control indicated that the former produced a DHFR of altered molecular weight (Melera et al., 1980).

Flintoff et al. (1976a) also recovered CHO cell lines, following EMS mutagenesis, with a DHFR possibly displaying altered MTX affinity, as judged by crude kinetic analyses (Gupta et al., 1977). The best support for this mechanism was the convincing evidence eliminating the other two most common modes of resistance mentioned above. These mutants also occurred at the rate of 2×10^{-9} per locus per generation and were expressed condominantly in somatic cell hybrids (Flintoff et al., 1976b). The case for the structural alteration in these workers' MTX resistant DHFR is less than solid on two accounts. First, they have not yet used accepted techniques of inhibition kinetic analysis to analyze the mutant (see above); and, second, the supposedly altered DHFR shows no differences from the wild type enzyme on two dimensional electrophoretic gels (Flintoff and Essani, 1980). However, the K_m for folate for their putatively altered DHFR is three fold higher than that of the wild type enzyme (Gupta et al., 1977). Such a change might be expected if a structural gene mutation had actually occurred. In the later report, the chromosome bearing the "altered" gene was revealed to have participated in a reciprocal translocation event (Worton et al., 1981). The possible position effect implications of this anomaly were not discussed. Nonetheless, a high DHFR derivative of this cell line,

recovered from a second step MTX selection, served as source material for the selection of DHFR null mutants (Flintoff and Weber, 1980) using the [^{3}H]-uriding suicide method previously reported by Chasin and Urlaub (1979) and Urlaub and Chasin (1980).

The most convincing data presented, to date, suggesting the recovery of an altered DHFR has recently appeared from Schimke's group (Haber et al., 1981). CHO cells were subjected to stepwise MTX selection. Selection at low MTX concentrations yielded resistant lines with elevated levels of normal DHFRs, as expected (see below). Further MTX selections within these "high DHFR" populations resulted in the recovery of a cell line displaying substantial MTX resistance, but normal levels of DHFR, as determined by FACS analysis. These workers proceeded to demonstrate an alteration in the DHFR of the variant cell line by several methods: 1) DHFR from the mutant bound less [^{3}H]-methotrexate than wild type DHFR in a sensitive binding assay (Kamen et al., 1976). 2) Its K_m for DHF was three fold higher than wild type's. 3) Its pH activity profile was substantially altered from that of wild type. 4) Purification of the enzyme from the variant cell line by affinity chromotography on MTX-Sepharose was inefficient, presumably owing to the enzyme's decreased affinity for MTX. 5) The turnover number for the altered DHFR was twenty fold lower than that of wild type. 6) The variant DHFR displayed altered sensitivity to other folic acid analogs. 7) The K_i for the altered DHFR's MTX binding was 270 higher than wild type's, as measured by equilibrium dialysis and Scatchard analysis (see above). 8) The enzyme displayed a significant basic shift in two dimensional electrophoretic migration (but no alteration in molecular weight). Given the extremely low K_i for MTX binding to the altered DHFR (normal $K_i = 2 \times 10^{-10}$ M; altered DHFR $K_i = 5.4 \times 10^{-8}$ M), the data obtained from equilibrium dialysis were at or very near the minimum level of sensitivity of the

assay. Despite their effort to engineer conditions to weaken the binding (and thus raise the data into the window of the assay's sensitivity), the Scatchard plots display considerable dispersion. Why the more theoretically sound methods (Greco and Hakala, 1979) of Ackermann and Potter (1949) or Morrison (1969) were not chosen was not explained. Nevertheless, Haber et al. raise the important question of how a cell line can arise with an amplified DHFR displaying decreased affinity for its natural substrate. Their data indicate that wild type DHFR's were reduced to a kinetically insignificant and barely electrophoretically detectable level in this variant line. Does a low MTX-affinity mutant, arising in a family of amplified normal DHFR genes, somehow replace all of the other members of the family? A "manuscript in preparation" promises to shed some light on this question (Haber et al., 1981).

The biochemical phenotype most frequently associated with MTX resistance is an increase in DHFR specific activity. The increased intracellular binding capacity for the drug results in more growth-supporting, free enzyme in these cells than in wild type cells, at any given ambient MTX concentration. The first direct demonstration of increased DHFR specific activity in MTX resistant was presented by Misra et al. (1961). The same mechanism was apparently operative in MTX resistant cultured hamster and mouse cells selected by Littlefield (1969) and Hakala et al. (1961), respectively. Hakla et al.'s AT3000 murine cell line exhibits resistance to 3000 fold more MTX than does the wild type S180 line. Alt et al. (1976) demonstrated that the 200 fold higher DHFR level in the AT3000 cell line was attributable solely to an increase in the synthesis of the DHFR enzyme. Hanggi and Littlefield (1976) found the same to be true for a MTX resistant baby hamster kidney cell line exhibiting a 140 fold elevated DHFR specific activity. DHFR mRNA levels were subsequently found to correspond to those of the enzyme in AT3000 cells (Kellems et al., 1976).

Finally, in a magnificent series of experiments, these workers demonstrated that in the AT3000 cell line, the DHFR structural gene is selectively multiplied about 200 fold (Alt et al., 1978). Partial phenotypic revertants of AT3000 displayed levels of DHFR enzyme, mRNA and structural genes which could wholly account for the revertants' reduction in MTX resistance. In the same communication, these workers also described selective DHFR gene amplification in a non-reverting MTX resistant murine lymphoma cell line, L1210S. cDNA-DNA hybridization was used to demonstrate the increased gene copy numbers in both cell lines. Schimke et al. (1978) have proposed that gene multiplication occurs by unequal crossing over, uptake of DNA from killed cells or disproportionate replication. Varshavsky (1981a) proposed the latter mechanism for tumor promotion, where tumor promoters ("firones") would cause replicon "misfiring". In a subsequent paper, Varshavsky (1981b) demonstrated how single step MTX-mediated DHFR gene multiplications could be promoted by 12-0-tetradecanoyl-phorbol-13-acetate (TPA), a proven and potent tumor promoter (Trosko et al., 1977). Confirmatory support for the "Firone Hypothesis" has yet to appear. In situ hybridization with radiolabeled DHFR cDNA has shown that stable MTX resistant lines contain amplified DHFR genes integrated into a unique chromosomal locus, whereas in unstable phenotypes, DHFR gene copies are localized in acentric "double minute" extrachromosomal elements (Nunberg et al., 1978; Kaufman et al., 1979). Loss or integration of the latter results in loss or stabilization, respectively, of the MTX resistant phenotype. Selective gene multiplication has since been shown to be responsible for toxic analog resistance in other systems (Wahl et al., 1979). A number of plant cell variants exhibiting a specific resistance correlated with elevated activities of one or more enzymes are candidates for a similar mechanism (Ohyama, 1976; Maretzki and Thom, 1978; Yamaya and Filner, 1981).

One additional mechanism of folate analog resistance naturally occurs in Aerobacter aerogenes (a.k.a. Klebsiella aerogenes) and in the yeast, Candida tropicalis. These organisms constitutively produce an enzyme which cleaves folate analogues, liberating a yellow pteridine compound which is subsequently assimilated (Webb, 1954). Resistance in C. tropicalis is not complete, the aminopterin I_{50} being 10^{-5} M. Breakdown of the drug was demonstrated by the appearance of a diazotizable amine, suggesting cleavage between the N^{10} and C^{9} (Nickerson and Webb, 1956).

Selection of Datura innoxia cells in vitro for resistance to aminopterin has been reported (Mastrangelo and Smith, 1977). A stepwise selection procedure yielded a number of variants resistant to 10^{-5} M aminopterin, whereas only 10^{-5} wild type cells could survive such a dose. Seeds were reportedly set on plants regenerated from one resistant clone, but neither Mendelian nor biochemical analyses have subsequently appeared. The report from Nielsen et al. (1979) of preliminary screening of several plant cell lines for methotrexate tolerance suggested that selections might be underway, but no variants have been reported. Other workers have begun selections in plant cell systems for mutants affected in upstream steps in folic acid metabolism (Merrick and Collins, 1980; Killmer et al., 1980).

Dihydrofolate Reductase: Enzymology and Regulation. The enzyme dihydrofolate reductase (DHFR; tetrahydrofolate dehydrogenase; 7,8-dihydrofolate:$NADP^{+}$ oxidoreductase, EC 1.5.1.3) catalyzes the second protonation in the two step reduction of folic acid to tetrahydrofolate (Figure 2). The latter product is subsequently converted to "C_1-activated" derivates which supply single carbon units in the biosynthesis of nucleotides and amino acids (Blakley, 1969). 7,8-Dihydrofolate (DHF, Figure 1), the product of the reduction of folic acid by folate reductase, is the substrate for DHFR, although other

folates can be less efficiently substituted in some systems. Reducing equivalents are most efficiently supplied by NADPH in all systems studied (Blakley, 1969). Neither pea nor soybean DHFR catalyzes the reduction of folate. Whereas NADH cannot serve as a cofactor for pea DHFR, soybean DHFR reduces DHF 90 percent less efficiently when NADH replaces NADPH (Suzuki and Iwai, 1970; Reddy and Rao, 1975). Michaelis constants of DHFR for DHF range from 0.2 to 35 μM, although values under 5 μM prevail for eukaryotic DHFR's (Blakley, 1969). The soybean and pea enzymes have K_m's for DHF of 35 and 4.3 μM respectively (Reddy and Rao, 1975; Suzuki and Iwai, 1970). Michaelis constants for the cofactor NADPH range from 1 to 45 μM for DHFR from most sources examined. However, the soybean enzyme displays a K_m of 415 μM for NADPH (Reddy and Rao, 1975), though the pea enzyme's NADPH K_m is 40 μM (Suzuki and Iwai, 1970). Turnover numbers for dihydrofolate reductase from different sources also display a remarkable range, from 57 (soybean; Reddy and Rao, 1976) to 8000 min^{-1} (Streptococcus faecalis; Nixon and Blakley, 1968). DHFR is activated in vitro by Mg^{++}, KCl, urea and mercurials (Perkins and Bertino, 1965; Greenberg et al., 1966; Kaufman, 1977; Domin et al., 1979), though no such effects have been reported in studies of plant enzymes.

Purified DHFR from most sources is a relatively small monomer of a molecular weight ranging from 15,000 to 34,000 daltons, although ~20,000 dalton is most prevalent. Apparent isozymic forms of DHFR have been described in bovine liver (Baumann and Wilson, 1975), cultured mouse (Jackson et al., 1975) and cultured hamster cells (Hanggi and Littlefield, 1974). The only multimeric DHFR occurs in soybean seedlings. It has at least three non-identical subunits (Reddy and Rao, 1976), but, like all other DHFR's studied, exhibits one active site per holoenzyme (Reddy and Rao, 1977; Purohit et al., 1981). No other molecular weight estimations have been reported for DHFRs of plant origin. The

enzyme usually exhibits a pH optimum near neutrality, though a second optimum near pH 5 is not uncommon in DHFR's of vertebrate origin (Bertino et al., 1964; Blakley, 1969). Pea and soybean DHFR's exhibit pH optima of 6.5 and 7.2, respectively (Suzuki and Iwai, 1970; Reddy and Rao, 1975).

The most commonly employed method for the assay of DHFR depends on the continuous measurement of the absorbance decrease at 340 nm (Osborn and Hunnenkens, 1958). Both DHF and NADPH have moderate extinctions at 340 nm, the combination of which gives the assay adequate sensitivity for many applications (Mathews et al., 1966). This procedure has been used in studies of pea (Suzuki and Iwai, 1970) and maize DHFR's (Crosti, 1981). Rothenberg (1966) introduced a radiometric DHFR assay which makes use of the capacity of vertebrate DHFR's to use folate as a substrate. This assay, as modified by Nakamura and Littlefield (1972) has been used extensively by researchers investigating the altered folate enzymology of variant mammalian cultured cell lines (described above). Its high sensitivity cannot be exploited in plant systems, however, owing to the inability of plant DHFRs to use folate as a substrate. Hayman et al. (1978) have presented an adaption of Rothenberg's (1966) assay, using [^{3}H]-DHF as a substrate. Instead, the spectrophotometric method of Reddy and Rao (1975), adapted from Rosenthal et al.'s (1965) tetrahydrofolate assay, has been used (see MATERIALS AND METHODS). The high extinction at 350 nm of the assay product, $N^{5}N^{10}$-methenyl tetrahydrofolic acid, makes this assay twice as sensitive as the other spectrophotometric assay described above. Kaufman et al. (1978) have developed a method of quantifying the level of DHFR in cultured cells based on the fluorescence of fluorescein-labeled, enzyme-bound MTX, using the fluorescence activated cell sorter (FACS).

Although a number of multistep purification procedures have been reported for DHFR of vertebrate and microbial origin, purification of the enzyme to

homogenaiety in two steps is possible by means of affinity chromatography (Erickson and Mathews, 1971; Kaufman, 1974; Whiteley et al., 1977). Reddy and Rao (1976) used the 10-formylaminopterin affinity method of Erickson and Mathews (1971) to purify soybean DHFR to homogenaiety (2000 fold), as judged by immunological methods and by its migration as a single band on non-denaturing polyacrylamide electrophoretic gels.

Little is known about the normal controls over the regulation of DHFR expression in prokaryotes or eukaryotes. Infection of E. coli by T-even bacteriophages results in a ten- to twentyfold induction in phage encoded DHFR inside the infected cells (Mathews, 1967b). Although it lies in a cluster of genes concerned with DNA percursor biosynthesis, the T_4 DHFR structural gene, "wh" (Hall et al., 1967) or "frd" (Hall, 1967), is apparently under a different temporal control than neighboring early phage genes (Mathews and Cohen, 1963; Warner and Lewis, 1966). Phage encoded DHFR is expendable for successful lytic infection (Mathews, 1967b). In a remarkable example of economy in nature, the enzyme dihydrofolate reductase, in addition to a small molecular weight pteridine residue, comprises part of the wedge subassembly of the T_4 phage baseplate (Kozloff et al., 1970). The baseplate DHFR appears to be identical in size and immunological crossreactivity to the DHFR eyzyme which participates in the synthesis of phage DNA precursors (Mosher and Matthews, 1979).

Sirotnak and coworkers (Sirotnak and McCuen, 1973) have intensively analyzed a class of trimethoprim resistant mutants of Diplocuccus pneumoniae. All ot their "ame^r" mutants map within the structural gene of DHFR and exhibit up to a 200 fold increase in the rate of de novo, transcriptionally controlled, DHFR synthesis. The conclusion of these workers, that DHFR is under autogenous control (Goldberger, 1974) in D. pneumoniae, must now be reexamined in the light of numerous reports (described above) that high levels of

DHFR in drug resistant mammalian cell cultures result from multiplication of the DHFR structural gene.

Sheldon and Brenner (1976) isolated a number of "fol" regulatory mutants of E. coli K-12 selected for resistance to trimethoprim. Cris-trans analysis (impossible in the Diplococcus system) of high DHFR mutants revealed that the mutations acted cis, once again in agreement with a selective gene multiplication mechanism. However, enzymological studies (Sheldon, 1977) suggested alterations in the structure of the DHFR protein in some "fol" mutants. Merodiploids constructed of some chromosomal "fol" mutations and episomal wild type alleles displayed up to 20 percent less activity that the episome-cured mutants. A trans-acting regulatory element was postulated, though not directly demonstrated. In addition, one mutant, "fol 60", exhibited both high (20 fold) DHFR activity and temperature sensitivity. Nineteen independent temperature stable revertants of "fol 60" also differed from "fol 60" in DHFR levels. Thus, the two phenotypes were manifestations of the same lesion. Smith and Calvo (1979) isolated "fol", the wild type E. coli DHFR structural gene, on the plasmid pBR322, and used it in a hydridization kinetic analysis of Sheldon and Brenner's "fol 60" mutant. They concluded that high DHFR results from constitutively high transcription of the DHFR structural gene in "fol 60". The basis of the mutant's pleiotropy has yet to be resolved.

Hospital strains of E. coli and Citrobacter sp. have been shown to achieve high constitutive (drug resistant) levels of DHFR by acquiring R-plasmids carrying genes encoding DHFR's which are virtually insensitive to trimethoprim, methotrexate and aminopterin (Pattishall et al., 1977). One of these plasmids codes for a hitherto unheardof tetrameric DHFR of twice the molecular weight of the chromosomal enzyme (Smith et al., 1979).

In non-synchronous cultures of Streptococcus faecilis, DHFR activity rises rapidly during lag and early exponential phases, and declines toward the end of the growth cycle (Nixon and Blakley, 1968). The availability of "high DHFR" mutants of cultured mammalian cells (discussed above) has provided researchers with the material to study the culture cycle regulation of this otherwise obscure gene product. Johnson and coworkers have shown that the in vitro regulation of DHFR expression in high DHFR (methotrexate resistant) mouse fibroblasts is identical to that in normal murine cells (Wiedemann and Johnson, 1979). Serum stimulation of confluent G_0 arrested 3T6 cells resulted in a three- to sixfold de novo induction of DHFR activity. Blockage of DNA synthesis had no effect on DHFR induction (Johnson et al., 1978b). Control appears to be at the level of translatable DHFR mRNA (Johnson, 1980). That DHFR activity should be maximal during S phase is not surprising. Thymidylate synthetase, which supplies precursors for DNA synthesis, is the only cellular consumer of activated folates which oxidizes C_1-THF back to the level of DHF. Therefore, the cell's need to recycle DHF via DHFR is greatest during periods of active DNA synthesis.

A recent communication from Schimke's laboratory has corroborated Johnson's findings (Mariani et al., 1981). High DHFR CHO cells were synchronized by mitotic selection. DNA and DHFR synthesis were simultaneously monitored by a double labelling modification of the FACS technique mentioned above (Kaufmann et al., 1978). DHFR and DNA were labeled with fluorescein-MTX and Hoechst 33342, respectively. A twofold induction in DHFR specific activity was reported, commencing two hours into the cells' 6.5 hour S phase. DHFR synthesis closely followed thymidine incorporation into DNA during S, both functions reaching a peak during the final hours of the DNA synthesis phase. These workers concluded that cell cycle

DHFR induction is probably under indirect control of a number of S phase parameters.

Kellems et al. (1979) used a high DHFR 3T6 mouse cell strain to corroborate Frearson et al.'s (1966) report of induction of DHFR by SV40 and polyoma virus infection. These workers also reported that high levels of intracellular cAMP blocked the rise in DHFR activity following serum stimulation, but not the rise following virus infection. Thus, two separable regulatory circuits control DHFR induction. More recently, Gudewicz et al. (1981) have used the cAMP analog, dibutyryl cAMP, to prevent entry of stimualted cells into S. A concomitant inhibition of DHFR induction was observed, demonstrating a tight correlation between S phase activities and DHFR induction.

Hillcoat has presented evidence for the transient induction of DHFR activity by treatment of cultured mammalian cells with methotrexate (Hillcoat et al., 1967) or folic acid (Hillcoat et al., 1973). The former study was based on the clinical observation by Bertino et al. (1963) that DHFR levels rose rapidly in patients receiving a single chemotheraputic infusion of methotrexate. Activity then returned to normal as serum MTX levels declined. Subsequent studies suggested that enhanced DHFR activity was attributable to the protection of the DHFR enzyme, from proteolytic degradation, by tightly bound MTX (Hillcoat et al., 1967; Bertino et al., 1977). Inhibitor studies suggested that a different, yet unresolved, mechanism is responsible for DHFR induction by folic acid (Hillcoat et al., 1973).

High DHFR cell lines have also provided a rare opportunity for investigation of the molecular biology of a normally minor gene product. The DHFR genes from Alt et al.'s (1978) AT3000 murine cell line and Flintoff et al.'s (1976) $Pro^{-}mtx^{RIII}$ CHO cell line have been cloned into the plasmid pBR322 by

Chang et al. (1978) and Wigler et al. (1980), respectively. The former group succeeded in demonstrating expression of a DHFR cDNA clone in E. coli. The mouse DHFR sequences came under the control of the plasmid's β-lactamase (ampicillin resistance) promotor, and produced an enzyme which both expressed immunological cross-reactivity with authentic mouse DHFR, and rendered the E. coli host resistant to trimethoprim. Wigler et al. (1980) introduced their (genomic) cloned CHO DHFR into mouse cells by the calcium phosphate precipitation method. They found transformants to be both methotrexate resistant and capable of amplifying both the DHFR sequences and their adjacent, non-selected, plasmid sequences. These two important gene transfer studies represent landmark events in the current biotechnology revolution. Preliminary comparisons of Chang et al.'s (1978) clones with genomic DHFR sequence clones indicate that considerable processing of the nascent transcript precedes its ultimate cytoplasmic translation (Schimke et al., 1979; Setzer et al., 1980). These and recent reports of the cloning of T_4 (Purohit et al., 1981) and E. coli (Rood and Williams, 1981) DHFR genes suggest that the molecular events surrounding the expression of this "housekeeping" gene will soon be coming to light.

In the only report approaching the question of the normal regulation of DHFR in plants, Reddy and Rao (1975) have shown that extractable DHFR specific activity reached a peak four days after seed imbibition in etiolated soybean seedlings and after five days in light-grown seedlings. It has been reported that a gene encoding a methotrexate resistant DHFR has been used as a selective marker in the first demonstration of in vitro plant cell transformation with the Agrobacterium tumefaciens Ti-plasmid (P. Ludden, personal communication).

Selections for Ethionine Resistance in vitro. Insufficient methionine content is the primary factor limiting the protein quality of grain legumes (Diekson and Hackler, 1975). Therefore, recovery of a selectable somatic cell variant of Phaseolus vulgaris displaying enhanced, adaptive levels of methionine would be valuable from a crop quality and somatic cell genetics standpoint. The problem thus becomes devising a selective scheme in which a methionine overproducing cell line would have a selective advantage in vitro. Adelberg (1958) has provided a model. Cultures of E. coli, subjected to growth inhibotory concentrations of the S-ethyl analog of methionine, L-ethionine (all amino acids discussed here are in the L-isomeric form), gave rise to resistant mutants. Analysis of the mutants revealed that they accumulated methionine to such high levels that it was excreted into the growth medium. In various organisms, ethionine has been shown to compete with methionine in the latter's role in primary protein structure, activation for transmethylation reactions (via S-adenosylmethionine), allosteric regulation of its own synthesis, and cell cycle control (Meister, 1965; Colombani et al., 1975; Singer et al., 1978). Presumably overproduction of the normal amino acid in the ethionine resistant E. coli strains provided a pool capable of out-competing the exogenous analog in methionine's physiological and biochemical roles. Similar physiological phenotypes have accompanied the expression of ethionine resistance in selections of yeast (Musilova and Fencl, 1965), Neurospora crassa (Kappy and Metzenberg, 1965), and Chlorella (Sloger and Owens, 1974). In the latter report, the overproduction phenotype was attributed to an altered feedback response to methionine's biosynthetic control normally exerted by both the analog and methionine. Other mechanisms of ethionine resistance include increased ability to degrade the analog (Spence et al., 1967), or the S-adenosylethionine formed from it (Cherest et al., 1968), enhanced discrimination by the tRNA charging enzyme for the analog and the

natural substrate (Lewis, 1963) and reduced transport of the analog (Sorsoli et al., 1964). Metzenberg et al. (1964) isolated a temperature sensitive ethionine resistant mutant of Neurospora which could not be rescued at high temperature by a variety of complex media, suggesting that acquisition of ethionine resistance, in this case, was at the expense of an indispensible function.

Three reports of higher plant somatic cell selections for ethionine resistance have appeared. Zenk (1974) briefly reported the gradual adaptaion of haploid Nicotiana sylvestris cultures to 10^{-2} M DL-ethionine, a level tenfold higher than that which completely arrests the growth of wild type. Although he reported the variant line overproduced methionine, no further analysis has been presented.

Widholm (1976) grew carrot suspension cultures in 0.5 mM ethionine (100 fold above the minimum I_{100}) and selected a resistant line at an estimated frequency of approximately 10^{-7}. EMS mutagenesis was used to increase the mutation rate, but the lack of controls precluded any evaluation of the efficacy of the mutagenic treatment. The ethionine resistant cell line displayed tolerance of 1000 times the wild type's I_{100}, although growth under non-selective conditions reduced its tolerance somewhat. The variant was also relatively resistant to Δ-hydroxylysine, α-methylmethionine and parafluorophenylalanine (analogs of lysine, methionine, and phenylalanine, respectively), but not to lysine + threonine. Amno acid analysis revealed a thirteenfold elevation in the methionine content (not specified free or bound) of the resistant cell line. Further analysis of this unusual pleiotropy has not been reported.

Reisch et al. (1981) have published the first report of an ethionine resistance selection in a crop plant culture system where methionine overproduction would be of practical value. Freshly initiated diploid alfalfa ovary cell suspensions were mutagenized with EMS and plated by the Bergmann

technique (Bergmann, 1960) in agar containing 0.02, 0.1, and 0.24 millimolar ethionine. In this single step selection, colonies only arose on the 0.02 mM ethionine plates. Mutagenesis slightly enhanced variant recorvery frequencies. On 1 mM ethionine, several of the variants grew significantly better than wild type, even after six months of relaxed selection. Some of these were also resistant to lysine and threonine. One resistant variant displayed both a tenfold enhancement of soluble methionine and overall increases in all free and protein amino acids. A number of plants were regenerated from these cell lines (Reisch and Bingham, 1981), but neither the inheritance, the physiology nor the whole plant expression of the ethionine resistance or methionine overproduction traits have been reported. A variety of morphological abnormalities appeared in the regenerated plants, but not in control regenerants originating from EMS (but not ethionine) treatment. It was suggested that ethionine was mutagenic in this system. It is also possible that the imbalance in methionine metabolism in some of the variants concomitantly upset ethylene metabolism (since methionine is a precursor of ethylene), resulting in hormonally mediated alterations in morphology (J. Saunders, personal communication).

Selections for Altered Carbohydrate Metabolism. The seeds of Phaseolus vulgaris contain factors which are digested in the human colon by processes accompanied by gas formation and its legendary discomfort. The α-galactosides raffinose, stachyose and verbascose are the substrates whose microbial fermentation in the gut is responsible for a significant proportion of the gas evolution (Murphy, 1964). It has been demonstrated that by selective chemical extraction of these sugars from bean seeds, the flatulence eliciting ability of the seeds is quantitatively reduced (Calloway, 1975). Despite both this clear demonstration of the causal factors and the discovery of limited genetic

diversity in their occurrence in bean seeds (Murphy, 1975), a reduction in the levels of these factors by selective breeding has not been achieved.

Little is known about the developmental control over the enzymatic activities responsible for the synthesis and degradation of the α-galactosides. The sugars are probably accumulated during the maturation stage of embryogenesis. They would then contribute to the osmoticum which counterbalances water accumulation in maintaining constant water potential before day 23 of embryogenesis in P. vulgaris. Thereafter, continued sugar inflow, in the absence of balancing water accumulation, contributes to the dramatic rise in axis water potential, characteristic of Phaseolus dormancy (Walbot, 1978). Germination does not rapidly deplete the seed of these sugars, since sprouted beans elicit a flatulence response equivalent to dry seeds (Calloway, 1975). Nevertheless, degradation must surely occur during the seedling stage, especially since six day etiolated pinto bean seedlings provide a particularly rich source of α-galactosidase (Agrawal and Bahl, 1968). Legume α-galactosidases have attracted some attention because they bind carbohydrates and thus have hemagglutinin properties (Hankins and Shannon, 1978; Hankins et al., 1980). That cleavage of the α-galactosidic bond is the first step in the assimilation of stored raffinose has been demonstrated for Vicia faba seeds (Pridham et al., 1969). The liberated galactosyl moiety is rapidly phosphorylated by galactokinase in germinating seedlings (Pazur et al., 1962). This efficient transformation of free galactose is apparently essential since this monosaccharide is toxic and inhibits the growth of a number of plant tissues (Dey, 1980). The mechanism of galactose toxicity is obscure. It has been shown (Roberts et al., 1971) that phosphoglucomutase, a downstream enzyme in hexose assimilation, is inhibited by the activated galactose-1-phosphate in maize roots. However, this mechanism is incompatible with the high activity of galactokinase

in germinating seeds. Maretzki and Thom (1978) have presented data which indicate that another intermediate, perhaps UDP-galactose, may be responsible for galactose inhibition of cultured sugarcane cell growth (see below). The stimulation is complicated by the fact that cucumber tissues are not inhibited by galactose (Gross et al., 1981) and that α-galactosidic oligosaccharides are major carbon transport forms in some cucurbits (Weidner, 1964).

Plant cell cultures are normally supplied with 2 to 3 percent (w/v) sucrose as a carbon and energy source. A few studies have been devoted to the understanding of the assimilation of other carbon sources. In some of the early plant cell cloning work, it was found that several individual single cell derived clones of Phylloxera, all derived from an earlier single cell clone, showed different efficiencies for carbon source utilization. Some even thrived on galactose (Arya, Hildebrandt and Riker, 1962). Nickell and Maretzki (1970, 1972) looked at the ability of sugarcane cultures to utilize a variety of carbon and energy sources. The only carbohydrate source which resulted in greater growth than sucrose was raffinose, a surprising finding since galactose supported only fifty percent of the dry weight increase that sucrose did.

Adaptation of cultured plant cell lines to growth on new carbon sources has been reported in a variety of systems. In some cases, the newly expressed metabolic phenotype appears to result from reliance on a constitutive assimilatory enzyme (Mitchell et al., 1980). Other systems respond to new carbon-energy sources with adaptive changes which resemble enzyme induction phenomena (Scala and Semensky, 1971; Meryl, Smith and Stone, 1973; Verma and Dougall, 1977; Gross et al., 1981). Still others acquire the new catabolic capacity at a frequency suggesting a mutational event. Those of the latter type are most pertinent to the present study. SB-1 soybean suspensions are normally maintained on 2 percent sucrose as a carbon-energy source (Gamborg et al.,

1968). When sucrose is substituted with maltose, the generation time increases from 24 hours to 8 days. Limberg et al. (1979) have reported that clones of shorter generation times, down to 24 hours, arise as soybean cultures are maintained on maltose for up to 200 generations. The improved maltose assimilation phenotype is stable, in that cultures returned to sucrose medium can be transferred back to maltose without a significant lag in generation time. The most striking physiological change accompanying the maltose assimilation phenotype is the vastly improved ability of the cells to take up maltose from the medium. The authors suggest that a multistep genetic change led to the new phenotype. Single step selections later indicated that the new assimilatory trait occurs with a spontaneous frequency of 1.2×10^{-7} (per incremental decrease in generation time on maltose). The mutagens EMS and hycanthone increased the frequency to 3.6×10^{-5} and 10^{-3} respectively. The authors were reluctant to speculate on the genetic nature of the variant phenotype (Weber and Lark, 1980). Chaleff and Parsons (1978) isolated a Nicotiana tabacum cell line which, unlike normal tobacco cultures, utilizes glycerol as a carbon-energy source. While a biochemical characterization of the variant is lacking, a relatively thorough Mendelian analysis of the trait established its Mendelian heritability (unique among plant cell variants of this class). The abberant segretation ratios suggested that the trait was dominant in its ability to confer the glycerol utilizing trait in the hemizygous condition, but recessive in conditioning embryo lethality. However, anther culture and somatic chromosome doubling produced a diploid homozygote, all of whose selfed progeny were also glycerol utilizing. Analysis in a more biochemical vein is underway (Chaleff, 1981). Finally, Maretzki and Thom (1978) recovered a galactose utilizing strain of cultured sugarcane cells. The variant line was selected after 6 to 8 weeks of incubation of cells in 18 g/l galactose, first in suspension culture, later on solid medium.

The variant displayed growth kinetics on galactose identical to those of the wild type grown on sucrose. Analysis of the levels of both the substrates and the enzyme activities of galactose assimilation revealed a ten fold elevation in the activity of UDP-galactose-4-epimerase over that of wild type cells grown in sucrose or freshly transferred to galactose. A concomitant decrease in the intermediate immediately upstream, UDP-galactose, was reported. Here is an attractive candidate for selective gene multiplication in a higher plant somatic cell system. However, since the variant grows on galactose at the same rate which is intrinsic to the wild type grown on sucrose, it would be difficult to impose a selection to further amplify the enzyme activity, and thus make the biochemistry as accessible as in the high DHFR mutants described above.

Flavanoid Pigmentation in Cultured Plant Cells. The study of flavanoid pigmentation in cultured plant cells has attracted plant scientists for several reasons. Many see plant cells as potential sources of drugs and metabolites for the pharmaceutical and chemical industries (Barz et al., 1977). Flavanoid compounds and the regulation of their synthesis provide model systems for understanding the elaboration of such secondary plant products in vitro. Visual screens for protoplast fusion hybrids (up to now based on differential chlorophyll contents of fusion partners) are becoming popular in plant somatic hybridization research (R. J. Greisbach, personal communication). An understanding of endogenous and exogenous factors controlling pigment accumulation is a necessary prerequisite to the rational use of such markers. The vacuole is an important subcellular compartment about which relatively little physiology is known. Since anthocyanin pigments accumulate in the vacuole, they provide a marker to aid in the intact isolation of this organelle for studies of its function (T. Boller and H. Kende, personal communication). Finally, the multistep phenylpropaniod pathway provides an incomparable system for fundamental

studies of the control of biosynthesis at the biochemical and genetic level (Hahlbrock and Grisebach, 1979). As a consequence of the requirements of these many applications, nearly all of the published studies on flavanoids in plant cell cultures have dealt with the factors which govern pigment synthesis: Developmental stage of the cells, pH, hormones, carbohydrate supply and, most commonly, light.

Several workers have reported the induction of phenylpropanoid enzymes (or the accumulation of their products) upon subculture of plant cell suspension cultures. Constabel et al. (1971) reported a positive correlation between the rate of pigment synthesis and the growth rate of Haplopappus gracilis suspension cultures. The first three enzymes of general phenylpropanoid metabolism (before branch points leading to lignin, flavanoids or cinammate esters) are induced in parsley and soybean cells by subculture into fresh medium or water (Ebel et al., 1974). Induction in the parsley system is 5 to 50 fold, and high activities persist for 12 to 15 hours before returning to the non-induced level (Hahlbrock and Wellmann, 1973; Hahlbrock and Schroder, 1975). The induction was later shown to be correlated with a rise in the levels of translatable mRNA's coding for the enzymes (Schroder et al., 1979). In addition to the developmental determinants of the suspension culture growth cycle, tissue and organ differences also impinge upon pigment expression. Whereas Colijn et al. (1981) suggested that anthocyanin pigmentation in their Petunia cultures qualitatively and quantitatively paralleled that found in whole Petunia plants (although their data is less than convincing), two other groups reported finding anthocyanin pigments in vitro not found in the whole plant (Slabecka-Szweykowska, 1952; Sugano and Hayashi, 1967). One is tempted to urge these latter workers to look harder. Hahlbrock et al. (1971) reported that the activities of the enzymes of flavone glycoside biosynthesis were maximal in young parsley seedling tissues,

declining continuously during seedling growth. The activities appeared to be coordinated both with each other and with seedling development. Berlin and Barz (1971) reported a correlation between flavanoid formation and the attainment of a visually distinct cellular differentiated state in callus and suspension cultures of mung bean. Kinnersley and Dougall (1981) reported an inverse relationship between clump size and anthocyanin production in carrot cell cultures. Physiological implications of this finding are discussed below. It is interesting to note that Colijn et al.'s (1981) pigmented Petunia cells were only observed to proliferate in the presence of the parental, non-pigmented cells. This may indicate that the pigmented phenotype is a developmental endpoint of the initially non-pigmented cells. It is not clear whether these authors eliminated the possibility that what appeared to be growth of the pigmented clones against a white background was actually differentiation of white cells within the background. However, Constabel et al. (1971) provided convincing photographic evidence of cell division of pigmented cells of Haplopappus gracilis.

A number of workers have described hormonal effects on anthocyanin accumulation in vitro. However, none have determined how far removed the pigmentation response is from the chemical stimulus of the hormone itself. Stickland and Sunderland (1972a) reported that 2,4-D inhibited both the growth and the production of cyanidin in Haplopappus cultures. This observation was in agreement with some previous reports on Haplopappus (Blakely and Steward, 1961; Constabel et al., 1971), but not with others (von Ardenne, 1961; Reinert et al., 1964). However, this discrepancy is not surprising in the light of Dougall et al.'s (1980) recent report of extreme and stable clonal variation in anthocyanin accumulation in carrot cell cultures. The notorious chromosomal (and consequently phenotypic) instability of these and most other cultured plant cell lines is perhaps responsible for this variation. Sunderland (1977) has carefully

studied the nuclear cytology of these and other Haplopappus cell lines and has reported a galaxy of abberations accumulated over the years. Stickland and Sunderland (1972a) also pointed out that the ability of a given Haplopappus cell line to form anthocyanin was governed by the hormonal constituents of the media used for primary callus initiation. A strain initiated in NAA, instead of 2,4-D, could not be coaxed into flavanoid production. Kinnersley and Dougall (1980) pointed out that many of these pigmentation responses could be secondary, due to the degree of aggregation caused by different hormonal regimes. This seems plausible in view of the unusual light requirement for flavanoid synthesis induction (see below). Berlin and Barz (1971) reported that IAA, kinetin and NAA dramatically enhanced production of coumestrol in mung bean cultures. This isoflavanoid is of particular phamacological importance due to its estrogenic activity (Harborne, 1967).

Dougall and Weyrauch (1980) studied the effects of pH and organic acids on the growth and pigmentation of cultured carrot cells supplied with ammonia as the sole nitrogen source. Of the fifteen organic acids evaluated, 14 to 20 mM succinate at an initial medium pH of 4.2 to 4.3 promoted optimum pigment yields. This was, however, at the expense of better growth at higher pH. Shepard (personal communication) reported that potato callus could be induced to form a red anthocyanin by the imposition of a "sucrose stress" consisting of 5 percent (w/v) sucrose. However, Colijn et al. (1981) reported that their Petunia line formed less pigment as sucrose was raised above the 3 percent level, in agreement with previous reports in other systems (Strauss, 1959; Ball et al., 1972; Ball and Arditti, 1974). Whether these workers' observation derived from sucrose's nutritional or osmotic role has not been determined.

By far the most intensely studied factor affecting flavanoid accumulation in plant cell culture is light. Only two cases have been reported where

anthocyanin production was not forestalled by a dark treatment. Sunderland (1977) observed that subclone A2, which originated in the light dependent, pigmented Haplopappus strain A1 (described below), produced copious amounts of anthocyanin in complete darkness. This alteration may have been related to A2's aberrant chromosomal behavior (Sunderland, 1977). Eriksson (1967) recovered several anthocyanin producing clones of Haplopappus following UV irradation of suspension cultured cells. One of these clones, 101s, was stable in its light-independent anthocyanin production for 4 years. This cell line's karyotype was also aberrant, displaying what appeared to be a single translocation. Stickland and Sunderland's (1972a, 1972b) Haplopappus strain A1 was wholly dependent on light for pigment formation. Any wavelength shorter than that of far-red was sufficient to elicit production of cyanidin containing anthocyanins. Light in the 400 to 500 nm range was the most stimulatory. UV light was not tested; perhaps a significant omission given the subsequent revelations about the action spectrum for pigment induction in the parsley system (see below). Interestingly enough, the quantitative light dependence of pigmentation and growth were inversely related. In this respect, pigment production resembled a stress response (cf. Shepard, above).

The most thorough exploration into the effect of light on the substrates and enzymes of flavanoid biosynthesis in vitro has been carried out by Hahlbrock and coworkers using parsley cell suspensions. Whereas the first three enzymes of general phenylpropanoid metabolism were induced by either media dilution or light, the downstream enzymes of the flavone glycoside pathway were specifically induced by UV light, an effect which was reversible by far-red light, through the phytochrome system (Hahlbrock and Grisebach, 1979). The UV receptor was not identified. In addition to their differential responses to dilution, further evidence was provided for two separate regulatory circuits

governing the up and downstream enzymes of flavanoid biosynthesis, based on their respective induction kinetics. The first three enzymes were induced about twice as rapidly as the last six (Hahlbrock, 1976). The enzymes are synthesized de novo upon illumination, their activities directly correlated with changes in specific mRNA activities (Schroder et al., 1979). The linear dose/response relationship between UV fluencne and the activities of some of the enzymes, in addition to the induction kinetics differences alluded to above, suggest subtle regulatory mechanisms are operative in attenuating the expression of these functions (Hahlbrock et al., 1978; Hahlbrock and Grisebach, 1979).

Ultraviolet Light Mutagenesis in Plant Cell Cultures. In spite of the fact that UV mutagenesis has been effective in generating heritable variability in cultured mammalian cells and microorganisms, the technique has not been used extensively in plant cell studies. The reasons for this are apparent if the plant cell mutagenesis system is evaluated in the light of Timofeeff-Ressovsky's (1934) five basic considerations for meaningful mutagenesis experiments (cited and discussed by Auerbach, 1962): 1) Purity of Material. Experimental units must be uniform from one replicate, treatment and population to the next. The notorious and spontaneous clonal variation in cultured plant cells introduces a considerable and as yet uncontrollable error variable into such studies. Further constraints on the uniformity of experimental material are discussed under 5), below. 2) Sufficiently large numbers of individuals or cultures in both the controls and the treated material. This is perhaps the only requirement more completely satisfied by in vitro than in vivo systems in higher plants. Populations of 10^8 units are easily manageable in plant cell systems. However, Mendelian analysis of mutations selected in vitro requires regenerating and sexually hybridizing large numbers of putative mutants, an undertaking which can consume considerable time and space. 3) Genetic methods suitable for the detection of

newly arisen mutations. Once again, inheritance of the trait, selected in vitro, by progeny of a plant regenerated from a putative mutant clone, is the best "genetic" method available for verifying the nature of in vitro variation. This is a tedious, time-consuming and not-always-possible (e.g., in grain legumes) process. However, if chromosomal damage is the endpoint one seeks to study, the "genetic" evaluation of variation is more straightforward, but no less tedious (cf. Eriksson, 1967). 4) Analysis of the variations which arise. This requirement unavoidably necessitates regeneration of in vitro variants, to determine the nuclear or cytoplasmic location of the mutation, the number of genes involved, dominance and complementation relationships within and among mutant phenotypes. Some of these questions might be approached by protoplast fusion studies (as in mammalian systems), but with little gain in precision or efficiency of analysis. This is not to suggest that future advances in protoplast technology will not render fusion methodologies more predictable and thus analytical. 5) Some knowledge concerning the manner in which the agent used can act on the (germ) cells of the treated material. This requirement highlights the most serious obstacles one encounters in UV studies with plant cells in vitro. Plant cell suspensions are invariably clumped, in such a way that a linear beam of UV light will only strike those cells on the side of a clump facing the light source. Agitating the culture during UV treatment does nothing to expose cells in the interior of the clumps. Furthermore, plant cells have cellulosic walls, numerous plastids (even when grown in the dark) and frequently pigmented vacuoles, all of which shield the nuclear chromatin from incoming irradiation. In the absence of a method to increase the intrinsic penetrating capacity of this particular mutagenic agent, some workers have resorted to seiving suspension cultures or working exclusively with protoplasts.

These obstacles notwithstanding, some data have been obtained on the effects and uses of UV irradiation in plant cell cultures (Howland and Hart, 1977). Klein (1963) was the first to quantify UV killing in cultured plant cells. He calculated an apparent D_{37} of 40 J/m^2 (without photoreactivation) for Ginkgo cells based on dry weight increase of survivors. No correction was made for cell aggregation, although Ginkgo cell suspensions are relatively well dispersed. In a very careful study of irradiation effects on Haplopappus suspensions, Eriksson's (1967) data indicated a D_{37} of 200 J/m^2 (with photoreactivation) based on colony forming ability. Eriksson suggests this estimate may have been inflated by aggregation and pigmentation factors. The frequency of cytologically abnormal metaphases, 16 hours following irridation, increased steadily with doses of 50 to 200 J/m^2. Isochromatid breaks predominated at all UV dosages. Ohyama et al.'s (1974) data, corrected by Howland and Hart (1977) for media and cytoplasmic interference, yielded a D_{37} of about 3 J/m^2 for soybean protoplasts (without photoreactivation) based on colony forming ability. For non-protoplasted cells, the D_{37} was five- to tenfold higher, demonstrating the protection afforded by the cell walls and aggregation. Macromolecular incorporation of labeled nucleotides closely paralleled cell survival in this study, although protein synthesis was less severely affected. Howland (Howland and Hart, 1977) calculated D_{37}'s of 80 and 260 J/m^2 for Haplopappus cultures with and without photoreactivation, respectively, applying a simple mathematical correction for aggregation. Most recently, Weber and Lark (1980) described quantitative mutagenesis studies with soybean cell cultures. Ultraviolet light was second only to the frameshift mutagen, hycanthone, in inducing maltose utilizing variants (the only phenotype selected). A dose of 90 J/m^2, delivered to cells and clumps plated on a solid surface, resulted in 86 percent killing, based on colony forming ability. Their data do not

permit estimation of a D_{37}. A dose of 225 J/m^2 increased the variant frequency 3250 fold over unirradiated controls. Variant frequencies following irradiation were also higher in haploid than in diploid soybean cell lines. The non-regenerability of cultured soybean cells precludes any formal genetic analysis of these variants. Furthermore, the lack of a well defined genetic or physiological basis for the maltose-utilizing phenotype (Limberg et al., 1979) calls for caution in interpreting these results.

Ultraviolet light induced lesions in DNA molecules are repairable by all organisms in which such a capacity has been sought (Hanawalt et al., 1978; Soyfer, 1979; Generoso et al., 1980). Thymine dimers are the predominant and most lethal photoproducts of UV-DNA interactions (Setlow, 1968). Klein (1963) demonstrated photoreactivation (PR), the light-induced process by which dimers are directly monomerized (Sutherland, 1981), in Ginkgo cells. The absorption spectrum for PR in this system revealed an efficiency maximum at 420 nm, a slightly longer wavelength than that which induces the PR system in E. coli. Whereas Klein's data were based on reversal of UV induced lethality, Trosko and Mansour (1968) were the first to demonstrate that pyrimidine dimers are readily produced by UV treatment of cultured tobacco XD cells and that the dimers could be monomerized by PR. However, Haplopappus cells could not be shown to have a PR or excision repair capacity, and tobacco cells lacked the latter. Howland (1975) suggested that these negative results may have been attributable to the high UV fluences employed by Trosko and Mansour (see below). These workers later demonstrated photoreactivation, but once again not excision repair, in haploid Ginkgo cells (Trosko and Mansour, 1969b). Returning to the tobacco XD system, they showed that UV inhibition of DNA synthesis could also be photoreactivated (Trosko and Mansour, 1969a). The search for dark (excision) repair in cultured plant cells finally ended with Howland's (1975) report of an

extremely low capacity system in carrot protoplasts. Removal of dimers was possible only following doses of less than 100 J/m^2. These results have yet to be corroborated by other workers with another plant system. In a study of the effects of irradiation on the totipotency of cultured tobacco cells, Eapen (1976) reported a D_{37} of approximately 360 J/m^2 for regeneration of whole plants. The irradiated populations consisted of filtered cell suspensions, comprised of mostly single cells. Regeneration of photoreactivated and non-photoreactivated cells followed the same pattern. Weber and Lark (1980) reported no photoreactivation could be observed in their soybean system. They suggested that, since their stock cultures were maintained in complete darkness, this and other light inducible adaptive functions may have been lost.

The underdeveloped state of the art of UV mutagenesis in cultured plant cell systems has prevented its exploitation by all but a few workers. In Eriksson's (1967) study of UV effects on Haplopappus cells, a stable anthocyanin-producing variant was recovered. That irradiation was directly responsible for the establishment of this phenotype was not shown, nor was a systematic search for other than cytological UV-induced phenotypes undertaken. Similarly, Widholm (cited by Howland and Hart, 1977) reportedly used UV irradiation to induce mutations to amino acid analog resistance in cultured carrot cells. Howland and Hart (1977) reported that by effecting a 50 percent UV-mediated kill, the mutation frequency was increased tenfold in Widholm's system. Widholm's failure to report these findings in any of his numerous publications would suggest that many of the "mutations" reportedly induced did not hold up under retest or further analysis. Bourgin (1978) irradiated haploid Nicotiana tabacum protoplasts with 100 J/m^2 of UV light (resulting in a 40 to 60 percent reduction in plating efficiency of unselected cells) and thereafter selected at least two true mutants expressing nuclear semidominant resistance to valine.

Once again, no direct connection was made between irradiation treatment and the recovery of the mutants. Still, his rigorous Mendelian analysis suggests that these may be the only UV-induced point mutations yet recovered in a cultured plant cell system. Finally, Lark's group, in addition to the numerous maltose-utilizing variants recovered in their mutagenesis studies, has used UV to enhance recovery of deficiency variants from a haploid soybean cell line. Some of the variants, requiring casein hydrolysate for growth, arose at a frequency of one percent following UV mutagenesis (Weber and Lark, 1980). Others, exhibiting Na^+ dependence, were recovered non-selectively at a frequency of $2\text{-}3 \times 10^{-3}$ following a UV treatment of 200 J/m^2 UV. Revertants could only be recovered following additional exposure of the variants to ultraviolet light (Zhou, personal communication).

MATERIALS AND METHODS

Cell Lines. Vigna radiata genotypes obtained from H. G. Park (Asian Vegetable Research and Development Center, Taiwan) were the following: Vr1 (cv. AVRDC #V1104); Vr2 (cv. AVRDC #V1399; Vr3 (cv. AVRDC #2184); Vr4 (cv. AVRDC #V2773); Vr5 (cv. AVRDC #V3476). N. C. Chen (Department of Horticulture, Michigan State University) kindly supplied seed of Vigna angularis cv. AVRDC #5124, from which Va2 was derived. The Phaseolus vulgaris cell line, Pv25, was derived from the navy bean cultivar "Saginaw" and was generously supplied by R. L. Malmberg (Cold Spring Harbor Laboratory, New York), as was Nt575, the Nicotina tabacum cv. Wisconsin 38 cell line. S. E. Barsel (Department of Botany and Plant Pathology, Michigan State University) kindly supplied LeW, a cell line of Lycopersicon esculentum cv. Walter. The genotypes of the pigmented Vigna sp. cell line and its parent are given in Part V. This material was supplied by J. F. Parrott (Department of Horticulture, Michigan State University).

Initiation and Maintenance of Cultures. The Vigna, Phaseolus and Lycopersicon cell lines originated from seedling hypocotyl explants. Seeds were surface disinfested by the following wash sequence: Sterile distilled water rinse, 20 minute soak in 10 percent commercial bleach + 1 percent sodium laurel sulfate (with agitation), 30 second wash in 95 percent ethanol, and two rinses in sterile distilled water. Seeds thus treated were germinated (hilum up in the case of the legumes) on 1 percent water agar in darkness at 26 to 28°C. Four to six day etiolated hypocotyls were sectioned and plated on maintenance medium (see

below). Callus, which grew at the cut ends of the sections, was transferred to and maintained on fresh maintenance medium by biweekly subculture thereafter. Nt575 callus was isolated from pith sections of an anther-derived plant by D. P. Ornsten and maintained by biweekly transfer on maintenance medium. The dates of origin of the cell lines were as follows: Vr1 - Vr5, July 1978; Va2, January 1979; Pv25, August 1976; Vr9, June 1978; Vr9a, October 1978; Nt575, May 1975; LeW, September 1978.

Suspension cultures were initiated by introducing 3 to 6 grams of friable callus of each genotype into 50 ml of agarless maintenance medium in a foam-stoppered 125 ml erlenmeyer flask. Cultures were maintained in darkness at 25 to 39°C on a 125 rpm gyratory shaker. Subculture was by 1:1 dilution with fresh medium at 4 to 7 day intervals depending on the genotype. Stock suspension cultures were eventually expanded into 200 ml liquid volumes in 500 ml erlenmeyer flasks to make larger populations available for growth tests and selections.

Media. The mainteiance medium R3 was used for all genotypes and consisted of the major and minor salts of Murashige and Skoog (1962) supplemented with 1 mg/l thiamine, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine, 0.5 mg/l 2,4-D, 5 mg/l IAA, 0.3 mg/l kinetin, 100 mg/l inositol and 30 g/l sucrose. Solid medium contained 0.9 percent agar. For plating of lawns, a modification of Kao and Michayluk's (1975) Medium 8 was used. This modification, called R3K, consisted of the salts, hormones, sucrose and agar of R3 (except R3K's iron source was 28 mg/l sequestrene) supplemented with the vitamins, organic acids, sugars (except sucrose and glucose) and sugar alcohols of Kao and Michayluk's Medium 8. The pH of R3 and R3K was adjusted to 6.0 with KOH and/or HCl prior to autoclaving for 15 minutes at 15 pounds pressure.

Growth Tests. The growth of calluses, suspension cultures and lawns was routinely measured by fresh weight, packed cell volume and photometric absorbance, respectively.

Callus growth tests were begun by dividing an exponential phase callus into a large number of sub-clones of equal size (by visual inspection). From this population, random samples were drawn for inoculating the various treatment and control plates, three callus inocula per 10 mm x 60 mm petri dish, each dish containing 15 ml of medium. In addition, a number were drawn for the determination of mean initial fresh weights. After a suitable period of time, the control and treatment calluses were weighed and growth rates were calculated as described below.

Suspension culture growth tests were begun by diluting a 200 ml stock suspension, in the appropriate phase of the growth cycle (usually late exponential), with 250 ml of fresh R3 medium. Sterilized, graduated, 50 ml conical centrifuge tubes (Corning Pyrex #8082) were inoculated with 45 to 50 ml of dilute culture by pipetting. Compounds to be tested for their effects on culture growth were then added to the tubes, and cells were permitted to settle at 1 x g for 20 minutes. The packed cell volume was then recorded. The cells were resuspended by inverting the screw-capped tubes, the cultures poured into sterile 125 ml flasks and returned to the shaker. This procedure was repeated daily or as appropriate until stationary phase was attained in all growing cultures.

For growth tests of lawns, 10 mm x 60 mm plastic petri dishes (Falcon #1007) were filled with 15 of the appropriate medium. Experimental dishes were randomized by the use of the random number table, except where the sequential plating technique ("sloshing", described below) would have lead to cross-contamination of the treatments. An aliquot (about 5 ml) of exponential phase

suspension was pipetted onto the first dish. The inoculum was then poured from this dish to the next, and so on, leaving behind a thin layer of cells and clumps on each dish's agar surface. A 5 ml aliquot would cover 5 to 10 dishes, depending on the density of the source culture. This technique, called "sloshing", was developed by R. L. Malmberg and produced relatively uniform, non-contaminated lawns very efficiently. The growth of the cultures thus created was measured photometrically. The photometer consisted of a flat-black painted wooden box, 9" wide, 10" deep and 20" high. The upper 4" was separable from the lower 16", the two portions being attached by a piano hinge at the back. A masonite skirt prevented stray light from entering through the interface of the upper and lower portions when closed. At the top of the lower (16" high) portion was a 9" x 10" masonite shelf, into the center of which a 62 mm diameter hole ("the sample holder") was cut, where a petri dish (cover secured with parafilm only around the circumference) fit snugly. In the bottom of the lower portion of the instrument were three 5 watt fluorescent lamps (General Electric F4T5) which were cooled by a fan. The lamps could be moved up and down as a unit by loosening wing nuts on the outside of the instrument. In the top of the upper (4" high) portion of the instrument was a photosensitive resistor, directed downward, along the axis defined by the center of the lamp assembly and the center of the sample holder. The resistor was wired to a Simpson 360 digital voltmeter. Frosted white plastic diffusers were positioned immediately below the resistor and the sample holder. Before each day's readings were to be taken, the instrument was warmed up for 30 minutes and the lamp assembly was adjusted to give a reading of 0.280 on the voltmeter (2 kΩ scale) when no sample was present in the sample holder. The reading of a particular culture was taken by placing the culture in the sample holder, closing the hinged upper portion, and reading the resistance on the voltmeter. The photometer blank for each treatment consisted of an

uninoculated dish in which liquid R3 has been poured and decanted. Absorbance was calculated as the natural logarithm of the quotient of the sample reading (resistance) divided by the blank reading (resistance). The instrument was calibrated using Nt575 suspensions of known cell densities and gave a linear response ($r^2 = .985$) up to a density of 2×10^6 cells per dish. Fogged petri dish covers were cleared before reading by warming with body heat or an electric hair dryer. Readings were taken at 1 to 3 day intervals, between which the cultures were maintained in darkness at 26 to 28°C.

Exponential growth parameters were calculated for all types of growth tests with the aid of the "Curve Fitting Program" (No. SD-03A) of a Hewlett-Packard HP-67 pocket calculator.

Cell counts were obtained by diluting suspension cultured cells sufficiently to make at least three counts of an entire field defined by an 18 mm x 18 mm cover glass, using a Zeiss Universal Research microscope. Such counts were then multiplied by appropriate dilution factors to obtain an estimate of the density of the source population.

UV Mutagenesis. Fresh lawns, plated onto R3K medium by sloshing, as described above, were positioned 10 inches beneath a 15 watt General Electric germicidal lamp in a laminar flow transfer hood. Irradiation was performed with the dishes uncovered. The UV flux at the lawn surface was 3.7 J/s/mm^2 (measured by a Black-Ray Ultraviolet Meter, Model J-225, Ultraviolet Products Inc., San Gabriel, CA). Treatment was terminated by covering both the petri dishes and the UV light, and sealing the former with Parafilm, in complete darkness. Cultures were then routinely maintained in complete darkness for 4 to 7 days. Cultures were photo-reactivated, when appropriate, by 24 hour/day exposure to cool white fluorescent light (41 $\mu Em^{-2}s^{-1}$) for specified periods of time.

Selection of Variants. Selective agents were filter sterilized and incorporated into solid R3K medium. Exponential phase cultures of Vr2 or Pv25 were sloshed onto selective media as described above. A 200 ml culture covered about 120 10 mm x 100 mm petri dishes. Where mutagenesis was employed, fresh lawns were irradiated immediately following inoculation and placed in complete darkness for 4 to 7 days. Cultures were inspected biweekly for 6 months following inoculation. Variant clones were purified by culturing subclones, taken from the center of each callus colony, onto media identical to the selection medium. All putative MTX resistant variants were eventually maintained on R3 + 1 uM MTX and on R3 without MTX by biweekly subculture of the healthiest tissue.

Extraction of Dihydrofolate Reductase. Suspension cultured cells were collected in a Buchner funnel, under vacuum, onto either Miracloth (Vr2) or Whatman #4 filter paper (all other cell lines). All subsequent steps were carried out at 0 to 4°C. Four ml of extraction buffer (100 mM Tris-HCl, 5 mM 2-mercaptheothanol, 2% (w/v) insoluble polyvinylpyrrolidone (PVP), pH 7.4) were added per gram of fresh weight. Cells in buffer were homogenized by 30 strokes with a teflon pestle tissue homogenizer. The homogenate was centrifuged at 20,000 x g for 20 minutes and decanted through glass wool (which had been boiled in 0.1 M EDTA and exhaustively rinsed) to remove lipids and suspended PVP. The filtrate was centrifuged at 50,000 x g for 30 minutes. The supernatant was used as a crude extract. When many samples were to be extracted, the first spin and the glass wool filtration were frequently omitted.

When higher activities were desireable, crude extracts were concentrated up to 10 fold by ultrafiltration through a PM-10 Diaflo membrane filter in an Amicon Ultrafiltration Cell (Model 52 or 202) under 1 to 60 pounds of nitrogen pressure. Concentrates were centrifuged at 50,000 x g for 30 minutes. Crude

extracts and concentrates, stored in ice at 0 to 4°C, maintained DHFR activity for 7 to 14 days.

To separate small molecules from enzymes, Sephadex G-25 column chromatography was employed. Columns were constructed of 5 ml plastic syringes, with two discs of Whatman #1 filter paper fitted at the bottom of the column bed. Total swelled bed volume was approximately 4 ml. The columns were loaded into 15 ml conical centrifuge tubes in an International Clinical bench top centrifuge. Columns were equilibrated by adding 2 ml of the appropriate buffer and centrifuging 15 seconds at a setting of 7, six times. The seventh equilibration run was at a setting of 5, for 15 seconds. Two ml of extract was then loaded and a 15 second spin, again at a seting of 5, effected a separation of enzyme from salts. The eluate was then used for enzyme assays. The columns could be reused several times if water-washed and stored at 0 to 4°C in 0.2% sodium azide following each use. A test mixture of blue dextran (MW = 2×10^6) and riboflavin (MW = 376), in an initial absorbance ratio of 1:2, respectively, at their respective maxima, was chromatographed by this procedure. The first eluate contained 77 percent of the input dextran and 2 percent of the riboflavin. However, recovery of 60 percent of input protein was more common.

Assay of Dihydrofolate Reductase. Two spectrophotometric assays were used to determine DHFR activity. The first was the continuous assay of Osborn and Huennekens (1958) as modified by Misra et al. (1961). The assay mixture consisted of 83 µM DHF, 83 µM NADPH, 4.2 mM 2-mercaptoethanol, 33 mM potassium phosphate buffer (pH 6.5) and enzyme extract in a total volume of 3 ml. After a 5 minute preincubation at room temperature, the reaction was initiated by the addition of NADPH. The decrease in absorbance at 340 nm was followed using a temperature controlled Gilford Model 240 spectrophotometer

and chart recorder. Plant enzymes were assayed at 30°C and bacteriophage T_4 enzyme at 37°C. Blanks included reaction mixtures lacking DHF or NADPH. One unit of enzyme activity was defined as the amount of extract causing a decrease in absorbance of 0.001 at 340 nm per minute under the conditions described above. In MTX inhibition studies, the inhibitor was included in the preincubation mixture and the reaction was initiated by the addition of DHF.

The DHFR assay of Reddy and Rao (1975) was used for most of this study. In this endpoint assay, the standard reaction mixture contained 0.4 mM DHF, 0.3 mM NADPH, 80 mM 2-meercaptoethanol, 80 mM buffer and enzyme extract to a final volume of 0.5 ml. For assays of Nt575 DHFR, the buffer was an equimolar mixture of ACES and TRIS-HCl, pH 6.8. For Vr2 enzyme, TRIS-HCl, pH 7.6, was used. All other plant DHFRs were assayed with TRIS-HCl at pH 7.2. After a 5 minute preincubation at room temperature, NADPH was added to start the reaction, which was carried out at 45°C. After 15 minutes, the reaction was stopped by the addition of 1 ml of 88% formic acid. Tubes were then placed in a boiling water bath for 5 minutes to convert the THF reaction product to N^5N^{10}methenyl THF. After cooling at room temperature, absorbance of the derivatized product was read at 350 nm on a Gilford Model 240 spectrophotometer. Zero time reactions were used as reaction blanks. In MTX inhibition studies, the inhibitor was included in the preincubation mixture and the reaction was initiated with the addition of DHF. In this assay, one unit of DHFR activity was defined as that amount of extract which converts one nanomole of DHF to THF in 15 minutes under the above conditions. An extinction of 26,500 $M^{-M}cm^{-1}$ was used for N^5N^{10}methenyl THF. As reported by Reddy and Rao, the boiling acid destroyed any unreacted NADPH. However, despite the low extinction of DHF at 350 nm, the high concentrations of this substrate employed in the assay generated a substantial background absorbance, stable to

boiling formic acid. Therefore, great care was taken in pipetting this reagent uniformly into the reaction and blank tubes.

Using either assay, specific activity was defined as units of enzyme activity per mg protein. Protein was determined by the Coomassie Blue method of Bradford (1976).

Qualitative Determination of Anthocyanidins. Pigment standards were as follows: Eggplant skin (delphinidin), Zea mays aleurone (cyanidin and pelargonidin) and Lythrum salicaria (malvidin). Maize seeds were kindly supplied by S. McCormick, eggplant was obtained at a local market and Lythrum was collected around Lake Lansing. All procedures were carried out in subdued light. Pigmented tissues were homogenized in 0.1% HCl in methanol at 0°C. The homogenate was centrifuged at 8000 x g for 15 minutes. The supernatant was combined with an equal volume of 2N HCl and boiled in a water bath in darkness for about 1 hour or until the volume was reduced by a half. After tubes were cooled, the flavanoids were extracted into n-amyl alcohol and spotted onto TLC plates (Polygram Cell-300 Cellulose, Brinkman #MN-300, 0.1 mm thickness). Chromatograms were run in foil-wrapped tanks pre-equilibrated with Forrestal solvent (acetic acid:HCl:water = 30:3:10), air dried at room temperature and visualized under UV and visible light. Absorption spectra were determined with a Cary 15 scanning spectrophotometer, using the appropriate solvent as a blank.

Quantitative Determination of Anthocyanins. The procedure of Scherf and Zenk (1967) was followed in subdued light. Vr9 and Vr9a cells were collected in a Buchner funnel and the pigment contained in approximately 1 gr of tissue fresh weight was extracted into 10 ml of 1N HCl by 45 minutes of gyratory agitation with 15 gr of 4 mm diameter glass beads in a 125 ml erlenmeyer flask. The extract was clarified by centrifugation at 8000 x g for 15 minutes. Under these

extraction conditions, the Vr9a pigment had an absorbtion maximum of 512 nm, as determined on a Cary 15 scanning spectrophotometer. Pigment concentration was therefore expressed as the A_{512} per gram fresh weight of the tissue extracted.

Protoplast Isolation. Exponential phase suspensions of light-grown Vr9a cells were sedimentated at 1 x g for 20 minutes. Ten ml of packed cell volume was added to 40 ml of filter sterilized (Nalgene 45 μ pore size) enzyme mixture consisting of 3% (w/v) cellulase, 1% (w/v) macerase, 1% (w/v) hemicellulase and 0.5 M mannitol at pH 6.0. The mixture was incubated in a 125 ml erlenmeyer flask slowly agitated on a gryatory shaker in darkness at room temperature for 5 hours. Protoplasts were collected through a 67 μ nylon mesh and pelleted at 130 x g for 5 minutes. The supernatant was drawn off and the protoplasts were washed twice and resuspended, all with isotonic R3 medium.

Gel Electrophoresis. Whole cell protein was extracted as described above for enzyme assays and precipitated with 9 volumes of cold acetone. After 15 minutes, the precipitate was collected by sedimentation at 20,000 x g for 20 minutes and resuspended in SDS sample buffer. This buffer and the remaining procedures were those described by Laemmli (1970). Molecular weight markers ranged from 14,000 to 330,000 daltons.

Sources and Handling of Reagents. All reagents were of the highest purity obtainable from Sigma (St. Louis, MO) unless otherwise indicated. Any media additives beyond the standard components described above were filter sterilized through 45 or 22 μ Nalgene or Millipore (Swinex) units and added to cooled liquid or agar media just above the gelling temperature. Difco Bacto agar was used throughout.

Methotrexate was stored dessicated at -20°C. It was dissolved in a minimal volume of 0.1 N KOH, diluted with water and its concentration determined from its extinction of 23,000 $M^{-1}cm^{-1}$ at 257 nm, immediately prior to each use. Folinic acid was stored dessicated at -20°C and dissolved in minimal 0.1 N KOH and water for use. Its concentration was determined from its absorbance at 282 nm based on an extinction of 26,922 $M^{-1}cm^{-1}$. Dihydrofolic acid was stored in a dark dessicated ampoule at -20°C. It was dissolved in 50 mM $KHCO_3$ immediately prior to use. DHF concentration was determined from its extinction of 19,000 $M^{-1}cm^{-1}$ at 282 nm. NADPH (chemically reduced) was stored dark and dessicated at -20°C and dissolved in slightly alkaline water immediately prior to use. Its concentration was determined from its absorption at 340 nm based on an extinction of 6220 $M^{-1}cm^{-1}$. The Coomassie Blue protein reagent was homemade from Coomassie Brilliant Blue G (Sigma) or obtained from Bio-Rad. Protoplast enzymes were obtained from Calbiochem (Cellulysin, macerase) and Sigma (hemicellulase), stored at -20°C and dissolved immediately before use. All water used was purified by cation exchange and glass distillation.

SELECTION OF VIGNA RADIATA CELLS RESISTANT TO METHOTREXATE

Introduction

The object of the initial portion of this study was to genetically mark a cultured cell line of Vigna radiata. Since the cell line's ultimate purpose was to participate in a protoplast fusion, the marker phenotype had to be stable, selectable and cell autonomous.

The first task was to establish a suspension cultured cell line with well defined growth characteristics. Thus, the early experiments reported herein involve the establishment, growth kinetics, morphology and nutritional requirements of such a V. radiata cell line -- Vr2.

The next step was to define a set of conditions under which a mutant cell of the desired characteristics could survive and wild type cells would die. Third, a large population of cells was screened under these selective conditions, resulting in the recovery of presumptive mutants. Finally, these clones were characterized and evaluated for their suitability as participants in parasexual hydridizations.

The reversion rate of a mutation is usually far lower than the forward mutation rate. Therefore, the most stable variants recovered from in vitro selections would be those whose molecular basis is an alteration in the primary sequence of the genomic DNA. Among the criteria used to assess the genetic nature of plant cell variants are (Maliga, 1976; Chaleff, 1981):

1. Recovery of variants at low rates or frequencies,
2. Enhancement of recovery rate or frequency by known mutagens,
3. Inheritance of the selected trait by progeny of plants regenerated from variant cells,
4. Phenotypic stability of variants in the presence and absence of continuous selection pressure,
5. Detection of a stable biochemical, physiological, or morphological alteration rigorously associated with the variant phenotype,
6. Demonstration of a change in the primary nucleotide sequence of a gene presumably responsible for the variant phenotype,
7. Satisfaction of criteria 1. - 6. by a phenotypic revertant of the variant in question.

Plants cannot yet be regenerated from single cultured cells of grain legumes. Nor are haploids readily available in the Leguminoseae. It was therefore necessary to devise selections to recover dominant mutants whose genetic basis could be demonstrated by other than Mendelian means.

The folic acid analogue, methotrexate (MTX), was chosen as a positive selective agent. Its cellular target is unique and ubiquitous: the enzyme dihydrofolate reductase (DHFR). Tight binding of MTX to the active site of DHFR prevents the cell from generating reduced folate derivatives, the suppliers of single carbon units for the synthesis of amino acids and nucleotides. The specificity of this agent's mode of action (see PROLOGUE) should render the biochemical characterization of variants sufficiently direct and convincing. A DHFR activity with reduced methotrexate sensitivity would be persuasive evidence for the biochemical basis of a variant cell line exhibiting MTX resistance, and would illuminate a path to the presumptive DNA sequence alteration.

In order to increase the probability of recovering a point mutation in the dihydrofolate reductase structural gene, ultraviolet mutgenesis was employed. A selection system on petri plates was devised so as to better quantify variant recovery frequencies.

Results and Discussion

Growth and Plating of the Vr2 Cell Line. Five cultivars of Vigna radiata were screened for primary callus induction capacity in a three-by-four factorial experiment of kinetin and 2,4-D, respectively. Cultivar Vr1 accumulated the greatest fresh weight of the five cultivars tested, across all media (Table 1).

Table 2 shows the overall response of the five cultivars to the various kinetin/2,4-D regimes, revealing a quantitative dependence on kinetin. This contrasts with soybean callus growth on B5, a medium containing no cytokinin supplement (Gamborg et al., 1968). Perhaps V. radiata is not induced to supply cytokinins endogenously by treatment with 2,4-D, as are other species (Witham, 1968). The data in Table 2 do not represent a response surface bounding an internal peak. Lower and higher concentrations of 2,4-D and kinetin, respectively, might have promoted greater fresh weight yields. However, a portion of the fresh weight accumulated at 1 mg/l kinetin was attributable to root proliferation on the primary explant. Cultures initiated on 0.3 mg/l kinetin were almost totally rootless. For this reason, the combination of 0.3 mg/l kinetin and 0.5 mg/l 2,4-D was used in all subsequent experimental and maintenance media and was designated R3.

As sufficient callus tissue became available, suspension cultures were initiated from each cultivar. Only the poorest callus producer, Vr2 (see Table 1), yielded dispersed suspension cultures. The other four cultivars would either not grow at all in liquid suspension or maintained a callus-like clumpiness, despite repeated attempts to remove large clumps by seiving and reinitiation from callus cultures. No attempt was made to determine whether the successful adaptation of only the slowest growing callus cultures to suspension growth resulted from relevant physiology or merely coincidence. Vr2 was chosen for further

Table 1: Callus growth of five Vigna radiata cultivars. Etiolated hypocotyl sections of five Vigna radiata cultivars were plated on twelve media, constituting a 3 by 4 factorial array of 2,4-D (0.5, 1.0 and 2.0 mg/l) and kinetin (0, 0.1, 0.3 and 1.0 mg/l) respectively. A treatment unit consisted of a single hypocotyl section. Replication consisted of 3 units per plate and 3 plates per treatment. All nine replicate units per treatment were weighed on day 0. The treatment inoculum average fresh weight was subtracted from each unit's 21 day fresh weight. The resulting fresh weight increases were averaged within cultivar across all media and are expressed as a percentage (± standard error) of the highest yielding cultivar, Vr1.

Table 1: Callus growth of five Vigna radiata cultivars.

CULTIVAR	FRESH WEIGHT
Vr1	100
Vr2	28.0 ± 8.3
Vr3	34.1 ± 9.1
Vr4	57.4 ± 12.8
Vr5	67.0 ± 17.0

Table 2: Performance of five Vigna radiata cultivars under various 2,4-D + kinetin regimes. Vigna radiata cultivar explants were treated as described in Table 1. Fresh weight accumulation within each cultivar was calculated as in Table 1 and scored here as a percentage of the value for the highest producing medium within that cultivar. Scores for all five cultivars thus computed were then averaged (± standard error) within each medium.

Table 3: Performance of Vr2 under various 2,4-D + kinetin regimes. Vigna radiata cv. Vr2 was treated as described in Table 1. Fresh weight accumulations are expressed as a percentage of that resulting from the highest yielding growth regulator combination, 0.5 mg/l 2,4-D + 1.0 mg/l kinetin.

Table 2: Performance of 5 Vigna radiata cultivars under various 2,4-D + kinetin regimes.

2,4-D (mg/l):		0.5	1.0	2.0
kinetin (mg/l)	0	11.4 ± 4.0	10.6 ± 2.4	11.2 ± 3.6
	0.1	24.2 ± 5.1	24.0 ± 7.5	16.8 ± 6.1
	0.3	63.4 ± 8.0	30.2 ± 7.0	29.2 ± 4.9
	1.0	97.0 ± 5.2	88.6 ± 15.9	70.2 ± 16.6

Table 3: Performance of Vr2 under various 2,4-D + kinetin regimes.

2,4-D (mg/l):		0.5	1.0	2.0
kinetin (mg/l)	0	12.7 ± 4.9	12.3 ± 11.2	8.1 ± 8.8
	0.1	17.0 ± 4.8	16.9 ± 9.1	19.6 ± 9.7
	0.3	68.6 ± 6.8	32.1 ± 3.6	27.3 ± 10.9
	1.0	100 ± 17	98.8 ± 37.1	47.2 ± 15.4

characterization and selections. The response of Vr2 (Table 3) to the various 2,4-D and kinetin combinations discussed above was not unlike the pooled responses of all cultivars tested (Table 2).

The overall mean doubling time for comparable untreated control Vr2 suspension cultures throughout the course of this study was 5.5 ± 2.1 days. Figure 3 illustrates the distribution of growth rates of these controls from the time the culture was initiated up to the present. The source of this inconsistancy has not been firmly established. The positive response of Vr2 callus initiation to a 3 1/3 fold increase in kinetin concentration above that of R3 (Table 3) suggested a means of achieving more robust suspension performance of Vr2. Three enrichments of R3 were tested: A doubling of the concentration of all micronutrients, of all three vitamins and a 3 1/3 fold increase in kinetin concentration. All three enrichments, tested separately, resulted in suspension culture growth rates which were virtually identical to that of the control grown in R3. Temperature was a factor to which Vr2 was particularly sensitive. Increasing the incubtion temperature to 33°C from the standard 26-28°C resulted in a 60% increase in the exponential growth rate (Figure 4). Vr2 suspensions were not normally maintained in controlled temperature incubators, and the laboratory's ambient temperature fluctuated between 16°C and 29°F. Vr2 suspensions frequently developed a brown color following ambient temperature fluctuations, recovering normal color and growth rate only after several weekly subcultures. This uncontrollable variable neccessitated a growth quantification and selection system on petri plates which could be maintained in controlled temperature incubators.

The morphology of Vr2 cells in suspension culture is not unlike that reported for other grain legumes, especially Phaseolus vulgaris (Lamport, 1964; Mehta et al., 1967; Liau and Boll, 1971). As Vr2 suspensions enter stationary

Figure 3: Growth rates of Vr2 suspension cultures over the course of this study. Exponential phase (log normal) growth rates were calculated for Vr2 suspensions grown in R3 medium. Each point represents the mean growth rate, expressed in doublings per day for untreated controls in growth experiments over a 3 year period. The 3-year mean (± standard error) is represented by the dashed line and equals 0.18 ± 0.07 doublings per day.

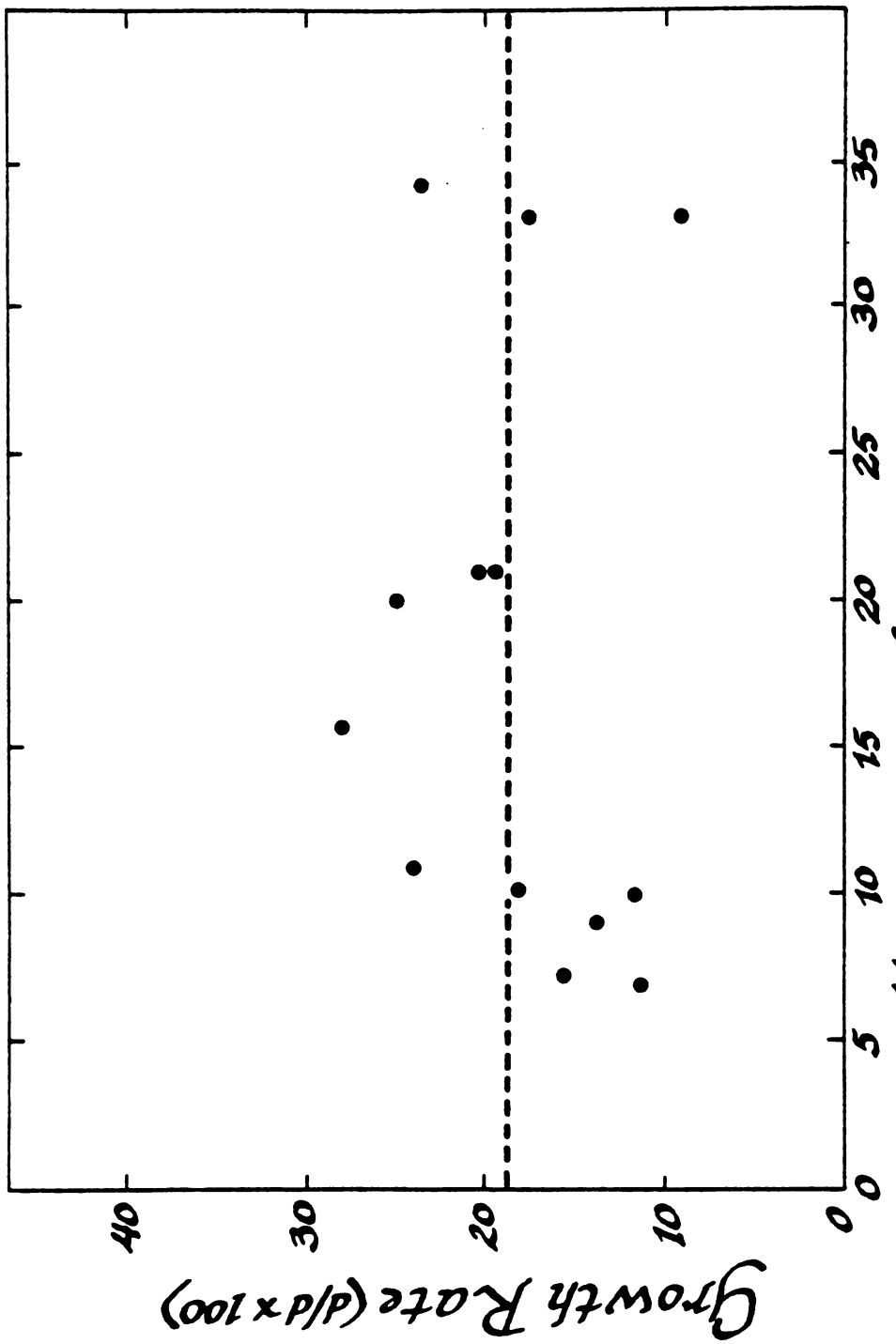


Figure 3: Growth rates of Vr2 suspension cultures over the course of this study.

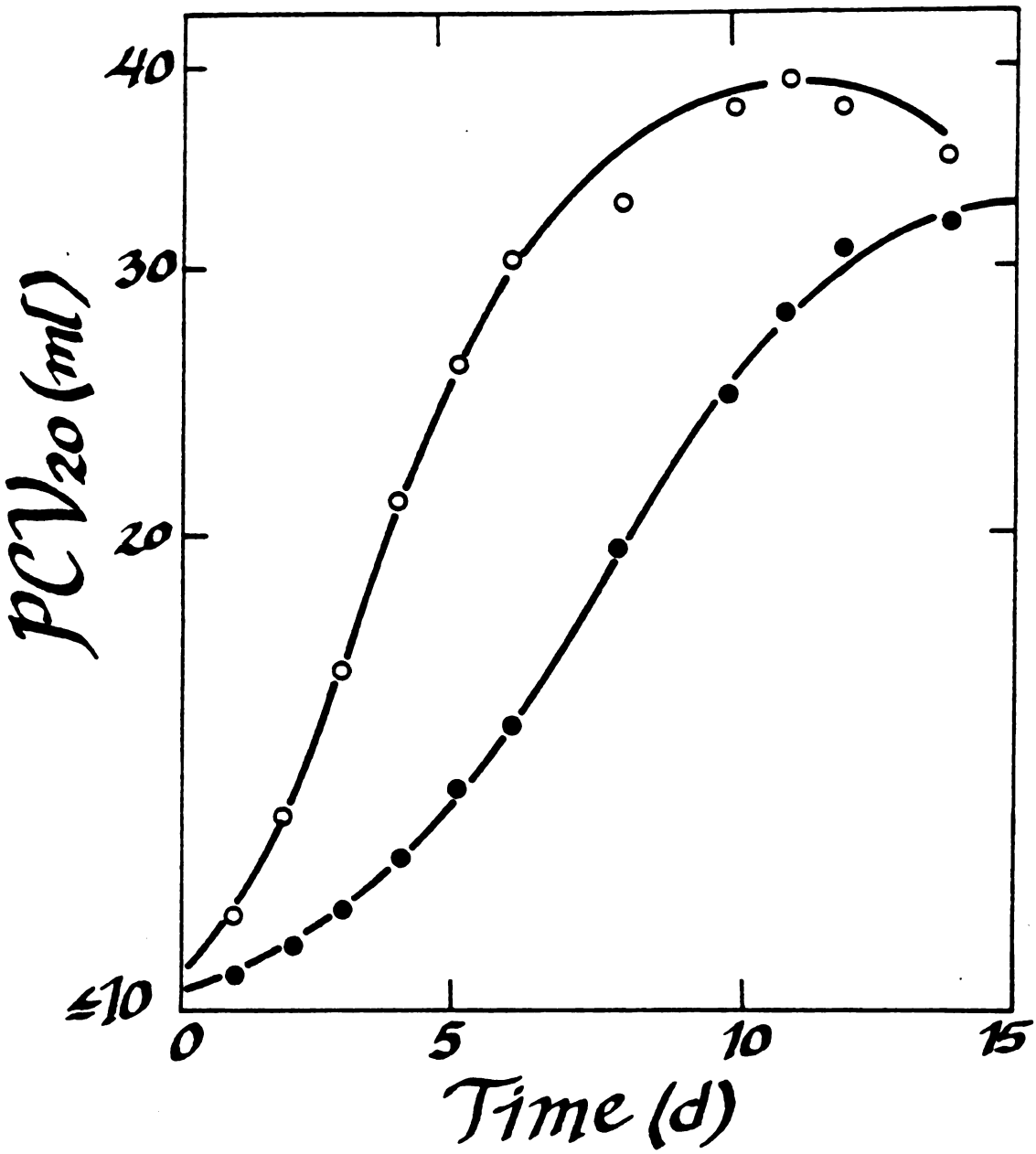


Figure 4: Temperature dependence of Vr2 suspension culture growth. Exponential phase doubling times are 4.1 days for growth at 33°C (O) and 5.4 days for growth at 22°C (●).

phase, giant isodiametric ($\geq$300 μ in diameter) and long filamentous non-septate ($\geq$600 μ in length) cells appear, in addition to aggregates of 100 cells or more. Upon subculture, the proportion of giant and elongated cells declines, presumably because they are partitioned by internal mitoses. This mechanism is supported by the appearance of many filamentous files of smaller, more isodiametric cells (40-80 μ in diameter) as the culture enters exponential growth. During this period, the proportion of "normal" free isodiametric cells, 40-80 μ in diameter, is maximal. Presumably, expansion of free cells and division (without separation) within the filamentous files gives rise to the giant cells and aggregates, respectively. The degree of aggregation of Vr2 stock suspensions fluctuated considerably during the course of this study. Frequent seiving was necessary, especially at the start of experiments requiring pipeting of suspensions. The stock culture maintenance routine adopted (see MATERIALS AND METHODS) was intended to maintain cultures in exponential growth most of the time, thus minimizing the proportion of giant cells and other abnormalities and imbalances characteristic of stationary phase cells.

Callus stocks of Vr2 are friable, yellow, somewhat shiny, and of a gross morphology, that frequently invited comparisons with the brain. Neither callus nor suspensions became green when grown on R3 under fluorescent lights, although under these conditions the color became a brighter yellow.

For maximum recovery of mutants, it was desirable to optimize plating efficiency of cells plated as lawns. An authentic plating efficiency estimate requires the determination of what proportion of plated cells divide and form colonies. This would require plating cells at a sufficiently low density for individual cells' fates to be followed. However, Vr2 cells would not plate at such a low density. Exponential growth rate was therefore measured on the assumption that, for a particular cell line, plating efficiency and growth rate are

positively correlated. Kao and Michayluk (1975) devised a medium which reportedly supported very low density growth of Vicia hajastana protoplasts. The response of Vr2 lawns to Kao and Michayluk's Medium 8, in addition to various compromises between R3 and Medium 8, is shown in Figure 5 and Table 4. Two conclusions were drawn from this experiment. First, Medium 8 supports a significantly higher growth rate than R3. Second, that improvement is not due to the effect of the undefined components, coconut water and casein hydrolysate, or to Medium 8's hormone composition. In the two treatments resulting in the highest growth rates, the lawns were of uniform density over the surface of the entire plate. In all other treatments, only in those regions of the plate where inoculation happened to be the heaviest did contiguous colonies form a lawn (the sloshing technique tends to leave a higher density of cells in a crescent shaped area along the side of the plate from which the suspension was poured onto the next plate). This may indicate that the enriched medium provides one of those factors, the diffusion and sharing of which necessitates high plating densities in plant cell culture systems (Chaleff, 1981). In all subsequent experiments where suspension cultured cells were to be plated, the modification of Medium 8 designated R3K (see MATERIALS AND METHODS) was used.

Mutagenesis. A mutagenesis system was devised to increase the mutation frequency in Vr2 cells. Ultraviolet light has been shown to cause point mutations in both prokaryotic and eukaryotic systems, via pyrimidine dimer formation and misrepair. Were its properties in such systems better defined, it would provide an inexpensive, clean and simple means of increasing genetic variability in cultured plant cells. Furthermore, the photometric growth measurement system provides a potentially precise method for quantification of UV dose lethality following irradiation of freshly prepared lawns. Three experiments were

Figure 5: Growth of Vr2 lawns on enriched media. The media employed were as follows: R3 (●), R3 + coconut milk (■), R3 + coconut milk + casein hydrolysate (▲), medium 8 (□) and medium 8 with R3 hormones (△). Experimental details are given in Table 4.

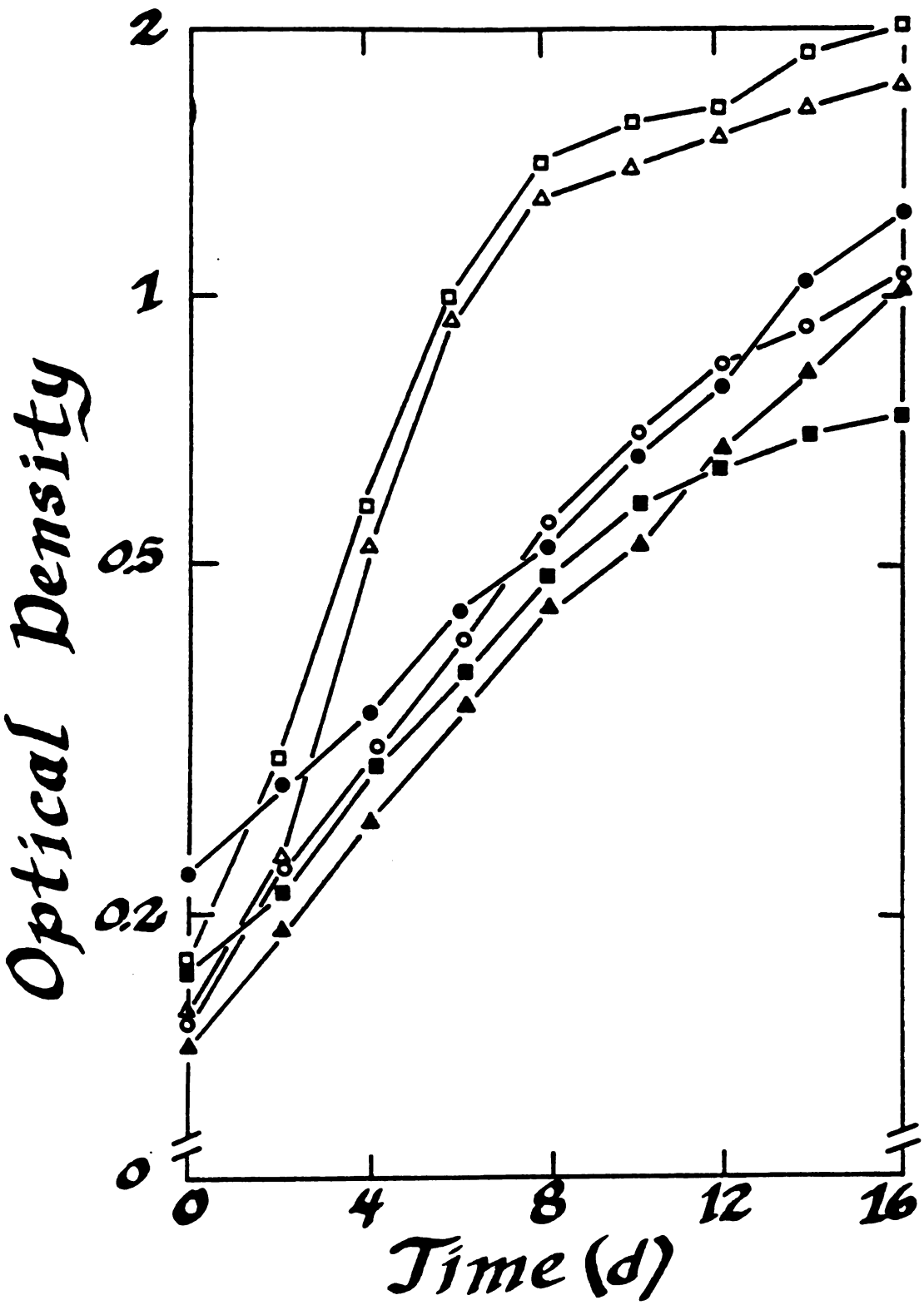


Figure 5: Growth of Vr2 lawns on enriched media.

Table 4: Effect of medium enrichments on Vr2 lawn growth. Growth measurements were made as described in MATERIALS AND METHODS. Medium 8 was that of Kao and Michayluk (1975). Medium 8 w/R3 hormones consisted of all but Kao and Michayluk's hormones, plus the IAA, 2,4-D and kinetin of R3. Doubling times followed by the same letter are not significantly different, $\alpha = 0.01$, $LSD_{.01} = 1.29$.

Table 4: Effect of medium enrichments on Vr2 lawn growth.

MEDIUM	DOUBLING TIME (d ± se)
R3	6.00 ± 0.83 a
R3 + Coconut Water (20ml/1)	4.90 ± 0.87 a b
R3 + Casein Hydrolysate (250 mg/1)	4.92 ± 0.13 a b
R3 + Coconut Water (20 ml/1) + Casein Hydrolysate (250 mg/1)	4.69 ± 0.76 b
Medium 8	2.37 ± 0.22 c
Medium 8 w/R3 hormones	2.22 ± 0.14 c

performed to characterize the response of Vr2 cells to UV light. The first experiment defined a UV dose-response relationship.

Lawns of Vr2 were initiated on 52 plates of R3K. Thirteen additional plates, to be used as photometer blanks, were sloshed over with uninoculatd liquid R3 medium. Irradiation was carried out as described in MATERIALS AND METHODS. Four lawns plus one blank per treatment were irradiated for periods ranging from 5 to 240 seconds. It was suspected that dessication of the thin layer of cells in a fresh lawn might result from some of the longer exposures in the laminar flow hood. Such a stress could inflate estimates of killing at higher irradiation doses. For this reason, controls were included in which lawns were uncovered in the hood for periods ranging from 30 to 240 seconds, in complete darkness. The growth of these lawns showed no detectable growth depression as a result of exposure to laminar flow hood air movement for up to four minutes. So as to prevent repair of UVL mediated DNA damage by the error-free photoreactivation pathway (Kimball, 1980) treatment and control cultures were placed in the dark immediately following irradiation, and photometric readings were taken on alternate days beginning on the fourth day (Figure 6).

Analysis of these data proceded as follows. When a population of cells is subjected to a treatment which proves lethal to only a fraction of the total, the growth rate of survivors may remain unaffected. Such fractional killing effectively lowers the inoculum size, resulting in an increased lag phase and lower (extrapolated) y-intercept of the plotted growth curve. UV induced lethality would be expected to yield a family of such curves, with nearly identical slopes and extrapolated y-intercepts proportional to the fractional survival of the treated population. Sung (1976) and Christianson and Chiscon (1976) used this method to quantify the lethality of EMS and NTG in soybean and tobacco suspension cultures, respectively. The curves in Figure 6 only partially

Figure 6: Growth of UV-irradiated lawns of Vr2. Freshly prepared lawns of Vr2, sloshed onto R3K, were irradiated with UV light of the following dosages (J/m^2): 0 (●), 19 (■), 37 (▲), 56 (O), 111 (+), 222 (□), 333 (△), 444 (⬮), and 888 (0). All cultures were placed in darkness for 4 days immediately following UV treatment.

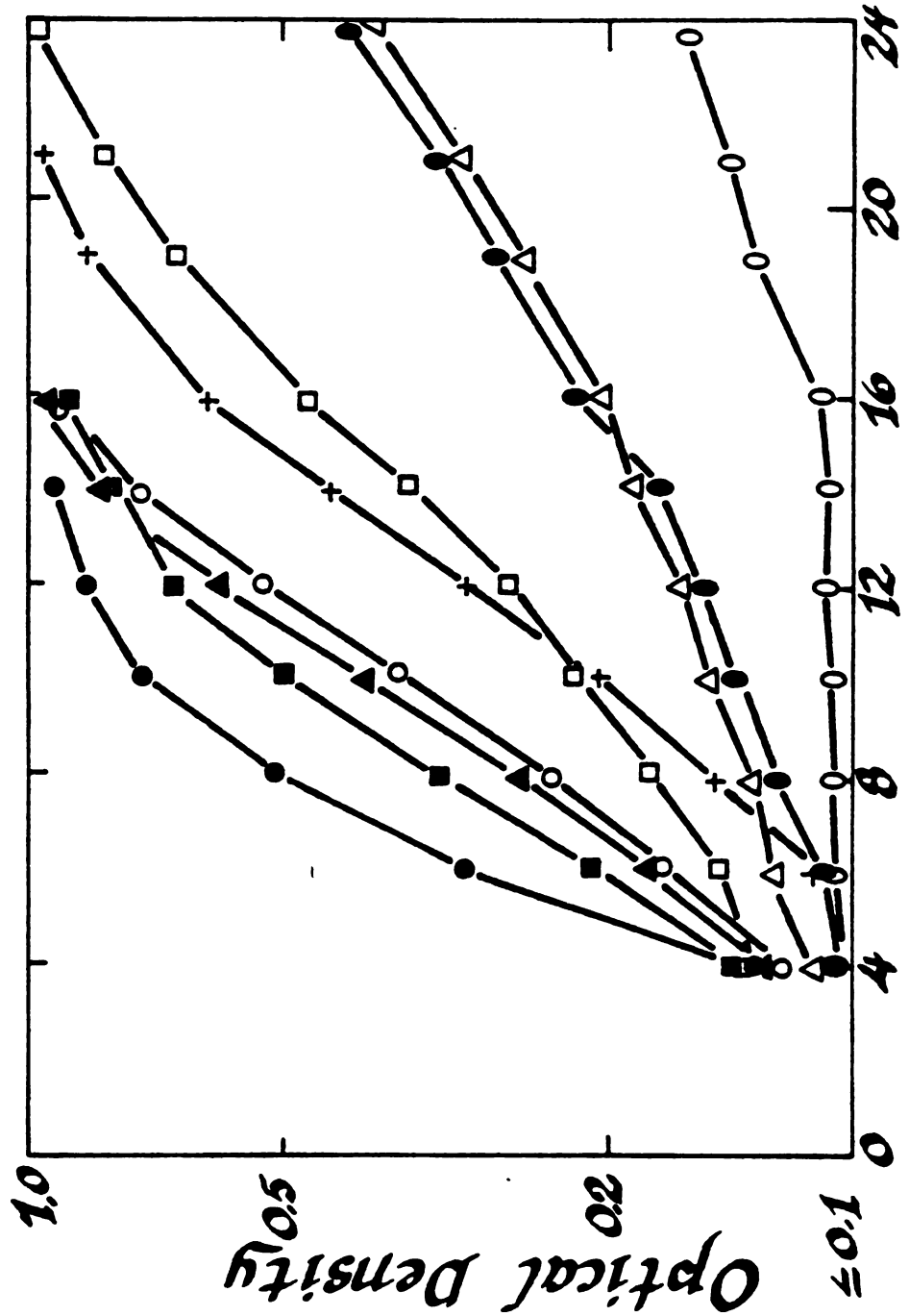


Figure 6: Growth of UV-irradiated lawns of Vr2.

satisfy this expectation. The slopes of the growth curves of more heavily irradiated cultures appear to be affected more than their intercepts. This deviation could be due to any or all of the following: 1) Surviving cells are nonetheless damaged, resulting in a population of sluggishly growing clones which never regain control growth rates; 2) The low viable population density in a UV decimated population introduces the constraint of low density growth inhibition; 3) The photometer may quantify the growth of confluent populations better than that of the discrete colonial populations which resulted from higher irradiation doses; 4) Cells killed by UV irradiation may leak toxic metabolites which retard the growth of survivors (although quite the opposite may be true; Horsch and Jones, 1980). Since growth rate may be proportional to survival under these conditions, the dose-response curves in Figure 7 represent both the intercept and slope methods of analysis. For the intercept method, the slopes were adjusted to that of the control (unirradiated) and back-extrapolated from a point determined to be the first day of exponential growth for that treatment.

Vr2 cells are more UV-sensitive as judged by the intercept method than by the slope method. Both curves are log-linear ($r^2 = .951$ and .987 for the intercept and slope methods, respectively), neither showing the shoulder at low irradiation doses which is usually indicative of a limited DNA repair capacity (Howland, 1977). Radiation sensitivity is frequently expressed as that dose which results in a 37 percent reduction in survival, or D_{37}. For UV irradiated Vr2 cells, the D_{37}'s are 74 J/m^2 and 184 J/m^2 by the intercept and slope methods, respectively (both calculated by regression, assuming log-linear dose-response). Taken as a range estimate of the true D_{37} for Vr2 cells, these are in reasonable agreement with previous reports. Erickson (1967) and Howland (1977) reported UVL D_{37}'s for Haplopappus gracilis cell suspensions of 200 J/m^2 and 80 J/m^2 respectively. Ohyama (1974) reported approximately 20 J/m^2 for soybean cells.

Figure 7: Growth response of Vr2 lawns to UV irradiation. Exponential phase (log normal) growth rates of UV treated lawns were calculated from the data presented in Figure 6, and were used to determine percent survival, by the slope (■) and intercept (●) methods, as described in the text.

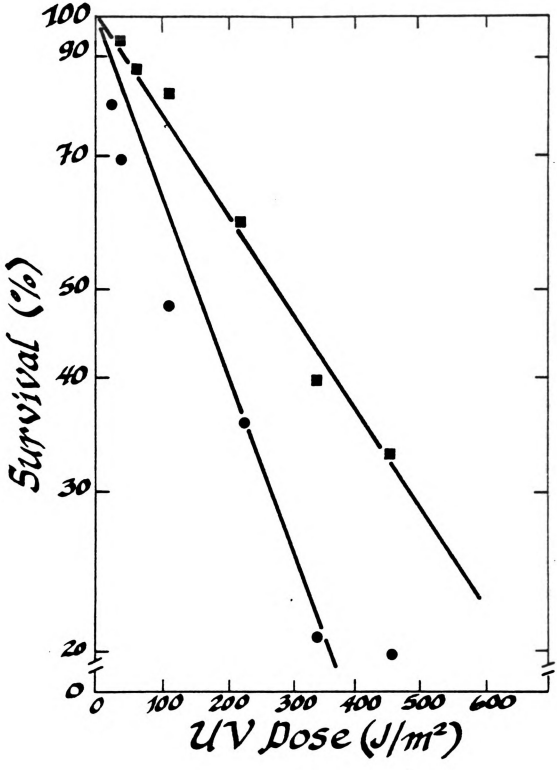


Figure 7: Growth response of Vr2 lawns to UV irradiation.

Survival was determined by colony forming ability of irradiated cells and aggregates in each of these studies. The differences in species, treatment conditions, methods of quantification and culture morphology in these studies preclude a rational comparison with the present data. Howland (1977) corrected his D_{37} estimate for the size distribution of aggregates in his Haplopappus cultures. However, the application of his correction would be practically untenable here since clump sizes range in Vr2 suspensions from 2 to over 100 cells.

In the mutagenesis experiments described above, cultures remained in complete darkness for four days immediately following irradiation to prevent error-free photoreactivation from reducing yields of mutants. This assumption of the existence of photoreactivation (and by association, mutagenesis; but see below) was tested in the following experiments.

In the first experiment, half of a preparation of fresh Vr2 lawns was treated with 900 J/m^2 of UVL and half was left unirradiated. Plates from each treatment were then divided into five groups, four of which were foil wrapped and one left unwrapped. All cultures were placed under 24h fluorescent lights (41 μ $Em^{-2}s^{-1}$). One set from each treatment was unwrapped each day for four days. Growth measurements began on the fourth day and continued for two weeks. Figure 8 reveals a linear relationship between mortality and the length of the dark incubation period, up to 3 days.

In the second experiment, fresh lawns of Vr2 were irradiated with 0, 38, 112, 336 and 900 J/m^2 of UV light. From each treatment, half of the cultures were placed directly under 24h fluorescent lights and half were wrapped in aluminum foil and placed next to the open set. After four days, the foil was removed and growth was monitored photometrically in all cultures for two weeks. Survival under each set of treatment conditions was calculated by the

Figure 8: Dependence of photoreactivation on length of dark incubation period following UV irradiation of Vr2 lawns. Fresh Vr2 lawns were irradiated with 900 J/m^2 of UV (●) and placed in darkness for 0, 1, 2, 3 or 4 days immediately thereafter. Optical densities were read on alternate days beginning on the fourth day. Survival was calculated by the intercept method. Control cultures were treated similarly, without UV irradiation (o). Survival of control lawns was normalized to 100 percent and survival of UV treated cultures was adjusted accordingly. Error bars represent standard errors of 5 replicate cultures.

Figure 9: UV dose dependence of photoreactivation of Vr2 lawn growth. Fresh Vr2 lawns were treated with 0, 38, 112, 336 and 900 J/m^2 UV. Half were placed directly in photoreactivating light (●), and half were placed in darkness (o). Growth and survival were determined and plotted as described in Figure 8.

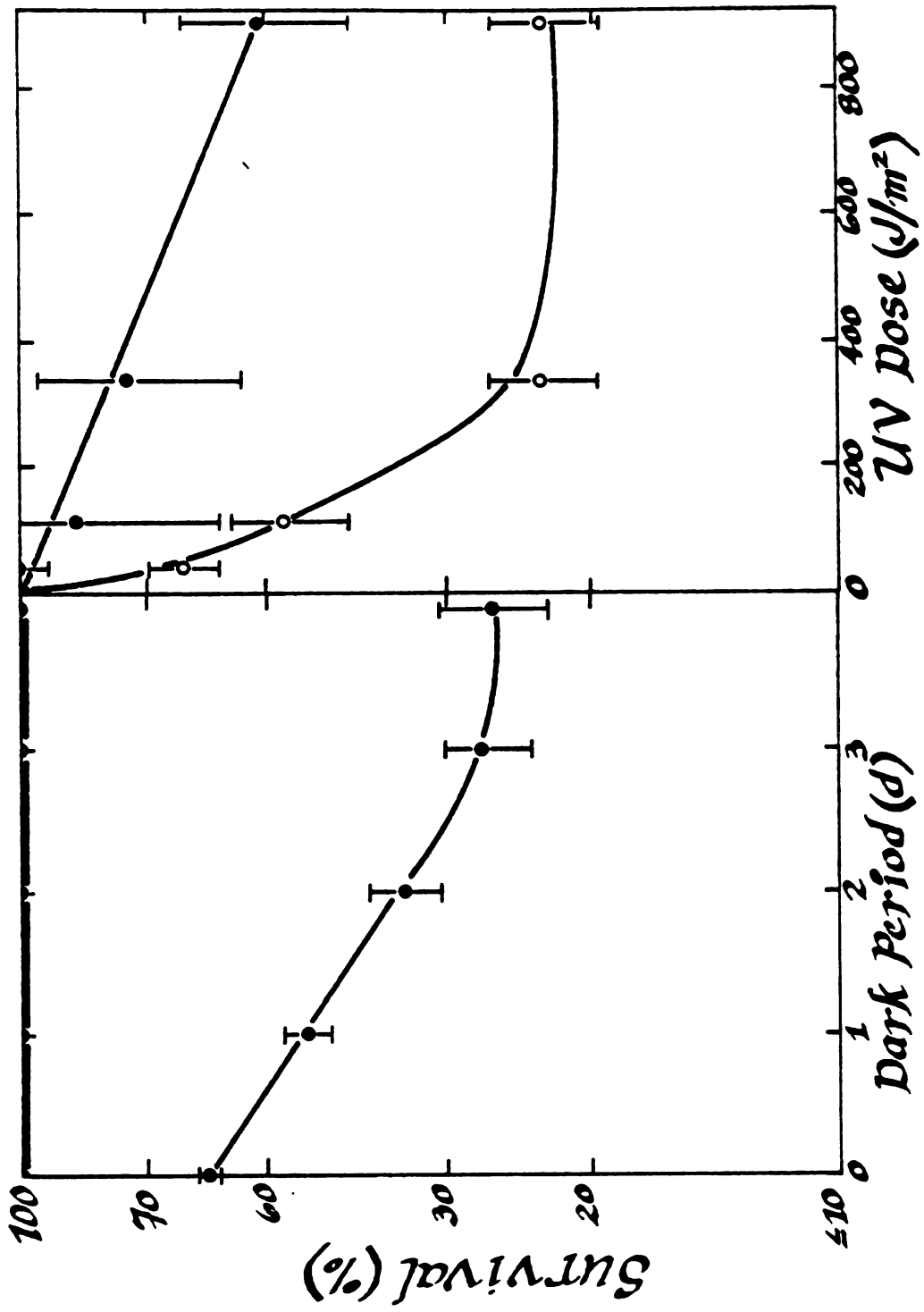


Figure 8: Dependence of photoreactivation on length of dark incubation period following UV irradiation of Vr2 lawns.

Figure 9: UV dose dependence of photoreactivation of Vr2 lawn growth.

intercept method and is shown in Figure 9. Incubation in the light following irradiation clearly enhanced survival. The leveling off of the survival response between 339 and 900 J/m^2 in the dark grown cultures may be due to aggregated cells remaining inaccessible to even the highest dose given. It should be noted that only at the lowest dose administered does it appear that complete restoration of growth is possible by photoreactivation. Above 112 J/m^2, the cells' capacity to photoreactivate DNA damage is saturated under these conditions and/or the cells suffer irreparably from other effects of UV irradiation such as the inhibition of protein (Murphy et al., 1975) and nucleic acid synthesis (Ohyama et al., 1974), or other metabolic perturbations (Reilly and Klarman, 1980).

Maximum response to UV mutagenesis would be expected to be expressed one generation following treatment, since a round of DNA replication and cell division is required for hereditary fixation of UV induced mutations. The requirement of at least one generation of darkness for maximum mortality of irradiated Vr2 cells supports, but does not prove, the proposition that the primary cause of UV induced mortality is DNA damage. Neither do any of the above experiments prove that UV light is mutagenic to Vr2 cells. I have only shown that its growth supressive effects are photoreversible and that its maximum effect is observed approximately one generation following treatment of Vr2 cell populations. Nonethelss, strong evidence suggests that UVL is indeed mutagenic in higher plants (see PROLOGUE). It will therefore be assumed that an error-prone repair mechanism is operative in UV irradiated Vr2 cells, as it is in all other organisms studied to date, and that its infidelity can be of service in the experiments which follow.

Response of Vr2 Cells to Methotrexate. The photometer was used to determine a concentration of methotrexate (MTX) that would be 100 percent growth

inhibitory to Vr2 cells. Exponentially growing Vr2 suspension cultures were sloshed onto solid R3K containing 10^{-9} to 10^{-4} M methotrexate, and absorbance readings were taken on alternate days for one month. One nanomolar methotrexate displaced the period of exponential growth approximately two weeks beyond that of untreated controls (Figure 10). Such potent toxicity was not forseen, and lower drug doses were not tested. The data displayed in Figure 10 were reduced by the intercept method and plotted in Figure 11. The I_{50} for methotrexate, under these conditions, appears to be about 2×10^{-10} M. During the 31 days of the experiment, no acceleration into exponential growth was observed in any cultures with methotrexate at or above 3×10^{-8} M. Had cultures been observed for a longer period, this might not have been the case (see discussion of selections which follows). The back extrapolation method is used here since the growth behavior (Figure 10) indicates that the drug arrests a subset of the population and leaves the remainder to proliferate, albeit at a reduced rate. This is in agreement with the well established mode of action of methotrexate (Blakely et al., 1981). Its tight binding to dihydrofolate reductase makes each cell's pool of this enzyme a sink for the drug, lowering the effective concentration to which surrounding cells are exposed. Intracellular derivatization of MTX also contributes to its accumulation within cells (Jacobs et al., 1975). The probability of a cell's survival is determined somewhat by aggregation displacing subsets of the population at variable distances from the medium. The undesirability of aggregated cells cannot be overemphasized here. Although fresh lawns appeared uniform to the eye, the population was obviously exposed non-uniformly to MTX, due to the vertical gradient of the drug that is formed by its being locked into the first (lowest) cells it encounters. This would not be as severe a detriment with a selective agent whose mode of action was more Michaelis-Menten and not "tight-binding", as is that of methotrexate.

Figure 10: Influence of methotrexate on Vr2 lawn growth. Vr2 lawns were initiated on solid media containing 0 (●), 10^{-9} (■), 3×10^{-9} (▲), 10^{-8} (○), and $\geq 3 \times 10^{-8}$ (□) M methotrexate. Optical densities were taken every 2 to 4 days for one month. Points represent means of 5 replicate cultures.

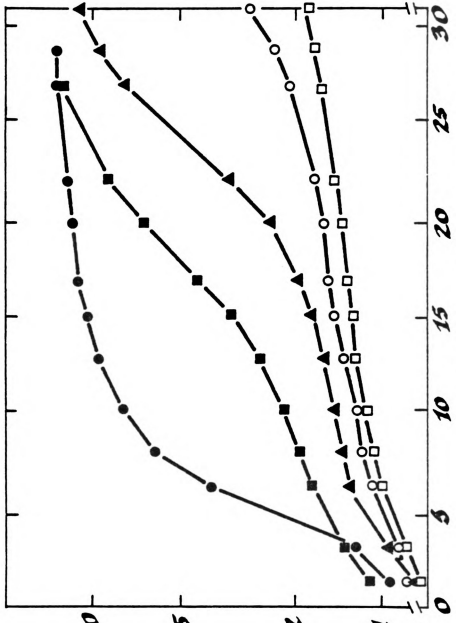


Figure 10: Influence of methotrexate on Vr2 lawn growth.

Figure 11: Response of Vr2 lawn growth to methotrexate. The data presented in Figure 10 was reduced by the intercept method to determine survival relative to the untreated control. Error bars represent standard errors of the mean of 5 normalized intercepts. Line determined by linear regression.

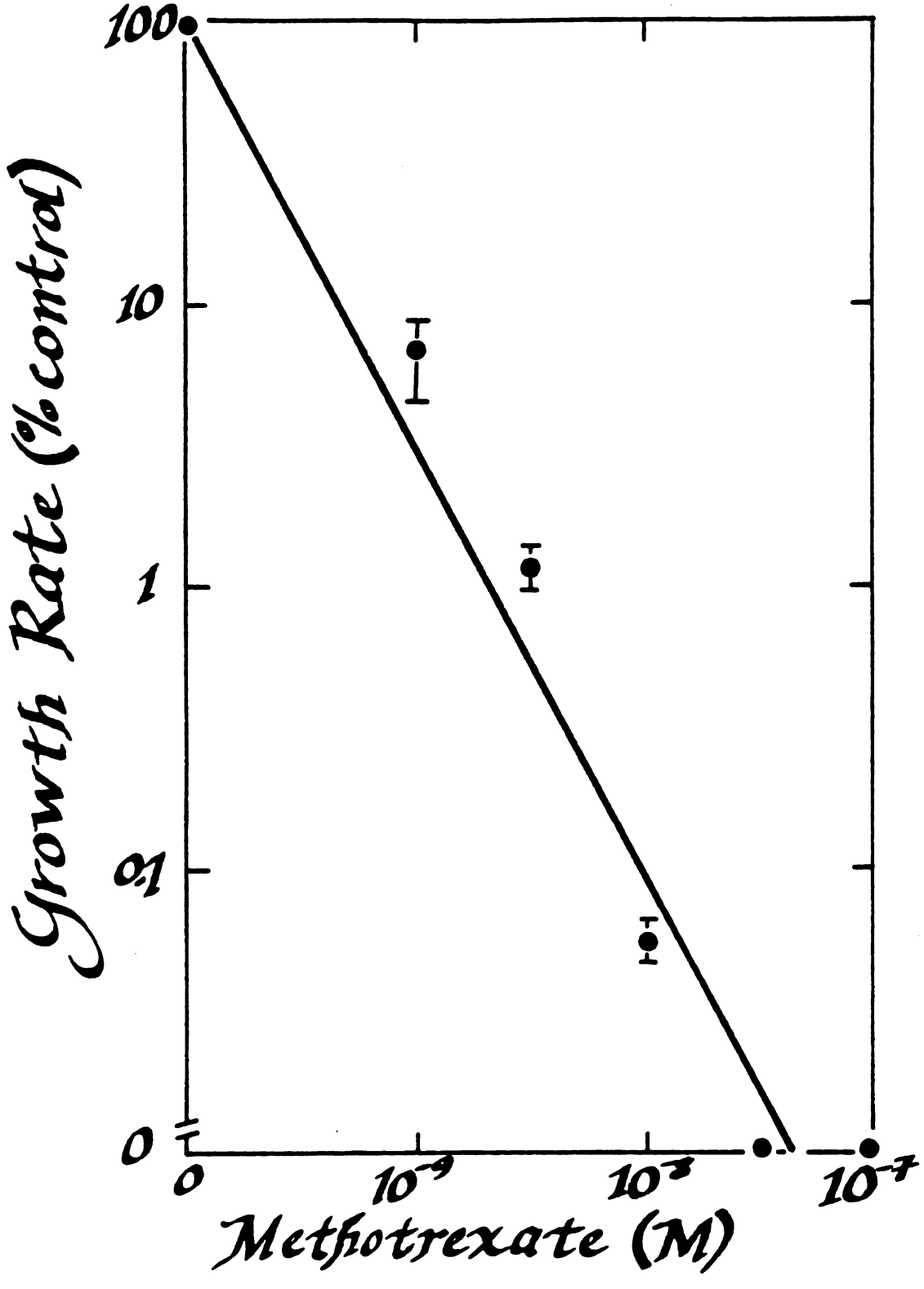


Figure 11: Response of Vr2 lawn growth to methotrexate.

The above considerations suggest that 2×10^{-10} M may be an underestimate of the I_{50} of MTX for Vr2 cells. Although the shapes of the curves in Figure 10 are identical to those generated by a similar analysis with E. coli (Webb, 1954), another folate-autotrophic organism, the I_{50} interpolated from Figure 11 is well below that estimated for other plant systems (see PROLOGUE). Such high sensitivity may be due to 1) Active transport of MTX into Vr2 cells coupled with a high intracellular binding capacity for the drug (and therefore, by inference, a high demand for the drug's target, DHFR), or 2) Inappropriate application of the back-extrapolation (intercept) method of growth curve analysis. With these considerations in mind, micromolar concentrations of methotrexate were used in all sections, well above the minimum I_{100} (Figure 11).

Folinic Acid Rescue. Development of one additional aspect of the system was required before selections could proceed. The predominant lesions (pyrimidine dimers) induced by UV light occur in only one strand of the DNA. Misrepair then leads to base substitutions. At least one round of DNA replication is required to fix such a lesion in both strands of the DNA, whereupon expression of the altered gene(s) is possible. It was therefore necessary to temporarily rescue cells from methotrexate toxicity for one generation (2 to 4 days) following plating and irradiation on MTX selective medium. It was reasoned that if a readily metabolizable antidote to methotrexate could be included in the medium, then its rapid assimilation and depletion would provide the desired effect. Folinic acid, the activated N^5-formyl derivative of tetrahydrofolic acid, is used as an antidote to methotrexate in cancer chemotherapy. This compound is the product of two enzymatic steps downstream from DHFR. Thus, the blockage of DHFR by MTX is bypassed by supplying folinic acid. In the following experiments, its capacity to temporarily rescue Vr2 cells was tested.

Table 5: Growth rate of Vr2 lawns on MTX + folinic acid. Fresh Vr2 lawns were initiated on R3K medium containing a factorial array of methotrexate (0, 10^{-7}, 10^{-6}, 10^{-5} M) and folinic acid (0, 10^{-6}, 10^{-5}, 10^{-4} M). For each MTX + folinic acid combination, the growth rate is given in doublings per day ± standard error of the mean of 5 replicate cultures. The mean rate expressed as a percentage of the 0M MTX control at that folinic acid level is given in parentheses. Data represent the growth rate for the first seven days.

Table 6: Final growth accumulation of Vr2 lawns on MTX + folinic acid. Data represent optical densities of 21 day old cultures grown under conditions described in Table 5. Standard errors of the means of 5 replicate cultures are given, as well as the means expressed as a percentage of the 0 MTX control of that level of folinic acid (in parentheses).

Table 5: Growth rate of Vr2 lawns on MTX + folinic acid.

MTX (M)	Folinic Acid (M): 0	10^{-6}	10^{-5}	10^{-4}
0	0.42 ± .03 (100)	0.37 ± .07 (100)	0.37 ± .04 (100)	0.35 ± .08 (100)
10^{-7}	0.06 ± .01 (14)	0.10 ± .02 (27)	0.13 ± .01 (35)	0.21 ± .06 (60)
10^{-6}	0.03 ± .01 (7)	0.04 ± .01 (11)	0.08 ± .02 (20)	0.16 ± .03 (44)
10^{-5}	0.05 ± .01 (12)	0.04 ± .01 (11)	0.06 ± .02 (16)	0.14 ± .02 (40)

Table 6: Final growth accumulation of Vr2 lawns on MTX + folinic acid.

MTX (M)	Folinic Acid (M): 0	10^{-6}	10^{-5}	10^{-4}
0	1.30 ± .02 (100)	1.23 ± .09 (100)	1.26 ± .04 (100)	1.23 ± .10 (100)
10^{-7}	0.19 ± .05 (14)	0.30 ± .07 (24)	0.33 ± .33 (26)	1.01 ± .38 (83)
10^{-6}	0.18 ± .07 (14)	0.18 ± .09 (14)	0.17 ± .03 (14)	0.39 ± .10 (31)
10^{-5}	0.17 ± .05 (13)	0.18 ± .04 (14)	0.14 ± .03 (11)	0.22 ± .07 (18)

A 4 x 4 factorial array of MTX (0, 0.1, 1.0, 10 uM) and folinic acid (0, 1, 10, 100 uM) was tested. Both the drug and its presumed antidote were incorporated into solid R3K and lawns of Vr2 plated thereon were measured photometrically for 3 weeks. An antidote:drug ratio of at least 10:1 was necessary to rescue Vr2 cells during the first week (Table 5). Restoration of 50% of the initial drug-free growth rate required over a thousand-fold excess of antidote. This is in agreement with the antidote:drug ratio required to effect significant rescue of permanent human lymphoblast cultures (Sauer et al., 1979). Inspection of the final absorbances attained (Table 6) reveals that initial growth rates and final growth accumulation were not quantitatively correlated, supporting the proposition that rescue could be temporary. However, visual inspection of these plates some weeks following the final readings revealed areas of substantial growth where the antidote:drug ratio had been 100:1 or less. Rescue had clearly not been temporary.

It was concluded that cells in this initial rescue experiment survived by using the diminishing, yet growth sustaining, supply of antidote diffusing up through the agar. The next experiment was an attempt to load the cells, and the liquid medium with which they were plated, with the antidote. The agarized plating medium here contained no folinic acid. By this method, cells would have to get by on what they brought with them to the selection. Based on the results of the previous experiment, Vr2 cells were preincubated in 1 mM folinic acid for 1, 12, 24, and 48 hours prior to plating onto R3K + 3×10^{-6} M MTX (antidote:drug = 333:1). The first week growth rates of these cultures and controls are shown in Table 7. A restoration of the initial growth rate to about 50% of the untreated control was achieved by 12 hours or more preincubation in antidote. A 12 hour antidote preincubtion treatment was therefore adopted as sufficient protection during the initial week of selection.

Table 7: Growth of Vr2 lawns following preincubation of cells in folinic acid. Suspension cultures were preincubated in 1 mM folinic acid for the times indicated prior to plating on R3K + 3 μM MTX. Growth rates are given in doublings per day (± standard error of the mean of 5 replicate cultures), followed by the mean growth rate expressed as a percentage of the untreated control (in parentheses).

Table 7: Growth of Vr2 lawns following preincubation of cells in folinic acid.

Treatment	Growth Rate (d/d)
-MTX, -FnA	0.35 ± .02 (100)
+MTX, -FnA	0.05 ± .01 (13)
+MTX, + 1h FnA	0.12 ± .01 (34)
+MTX, + 12h FnA	0.17 ± .01 (47)
+MTX, + 24h FnA	0.17 ± .01 (47)
+MTX, + 48h FnA	0.18 ± .02 (50)

Selection of Vr2 Cells Resistant to Methotrexate. Five selection experiments were carried out in an attempt to recover Vr2 cells genetically resistant to methotrexate. Three experiments employed mutagenesis, two relied on spontaneous rates of variation.

The two experiments without mutagenesis merit only brief mention. In the first, 1.7×10^8 late exponential phase cells were sloshed onto 100 plates of agarized R3K + 10^{-7}M MTX (1.7×10^6 cells/plate). This drug concentration was chosen on the basis of preliminary results of the MTX dose-response experiment discussed above, and on reported selective concentrations for model experiments with chinese hamster ovary cells (Flintoff et al., 1976). Lawns initiated concurrently on non-selective plates grew vigorously, as did control cultures in all of the selections described here. After six weeks, confluent colonization was in evidence on nearly all selection plates, particularly in a crescent shaped region along the side from which excess suspension had been poured during plating. This experiment was discontinued. It was concluded that, 1) the MTX concentration would have to be increased in future selections, and, 2) the ability of a freshly plated cell to tolerate a given MTX concentration depends on the cell population density in its immediate vicinity. The marginal pileup of cells resulting from the slosh plating technique of lawn inoculation creates a favorable environment for some cells to survive this selection.

In addition to within-plate variations in plating density, the geometry of the petri plates may have contributed to the unexpected tolerance of MTX displayed in this experiment. In the photometric dose-response experiments, petri plates of 6 cm diameter had been poured with 15 ml of media, resulting in a surface to volume ratio of 1.88:1 (cm^2:ml). In the actual selections, 10 cm diameter plates contained 33 ml of medium, giving a surface to volume ratio of 2.38:1 (cm^2:ml). Since inoculum density was constant between plates and experiments, the cells in

the selection experiments were exposed to a lesser absolute quantity of drug than those in the dose-response characterizations. Whether this 25% increase in cell:drug ratio was solely responsible for the survival reported above was not rigorously tested.

In the second selection of non-mutagenized cells, the concentration of methotrexate was increased to 10^{-5} M. The results of this experiment were virtually identical to those of the non-mutagenized control treatments of the selections with mutagenesis reported below. No useful information if forfeited by omitting their description here.

In each of the remaining three selection experiments, 120 fresh lawns were initiated on R3K + MTX. Sixty cultures were left unmutagenized, while an equal number were irradiated with ultraviolet light. All cultures were promptly sealed and stored in complete darkness. One week later, they were resealed and placed in a dark 25-27^{o}C incubator. The conditions and results of these selections are detailed in Table 8.

A large number of colonies was recovered from the three selections. A few colonies continued to appear after 64 days on some selection plates, but not enough to significantly alter the frequencies reported in Table 8. In each experiment, confluent lawns grew up to a limited extent, stopped, and became necrotic. White colonies began to appear above the necrotic layer 26 days after plating (Figure 12). The stringency of selective conditions was increased in succeeding selections, in case the profusion of colonies in experiment 8072 was due to escapes (wild-type clones which escaped the lethal effect of MTX).

Some preliminary arguments can be made for and against the proposition that most of the surviving colonies in all three experiments were indeed escapes. The earliness with which colonies appeared strongly suggests that they could not have arisen from single cells at the time of plating. Given the Vr2 doubling time of 2.5

Table 8: Conditions and results of methotrexate resistance selections.

Experiment No.	8072		8077		8080	
MTX (M)	3×10^{-6}		10^{-5}		10^{-5}	
Folinic Acid (M)	10^{-3}		10^{-4}		3×10^{-5}	
Folinic Acid : MTX	333 : 1		10 : 1		3 : 1	
Preincubation (h)	12		6		11	
UV Dose (J/m^2)	0	225	0	225	0	300
Population Size	1.2×10^8	1.2×10^8	1.2×10^8	1.2×10^8	1.2×10^8	1.2×10^8
Colonies after 26d	61	33	-	-	-	-
Colonies after 64d	555	1052	48	60	52	286
Variant Frequency	2.3×10^{-6}	4.4×10^{-6}	2.0×10^{-7}	2.5×10^{-7}	2.2×10^{-7}	1.2×10^{-6}

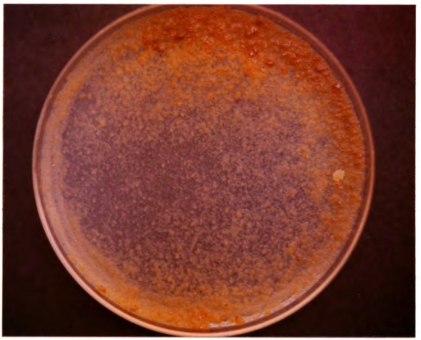


Figure 12: Typical putative MTX-resistant colony arising above necrotic layer.

days (on plates), a 2 mm colony (the minimum visibly discernable size) would require about 36 days to arise from a single cell (assuming cells are cubical, 70 microns/side). Slower growing variants would, of course, take longer. Although appropriate data were only taken in experiment 8072, colonies appeared in all selections well before 36 days. The absolute number of colonies, especially in experiment 8072, is rather high. Furthermore, it was known beforehand that Vr2's clumpiness, and the slosh plating technique, can permit survival due to protection afforded by localized high population densities. Even in the absence of aggregation, it can be calculated that at least 20 percent of the cells plated had another cell between them and the agar surface, if the average cell's contact area, the petri plate surface area and the plating density are .0049 mm^2, 7854 mm^2 and 2.0×10^6 cells/plate, respectively.

The apparent enhancement of variant recovery by UV light may be an artifact. Cells killed by irradiation might leak beneficial metabolites thereby promoting higher plating efficiencies of those aggregated cells which received marginally lethal doses of UV light (Horsch and Jones, 1980). Cells killed by methotrexate might also benignly leak nutrients -- but not the toxin itself -- into the medium. UV light is known to reduce DNA synthesis and thereby delay mitosis in cultured plant cells (Erickson, 1967a, 1967b; Trosko and Mansour, 1969b). If cells undergoing mitosis have an enhanced sensitivity to MTX, then mitotic delay might permit a sublethally irradiated population to weather the storm while dividing cells deplete the medium of the drug. Similarly, the large number of survivors on unirradiated plates could have been quiescent during the early days of selection. Chu and Lark (1976) found that 20-30 percent of the cells in soybean suspension cultures were non-dividing, synthesizing protein and RNA at extremely low rates. Significantly, they reported a positive correlation between the size of an aggregate and the proportion of the cells comprising it which were quiescent.

Cells in a clump would have a selective advantage were the selective agent specific for cycling, DNA synthesizing cells. This point will be explored further in Part V.

Several observations support the proposition that a significant proportion of the colonies comprises authentic variants from wild type with respect to MTX tolerance. The variant frequencies (Table 8) are within the range previously reported for plant cell variants (Maliga, 1976). Due to variations in plating efficiency and selection protocols, comparison of variant frequencies is not a particularly illuminating enterprise. However, clonal variation rates are comparable, due to the uniformity of experimental protocol which their calculation requires. Such a determination requires that a number of parallel base populations be initiated separately, several generations prior to selection. The established rate estimation procedures (Luria and Delbruck, 1943; Lea and Coulsen, 1949) are based on the distribution of variants arising from one culture to the next. Unfortunately, each selection's base population in this study came from one large stock culture at the time of plating. The one generation lag (engendered by temporary folinic acid rescue) between plating and MTX selection does not satisfy the conditions necessary for rate estimation.

The argument that colonies arose too early can be countered with the suggestion that resistant clones had arisen in the base population several generations prior to plating, and that these had grown into multicellular aggregates by the time selection began. This, in turn, might be countered by the observation that UV irradiation had a pronounced effect on variant frequency. The UV treatment was applied well after pre-existing clones would have airsen. However, it can be seen in experiment 8072 that the majority of the "early" colonies appeared on unirradiated plates. This supports the possibility of pre-existing resistant clonal aggregates. Irradiation, before its mutagenic effects were

expressed, may have simply killed 28 of the pre-existing resistant clones [28 = (2 x 61) - (61 + 33) = potential number of clones without irradiation -observed number with irradiation].

The enhancement of colony formation by UV irradiation is especially apparent in experiments 8072 and 8080. Colonies arising from UV treated cultures consistently appeared whiter, more friable and generally more robust than those from untreated cultures. The colonies appeared at random locations over the plates' surfaces, not in the high density crescent described earlier. Where the dose was increased, in experiment 8080, the effect was also increased. The distribution of colonies from plate to plate in 8080 is shown in Figure 13.

Characterization of Variants. In the foregoing discussion, it was argued that these variants can be shown to satisfy -- though not unequivocally -- the first two criteria for judging plant cell variants listed in the Introduction to this part. Inability to regenerate V. radiata plants from cultured cells precludes a Mendelian analysis of the variant phenotypes, the third criterion listed. The stability of the selected phenotypes was evaluated in the following experiments.

From each selection, colonies were transferred to fresh medium containing the same MTX concentration as in the selection plates. In this way, each propagative transfer was a retest of each clone's MTX tolerance. All clones grew slowly on MTX maintenance medium. A large number did not survive the first few transfers. Periodic culling reduced the number of presumptive variants to a collection of manageable size and apparently superior tolerance to the drug.

In the early transfers, clones maintained by transferring large inocula grew better than those from small inocula. This is in agreement with the observations above concerning the obstruction of selection efficiency by high plating density and clonal aggregation.

Figure 13: Irradiation dependence of colony frequency distribution in a methotrexate resistance selection. Data were taken from experiment 8080 (see Table 8). Open bars represent UV irradiated cultures; filled bars, unirradiated cultures.

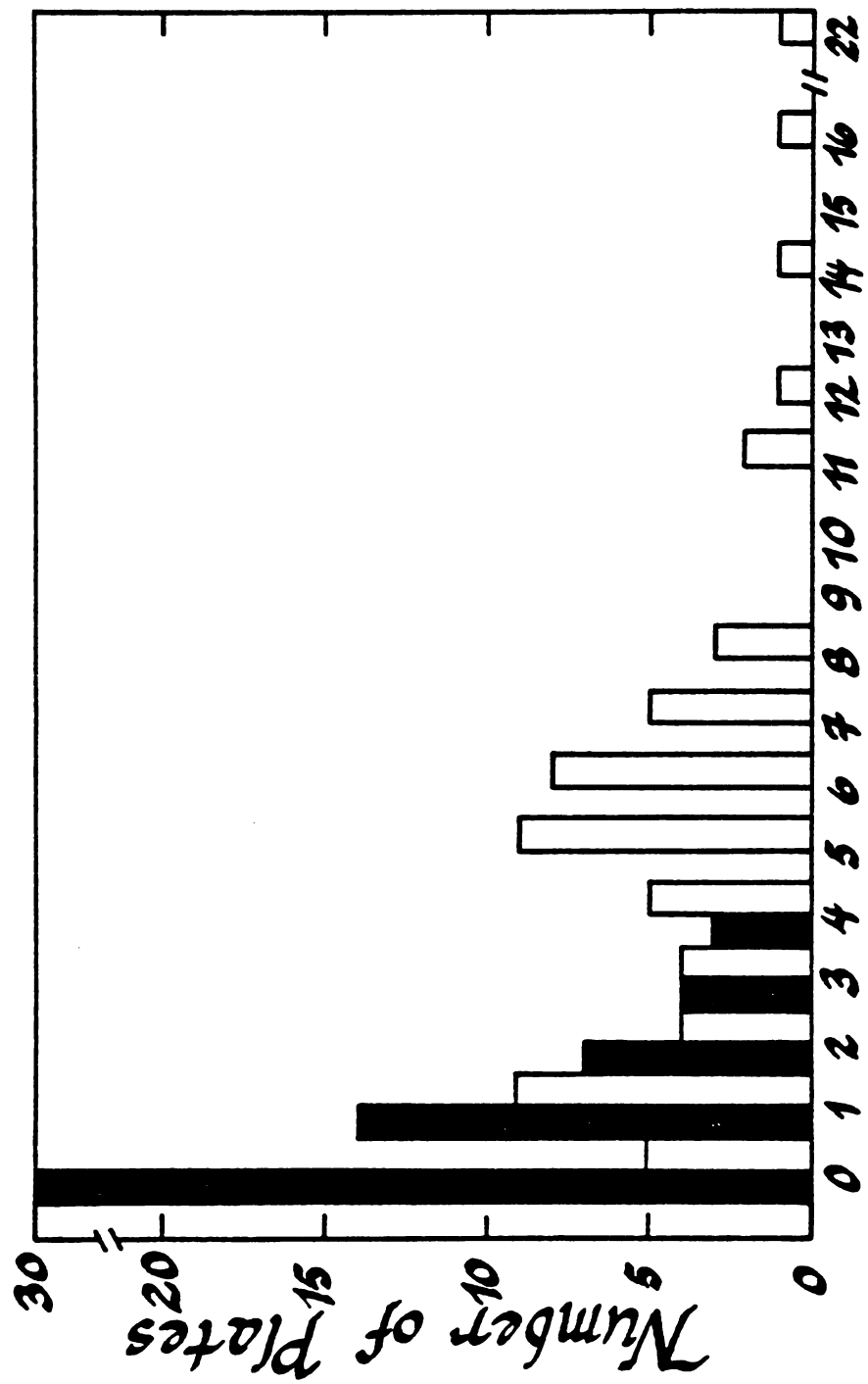


Figure 13: Irradiation dependence of colony frequency distribution in a methotrexate resistance selection.

Figure 14. Inoculum size dependence of growth of presumptive MTX resistant clones. Each number represents an individual presumptive MTX resistant clone; its location corresponds to its growth rate from the corresponding inoculum size. Measurements were made photometrically. Growth rates of control cultures of Vr2 on R3K (●) and Vr2 on R3K + 3×10^{-6} M MTX (■) are also shown. The diagonal line is the least squares regression of the data for the presumptive mutants ($r = +0.47$).

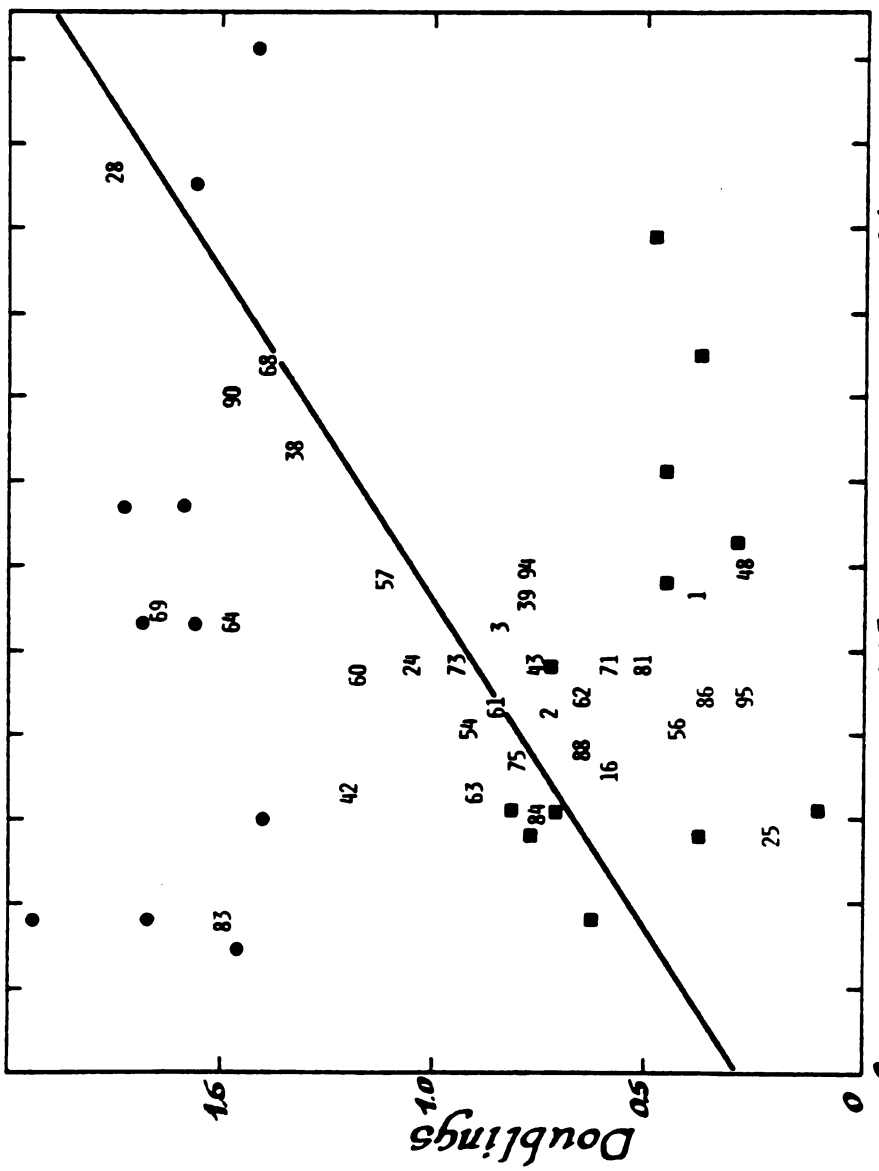


Figure 14. Inoculum size dependence of growth of presumptive MTX resistant clones.

In the first quantitative assessment of MTX tolerance, calluses of 34 presumptive resistant clones from selection experiment 8072 were monitored photometrically during growth on R3K + 3 μM MTX. Since the photometer was not designed to measure callus growth (but lawn growth), a photographic record was made of clones on the first and last days of the experiment. This qualitative evidence largely supported the photometric data (see below). Plotted in Figure 14, the data show a moderate correlation between inoculum size and ultimate growth. About two-thirds of the clones grew as much as Vr2 on 3 μM MTX; six grew as much as Vr2 on R3K. On the basis of this experiment and continued visual observation of growth and morphology, seven clones from 8072 were chosen for further study. Three of these (M^r-42, M^r-64, M^r-69) were "outliers" in that they deviated positively from the inoculum size - growth rate correlation shown in Figure 14. Four had followed the correlation more closely (M^r-28, M^r-38, M^r-68, M^r-90), but deviated positively nonetheless. Clone M^r-83, although it appears to "lie the furthest out" in Figure 14, fooled the photometer by necrosing to opacity, but not growing.

During the period of clone propagation preceeding the second quantitative growth experiment, another test of the inoculum size effect was made. During routine transfer of the clones, one plate of R3K + 1 μM MTX (selection pressure had been relaxed to permit more rapid growth) was inoculated with a series of successively smaller callus inocula, from 15 mm in diameter to 2 mm in diameter for each clone, plus wild type controls. After twenty days, visual observation revealed that no presumptive resistant clones has survived from inocula below 5 mm in diameter, even under such mildly selective conditions. Not only did control Vr2 callus survive from the 2 mm inocula on non-selective plates, but wild type Vr2 survived on R3K + 1 μM MTX selective plates, where the inoculum had been large enough ($\geq$ 15 mm). These observations once again suggested that, in this

system, the variable determining drug response is actually moles of MTX *per gram of tissue* not per liter of media. Therefore, in the growth test which follows, uniform inocula (= 60 mg, 7 mm in diameter) were carefully chosen.

Ten weeks prior to the start of the second growth test, stock cultures of all presumptive variants had been divided, and thence maintained under selective (R3K + 1 μM MTX) and non-selective (R3K) conditions. This permitted a test of the phenotypic stability of the variants in the absence of continuous selection.

In addition to the seven clones from 8072, seven qualitatively superior clones from 8080 were included in this experiment. The fourteen presumptive variant clones, plus a Vr2 wild type control were grown on 0, 1 and 10 μM MTX for 2 weeks. A random sample of each was weighed on the first day to determine the mean inoculum size for each clone. These data and the 15 day fresh weights were used to calculate growth rates for each clone and wild type Vr2 (Table 9). All fourteen clones displayed significantly greater MTX tolerance than the unselected control. Selectively maintained M^r-42, M^r-64, M^r-201, M^r-203 and M^r-204 were relatively uninhibited by 1 μM MTX, although all clones showed some degree of inhibition by 10 μM MTX. Two anomalies appeared. Non-selectively maintained clone M^r-38 displayed greater tolerance than the same clone maintained under continuous selection. Variants M^r-206 and M^r-207 appeared more tolerant of 10 μM MTX than of 1 μM MTX, compared to controls. The former anomaly could have been due to non-adaptive somatic variation in M^r-38 callus during propagation (Shepherd, 1981). In neither of the latter cases did clones perform better on higher than on lower methotrexate concentrations when compared within clone, but only with respect to controls.

Seven of the variants tested demonstrated clear phenotypic stability in the absence of propagation under selection (M^r-41, M^r-68, M^r-201, M^r-203, M^r-204, M^r-205 and M^r-209). M^r-64 and M^r-90 had lost the tolerance displayed by

Table 9: Growth of presumptive MTX resistant clones. Each datum is the growth rate in doublings per day (± se). For each clone/MTX combination, the upper figure is the growth rate of the clone maintained under selective conditions; the lower, its sibclone maintained under non-selective conditions for 10 weeks prior to test. **,* = significantly different from wild type Vr2 at same MTX concentration at $\alpha = .01$ and .05 respectively by Dunnett's Test ($m = 15$, $\nu = 120$). +, - = Clone selected with, without UV treatment. Each point is mean of nine replicate calluses.

Table 9: Growth of presumptive MTX resistant clones.

Clone	UV	Methotrexate (μM)		
		0	1.0	10.0
M^r-28	-	0.164 ± .030	0.114 ± .025**	0.110 ± .046**
		0.161 ± .042	0.073 ± .035*	0.042 ± .024
M^r-38	-	0.145 ± .019	0.081 ± .026**	0.038 ± .022
		0.169 ± .017	0.098 ± .015**	0.054 ± .028**
M^r-42	-	0.190 ± .020**	0.162 ± .036**	0.062 ± .029**
		0.165 ± .043	0.092 ± .031**	0.064 ± .017**
M^r-64	+	0.187 ± .030*	0.128 ± .041**	0.060 ± .013**
		0.142 ± .009	0.041 ± .031	0.028 ± .010
M^r-68	+	0.174 ± .022	0.068 ± .007**	0.053 ± .027**
		0.183 ± .035*	0.087 ± .030**	0.059 ± .022**
M^r-69	+	0.159 ± .033	0.070 ± .047*	0.073 ± .033**
		0.147 ± .022	0.070 ± .027*	0.034 ± .019
M^r-90	+	0.157 ± .018	0.105 ± .023**	0.057 ± .020**
		0.131 ± .038	0.063 ± .034	0.040 ± .036
M^r-201	-	0.146 ± .027	0.137 ± .042**	0.069 ± .030**
		0.161 ± .025	0.090 ± .027**	0.066 ± .023**
M^r-203	-	0.203 ± .013**	0.126 ± .050**	0.097 ± .026**
		0.159 ± .032	0.081 ± .040**	0.047 ± .027*
M^r-204	-	0.194 ± .028**	0.192 ± .025**	0.076 ± .027**
		0.166 ± .035	0.117 ± .060**	0.054 ± .034**
M^r-205	+	0.175 ± .018*	0.098 ± .029**	0.046 ± .024*
		0.174 ± .023	0.085 ± .051**	0.061 ± .021**
M^r-206	+	0.172 ± .026	0.088 ± .029**	0.063 ± .028**
		0.166 ± .024	0.061 ± .020	0.060 ± .016**
M^r-207	+	0.152 ± .022	0.050 ± .016	0.048 ± .026*
		0.102 ± .044	0.022 ± .010	0.018 ± .014
M^r-209	+	0.180 ± .019*	0.095 ± .010**	0.054 ± .024**
		0.171 ± .015	0.133 ± .027**	0.093 ± .024**
Vr2		0.145 ± .021	0.031 ± .026	0.017 ± .018

selectively maintained sib-clones, and M^r-207 had lost what little (anomalous) tolerance its selectively maintained sib could demonstrate. The remaining four (M^r-28, M^r-38, M^r-69 and M^r-206) had retained an intermediate level of tolerance. Strong phenotypic stability was demonstrated in 3/8 and 4/6 of the clones selected with and without UV treatment respectively. The small sampling of the total number of variant colonies recovered precludes a rigorous comparison of the stabilities of variants recovered with vs without UV treatment.

The foregoing data and observations indicate that Vr2 clones demonstrating a very low but measureable and frequently stable level of MTX resistance were recovered. The minimum inoculum size requirement for phenotypic expression limits the utility of these variants in protoplast fusion selections, unless both the drug concentration and cell aggregation could be accurately reduced to lower levels than those employed in this study. Plant cell variants demonstrating very high level MTX resistance should be selectable under the appropriate conditions. It was concluded that a better understanding of the biochemical interaction between methotrexte and cultured plant cells would be advisable before embarking on further mutant searches.

Conclusions

The Vr2 cell line is not a model system for plant cell genetic studies. Its erratic growth and clumpy morphology must be improved before further selections are attempted. Both of these problems might be eliminated if a protocol for the isolation, cell wall regeneration and multiplication of V. radiata leaf protoplasts were developed. The uniformity of material from one experiment to the next could be better controlled, cells could be more uniformly exposed to a chemical or

physical selective agent and cell counts would be easier. The physiological, morphological and cytogenetic changes to which long term cell cultures are prone would be avoided. In addition, initial characterization of protoplast survival response would be especially appropriate where selection of protoplast fusion hybrids is the ultimate use to which a variant would be put.

The doubling time for Vr2 suspension cultures in R3 medium measured by packed cell volume ranged from 3.5 to 10.8 days. Ambient temperature fluctuation was partially responsible for this variability. In contrast, lawns of Vr2 on R3K doubled reproducibly once every 2.3 days, when measured photometrically. The accumulation of intracellular solutes by actively growing cells could, however, inflate absorbance readings in the absence of active population growth. Likewise, since freshly subcultured Vr2 giant cells divide by internal partitioning, packed cell volume measurements may not reflect true population growth either. Both methods of growth measurement, although not comparable *inter se*, are internally consistent. Remaining untested remedies to improve suspension culture performance might include lowering liquid volume (relieving anoxia) or reducing major salts concentrations.

Kao and Michayluk's (1975) Medium 8 was shown to improve the growth of Vr2 lawns. Undefined additives were not essential to bring about this improvement. Further studies of complex additives might reveal a medium capable of supporting growth of cells and protoplasts at very low density.

The "slosh" method of lawn inoculation is quick and convenient, but results in non-uniform population densities over the plate surface, especially with clumpy suspension cultures. Contamination was never a problem. Once again, protoplasts in liquid droplets or soft agar might be more uniformly exposed to the medium.

Photometric measurement of lawn growth provided an efficient, reproducible and non-destructive solution to the perennial problem of growth measurement in

plant cell cultures. Improvements in the relatively unsophisticated optics of the system are certainly possible. However, treatments inducing a pigmentation or necrotic response can yield anomalous results, detracting from the general applicability of the technique. Another disadvantage is the photometer's apparent insensitivity to the growth of tiny dispersed colonies. Indeed, an ideal dose-response assay for a potential selective agent should evaluate plating efficiency, not lawn growth, since it is the former which constitutes the basis of the selection itself. Such an approach has been reported recently (Ranch and Giles, 1980; Strauss and King, 1981). However, estimates of plating efficiency require lower density plating than is currently feasible with most cell lines.

Although ultraviolet light irradiation was shown to be cytotoxic, its mutagenicity was not proven. The D_{37} of 74 - 184 J/m^2 for Vr2 cells is in agreement with studies in other plant cell systems. That UV damage was one effect leading to cytotoxicity was strongly suggested by the photoreactivation response displayed. Mortality of UV irradiated lawns was increased by up to four days of post-irradiation dark treatment. Still, a biochemical connection between light-enhanced recovery and DNA repair was not sought. Although irradiation of lawns of cells made dosage easy to control, it created problems not convincingly solved in this study. A one generation rescuing effect of an antidote to the selective agent was sought but not precisely demonstrated, although some rescue by folinic acid was evident. The necessity of adequate expression time requires perhaps a longer period between mutagenesis and selection than that achieved here. Irradiation of suspension cultures in quartz flasks followed by a suitable (dark) mutation fixation period, would not only solve this problem but would provide for the application of Lea and Coulsen's (1949) P_0 mutation rate estimation (Malmberg, 1981). Cytotoxic levels of ozone generated inside irradiated flasks could, however, inflate estimates of UV lethality.

Methotrexate was shown to be a potent toxin in the Vr2 cell culture system. An I_{50} of 2×10^{-10} M was estimated for Vr2 lawn growth inhibition. However, 10^{-6} M MTX was necessary to avoid low level wild type survival in selection experiments. Aggregation of cells, plating density and callus inoculum size persistently confounded precise estimations of dose-response relationships. This may be a problem peculiar to the evaluation of "suicide" inhibitors such as methotrexate, which do not diffuse out of cells in a cytotoxic form once they have acted. Non-dividing cells associated with aggregates may have contributed a subpopulation of cells relatively resistant to MTX.

Folinic acid was shown to rescue cells from methotrexate toxicity, suggesting that the latter acts, in this system as in others, by inhibiting dihydrofolate reductase. Although this capacity for antidote activity had been demonstrated in plant systems long ago (Rudenberg et al., 1955), its like capacity in plant cell culture had not. A thousand fold molar excess of folinic acid over MTX was necessary to achieve a fifty percent recovery in growth rate. This suggests that Vr2 cells transport folinic acid by diffusion or at least by a different active system than that by which methotrexate enters the cell.

The selective system employing UV light, folinic acid and MTX yielded a large number of presumptive resistant colonies. The unexpectedly high variant yields suggested that most colonies may have escaped mortality via a physiological adaptation engendered by UV light, methotrexate, folinic acid, a combination thereof, or by insufficient drug dosage to eliminate wild type cells at the center of aggregates. The latter mode of escape was suggested by repeated observations that tolerance to the drug was proportional to the inoculum clump size. Nonetheless, variant recovery was increased by UV irradiation of lawns at the time of plating. Irradiation may have simply increased the plating efficiency of "escapes" (Werry and Stoffelsen, 1981). Other reportedly mutagenic effects in

plant cell cultures may similarly arise from the post mortem altruism of mutagen-killed cells (Weber and Lark, 1980). Attempts to quantitatively retest the putative resistant variants were once again hampered by the inoculum size dependence of drug response. Suspension cultures made from the variants might have provided more precise methods for quantifying resistance. However, when inoculum size was carefully controlled, all of the variants tested demonstrated MTX tolerance significantly greater than that of wild type. This by no means indicates that all or most of the colonies which had appeared on selection plates represented genuinely resistant phenotypes. First, those ultimately tested were judged to be the most promising by several months of maintenance under mild selective conditions and visual selection. Hundreds of possible "escapes" were culled. Second, there remains the possibility that the wild type control in the final growth test was uncharacteristically MTX-sensitive. The instability of the Vr2 cell line makes necessary such a caution, although the cell line appeared healthy at the outset of the experiment.

Three mechanisms of stable MTX resistance are known to function in eukaryotic cells (Bertino et al., 1981): 1) Diminished transport of MTX into resistant cells; 2) Reduced affinity of the intracellular target, dihydrofolate reductase, for the drug; 3) Increased intracellular MTX binding capacity (i.e., DHFR) above the concentration of methotrexate. That the MTX resistant Vr2 lines transport the drug poorly is unlikely on two accounts. First, plant cells in vitro and in vivo are autotrophic for folates and consequently probably do not actively transport them (Baker, 1967). A viable mutant with altered passive permeability is difficult to rationalize. Second, Vr2 cells are at least diploid, if not polyploid, and a transport mutant would be expected to be recessive. The variant frequencies were too high for double mutants.

Although a mutant displaying reduced DHFR-MTX affinity was sought in this study, none was probably found. The extremely low-level resistance displayed by the variants argues against this explanation. Such mutants are rare among the MTX resistant phenotypes reported in the literature. The high frequency of variant recovery here suggests some other mechanism must be operative.

Selective amplification of the DHFR structural gene renders cultured vertebrate cells resistant to methotrexate (see PROLOGUE). However, stepwise long term selections have usually been required to obtain these genotypes. A recent report suggests that single step DHFR amplification is possible and that its frequency can be enhanced with tumor promotors (Varshavsky, 1981). In addition, both folic acid (Hillcoat et al., 1973) and methotrexate (Hillcoat et al., 1967) have been reported to promote transient increases in DHFR activity in cultured mammalian cells, due to a reduction in degradation of DHFR in the latter case. Selective gene amplification in cultured plant cells has been suggested, though not proven, by reports from several laboratories (Ohyama, 1976; Maretzki and Thom, 1978; Skokut and Filner, 1980; Yamaya and Filner, 1981). Grosser amplification of genomic plant DNA has been better documented (Buiatti, 1976). Amplification mediated phenotypes can be stabilized by chromosomal integration of the amplified genes (Kaufman et al., 1979).

The possibility remains that the MTX resistant phenotypes described in this study are one-step amplification events. If this were the case, variants should display higher DHFR activity than wild type controls. If so, then greater-fold amplification might be expected by resubjecting these clones to selection at higher MTX concentrations.

A stable epigenetic mechanism may also have rendered the selected clones resistant to methotrexate. The selective system may have generated either a physiological derepression of DHFR gene expression or a cellular incapacity to

degrade it. Finally, Vr2 cells may have a limited capacity to detoxify MTX, by degradation or derivatization (besides non-covalently binding it to DHFR), which was physiologically or genetically enhanced by the selective system. Only further selections and biochemical studies will decide this issue.

PHYSIOLOGY AND BIOCHEMISTRY OF METHOTREXATE INHIBITION IN CULTURED PLANT CELLS

Introduction

The suspension cultured higher plant cell is a unique organism. Assumptions about its physiological or biochemical similarity to other cultured eukaryotic cells or to the organism from which its ancestors were explanted are not always warranted. It was concluded that a better characterization of the activity of methotrexate in plant cell systems might suggest more rational designs for future selections of resistant mutants. The approach taken was to first determine whether interspecific variation exists in sensitivity to MTX in vitro. If cell lines displaying differential drug sensitivity were found, the next appropriate task would be to ascertain the physiological or biochemical basis of the difference. Such an analysis would also reveal whether the drug's mode of action is the same in plant cells as it is in other systems.

Goldman et al. (1979) have provided a framework for this inquiry by elaborating five determinants of methotrexate toxicity, viz.,

1. Methotrexate membrane transport: By what mechanism and at what rate does MTX enter the cell?
2. Dihydrofolate reductase (DHFR) level: Does the cell have DHFR activity (i.e., MTX binding capacity) above its minimal biosynthetic requirement?
3. The K_i for MTX: How tightly does DHFR bind MTX?
4. The basal rate of thymidylate synthesis from deoxyuridylate: What is the cell's basal requirement for reduced folate derivatives?
5. Polyglutamation of MTX: To what extent is MTX derivatized (polyglutamated) in plant cells?

Early results suggested that factors other than transport were probably more critical in determining MTX toxicity. Furthermore, folate transport is probably diffusion limited in cultured plant cells, since they are folate autotrophic (Baker, 1967). This assumption remains untested and merits study. Determination of the DHFR level in cultured cells entailed the development of an assay for the enzyme activity, which was assumed to reflect the MTX binding capacity of the cell. A method for the precise determination of the K_i (or K_D, the dissociation constant) for a tight binding inhibitor has not yet been formulated. Available techniques (Greco and Hakala, 1979) provide estimates with insufficient precision to detect the slight differences expected here. A qualitative demonstration of the stoichiometric (very tight binding) nature of MTX inhibition of DHFR in the cell lines was satisfactory for the present purposes. Intrinsic growth rate was used as an indirect measure of the basal rate of thymidylate synthesis (this assumes, of course, equal genome sizes among the species tested). Polyglutamation of methotrexate may occur in plant cells. Such derivatives of natural folates have been demonstrated in plant tissues (Shah et al., 1970). However, such an analysis was beyond the scope of this study.

Results and Discussion

Species Sensitivity to Methotrexate. Rationalization of interspecific differences in methotrexate tolerance could suggest what forms genetic resistance takes and how it might be selected in vitro. Suspension cultures of tomato (LeW), tobacco (Nt575), common bean (Pv25), mung bean (Vr2) and adzuki bean (Va2) were tested for their natural tolerance of the drug. Substantial interspecific variability exists for this character (Figure 15). Calculated by linear regression through the

Figure 15. Growth response of 5 plant cell suspension culture lines to methotrexate. The cell lines represented by the curves are, from left to right, LeW (●), Va2 (▲), Nt575 (■), Pv25 (o), and Vr2 (△). Each point represents a single culture in one of three separate growth experiments run with each cell line. The linear portion of each curve was drawn by regression. The horizontal dashed line represents a growth rate of 50% of that of the control.

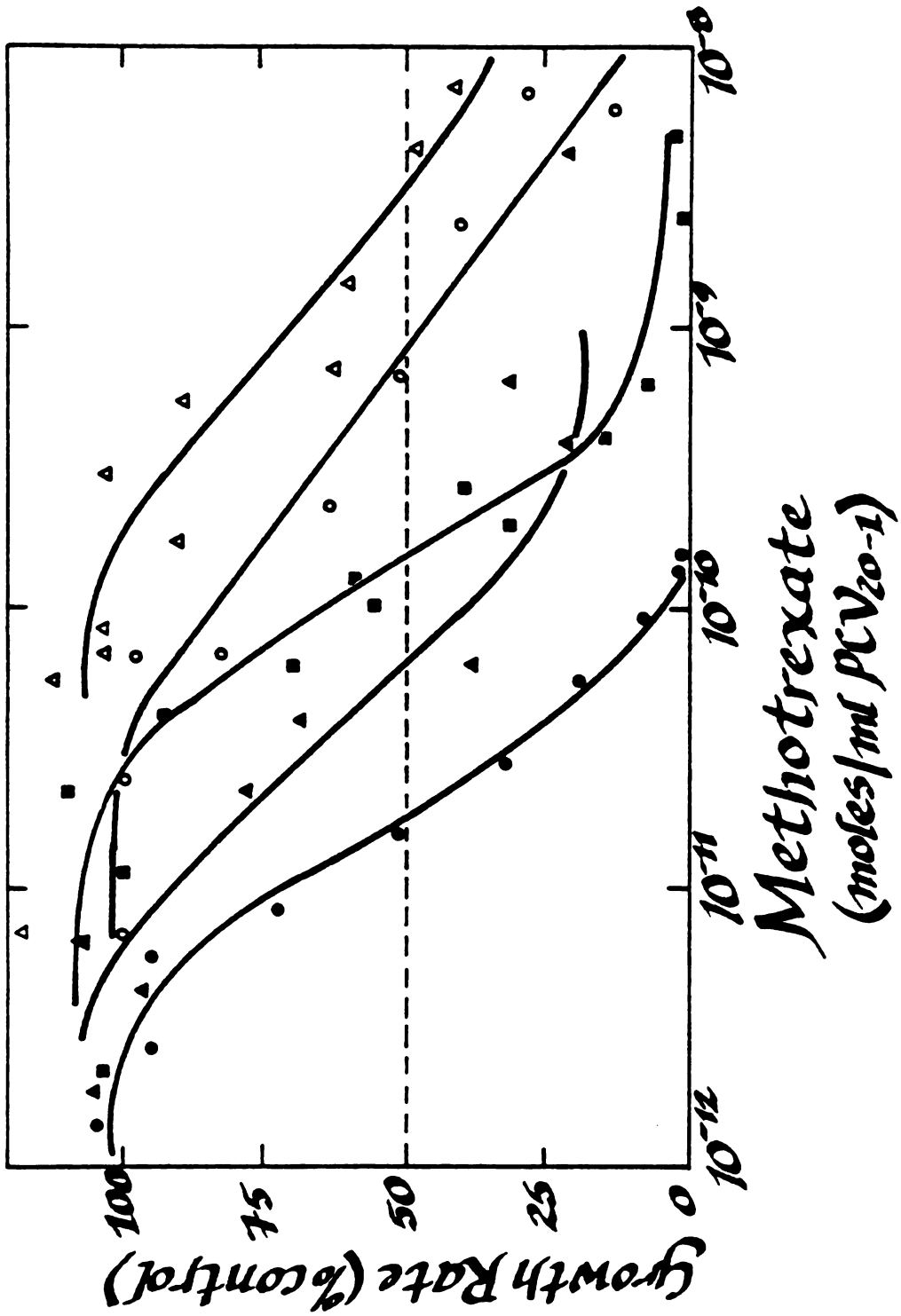


Figure 15. Growth response of 5 plant cell suspension culture lines to methotrexate.

linear portion of each curve, the I_{50}'s vary over a 200 fold range: 1.6×10^{-11} (LeW), 6.2×10^{-11} (Va2), 1.5×10^{-10} (Nt575), 8.0×10^{-10} (Pv25) and 3×10^{-9} (Vr2) moles MTX/ml PCV_{20} (day 1). The units of moles MTX per ml PCV_{20} (day 1) are appropriate here because the amount of drug per cell, not per ml of culture, determines toxicity. Tight binding of the drug to intracellular DHFR (and MTX polyglutamation, if it occurs) reduces egress of the drug from cells. Therefore, the amount of MTX to which a given cell is exposed is dependent upon how many neighboring cells are simultaneously depleting the medium of it. MTX doses in moles per ml PCV_{20} can be roughly converted to moles/liter by multiplying the former by 200, since the initial PCV_{20} in a 50 ml culture averaged about 10 ml. Using this conversion, MTX is 3000 fold more toxic to Vr2 cells grown in lawns (see Part III, Figure 11) than in suspension cultures. This difference may be attributable to 1) the different methods of quantification employed to measure growth in solid and liquid culture, 2) more efficient depletion of the drug from the medium by cells in suspension culture, 3) the compounded stress imposed by lawn plating of cells onto solid medium, 4) variation in the Vr2 cell line intself. The I_{50}'s obtained for these cell lines, when converted to MTX concentrations, range from 3.2×10^{-9}M (LeW) to 6×10^{-7}M (Vr2). These are well within the range reported for plant and animal cells cultured in vitro (see PROLOGUE).

The intrinsic growth rates and methotrexate sensitivities of the cell lines were highly correlated (R = 0.90; Figure 16). This is in agreement with Goldman et al.'s (1979) fourth determinant of MTX toxicity, the basal rate of thymidylate synthesis. Over any given time period, less DNA is being synthesized in a slow growing non-synchronous culture than in a more rapidly proliferating one. The level of DNA precursor synthesis is coordinately regulated with DNA synthesis (Klevecz and Gerald, 1977; Weidemann and Johnson, 1979). Rapidly growing cells cultures have a higher demand for DNA precursors (e.g., thymidylate) and

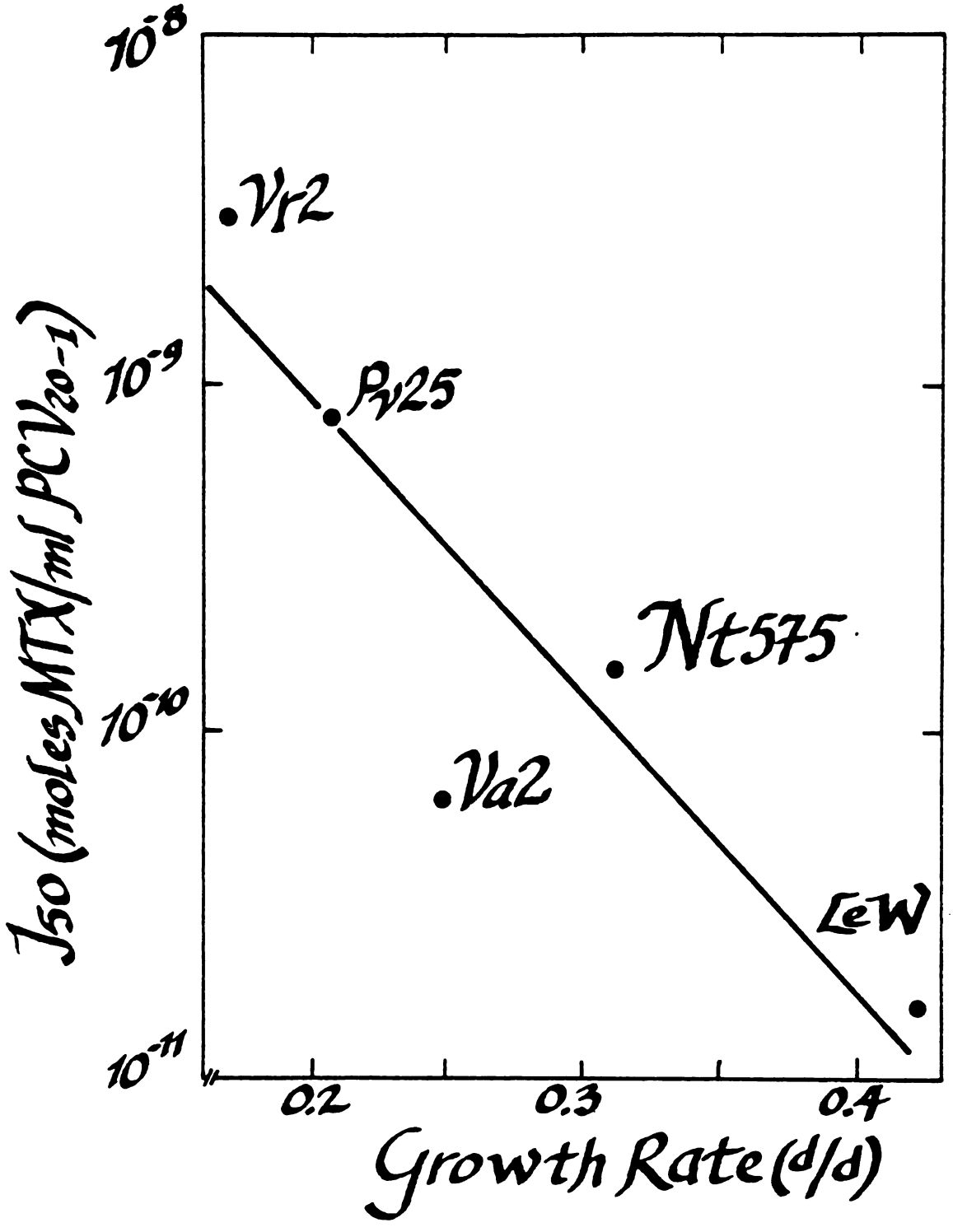


Figure 16. Methotrexate I_{50} vs growth rate for 5 cell lines. Data taken from Figure 15.

consequently are more sensitive to the depletion of a cofactor (e.g., reduced folates) required for precursor synthesis.

A positive correlation also exists between methotrexate tolerance and the degree of cell aggregation. Vr2 and Pv25 suspensions are composed of a higher proportion of cell clumps than LeW, Va2 of Nt575. This correlation may result from the drug not readily penetrating into the center of clumps. In addition, it has been observed that the non-dividing "quiescent" state is more common in clumped than among free soybean cells (Chu and Lark, 1976). Cells within aggregates may be non-dividing because of unfavorable competition for nutrients by cells on the periphery. Whatever its cause, quiescence could render aggregated cells more resistant to MTX by virtue of their relatively low requirement for DNA precursors. Va2 is a finely divided yet slow growing culture. Its divergence from linear correlation in Figure 16 suggests that the degree of aggregation is at least as important as growth rate in determining MTX sensitivity.

An attempt was made to test the hypothesis that MTX sensitivity is causally related to growth rate. Suspension cultures frequently grow faster at slightly elevated temperatures (see Part III and Malmberg, 1979). Pv25, a slow growing, relatively MTX resistant line, was grown at 26°C and at 33°C on a range of MTX concentrations. Although growth at 33°C rendered Pv25 more sensitive to MTX below 10^{-9} moles/ml PCV_{20} (day 1), it did not accelerate growth (Figure 17).

Because of its superior attributes in vitro, the tobacco cell line Nt575 is used as a model system in our laboratory. It provided a fast growing contrast to Vr2 in these comparative studies. Although LeW might have provided a more striking difference in MTX sensitivity and growth rate, its performance is generally more variable and less well characterized than that of Nt575.

Figure 17. Temperature dependence of methotrexate toxicity in Pv25 cell suspensions. Filled symbols represent growth rate (circles) and PCV_{20} (squares) at 26°C. Open symbols represent growth rate (circles) and PCV_{20} (squares) at 33°C.

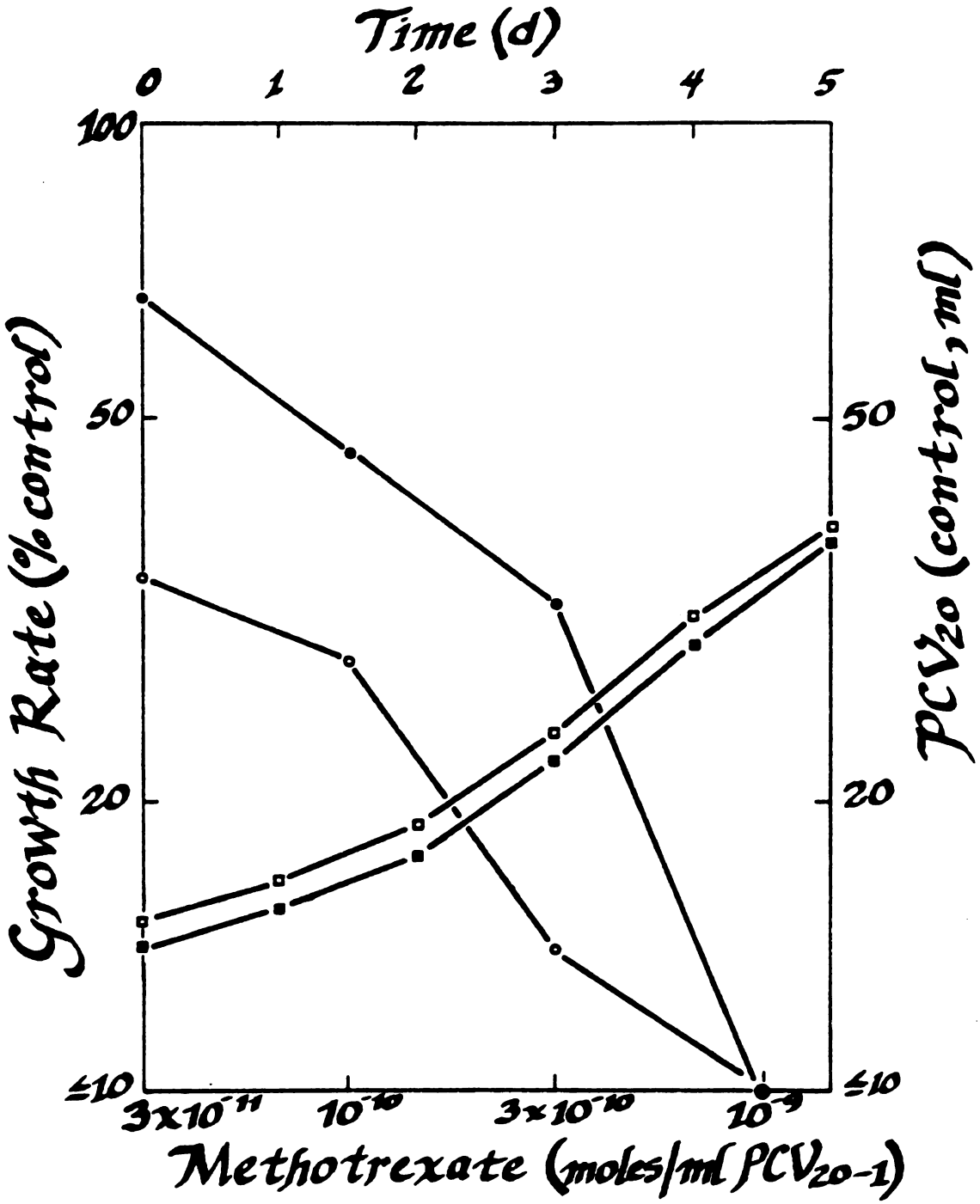


Figure 17. Temperature dependence of methotrexate toxicity in Pv25 cell suspensions.

Folate reduction is also the primary target for MTX inhibition in Nt575. When the minimum I_{100} for MTX and a 1000-fold molar excess of folinic acid were simultaneously incorporated into Nt575 suspension cultures, growth was virtually unaffected compared to that of untreated controls (Figure 18). The 1000-fold molar excess of FnA required (10 and 100 fold had no rescuing effect) indicates that FnA and MTX are probably not competing for active transport into Nt575 cells. When FnA administration followed that of MTX by 12 hours, a 2 day lag preceeded rescue (Figure 18). This result suggests that 1) MTX works rapidly, killing a large portion of the population within 12 hours, or 2) exhaustion of reduced folates leads to the coordinate depletion of other cell division factors. In the latter case, cell division may only recommence following FnA administration when the other depleted pools are replenished.

Assay of Dihydrofolate Reductase. The intracellular level of DNFR and its kinetics of inhibition by MTX determine, in part, a cell's tolerance of the drug. Development of an assay for DHFR activity in these systems was therefore necessary. Two assays are widely used for DHFR activity determinations. One is simple and spectrophotometric (Osborn and Huennekens, 1958), the other radiochemical and more sensitive (Rothenberg, 1966). The latter method requires the use of folate, not dihydrofolate (DHF), as a substrate. Mammalian DHFR's can use either substrate (Blakley, 1969), but plant DHFR's are specific for dihydrofolate (Suzuki and Iwai, 1970; Reddy and Rao, 1975). The former method has been used to detect DHFR activity in pea seedlings (Suzuki and Iwai, 1970) and cultured soybean cells (Ohyama, 1976). However, little activity in crude Vr2 extracts could be detected at neutral pH, even though pH optima of 6.5 and 7.2 have been reported for pea (Suzuki and Iwai, 1970) and soybean (Reddy and Rao, 1975) DHFR's. On the assumption that the mung bean enzyme might have a different pH optimum, the pH dependence of Vr2 DHFR activity

Figure 18: Folinic acid rescue of methotrexate inhibited Nt575 cell suspensions. Symbols represent Nt575 cultures treated as follows: Untreated control (●), 3 x 10^{-7}M MTX (○), 3 x 10^{-7}M MTX + 3 x 10^{-4}M FnA after 12 hours (▲) and 3 x 10^{-7}M MTX + 3 x 10^{-4}M FnA at time 0 (△). Points represent means of 2 replicate cultures.

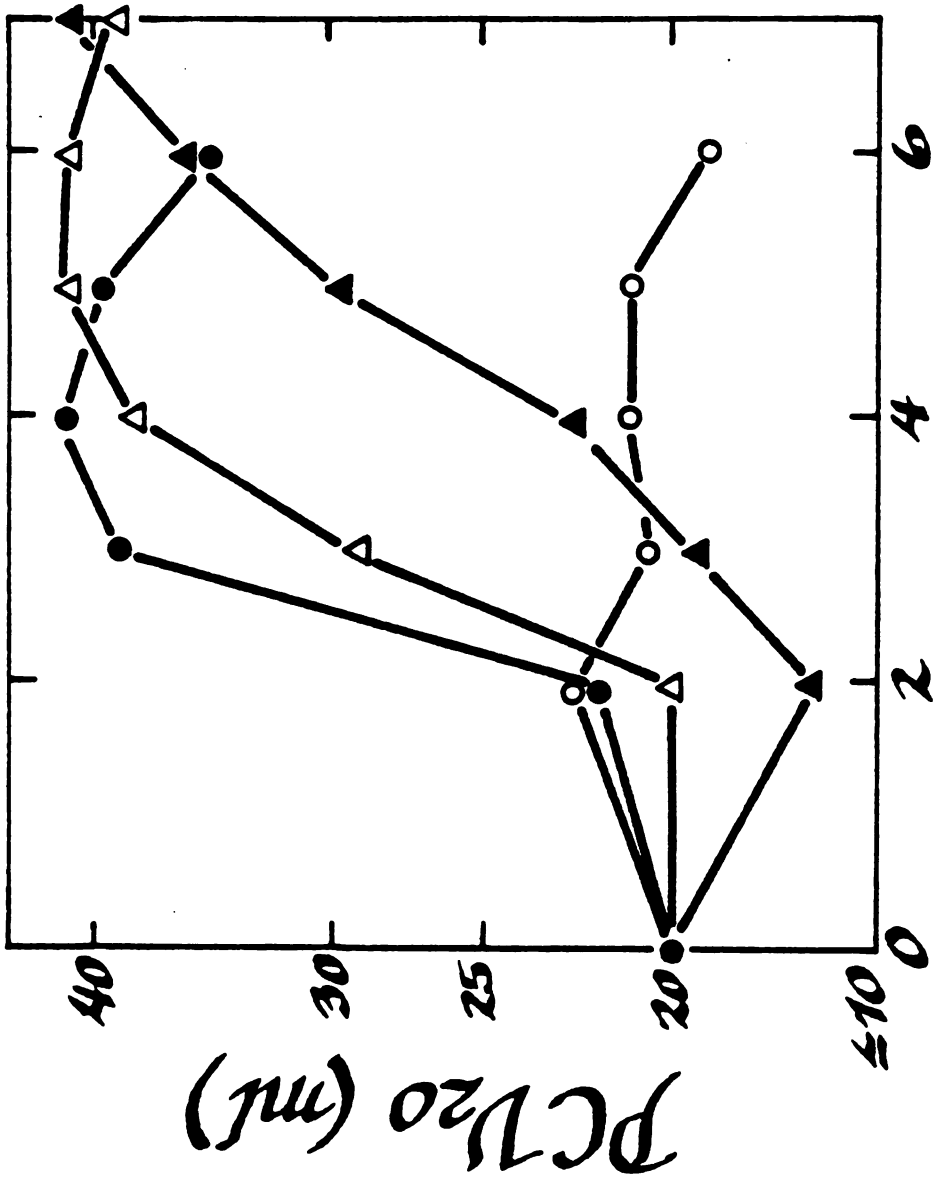


Figure 18: Folinic acid rescue of methotrexate inhibited Nt575 cell suspensions.

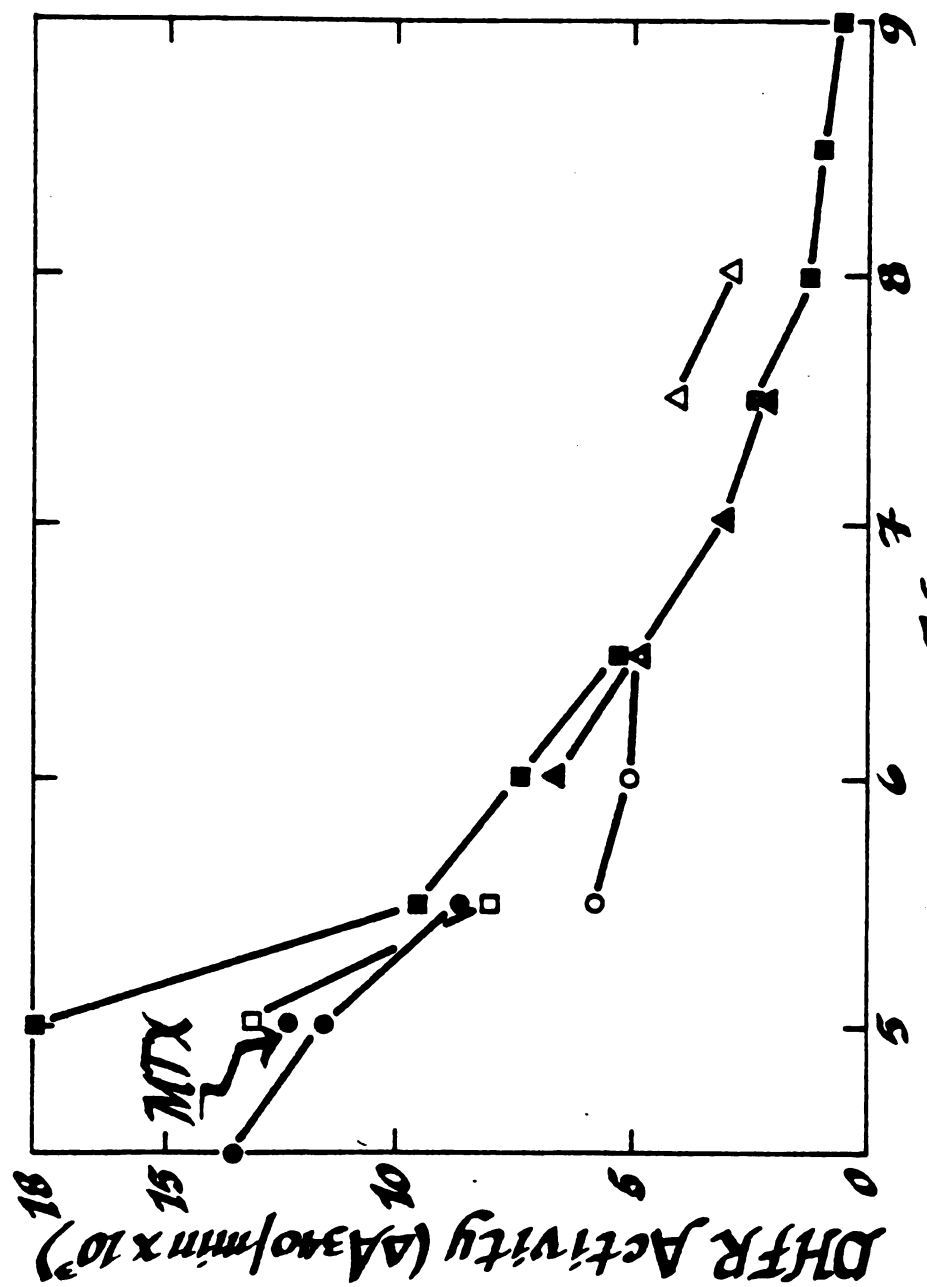


Figure 19: Activity vs pH for crude Vr2 DHFR. Assay of Osborn and Huennekens (1958) was used with the following buffers: acetate (●), orthophosphate (▲), pyrophosphate (■), MES (O), TRIS (Δ) and citrate (□). Methotrexate included at pH 5 (acetate) was at a concentration of 10^{-6}M.

was investigated. The results are graphically presented in Figure 19. This pH-activity relationship was not unlike that described for E. Coli DHFR (Mathews and Sutherland, 1965), although the almost exponential rise in activity with increased acidity was unprecedented. However, 10^{-6}M methotrexate included in the assay mixture had no effect on DHFR activity determined by this assay. This Vr2 DHFR is naturally resistant to MTX was ruled out by the in vitro studies and rescue by folinic acid reported in Part III. No configuration of control blank reactions could be devised to subtract this apparently anomalous background activity. Nor could reported activators such as Mg^{++} (Greenberg et al., 1966), KCl (Domin et al., 1979), urea (Kaufman, 1977) or p-mercuribenzoate (Blakley, 1969) promote MTX inhibitable activity at or near neutrality. Substitution of NADPH with NADH had no effect. Since crude enzyme extracts might contain small molecular weight inhibitors or competing enzymatic activities, the enzyme preparations were desalted and partially purified by dialysis, Sephadex G-25 chromatography, ammonium sulfate and acetone precipitation. None of these methods yielded a MTX inhibitable activity. The validity of the assay was tested on a reliable source of enzyme. DHFR was prepared from crude lysates of T4 bacteriophage infected E. Coli B834, a particularly abundant source (Mathews and Cohen, 1963). The pH-activity relationship (Figure 20) exhibited by this enzyme was precisely as reported (Erickson and Mathews, 1971) and activity was 100 percent inhibited at pH's 5.0 and 7.0 by 30 μM MTX. It was concluded that this assay was apparently inappropriate for crude Vr2 extracts.

Reddy and Rao (1975) have described a spectrophotometric DHFR assay in which the product of DHF reduction, tetrahydrofolate, is quantitatively converted to N^5N^{10}-methenyl THF by treatment with formic acid at 100°C. The high molar extinction coefficient of this product at 350 nm (26500 $cm^{-1}M^{-1}$)

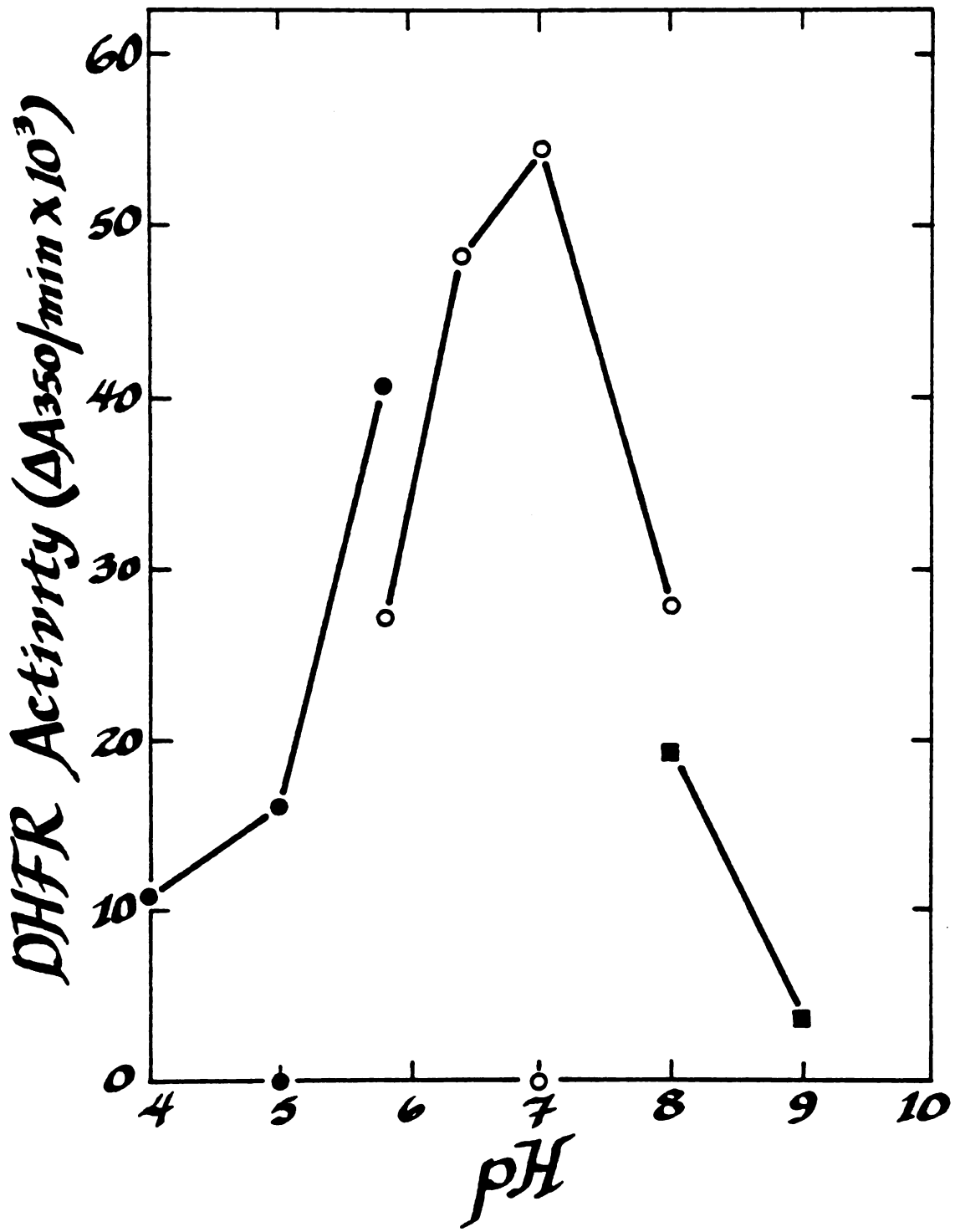


Figure 20: pH dependence of T4 DHFR activity. Assay of Osborn and Huennekens (1958) was used with acetate (●), orthophosphate (O) and TRIS (■). Points at pH's 5 and 7 on abscissa represent activity with 3×10^{-4}M MTX.

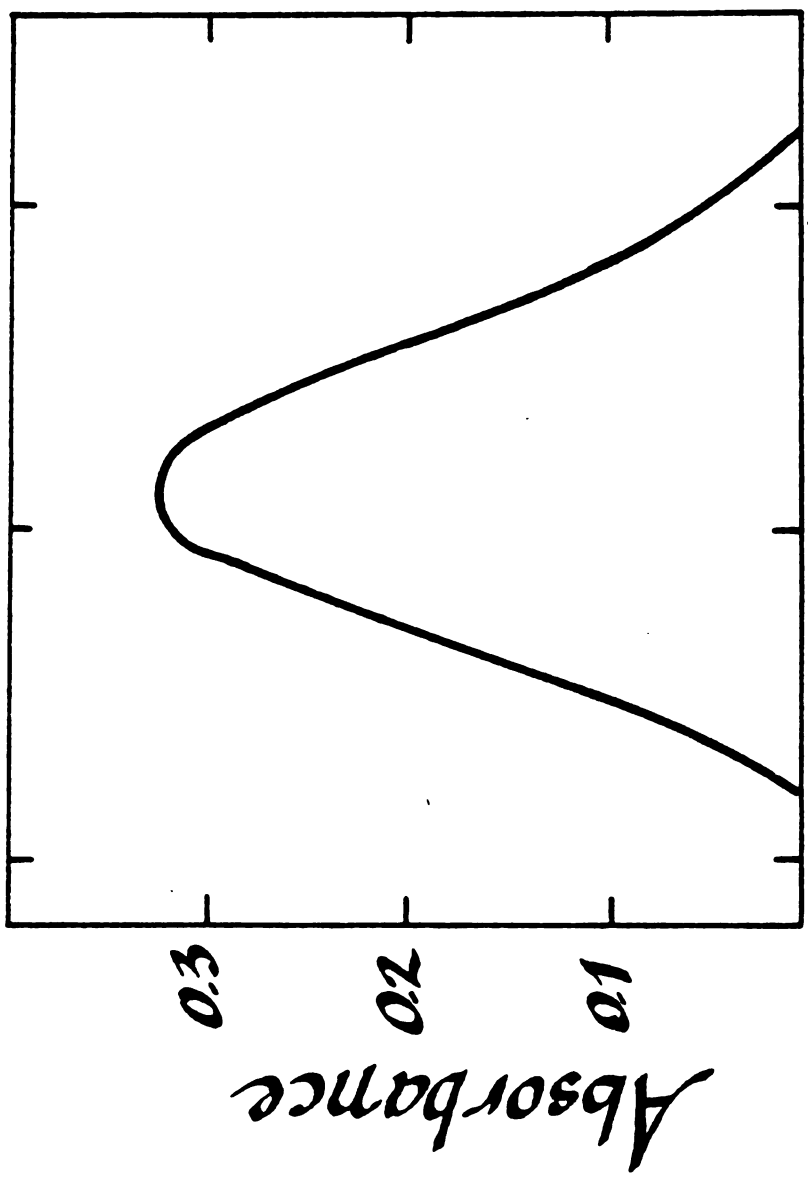


Figure 21. Absorption spectrum of product of boiling formic acid treatment of Nt575 DHFR reaction product.

renders this assay twice as sensitive as that described above. The validity of the assay for measuring DHFR activity in crude extracts was demonstrated. The product of formic acid treatment of the Nt575 DHFR reaction mixture displayed spectral properties (Figure 21) identical to those reported for N^5N^{10}-methenyl THF (Rosenthal et al., 1965). The activated THF product is very stable (Table 10) compared to DHF or THF, both of which decompose rapidly at room temperature in the light (Rabinowitz, 1960). The pH dependence of the activity detected by this assay is shown in Figure 22. Crude Vr2 enzyme displays a single optimum at pH 7.6. The optimum for Nt575 enzyme is pH 6.8. DHFR activity in LeW and Va2 was not pH optimized, although crude extracts yielded sufficient activity at pH 7.2 for the experiments reported below. Pv25 extracts never displayed enough activity by this assay, under any conditions tested, to determine pH or temperature optima. This represented the most striking interspecific difference found in DHFR activity. The unusual requirements for Pv25 DHFR activity warrant further investigation. Nt575 DHFR was not activated by KCl, Mg^{++}, urea or p-CMB. Under standard assay conditions (see MATERIALS AND METHODS), with 259 units of DHFR activity in the reaction mixture, product formation proceeded linearly for 20 minutes (Figure 23). Absorbance at 350 nm vs mg of protein added to the assay mixture also displayed a linear relationship (Figure 24). Where higher activities were desirable, such as in the inhibition kinetics studies, the crude enzyme was concentrated by ultrafiltration (Tables 11, 12, 13). A 2-3 fold purification could be achieved by the removal of base precipitable material with streptomycin sulfate (Tables 11 and 12). A final high-speed centrifugation efficiently removed contaminating particulate proteins from crude extracts or ultrafiltration concentrates (Table 13). The DHFR activity of extracts processed as in Table 13 was stable at $0^{\circ}C$ for two weeks, after which the extract darkened and activity declined.

Table 10: Stability of N^5N^{10}-methenyl THF. Each datum is the mean (± se) of five replicate assays of DHFR from Nt575 crude extracts.

TIME (h)	STORAGE CONDITIONS	
	DARK, 26°C	DARK, 4°C
	(ΔA_{350})	
0	0.325 ± 0.001	0.335 ± 0.009
3	0.324 ± 0.005	0.335 ± 0.009
9	0.327 ± 0.003	0.333 ± 0.009
19	0.320 ± 0.002	0.333 ± 0.009

Figure 22: pH dependence of Vr2 and Nt575 DHFR activity. Assay of Reddy and Rao (1975) was used for Vr2 DHFR (●, left ordinate) and Nt575 DHFR (o, right ordinate). Buffers included MES, pyrophosphate, ACES (Nt575 peak), BTP, TRIS (Vr2 peak) and TES.

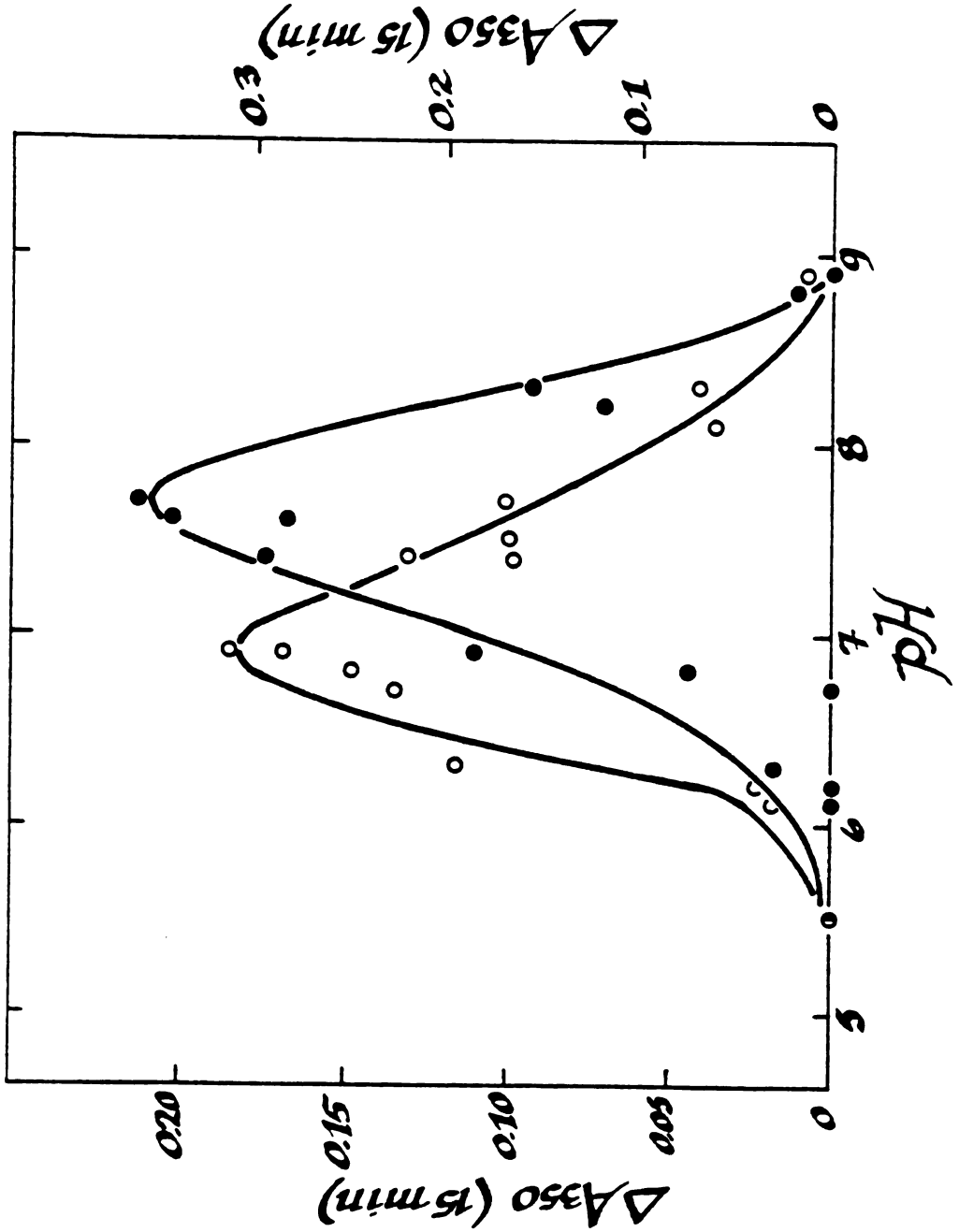


Figure 22: pH dependence of Vr2 and Nt575 DHFR activity.

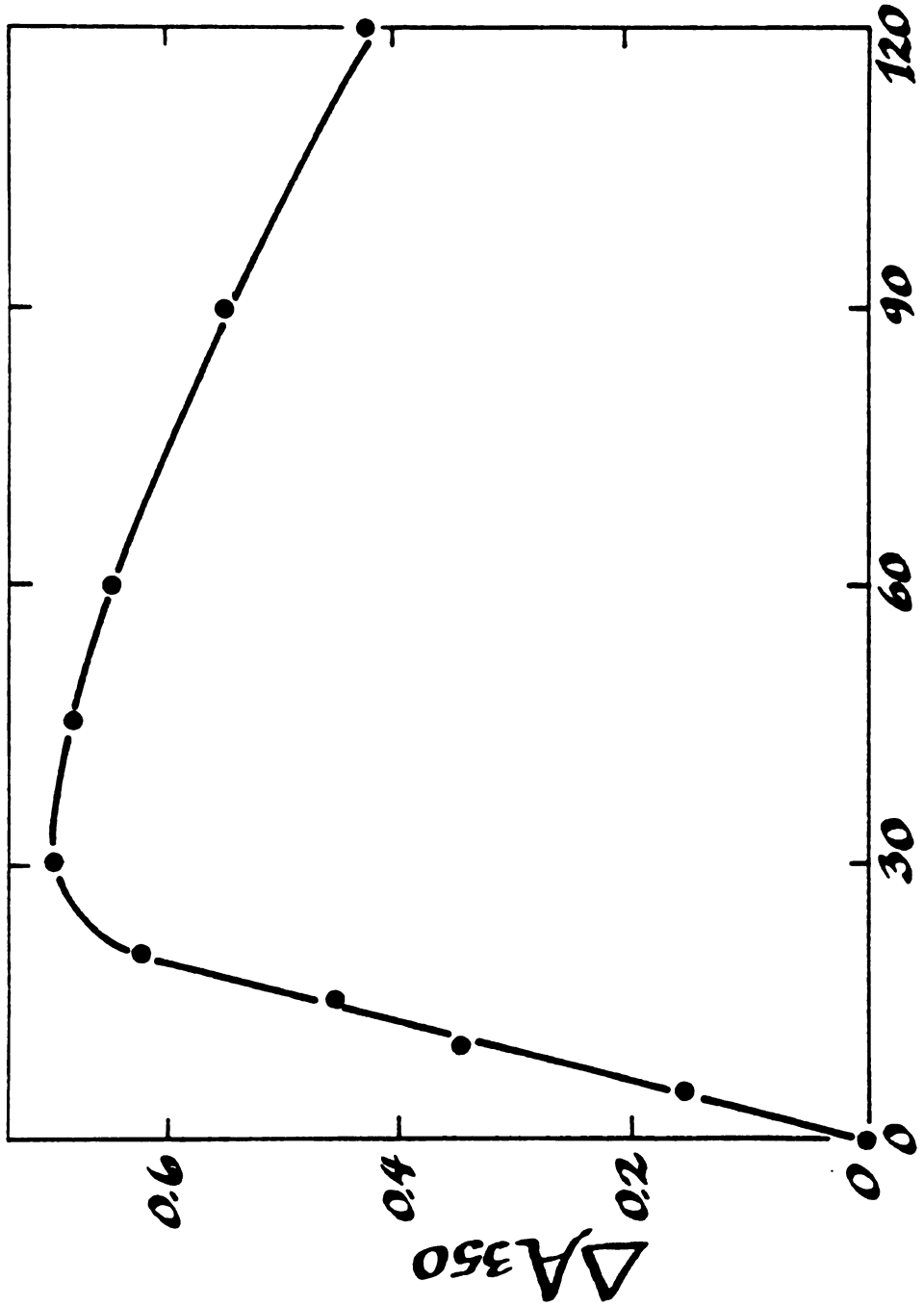


Figure 23: Time dependence on Nt575 DHFR assay. Assay was that of Reddy and Rao (1975).

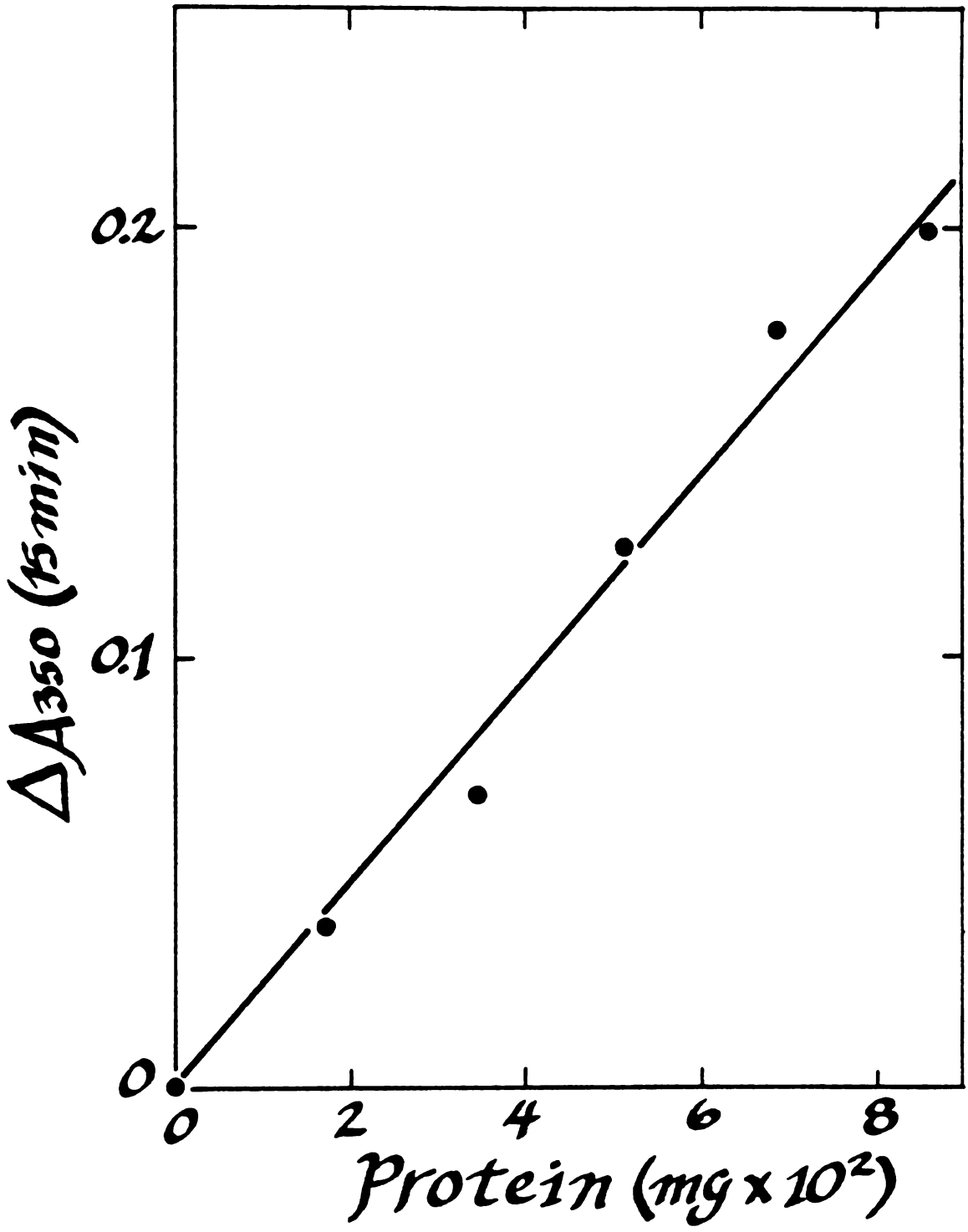


Figure 24: Nt575 DHFR activity vs protein added. Assay of Reddy and Rao (1975) was used.

Table 11: Purification summary for Va2 DHFR. DHFR activity units are defined as nanomoles of N^5N^{10}-methenyl THF formed in 15 minutes at 45°C.

Table 12: Purification summary for Nt575 DHFR. Only 47 percent of the streptomycin supernatant was actually concentrated. Values in the table have been corrected to 100 percent. DHFR units are defined as in Table 11.

Table 13. Purification summary for Vr2 DHFR. Only 54 percent of the crude extract was concentrated. Values in the table have been corrected to 100 percent. DHFR units are defined as in Table 11.

Table 11: Purification summary for Va2 DHFR.

Fraction	Volume	Act/ml	Total Act.	Protein/ml	Specific Act.
	(ml)	(U/ml)	(U)	(mg/ml)	(U/mg Prot.)
Crude Extract	50	13.6	680	0.53	25.7
Streptomycin Supernatant	50	9.1	453	0.29	31.2
Concentrated Crude	4	174.0	696	4.02	43.3
Concentrated Strep. Super.	5	97.4	487	1.45	67.2

Table 12: Purification summary for Nt575 DHFR.

Fraction	Volume	Act/ml	Total Act.	Protein/ml	Specific Act.
	(ml)	(U/ml)	(U)	(mg/ml)	(U/mg Prot.)
Crude Extract	162	24.3	3936	0.43	56.8
Streptomycin Supernatant	211	19.2	4051	0.23	85.0
Concentrated Strep. Super	20	165.0	3333	1.72	95.9

Table 13: Purification summary for Vr2 DHFR.

Fraction	Volume	Act/ml	Total Act.	Protein/ml	Specific Act.
	(ml)	(U/ml)	(U)	(mg/ml)	(U/mg Prot.)
Crude Extract	92	6.8	626	0.53	12.8
Concentrated Crude	11	113.0	1243	3.84	29.4
50000 x g Supernatant	11	115.0	1265	2.88	39.9

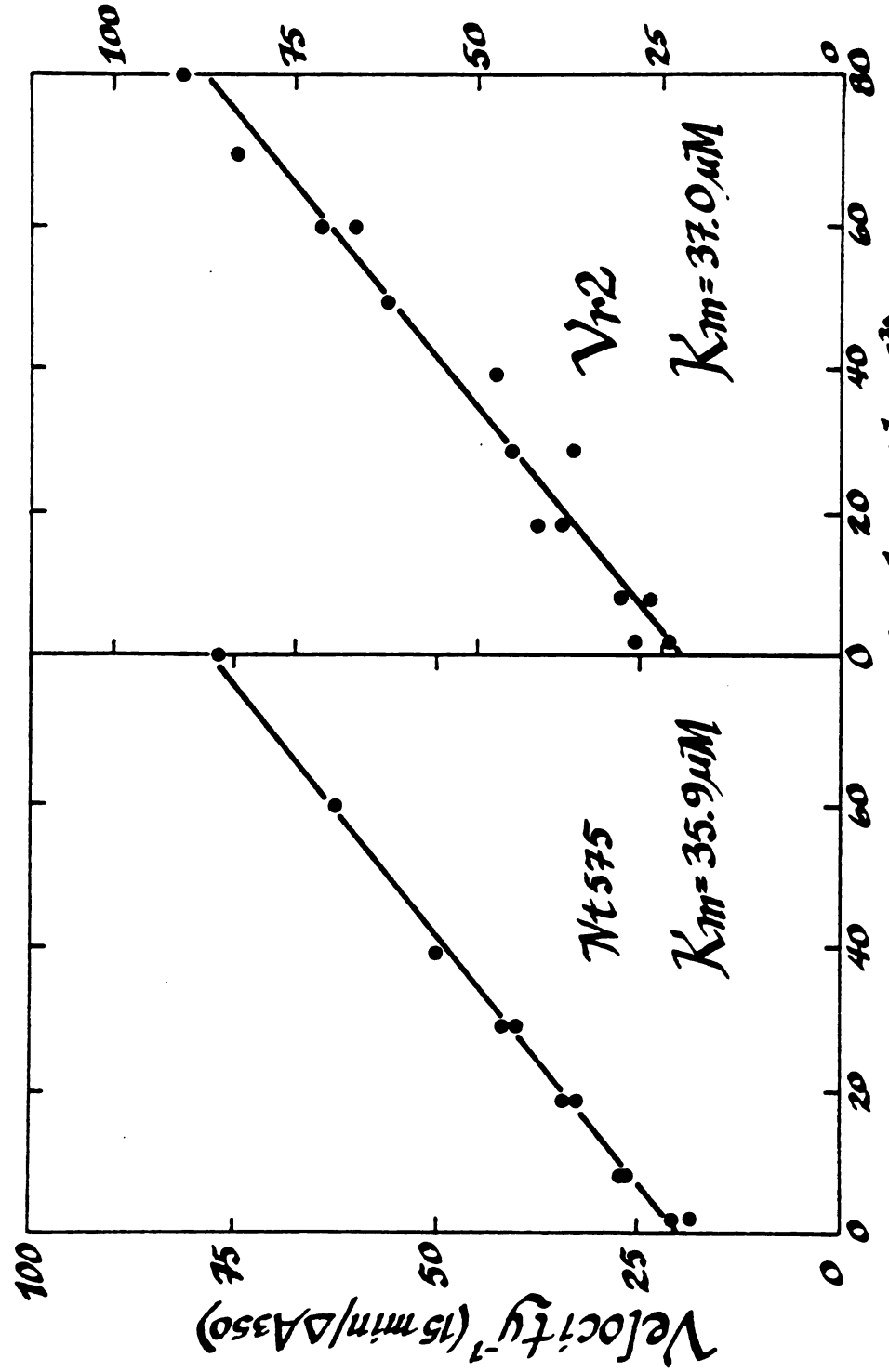


Figure 25: Lineweaver-Burk plots for determination of DHF K_m's for DHFR from Nt575 and Vr2.

Kinetic Parameters of Nt575 and Vr2 Dihydrofolate Reductases. The differential MTX sensitivity displayed by Vr2 and Nt575 (Figure 15) might be due to the former's being a better competitor (lower K_m) for its natural substrate, dihydrofolate. The apparent K_m's are 37.0 µM and 35.9 µM for Vr2 and Nt575 respectively (Figure 25). The apparent K_m's for NADPH are 5.0 µM and 5.1 µM for Vr2 and Nt575 respectively (Figure 26). These results were typical for three experiments designed to verify these observations. Thus, it appears that DHFR's from Vr2 and Nt575 are kinetically indistinguishable in crude extracts and that MTX tolerance is not associated, in this case, with increased DHFR:DHF affinity. The K_m's for DHF are in good agreement with the value of 35.5 µM reported for soybean DHFR (Reddy and Rao, 1975). Suzuki and Iwai (1970) reported a value of 4.5 µM for the pea enzyme. These two groups reported NADPH K_m's of 425 µM and 40 µm for soybean and pea DHFR's respectively. These substantial differences between the present data and the published parameters for soybean and pea could be accounted for by 1) Genuine differences in the substrate affinities of DHFR from different plant sources, 2) Isozymic variability due to differences in tissue source (the pea and soybean enzymes were extracted from seedlings) or 3) The unpurified condition of the enzyme in the present studies, compared to the relatively clean enzyme preparations in the pea (10-fold purified) and soybean (25-fold purified) reports. Endogenous pyridine nucleotides in the undialyzed extracts employed here may have resulted in anomalously low K_m's for NADPH. The DHF K_m's reported here are 3 to 30 fold higher than those reported for mammalian and microbial DHFR's. This may be a reflection of the folate autotrophy of plant cells.

Culture Cycle Regulation of DHFR Activity. The intracellular level of DHFR determines how much methotrexate is required to completely inhibit the biosynthesis of reduced folates. Cells with very high intracellular DHFR levels

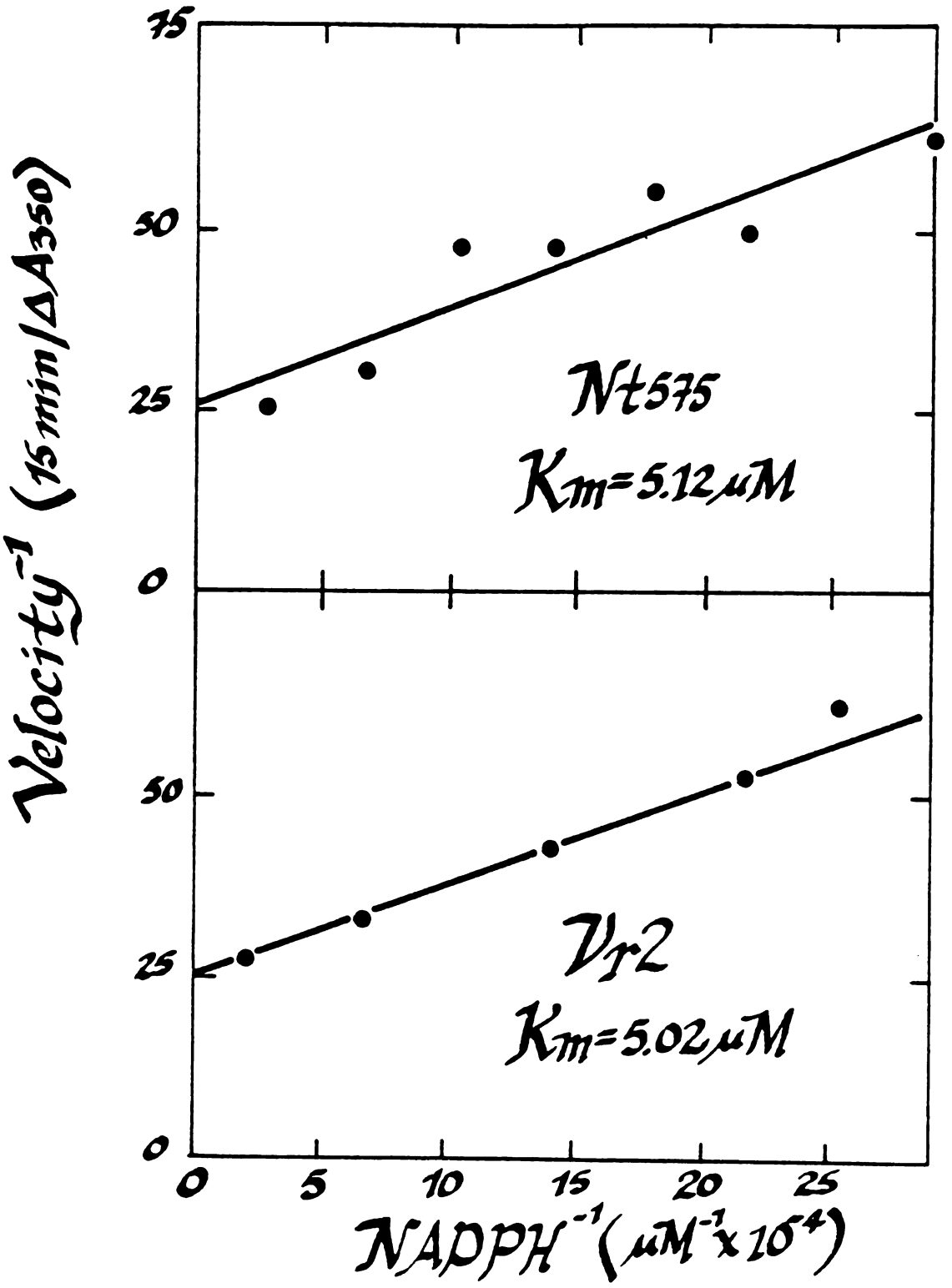


Figure 26: Lineweaver-Burk plots for determination of NADPH K_m's for DHFR from Nt575 and Vr2.

Figure 27: Culture cycle dependence of DHFR specific activity. DHFR specific activity of Va2 (●), Nt575 (▲) and LeW (■) was measured over the culture cycle. Growth of Va2 (○), Nt575 (△) and LeW (□) was measured by weighing retentate of Buchner funnel harvest. Inset shows least squares regression of exponential growth rate (d/d) vs DHFR specific activity (S.A.) for the three cell lines.

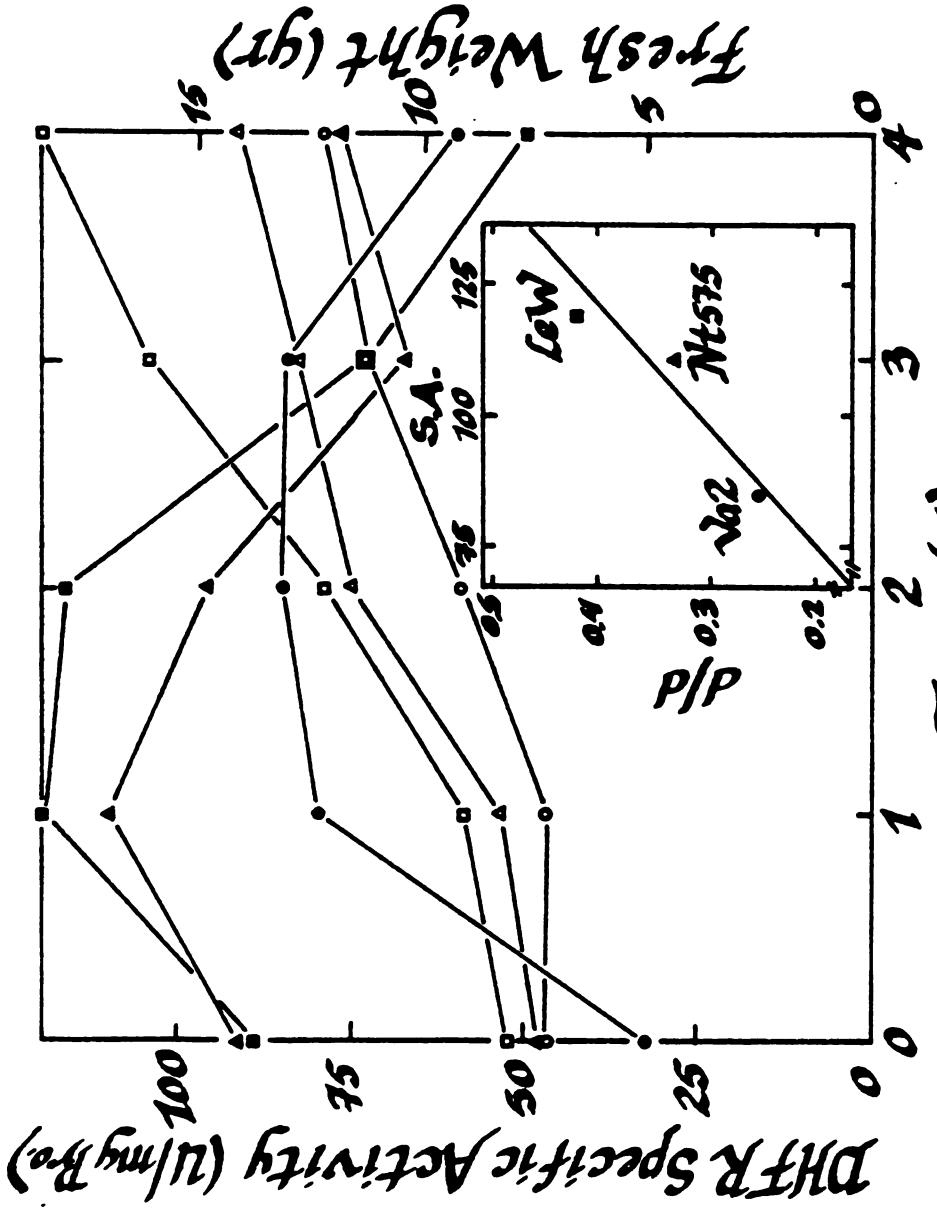


Figure 27: Culture cycle dependence of DHFR specific activity.

are correspondingly resistant to MTX (Alt et al., 1976). To test whether tolerance of MTX is conditioned by higher intracellular MTX binding capacity in plant cells, the level of DHFR activity was measured over a routine maintenance culture cycle of Nt575, LeW and Va2 (Figure 27). As might be expected for an enzyme supplying cofactors for DNA nucleotide biosynthesis, DHFR activity rises to a peak during early to mid-exponential growth and falls as stationary phase is approached, in each cell line. The levels of culture cycle variation in DHFR activity in LeW, Nt575 and Va2 are 2.4, 1.7 and 2.6 fold, respectively. Greater fold differences would have been observed if the cultures had been permitted to enter "deep" stationary phase immediately before and toward the end of this experiment (see Figure 32). This growth cycle regulation is reminiscent of that in cultured murine fibroblasts reported by Johnson (1980). As in the present system, the onset of active growth in serum-replenished mouse cells is accompanied by a 3 to 5 fold increase in DHFR activity (Johnson et al., 1978a). De novo synthesis has been shown to be responsible for the activity induction (Johnson et al., 1978b). Mariani et al. (1981) have demonstrated S-phase specific synthesis of DHFR in synchronized chinese hamster ovary cells. This is in agreement with the observtion (Figure 27) that, in Nt575 and LeW, DHFR activity reaches a peak before the onset of exponential growth.

Peak DHFR activity is positively correlated with the growth rates of LeW, Nt575, and Va2 (inset, Figure 27). Although high DHFR activity can confer MTX resistance in variant mammalian cell lines, high natural DHFR levels in cultured plant cells are associated here with enhanced MTX sensitivity. Thus, it appears that sensitivity is dependent upon the cell's requirement for reduced folates. Rapidly growing cultures have a high demand for C_1-THF and are correspondingly sensitive to MTX. The converse is true for slower growing cell lines (Figure 15), "quisecent" cells (Chu and Lark, 1976) or resting cells (Johnson

et al., 1978a). Goldman et al. (1979) have argued that MTX inhibition of DHFR can be competitive with respect to DHF when sufficiently high ratios of DHF/MTX are obtained. Depletion of the DHF pool by elevated DHFR activity during active growth would virtually eliminate the competetive component of MTX inhibition, resulting in more acute MTX sensitivity in the high DHFR cell lines here. Such kinetics would, of course, probably not apply to variant cell lines with very high DHFR levels, far in excess of the cell's basal metabolic requirement.

Inhibition of Plant DHFR's by Methotrexate. Under appropriate conditions, methotrexate stoichometrically titrates mammalian and microbial DHFR activity in vitro (Werkheiser, 1961; Williams et al., 1973). That such an interaction can occur between MTX and DHFR from plant sources was tested by incubating Vr2 and Nt575 DHFR's with increasing concentrations of MTX (Figure 28). The ternary complex of DHFR:NADPH:MTX is stable in mammalian systems (Perkins and Bertino, 1966), although soybean DHFR forms a stable binary complex with the inhibitor in the absence of NADPH (Reddy and Rao, 1977). Nonetheless, extracts were pre-incubated with MTX and NADPH here, prior to initiating the reaction by the addition of DHF. By this analysis, both mung bean and tobacco DHFRs display stoichiometric inhibition by methotrexate. The lack of complete titration of Nt575 DHFR activity, even at high MTX concentrations, may have resulted from insufficiently long pre-incubations of enzyme and inhibitor. Pre-incubations in the tobacco and mung bean experiments were 5 and 10 minutes, respectively. Figure 29 shows that, even at molar equivalence, enzyme-inhibitor complexing is only 90% complete after 5 minutes (see discussion of Figure 29 which follows). Alternatively, the K_i for Nt575 DHFR:MTX dissociation may be sufficiently high that, under the conditions of this assay ([DHF]/[MTX] = 50,000 to 4 million), inhibition was

Figure 28: Titration of Nt575 and Vr2 DHFR activity with methotrexate. Crude extracts of Nt575 (left) and Vr2 (right) DHFR were incubted for 5 and 10 minutes, respectively, with various concentrations of methotrexate prior to assay. Dashed lines represent least squares extrapolation of linear portion of each curve to the abscissa. Turnover numbers (kp) were calculated as the ordinate intercept (in activity units) divided by the abscissa intercept.

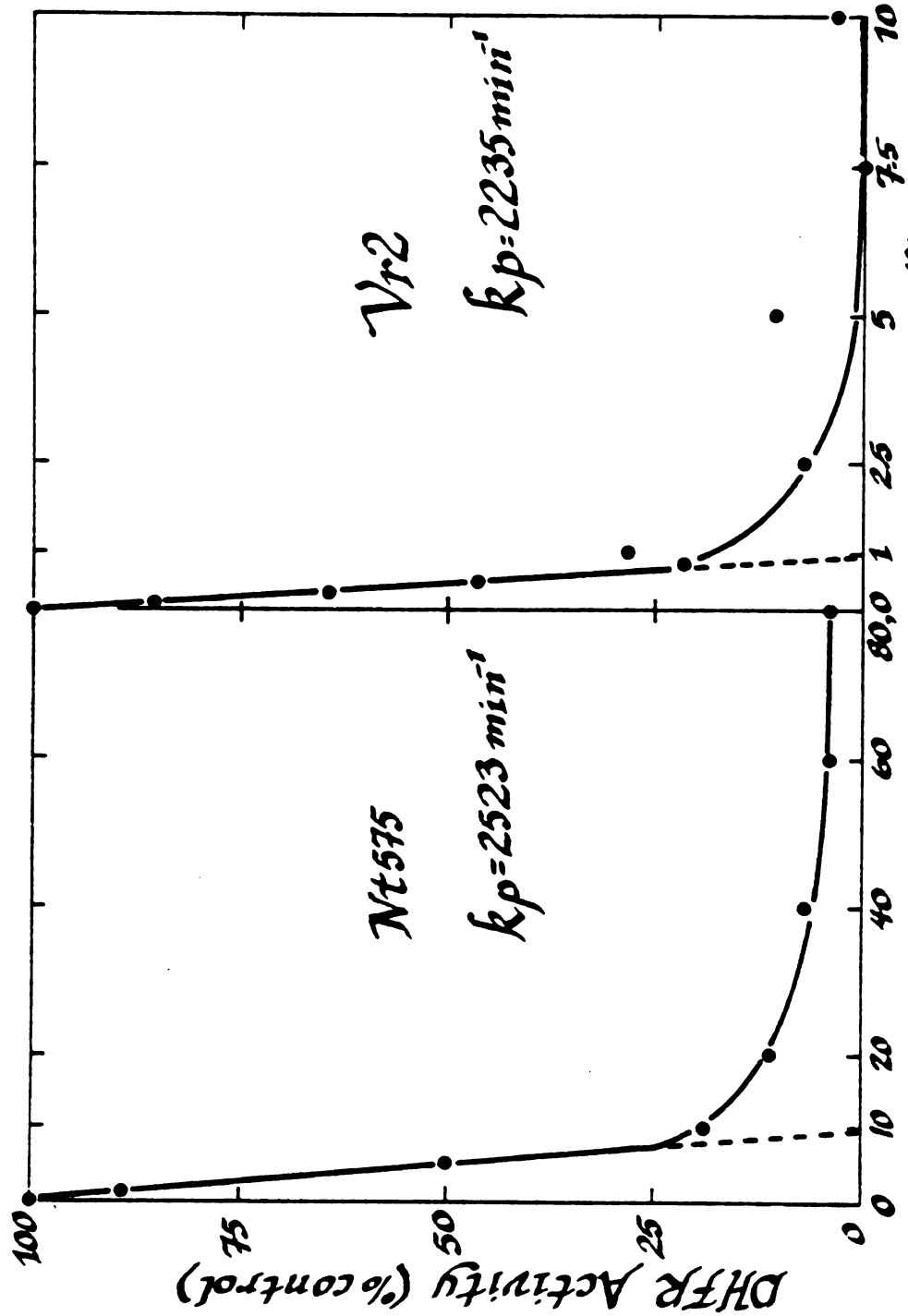


Figure 28: Titration of Nt575 and Vr2 DHFR activity with methotrexate.

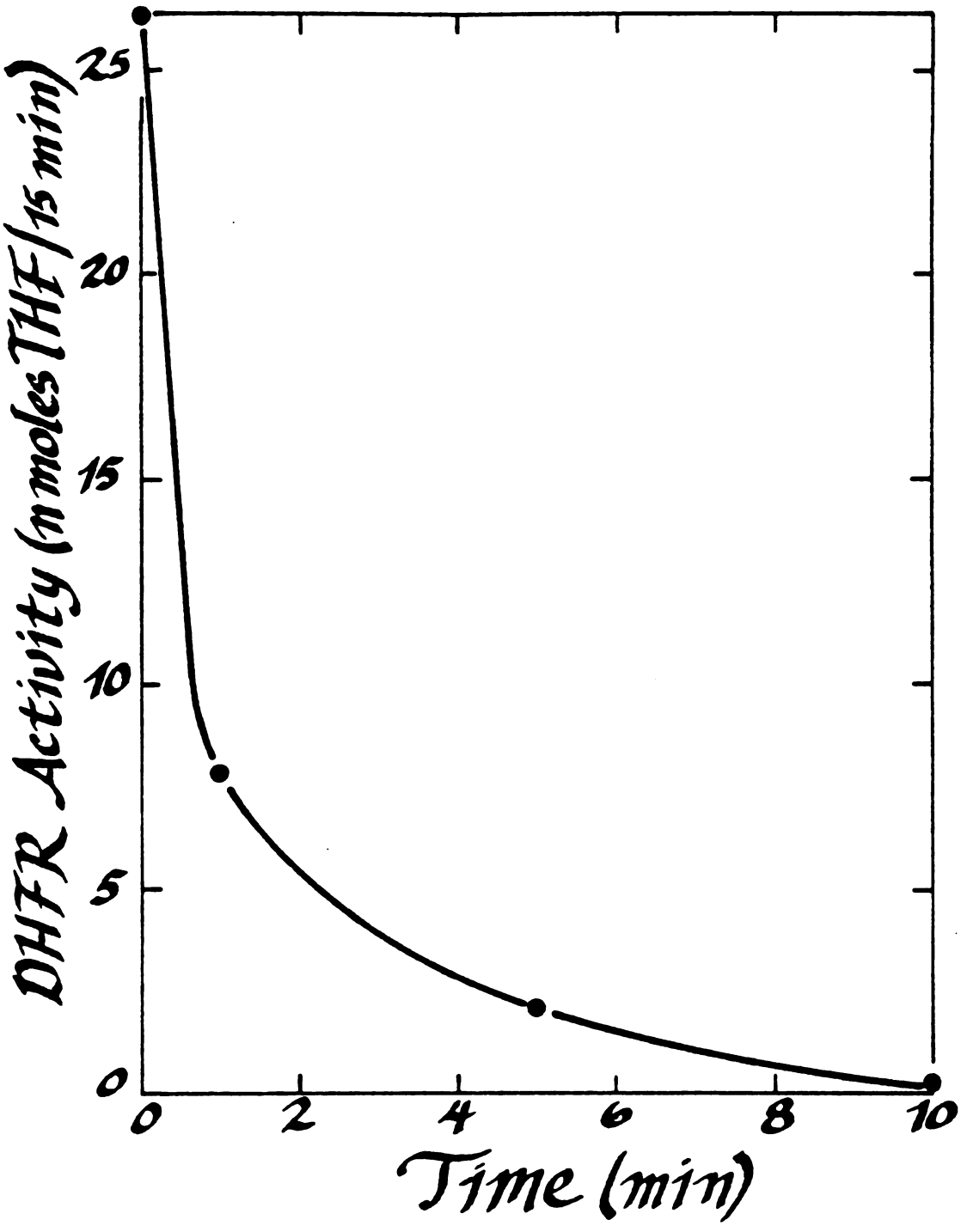


Figure 29: Time dependence of methotrexate titration of Nt575 DHFR activity. See text for details of procedure.

competitive. The intercept of the extrapolated linear (unsaturated) portion of each curve to the abscissa represents the minimum concentration of methotrexate required to completely titrate the DHFR activity in the assay. From this value and the uninhibited V_{max} (ordinate intercept), the apparent "catalytic center activity", k_p, can be calculated. The k_p is equivalent to the apparent "turnover number" or "molecular activity" (Segal, 1978) if each enzyme has a single active site. The only case of a multimeric DHFR is the soybean enzyme (Reddy and Rao, 1976; C. K. Mathews, personal communication) although its three non-identical subunits appear to possess a single MTX binding site among them (Reddy and Rao, 1977). The calculated apparent k_p's for mung bean and tobacco DHFRs are 2235 min^{-1} and 2523 min^{-1} respectively, once again indicating a strong kinetic similarity. These values are comparable to those reported for mammalian and microbial DHFR's, with k_p's ranging from 3000 to 8000 min^{-1} (Blakley, 1969). Soybean DHFR had a k_p of 57 min^{-1}, the lowest reported for any source (Reddy and Rao, 1976).

Another striking demonstration of MTX titration of DHFR activity was made in an attempt to determine the minimum pre-incubation time necessary to achieve equilibrium inhibitor binding. Equimolar amounts of Nt575 DHFR (assuming one MTX binding site per enzyme molecule) and MTX were preincubated for 1, 5, 10, and 30 minutes, prior to rapid separation of total enzyme (MTX-bound and unbound) from free MTX by centrifugal Sephadex G-25 chromatography (see MATERIALS AND METHODS). Complete titration required 10 minutes of pre-incubation (Figure 29). The apparent elution of inhibitor-bound enzyme from the columns once again demonstrates the tightness of MTX's binding to DHFR.

The tenacity of the bond between MTX and microbial DHFR's has been attributed to enhanced hydrogen bond formation between the N-1 of the MTX

Figure 30: pH dependence of MTX inhibition of Nt575 DHFR activity. Nt575 DHFR activity was measured at pH 5.8 to pH 8.0, with (■) and without (o) 10^{-9}M methotrexate. Percent inhibition by 10^{-9}M methotrexate, as a function of pH, is shown by the least squares line (●, $r^2 = 0.83$).

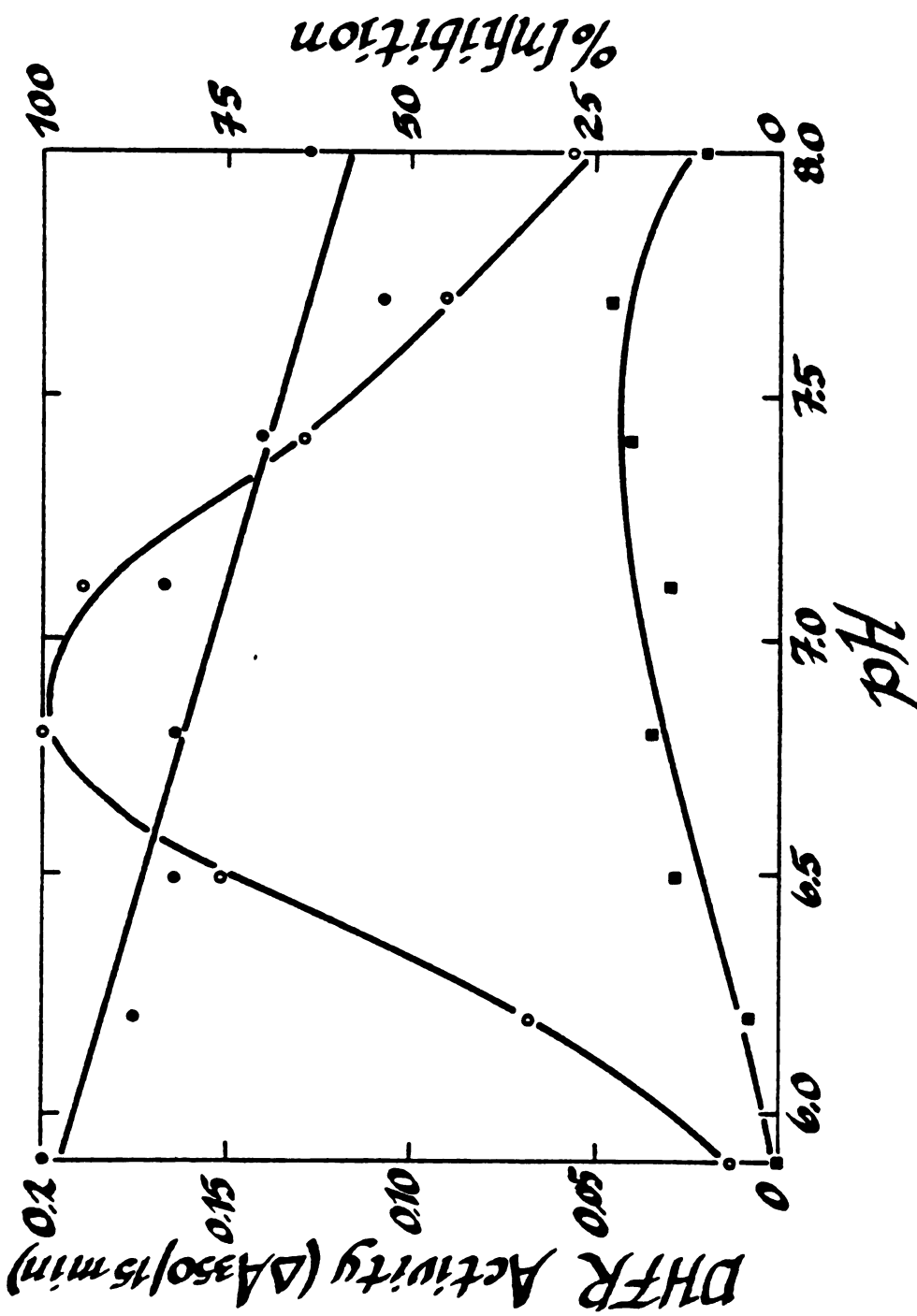


Figure 30: pH dependence of MTX inhibition of Nt575 DHFR activity.

pteridine ring and an aspartate residue (No. 26) within a hydrophobic cavity of the enzyme (Baker, 1959). If such an interaction obtains in plant DHFR's, then MTX inhibition would be expected to decline with increasing pH. Figure 30 shows the pH dependence of MTX inhibition of Nt575 DHFR. The direct relationship between proton concentration and percent inhibition is in agreement with Bertino et al. (1964). These workers reported dual pH optima, at pH's 5.9 and 7.6, for mouse tumor cell DHFR. At a saturating MTX concentration, inhibition at pH 7.6 was only 60% of that at pH 5.9. That MTX inhibition does not decline more drastically with increasing pH has been attributed to NADPH's preventing solvent access to the hydrophobic cavity where the critical hydrogen bonding occurs (Blakley, 1981).

A relaxation of the MTX:DHFR bond at higher pH might be expected to diminish the stoichiometric nature of MTX inhibition. Figure 31 shows how inhibition becomes more competitive when the pH is raised from 6.8 to 7.8 in assays of Nt575 activity in the presence of methotrexate.

Inducibility of Nt575 DHFR. In Part III, I described how a large number of colonies had appeared on selection plates where presumably stringent selective conditions had been expected to permit the growth of only methotrexate resistant cells. The possibility remained that the selective conditions could have induced sufficient DHFR activity to allow a cell to survive a strong methotrexte challenge. Three components of the selective medium -- folic acid, folinic acid, and methotrexate -- might play physiological or gratuitous roles in the regulation of plant folate metabolism, resulting in DHFR induction. The enzyme's induction by folate and methotrexate has been reported (Hillcoat et al., 1967; Hillcoat et al., 1973). In addition, UV irradiation, another component of the selective system, might have enhanced DHFR synthesis. Thymine dimers are the most common photoproducts of UV absorption by DNA. If these lesions are

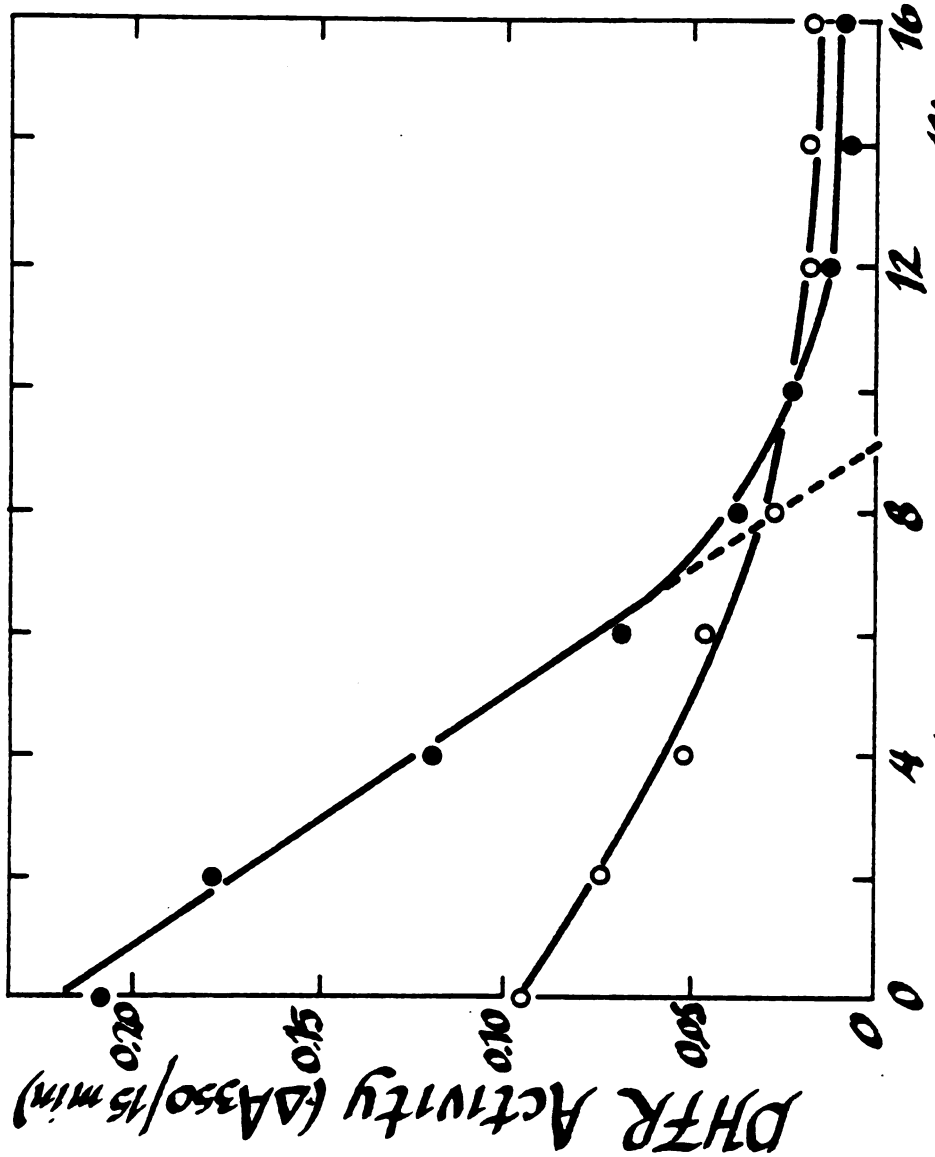


Figure 31: Effect of pH on methotrexate titration of Nt575 DHFR activity. Assays performed at pH 6.8 (●) and pH 7.8 (○).

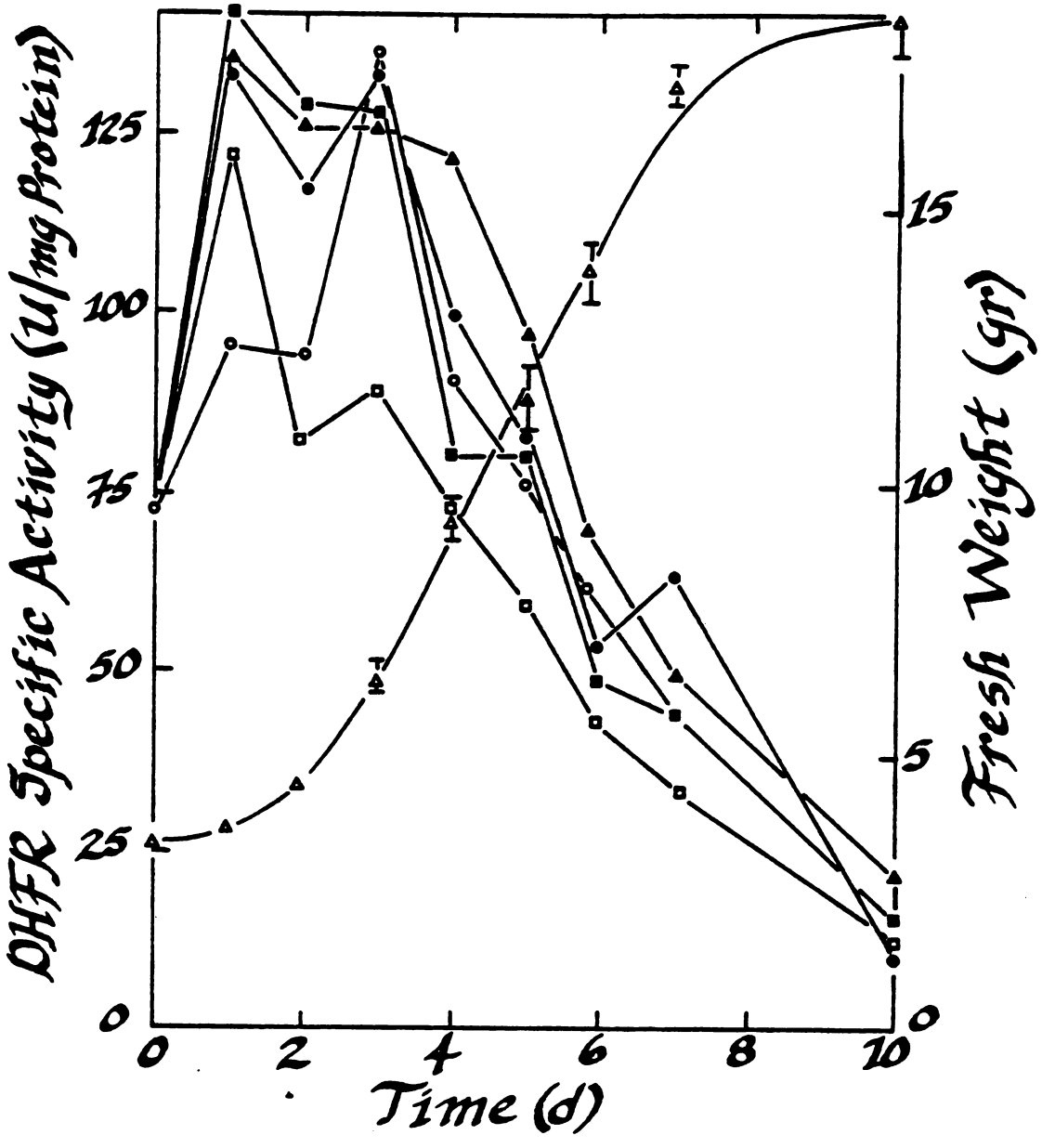


Figure 32: Inducibility of Nt575 DHFR. Treatments were: control (●), 5×10^{-4}M FnA (▲), 75 J/m^2 UV (■), 5×10^{-4}M folic acid (○) and 5×10^{-8}M MTX. Growth measured by weighing Buchner funnel retentate (△).

corrected by excision repair, then thymidylate demand would increase in proportion to the level of dimer formation in sublethally irradiated cells. Since reduced folates participate in thymidylate synthesis, it follows that UV absorption by DNA might lead to the induction of folate biosynthetic enzymes, including DHFR.

Figure 32 shows the results of an experiment designed to test the effects of methotrexate, folic acid, folinic acid and UV light on DHFR specific activity in tobacco (Nt575) cell suspensions. Each treatment was administered singly, upon subculture from stationary phase, in the highest possible dose without being growth inhibitory. No treatment significantly enhanced DHFR activity over that of the control. Methotrexate depressed DHFR activity without affecting growth rate. However, the reduced DHFR activity measured in MTX grown cultures may have resulted from compartmentalized (vacuolar?) MTX being released during homogenization. Hillcoat et al. (1967) took advantage of the relative insensitivity of mouse DHFR to MTX at that enzyme's higher pH optimum (pH 7.6) to detect DHFR activity in MTX-grown cells. The single optimum at pH 6.8 in tobacco cells precluded such an approach here. Folic acid seems to have delayed the peak of DHFR activity, while not reducing it. The growth cycle variation in DHFR specific activity in the control was 13.7 fold. This was considerably greater than in a previous experiment (Figure 27), owing to the fact that the cultures in this experiment were left to enter "deep" stationary phase toward the end of the experiment.

Conclusions

The sensitivity of plant cell suspension cultures to growth inhibition by methotrexate was found to be directly proportional to their intrinsic growth rates. A practical corollary of this conclusion might be that non-dividing cells are virtually impervious to MTX toxicity and that the drug could be used as a negative selective agent in plant cell cultures. 5-bromodeoxyuridine (Malmberg, 1981) and arsenate (Polacco, 1979) have served this purpose in the past. It should be noted here that none of the putative MTX resistant clones listed in Table 9 (Part III) achieved MTX tolerance by reducing their intrinsic growth rates. Some survivors of the selection may have been "quiescent" (Chu and Lark, 1976) during the initial period of selection. An attempt to further demonstrate the correlation between growth rate and methotrexate sensitivity was made by culturing Pv25 at an elevated temperature. Enhanced MTX sensitivity in the absence of an increased growth rate may have resulted from the compounded temperature and metabolic stresses. High temperature may have permitted greater entry of MTX into the cells by thermally enhanced diffusion or by disaggregating clumps. These findings are in agreement with the pharmacological basis for the use of methotrexate in cancer chemotherapy. Highly mitotic tumor tissue is relatively more sensitive to the drug than normal somatic tissue. Methotrexate's utility is, in fact, limited by the sensitivity to the drug of normal mitotic tissues such as the intestinal mucosa and bone marrow.

Tobacco cells could be rescued from MTX growth inhibition by folinic acid, demonstrating that MTX acts in tobacco by inhibiting reduced folate biosynthesis. Briefly delayed administration of the antidote resulted in delayed rescue, suggesting that MTX's action is rapid in tobacco cells. These results

suggest two potentially useful lines of investigation. First, inhibition and rescue of cell suspensions by MTX and folinic acid might be designed to synchronize mitoses in vitro. Since MTX blocks both DNA and protein synthesis, growth inhibition by this drug does not lead to "unbalanced growth", which is thought to be detrimental to plant cell suspension cultures for even short periods of time (King and Street, 1977). Mitotic inhibition by MTX would be expected to arrest cells at the G_1 - S phase interface. Properly timed administration of a massive dose of folinic acid might relieve inhibition and result in synchronous passage through mitosis. Thirty percent synchrony of mammalian cell cultures has been achieved by MTX arrest (Blakley, 1969). Second, experiments could be performed to determine the minimum interval by which antidote administration must be delayed for no rescue to occur. Feeding the antidote and appropriately supplemented media after such an interval might constitute a negative selection protocol for recovery of auxotrophic mutants. DeMars and Hopper (1960) introduced a similar method for the recovery of auxotrophic mutants of HeLa cells.

The widely used assay of dihydrofolate reductase which employs continuous spectrophotometric monitoring of DHF reduction and NADPH oxidation (Osborn and Huennekens, 1958) was found to be inappropriate for crude enzyme preparations from plant cell cultures. High apparent activities at very acidic pH were uninhibitable by methotrexate. Suzuki and Iwai (1970) successfully used this assay in their characterization of pea DHFR, but failed to report assays with crude extracts. A recent report by Crosti (1981) suggests that by 1) ultracentrifugation, 2) 30-60% $NH_4(SO_4)_2$ precipitation and 3) dialysis, extraction of artifact-free DHFR activity, measured by this assay, may be possible.

The validity of an alternative assay was demonstrated. In this procedure, the endpoint level of the reaction product, tetrahydrofolate, was measured. The reaction was linear with time and protein concentration, and yielded a stable derivative, N^5N^{10}-methenyl THF, which conformed to published absorption spectra. Most importantly, product formation was inhibited by nanomolar concentrations of methotrexate. One drawback to this assay is the unpleasant odor which accompanies the addition of formic acid to the 80 mM 2-mercaptoethanol reaction mixture. Total and specific DHFR activities could be enhanced by ultrafiltration-concentration and streptomycin precipitation, respectively. Of the five cell lines surveyed, only the Pv25 cell line of Phaseolus vulgaris proved intractable to the DHFR assay.

The pH optima of Vr2 and Nt575 are 7.6 and 6.8, respectively. The kinetic parameters of DHFR from these sources were remarkably similar. The K_m's for DHF were 37.0 µM and 35.9 µM, and for NADPH, 5.0 µM and 5.1 µM for Vr2 and Nt575 respectively. The low activities required for the determination of the K_m's for NADPH approached the level of sensitivity of the assay. However, the values were reproducible and have been confirmed by a variation of the Lineweaver-Burk procedure which applies to cases of substantial substrate depletion (Lee and Wilson, 1971). It was concluded that Vr2's greater tolerance of MTX is not due to its greater affinity for its natural substrate, DHF.

Dihydrofolate reductase activity is regulated over the cell culture growth cycle. In the three cell lines studied, Va2, Nt575 and LeW, DHFR activity increased following subculture, attained a maximum during early to mid-exponential growth, and declined as stationary phase approached. Induction was shown to be 14 fold in one cell line. It would be interesting to know what signal (relief from oxygen, water or nutrient depletion?) triggers this induction and how it may be coregulated with other cell division enzyme activities. Whether

induction is controlled at the level of transcription, translation or post-translation is an open question in this system. The maximum DHFR activity attained by a particular cell line was proportional to its intrinsic growth rate. That rapidly growing cultures require higher levels of reduced folates is in agreement with their higher sensitivity to methotrexate (Figure 16). High DHFR activity is probably the effect, not the cause, of high mitotic activity. This proposition is supported by the observation that a 30 percent inhibition of DHFR activity in vivo had no demonstrable effect on the growth rate of Nt575 cultures (Figure 32). Furthermore, mouse cell lines with enormously elevated DHFR activities do not grow any faster than wild type, when maintained in the absence of MTX selection pressure (Hakala et al., 1972).

Reddy and Rao (1975) reported that a DHFR activity maximum was reached in germinating soybean seedlings six days following sowing. The relatively high mitotic index of an exponential phase plant cell culture makes it a particularly rich source of the plant enzyme.

Stoichiometric inhibition by methotrexate was demonstrated for DHFR's from Nt575 and Vr2. Apparent turnover numbers for DHFR, determined from activity titration curves, are 2235 min^{-1} and 2523 min^{-1} for the tobacco and mung bean enzyme respectively. These values represent moles of product formed per mole of methotrexte bound, and are therefore dependent upon the equilibrium condition of MTX binding. A DHFR with a lower K_i for MTX would have a lower apparent turnover number. Therefore, determination of actual DHFR turnover numbers by MTX titration would require knowledge of the K_i for MTX:DHFR. Development of methods for the determination of the K_i for a tight binding inhibitor ($K_i \leq 10^{-9}M$) such as methotrexate is an area of active debate and research (Greco and Hakala, 1979). The best available computer procedures yield K_i estimates of inadequate precision to differentiate between

two K_i's which differ by less than a factor of 5 (C. Suelter, personal communication). Tight binding of MTX to tobacco DHFR was further demonstrated by elution of inhibitor bound enzyme from Sephadex G-25 columns. An initial input activity of 89 units of DHFR activity could be titrated by an equimolar amount of MTX in 10 minutes. This rate of enzyme-inhibitor association is diffusion limited and does not necessarily relate to the dissociation kinetics of the complex.

MTX inhibition of Nt575 DHFR activity was shown to be pH dependent. The decline in percent inhibition with increasing pH suggests that MTX binding to DHFR may involve the same hydrogen bond interactions reported in other systems (Blakley, 1981). The pH dependence of inhibition type (Figure 31) demonstrates the importance of characterizing inhibition conditions before proceeding with a kinetic analysis.

Neither folic acid, folinic acid, methotrexate nor UV irradiation affected the kinetics of DHFR induction during the Nt575 growth cycle. None of these potential inducers were tested in combinations. The former three might have been expected to increase the DHF pool size within the cells, the first by conversion to DHF via folate reductase, the second by cycling through thymidylate biosynthesis and the third by blocking DHF reduction. UV light might have stimulated a requirement for reduced folates by enhancing thymidylate demand. Even if these treatments elicited the proposed metabolic effects, elevated DHFR activity did not result. Regulation of the enzyme's activity must therefore be under the control of metabolic signals not tested in this experiment.

SELECTIONS AND VARIANTS IN CULTURED GRAIN LEGUMES

Introduction

Grain legumes exhibit a plethora of nutritional characteristics which limit their food value and consumer acceptance (Milner, 1975). All food legumes are deficient in sulfur-containing amino acids, with respect to the optimum amino acid profile for human nutrition (Dickson and Hackler, 1975). Elimination of the intestinal gas-forming factors present in the seeds of Phaseolus vulgaris represents a difficult breeding objective toward which little effort has been applied in the past (Murphy, 1975). Both of these characteristics, however, constitute biochemical phenotypes which may be approachable at the cellular level. In addition, a variant phenotype in either of these areas of metabolism can provide a complementing marker for the selection of somatic cell fusion hybrids.

Methionine is the amino acid which limits the human nutritional value of P. vulgaris seeds. Ethionine, the δ-ethyl analog of methionine, has been shown in various systems to compete with the latter in tRNA charging, protein structure, polypeptide chain initiation, biological methylation reactions and in the control of the cell cycle and biosynthetic pathways (Umbarger, 1971; Lea and Norris, 1976), usually with cytotoxic consequences. Plant cells selected in vitro for resistance to ethionine have displayed elevated levels of free methionine (Reisch et al., 1981; Zenk, 1974). Once a protocol is established for the

regeneration of grain legume plants from cultured cells, the expression in seeds of traits selected in vitro can be correlated. Until such time, we must operate on the assumption that at least some of the phenotypes expressed in vitro can also be expressed in the seeds of mature plants. In this section, selections are reported which were designed to recover variant cell lines of P. vulgaris exhibiting resistance to ethionine.

The α-galactosides raffinose, stachyose and verbascose are believed to be the substrates whose microbial fermentation in the human colon leads to digestive discomfort and flatulence (Murphy, 1964). Chemical or enzymatic extraction of these oligosaccharides from legume seeds effectively reduces the flatulence inducing ability of bean seeds (Calloway, 1975). If P. vulgaris cells, cultured in vitro, could be selected for the ability to perform this degradation with vigorous enzymatic efficiency, then it is conceivable that developing seeds on plants regenerated from the selected cells might be hard put to accumulate α-galactosides. Furthermore, if the ability to respire the sugars is not characteristic of cultured plant cells, then acquisition of such a capacity would also render a variant genome selectable in heterogeneous protoplast fusion populations. Little genetic variability has been uncovered in α-galactoside levels of P. vulgaris seeds (Murphy, 1975). How detrimental a severe reduction in the seed's α-galactoside stores would be to germination and seedling vigor is an open question. It is therefore assumed in this study that there exists an intermediate storage level of these compounds which is compatible with the needs of both the germinating bean seed and the human intestinal tract.

A serendipitous observation during the course of this study yielded an interesting variant cell line of Vigna sp. Callus tissue originating from an embryonic explant of a complex Vigna species hybrid produced a deeply pigmented sector. These cells have been isolated in as homogeneous a culture as

possible. Indeed, they represented the only unassailable variant cell line recovered in this entire study. A brief investigation of the nature of their pigmentation was justified by their potential as markers in protoplast fusion experiments and by their intrinsic physiological and genetic interest.

Results and Discussion

Selection for Ethionine Resistant Phaseolus vulgaris. The Pv25 cell line of P. vulgaris grows relatively slowly with a doubling time in suspension culture of 5 to 5.5 days (Figure 33). It is the most highly aggregated among the cell lines used in these studies. Clumps of ~2 mm in diameter comprise a substantial portion of the total cell volume of a Pv25 suspension culture at any stage in the growth cycle. Its color is uniformly white. Stressful treatments which cause most other cell lines to darken (toxins, temperature shifts, deep stationary phase) have little or no effect on the color of Pv25. This cell line has a strict minimum density requirement. Suspension cultures will not survive inoculum densities of less than 10 percent (PCV_{20}/V_T x 100).

Ultraviolet irradiation was used to increase the probability of recovering point mutations in genes encoding methionine biosynthetic enzymes. Figure 34 shows that, as with Vr2 (see Part III), UV treatment affected both the slopes and the extrapolated ordinate intercepts of the growth curves of treated Pv25 lawns. Dose-response curves generated by both the slope and intercept methods (see Part III) are shown in Figure 35. These log-linear survival curves intersect, coincidently, at the D_{37} of 204 J/m^2. This dose is slightly higher than that required to elicit the same response in Vr2 cultures, perhaps due to the greater aggregation of Pv25 cells. Photoreactivation was not examined in Pv25, but

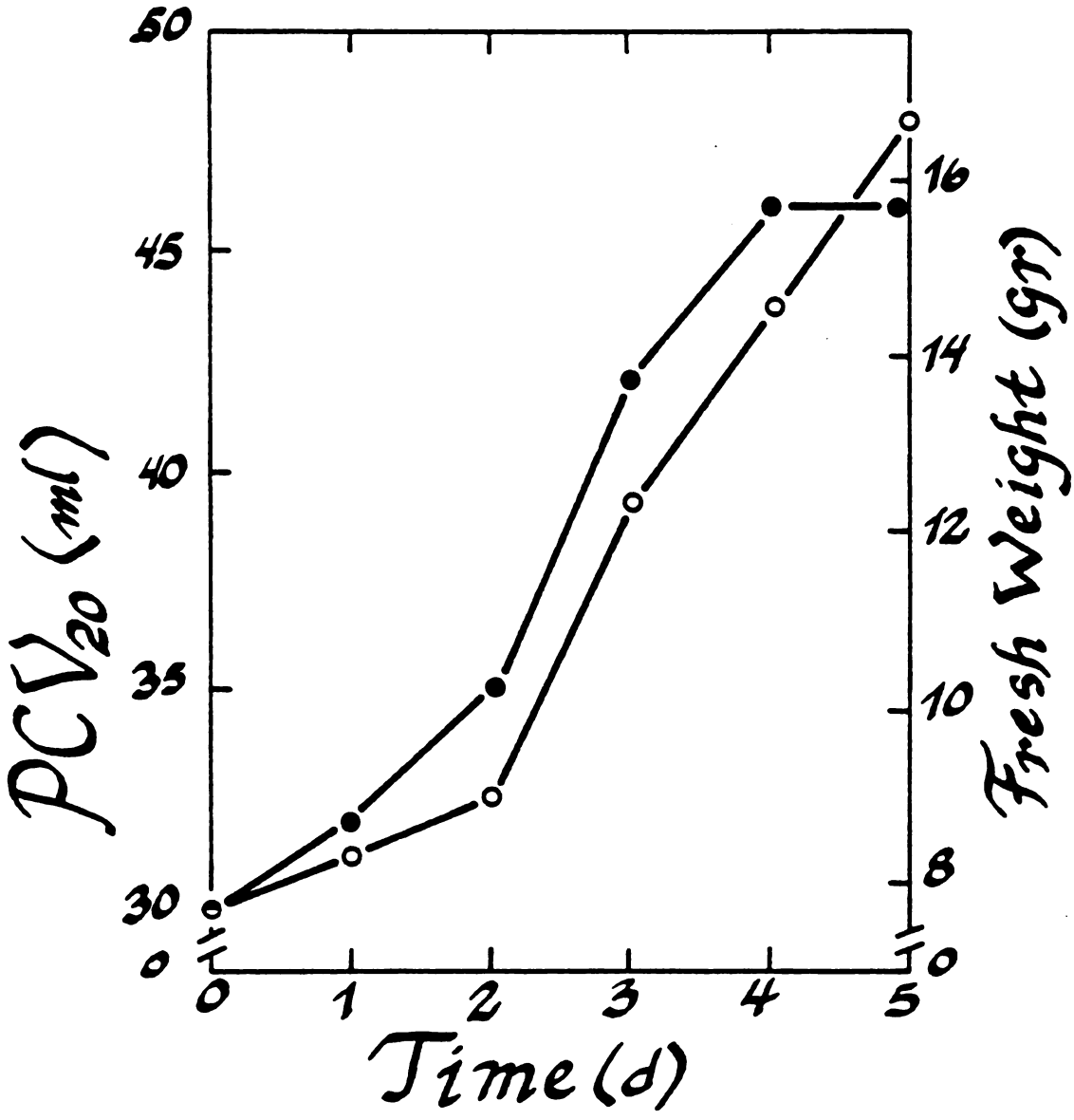


Figure 33: Growth of Pv25 suspension cultures. Growth measured by PCV_{20} (●) and by weighing Buchner funnel retentate (o).

Figure 34: Growth of UV irradiated Pv25 lawns. Fresh Pv25 lawns were irradiated with 9 (●), 19 (▲), 38 (■), 75 (o), 150 (Δ), 225 (□), 338 (+), 563 (O) and 900 (0) J/m^2 UV, and placed in darkness for 4 days. Photometric readings were then taken periodically for one month.

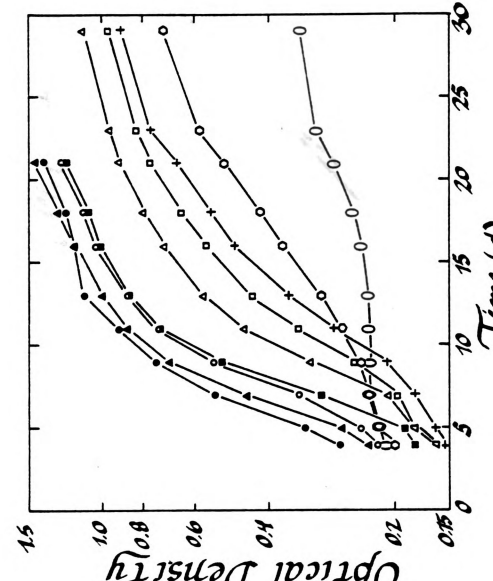


Figure 34: Growth of UV irradiated Pv25 lawns.

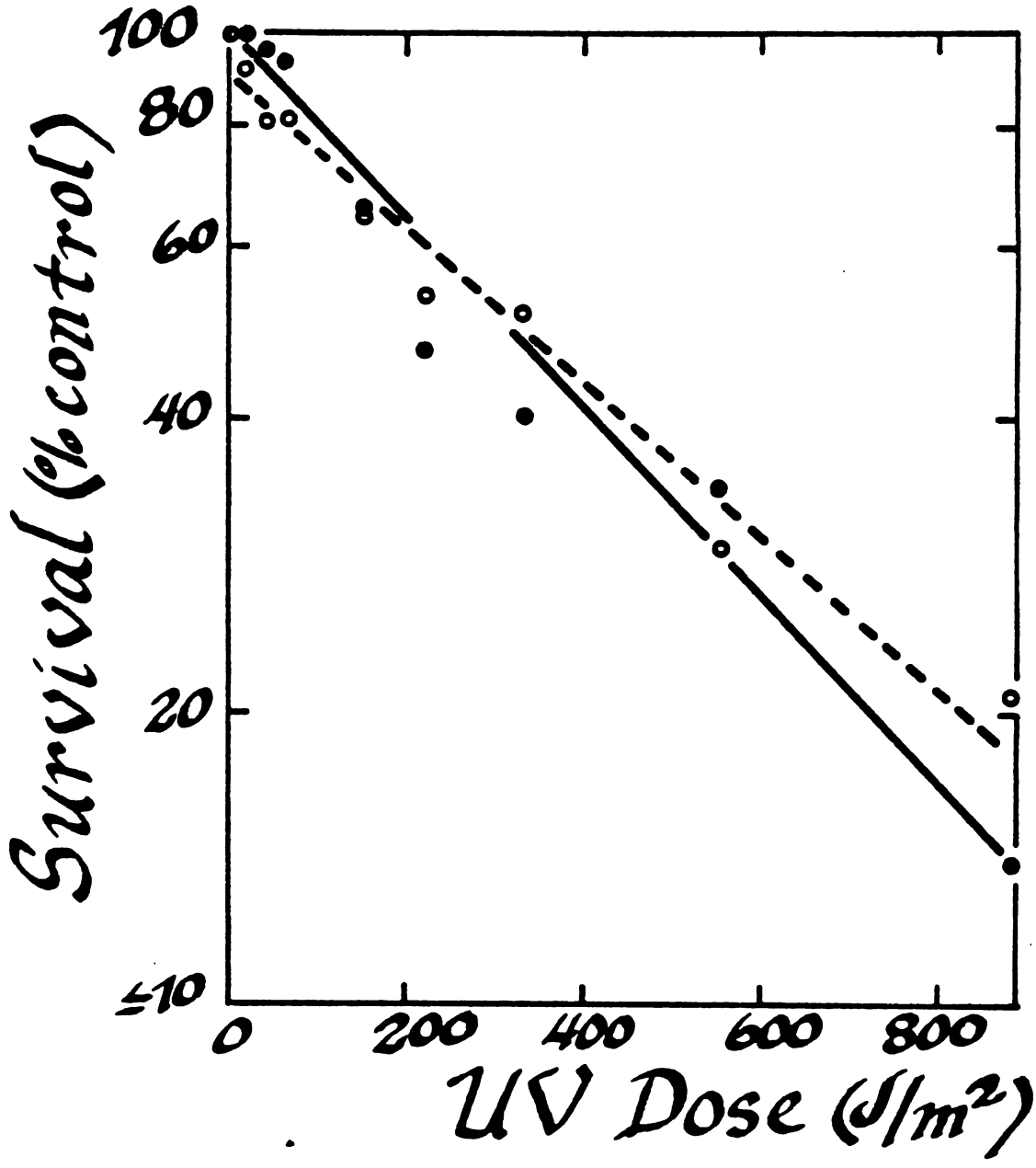


Figure 35: Growth response of Pv25 lawns to UV irradiation. Exponential phase (log normal) growth rates of UV treated lawns were calculated from the data in Figure 34 and were used to determine percent survival by the slope (●) and intercept (O) methods, as described in the text.

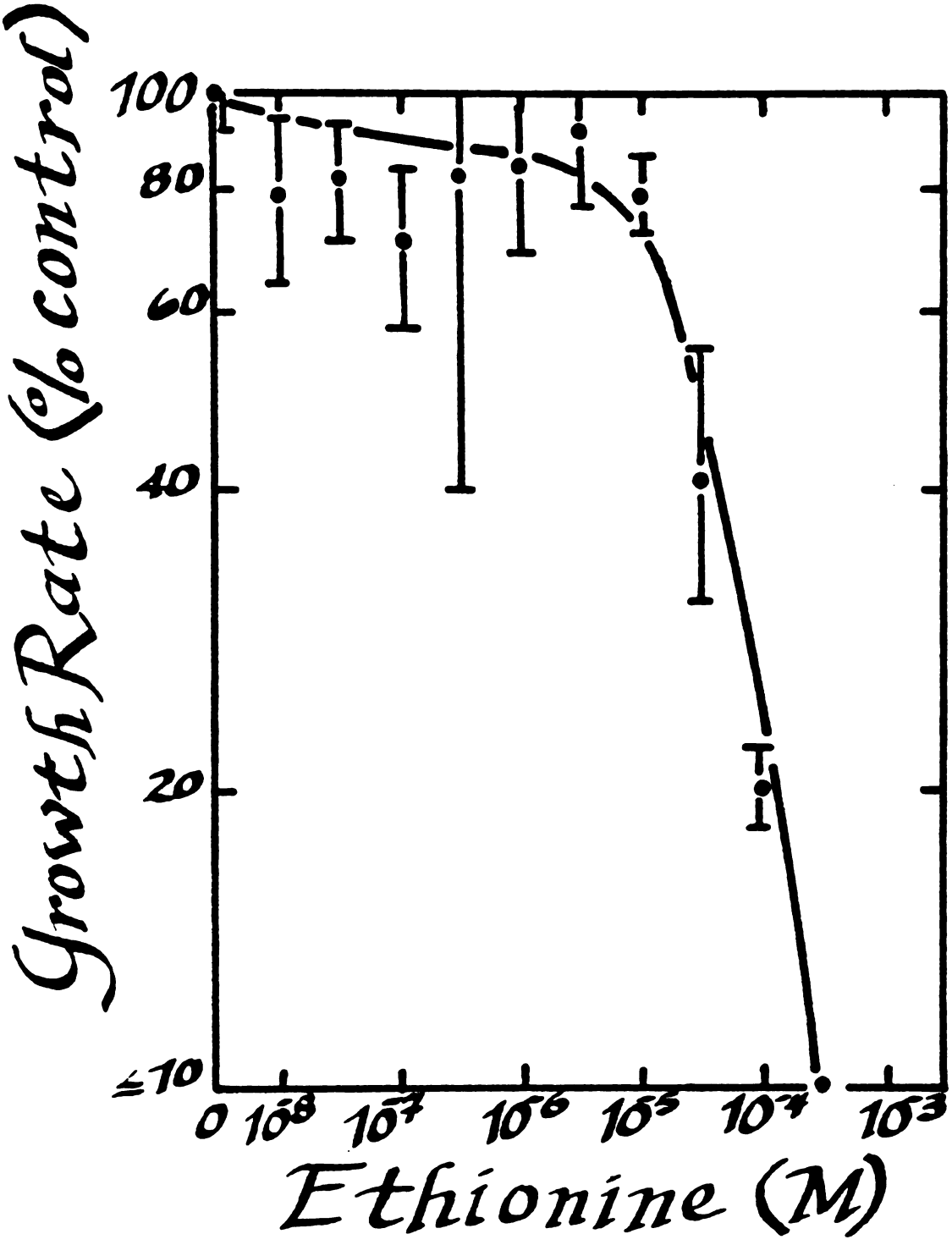


Figure 36: Growth response of Pv25 cells to ethionine. Error bars represent standard error of 5 replicte cultures.

cultures were maintained in complete darkness for one week immediately following UV treatment.

The I_{50} for Pv25 lawns grown on R3K + ethionine was approximately 20 µM ethionine (Figure 36). Colony growth was completely inhibited by 300 µM ethionine. Unlike the response of Vr2 to high methotrexate doses, Pv25 lawns grown on 300 to 1000 µM ethionine never produced a single "escaped" colony in these dose-response tests, even after two to three months of incubation. This may be a reflection of the numerous effects of ethionine, and the kinetics (not tight binding) of its interaction with its many cellular targets. The ethionine I_{50}'s reported for yeast and carrot cell suspensions are 5 µM and 1 µM, respectively (Colombani et al., 1975; Widholm, 1976). Slow growth and aggregation may contribute to Pv25's higher apparent I_{50} for ethionine.

For two reasons it was necessary to establish conditions for the reversal of ethionine growth inhibition of Pv25 cultures. First, if a selection procedure was to be designed to recover methionine overproducing cell lines, then exogenous methionine must be shown to rescue ethionine inhibited cultures. Of equal importance, added methionine in the absence of ethionine should not be growth inhibitory; otherwise, the resistant phenotype could be either impossible or lethal in the absence of selection. Secondly, conditions for the temporary rescue of ethionine inhibited cultures were needed to permit the obligatory one generation of non-selective growth for mutation fixation following UV mutagenesis (see Part III). Figure 37 shows the results of an experiment designed to clarify these conditions. Several interactions were revealed. Methionine alone (1 mM) caused a 50 percent growth inhibition. The apparent growth of Pv25 on 1 mM ethionine is misleading, since no colonies ever survived this treatment. Likewise, methionine at one-half, equal and twice the concentration of ethionine could not effect detectable reversal of the latter's toxicity. However, if lysine and

Figure 37: Rescue of Pv25 from ethionine toxicity. All treatments were applied simultaneously, at the start of the experiment. Abbreviations are as follows: ETH, ethionine; MET, methionine; MLT, methionine + threonine + lysine, at the mM concentrations given above each, respectively; CH, casein hydrolysate at the g/l concentration given above each. Lawn growth was measured photometrically. Bars with the same letter above are not significantly different at $\alpha = 0.01$ ($LSD_{.01} = .058$). Variances were homogenized by lnx transformation for ANOVA as CRD.

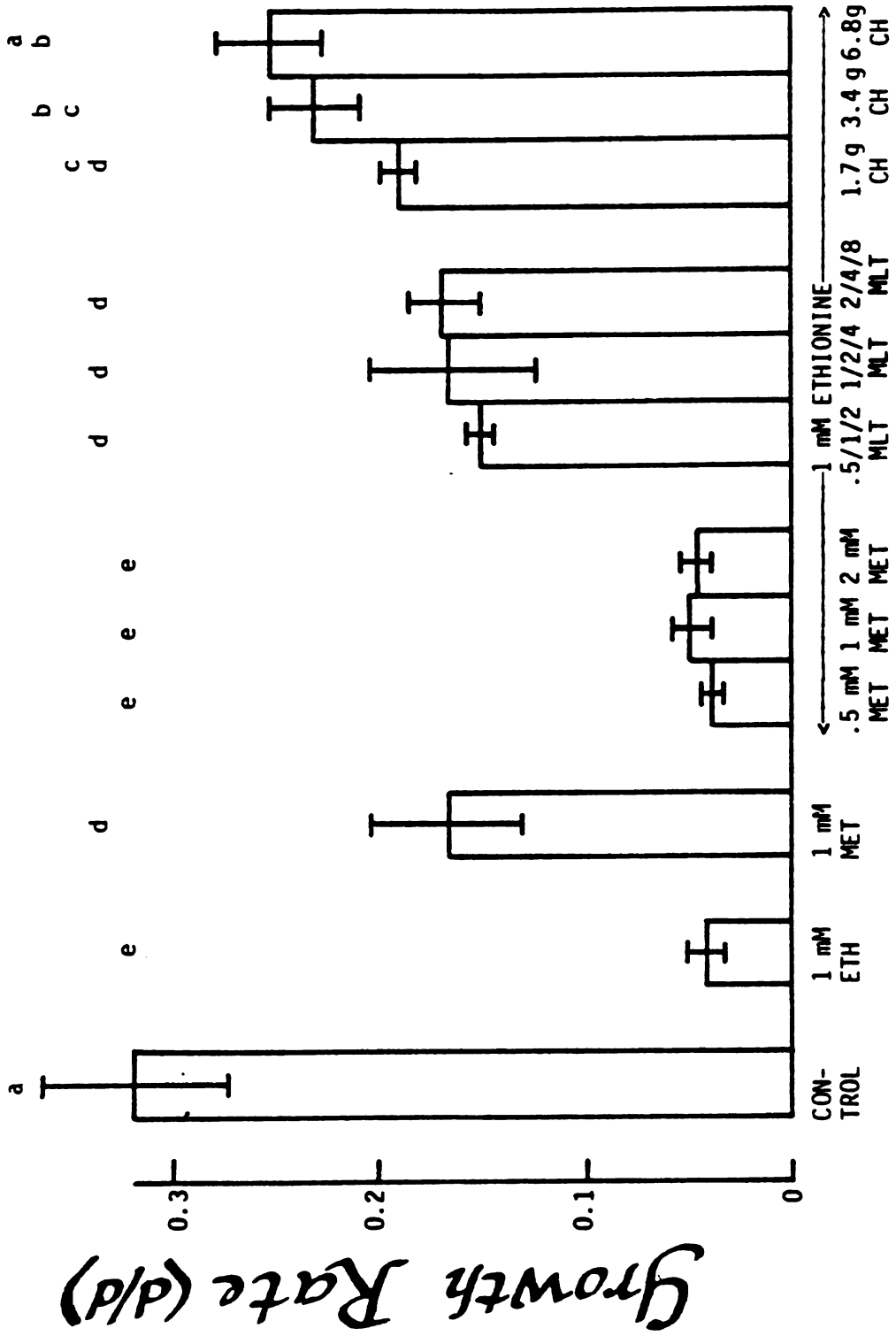


Figure 37: Rescue of Pv25 from ethionine toxicity.

threonine were introduced into the rescue cocktail, at twofold and threefold the methionine concentration respectively, a 50 percent restoration in growth rate was achievable in the presence of 1 mM ethionine. These three simultaneously administered antidotes did not exhibit a dose-response relationship over the concentration range tested. Confluent growth was visibly apparent on these plates. The inability of methionine alone to reverse ethionine inhibition in this system is incompatible with the observations of Widholm (1976) who reported a 50 percent growth restoration in 0.05 mM ethionine inhibited carrot cultures by 1 mM methionine. One millimolar methionine, in the absence of ethionine, was 50 percent growth inhibitory in the carrot system. Methionine (1 mM), lysine (1 mM) and threonine (1 mM) restored carrot culture growth to 80 percent of control levels when 0.05 mM ethionine was present. Thus, the only discrepancy between the present results and Widholm's may be attributable to the twentyfold higher concentration of ethionine employed here. Methionine completely reverses ethionine inhibition in yeast (Colombani et al., 1975). The requirement for these three aspartate family amino acids in the reversal of ethionine inhibition is possibly due to the latter's substitution of methionine in feedback inhibition of aspartate semialdehyde dehydrogenase, an enzyme required for methionine, lysine and threonine biosynthesis (Gengenbach et al., 1978). If this were the case, then methionine supplementation of ethionine inhibited cultures would only aggravate the conditions of lysine, threonine and possibly leucine depletion.

Figure 37 also shows that casein hydrolysate quantitatively restores growth to 1 mM ethionine inhibited Pv25 cultures. Casein hydrolysate at 6.8 g/l restored growth to a level not significantly different from that of the control at the $\alpha = 0.1$ level. This treatment, therefore, provided an option for the one

generation rescue necessary following UV mutagenesis. However, due to its specificity, methionine-lysine-threonine rescue was preferred.

Since methionine-lysine-threonine treatment was shown to effect permanent reversal of ethionine growth inhibition (Figure 37), an experiment was performed to test a method for effecting transitory rescue. Figure 38 shows the growth of Pv25 lawns on ethionine (1 mM) + threonine (1.5 mM) + lysine (3 mM) following pre-incubation in suspension cultures at 1 mM methionine for 1, 6, 12, 24, and 36 hours prior to plating. The 12 hour pre-incubation provided the most effective temporary reversal of inhibition.

Five selection experiments were performed in an effort to recover methionine overproducing Pv25 cells (Table 14). No colonies were recovered from a total of approximately 1.3×10^9 cells subjected to the various forms of ethionine resistance selection attempted. Three of the five selections (8065, 8070, 8074) employed UV mutagenesis and two of these used pre-incubation in 1 mM methionine to permit a generation of non-selective growth following mutagenesis. Prolific lawns formed quickly on non-selective control plates in each experiment. This failure to recover even "escapes" could be ascribed to Pv25's having a very low plating efficiency under these conditions, although it was never quantified in this system. As mentioned above, Pv25 has a stringent minimum density requirement in suspension culture. The R3K enriched medium (see MATERIALS AND METHODS) was used here to promote low density plating efficiency. Nonetheless, rare variants would have had to proliferate initially at extremely low density in these selections. "cooperative death" (Weber and Lark, 1979) may have been more of a problem in these selections than in the MTX resistance selections discussed in Part III. Ethionine + lysine + threonine might arrest cells in a metabolically unbalanced state, leading to the continued elaboration of normal metabolites which accumulate and are secreted at

Figure 38: Effect of preincubation time in methionine on growth of Pv25 lawns on ethionine + lysine + threonine. Preincubation times were as follows: no additives (●), 0h (▲), 36h (■), 24h (○), 12h (△), 6h (□) and 1h (+) in 1mM methionine. Other additives were ethionine (1 mM), threonine (1.5 mM) and lysine (3 mM). Each point represents the mean of 5 replicate cultures. The average coefficient of variation for all points was 12 percent.

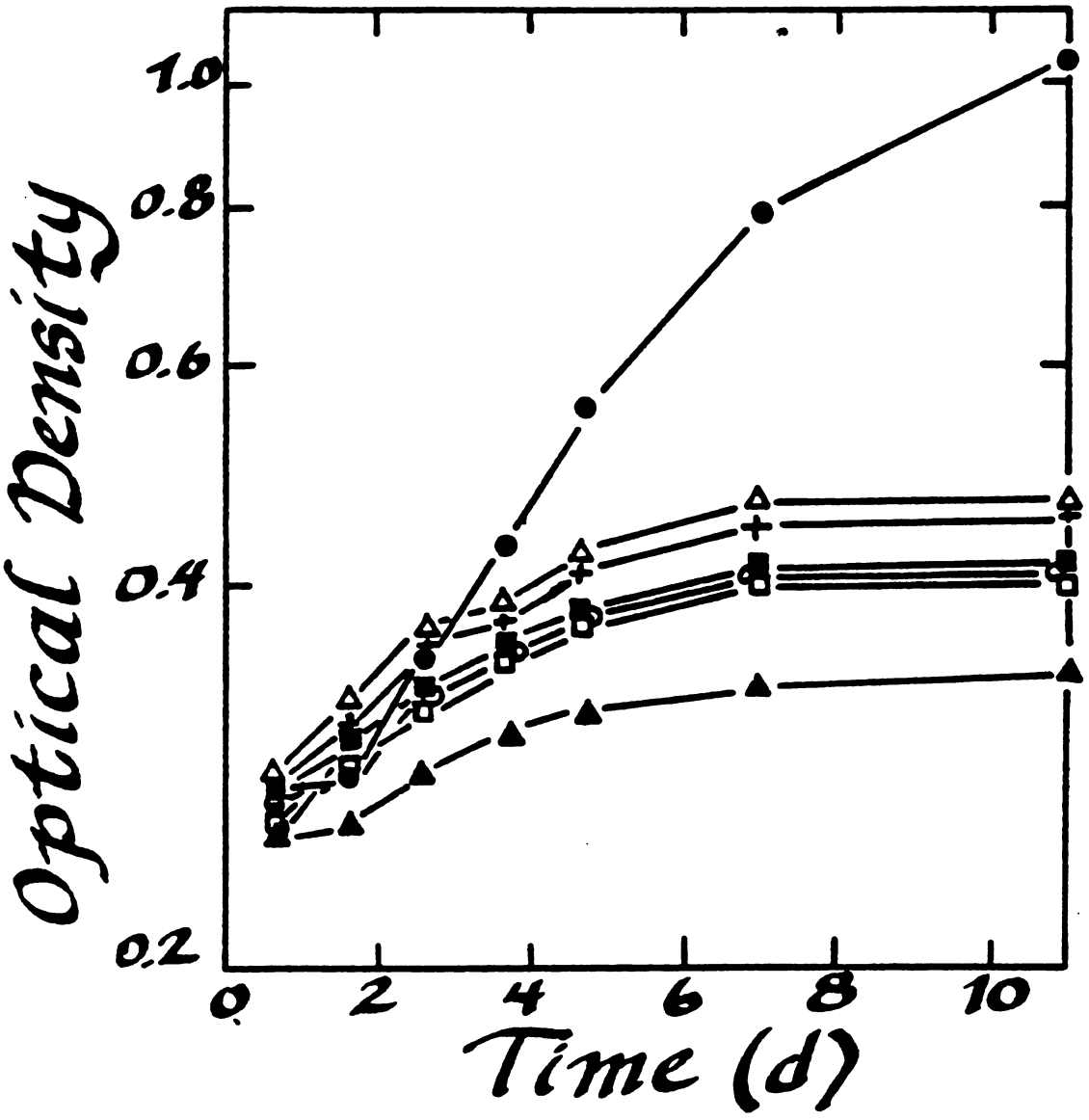


Figure 38: Effect of preincubation time in methionine on growth of Pv25 lawns on ethionine + lysine + threonine.

Table 14: Selections for methionine overproducing Pv25 cells.

Experiment No.	8061	8065	8070	8070	8074	8074	8078
No. of Cells Under Selection	2.1×10^8	2.5×10^8	1.3×10^8	1.3×10^8	1.3×10^8	1.3×10^8	2.5×10^8
L-Ethionine (mM)	0.3	1.0	1.0	1.0	1.0	1.0	1.0
L-Lysine (mM)	0	0	3.0	3.0	3.0	3.0	3.0
L-Threonine (mM)	0	0	1.5	1.5	1.5	1.5	1.5
Ultraviolet Light Treatment (J/m^2)	0	225	0	225	0	225	0
Preincubation in 1 mM L-Met. (hours)	0	0	0	48	0	12	0
No. of Colonies Recovered	0	0	0	0	0	0	0

supranormal, toxic levels. Finally, although selections for resistance to either ethionine (Reisch et al., 1981) or to lysine + threonine (Hibbard et al., 1980) have been successful in plants and microorganisms (Bright et al., 1980), this attempt to combine these agents into a single selective system may be unprecedented. It is possible that plant cells are incapable of undergoing the physiological or genetic changes necessary to survive this compound stress.

Selection for Altered Carbohydrate Metabolism in Pv25. Pv25 cells are normally maintained on 30 grams per liter sucrose. Lawns were inoculated onto solid media containing raffinose (30 g/l), its substituent monosaccharides (30 g/l) and a range of sucrose concentrations (0, 3, 5, 10, 30 g/l) for comparison (Figure 39). Glucose was the most efficiently utilized of the carbon sources provided, as judged by growth rate and final OD. Reduced sucrose supply affected the final growth accumulation more than it did growth rate. With final growth rate attained at various sucrose levels as a gauge, 30 g/l raffinose supplies a quantity of carbohydrate equivalent to 5 to 10 g/l sucrose. Since stationary phase was attained in the raffinose grown cultures, it was concluded that not all of the raffinose molecule is used as a carbon source. Thirty g/l fructose provided carbon roughly equivalent to 30 g/l sucrose, although assimilated at a slightly slower rate. The final and most important conclusion from this experiment was that 30 g/l galactose did not support growth at all. In terms of growth rates, galactose appeared to be inhibitory, in that cultures inoculated onto carbohydrate-free media grew faster than those with galactose. Thus, it appeared that the reduced efficiency of raffinose utilization was either due to Pv25's inability to assimilate the galactose moiety or to its inability to cleave the α-galactosidic linkage joining galactose and glucose in raffinose, or both.

In a second experiment, the substituent monosaccharides of raffinose, plus its galactose-(α1→6)-glucose disaccharide substituent, melibiose, were tested

Figure 39: Growth of Pv25 on various carbon sources. Pv25 lawns were grown on 30 g/l sucrose (●), 10 g/l sucrose (▲), 5 g/l sucrose (■), 3 g/l sucrose (o), 30 g/l raffinose (△), 30 g/l glucose (□), 30 g/l galactose (+), 30 g/l fructose (○) and no carbon source (0). Each point represents the mean of 5 replicate cultures. Average coefficient of variation for all points is 16 percent.

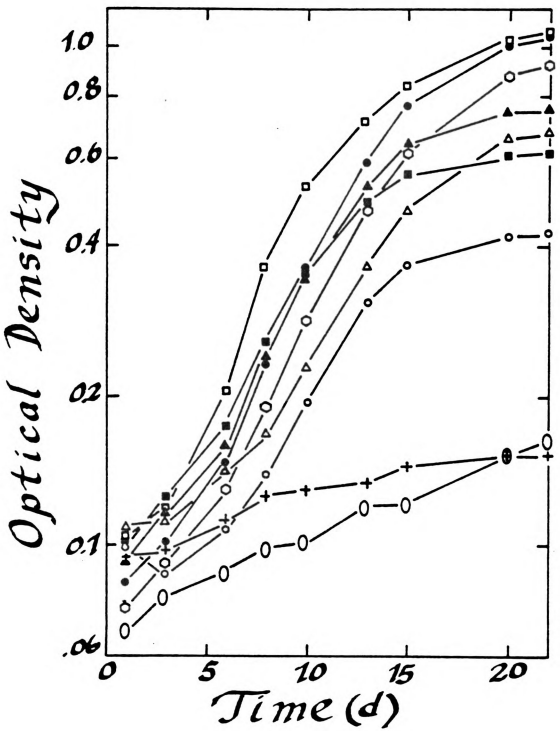


Figure 39: Growth of Pv25 on various carbon sources.

Figure 40: Growth of Pv25 on raffinose and its substituents. Pv25 lawns were grown on 30 g/l raffinose (●), 20 g/l melibiose + 10 g/l fructose (▲), 20 g/l melibiose (■), 10 g/l glucose + 10 g/l fructose + 10 g/l galactose (○), 10 g/l glucose + 10 g/l galactose (△), 10 g/l glucose + 10 g/l fructose (□), 10 g/l galactose (+), 10 g/l glucose (○), 10 g/l fructose (○), and no carbon (×). Each point represents the mean of 5 replicate cultures. Average coefficient of variation for all points is 14 percent.

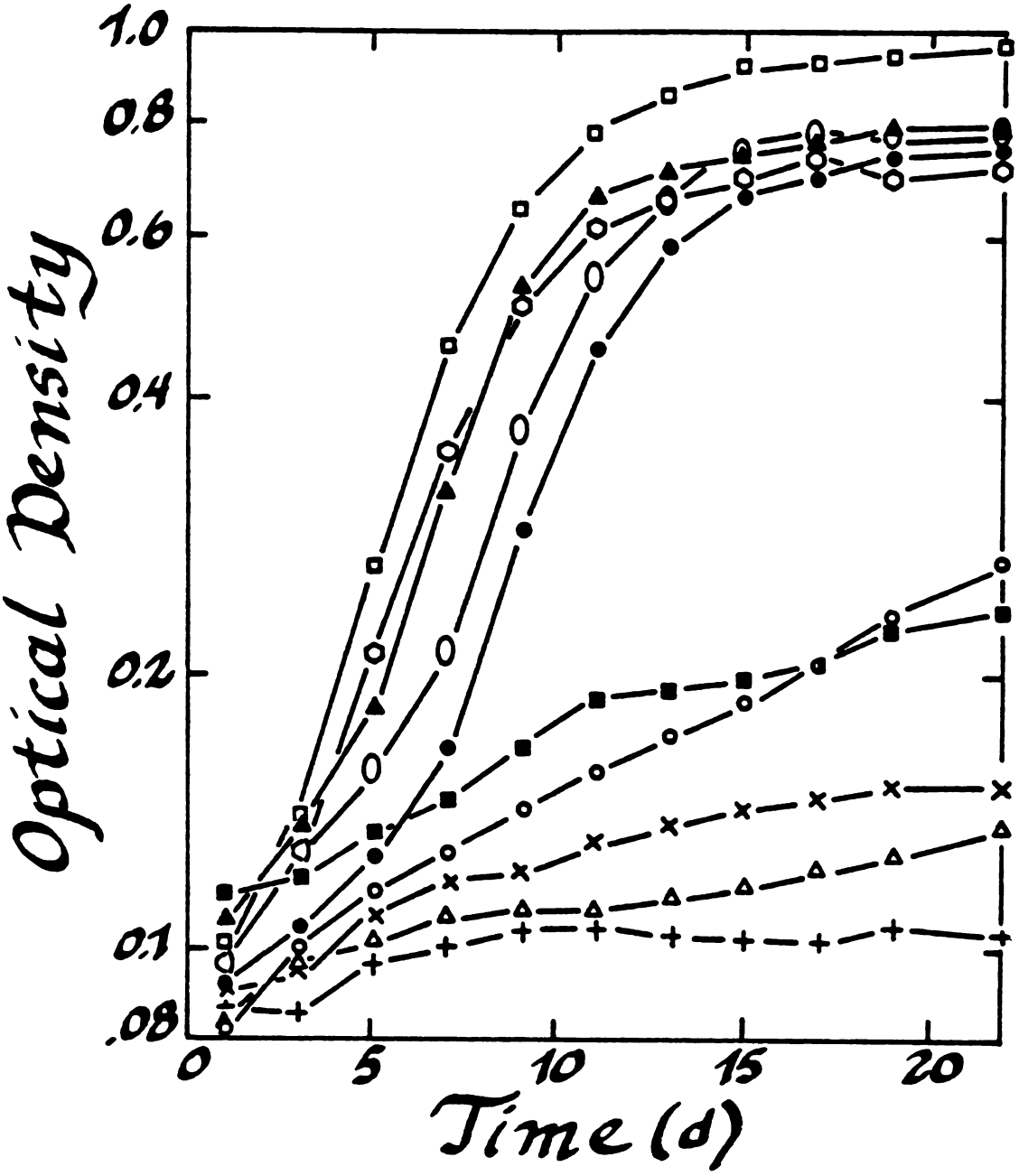


Figure 40: Growth of Pv25 on raffinose and its substituents.

more thoroughly for their ability to support Pv25 lawn growth (Figure 40). Several conclusions can be drawn from comparisons of the treatments. A comparison of growth on melibiose (20 g/l) + fructose (10 g/l), on raffinose (30 g/l) and on fructose (10 g/l) alone suggests that only the fructose unit of raffinose is assimilated, since all three treatments yielded essentially identical growth curves. This is supported by the observation that the growth rate on melibiose is apparently identical to that of the 0 carbon control. That free galactose is inhibitory is clearly indicated by comparing growth on glucose (10 g/l) alone and glucose (10 g/l) + galactose (10 g/l). The presence of galactose severely depressed growth in the latter treatment. The same conclusion can be drawn from a comparison of growth on a mixture of all three monosaccharides and growth on glucose (10 g/l) + fructose (10 g/l) without galactose. It is clear that when galactose is present, growth is greatly reduced. However, it is not clear from the data whether poor growth on melibiose is due to the inability of Pv25 cells to cleave the α-galactosidic linkage, thereby freeing glucose, or to the inhibitory effect of the concomitantly freed galactose. A comparison of the melibiose (10 g/l) with the glucose (10 g/l) + galactose (10 g/l) treatment suggests that melibiose may be slowly cleaved to yield an inocuous level of galactose and a useable quantity of assimilable glucose. On the other hand, superior growth on raffinose (30 g/l) and on melibiose (10 g/l) + fructose (10 g/l) to that on the mixture of all three hexoses strongly suggests that Pv25 is rescued in the former treatments (and possibly in vivo) from galactose toxicity by its limited ability to liberate the toxic monomer. This is in agreement with the finding (Figure 39) that raffinose is utilized approximately one-third as efficiently as sucrose. Finally, a comparison of the galactose (10 g/l), galactose (10 g/l) + glucose (10 g/l), and galactose (10 g/l) + glucose (10 g/l) + fructose

(10 g/l) treatments indicate that addition of assimilable hexoses can at least partially overcome galactose inhibition.

These results are in agreement with numerous reports of galactose inhibition in plant systems (Dey, 1980). Galactose toxicity at 1 g/l has been reported (Roberts et al., 1971), so the pronounced effects of tenfold higher levels here are not extraordinary. Whether free galactose toxicity results from its role in energy, glycoprotein or cell wall metabolism is not known. It became clear that the task of recovering a cell line which efficiently assimilates raffinose family oligosaccharides must begin with the selection of a line which at least tolerates, if not assimilates, galactose. Maretzki and Thom (1978) have selected a galactose utilizing cell line of Saccharum sp.

In the first galactose adaptation experiment, several late exponential phase 200 ml suspension cultures of Pv25 were subcultured by one to one dilution into R3 containing 20 g/l galactose as the sole carbon source. The medium was replenished every 2½ weeks for 7½ weeks, after which the cultures were plated onto solid R3K with galactose (20 g/l) as the sole carbon source. By this time, the cultures had become extremely necrotic, so some plates of R3K + sucrose (30 g/l) were also inoculated as viability controls. Several month's observation of the plated cultures revealed a complete absence of colony formation on galactose or sucrose media. Once again, either the selection had been too stringent or low plating efficiency had precluded the recovery of rare survivors.

It was concluded that a less stringent selection might yield positive results. If Pv25 could be selected for *tolerance* of free galactose in a first round of selection, then ability to assimilate the sugar could be selected for in a second round. Several Pv25 suspensions were subcultured one-to-one with R3 containing 5 g/l galactose and 25 g/l sucrose. All cultures grew slowly through two bi-weekly subcultures in this medium. However, when the first step-up in

galactose concentration was attempted, (to 10 g/l galactose + 20 g/l sucrose), the experiment was lost to microbial contamination. The viscous concentrated galactose additives were filter sterilized only with difficulty and this step was probably the source of the contamination. Nevertheless, the ability of Pv25 to tolerate 5 g/l galactose in the presence of a growth sustaining level of sucrose suggests a good starting point for experiments designed to adapt Pv25 to growth on galactose. The failure to recover a galactose utilizing line from the first experiment may have been attributable to the infrequent subculture schedule adopted. Maretzki and Thom (1978) subjected their galactose non-adapted sugarcane cell line to only thirteen days of 18 g/l galactose suspension culture prior to plating on the same, solidified, medium. Recovery of galactose adapted clones then required a further five months of observation and replating. Another approach might be to grow Pv25 suspensions under carbon limitation in batch or continuous culture with raffinose (10 to 20 g/l) as the sole carbon source. Under these conditions, a clone which could use the entire raffinose trisaccharide molecule would have a competitive advantage.

Characterization of a Pigmented Cell Line of Vigna spp. During the course of this study, a number of callus cultures were initiated from embryonic and seedling tissues of interspecific Vigna hybrids. These were the by-products of attempts (Parrott, 1981) to rescue aborting embryos from pods set on Vigna plants which had been crossed with heterologous Vigna species (Chen, 1980). One such culture, Vr9, originated from root tissue of a precociously germinating embryo of the following genotype: (V. radiata cv. M884 x V. umbellata cv. 4065)$_{II}$ x V. radiata cv. TN #1. The female parent had been a spontaneous amphidiploid, making this explant presumably triploid (Vr9's ploidy has not been cytologically verified). Four months following explanting, a purple pigmented sector appeared in a Vr9 callus maintained on R3. The pigmented subclone was

excised from the parent callus, propagated separately and named Vr9a ("a" for "anthocyanin"). Numerous attempts to isolate a clone of solely purple cells from Vr9a have been unsuccessful. Single cell cloning on nurse callus, enriched and conditioned medium has not promoted cell division in isolated pigmented cells. It has not been established, to date, whether the pigmented cells themselves are capable of division; they may represent developmental endpoints of a variant class of Vr9 cells. Vr9a morphologies range from intensely pigmented small (40 μ diameter) isodiametric cells to extremely elongate (40 x 600 μ), more diffusely pigmented types. Visibly dark purple Vr9a callus is actually a heterogeneous mixture of mostly nonpigmented cells and up to 20 percent purple cells. Suspension cultures of Vr9a (Figure 41) contain an even lower proportion of pigmented cells. Callus cultures must be continuously selected, at each subculture, for cells of high pigmentation, lest non-pigmented cells outgrow them. Likewise, suspension cultures must be periodically reinitiated from selected callus clones.

The physiological or genetic event which elicited pigment expression in Vr9a is extremely rare. Parental Vr9 callus cultures have never produced another pigmented sector in approximately 250 generations since Vr9a appeared. Vr9 is an interspecific hybrid and presumably triploid. Therefore, the event which mediated the Vr9a phenotype may have been a chromosomal anomaly resulting from an aberrant somatic interaction between the heterologous genomes. Such a position effect mechanism is neither genetically nor cytologically verifiable in the present system. Culture filtrates of Vr9a suspensions do not elicit pigment formation in Vr9 cultures, suggesting that pigment production is not mediated by a diffusable factor. This novel phenotype provided a potential visual marker for protoplast fusion hybridization and invited a number of inquiries regarding its nature and origin.

Figure 41: Suspension cultures of Vr9a and Vr9. Two left cultures are Vr9a and to right cultures are its parent line, Vr9.

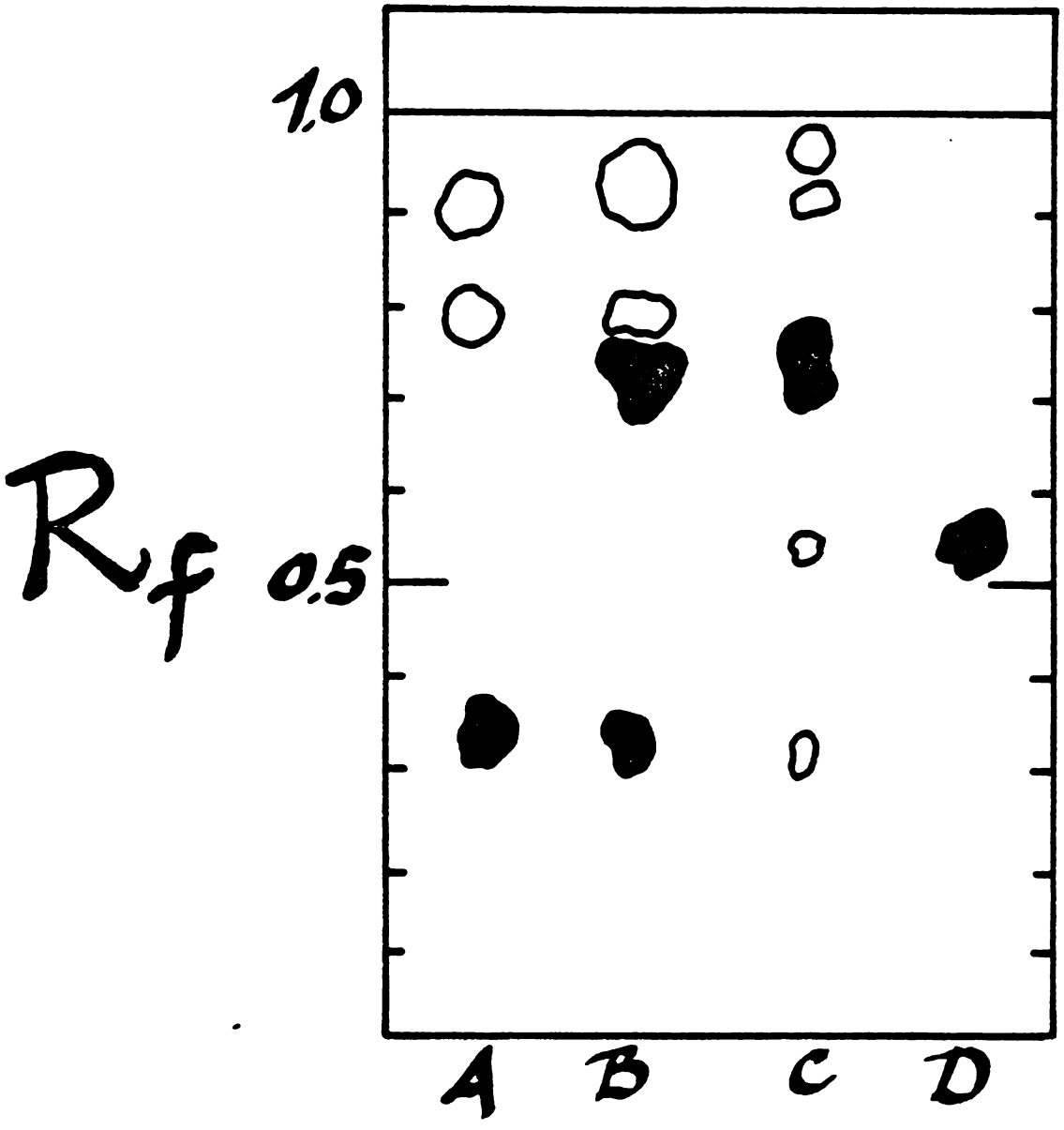


Figure 42: Thin layer chromatogram of Vr9a pigment and standards. Lanes are delphinidin standard (A), Vr9a + delphinidin standard (B), Vr9a (C), cyanidin standard (D). Forrestal solvent system was used. Dark spots are purple, open spots visible only by UV fluorescence.

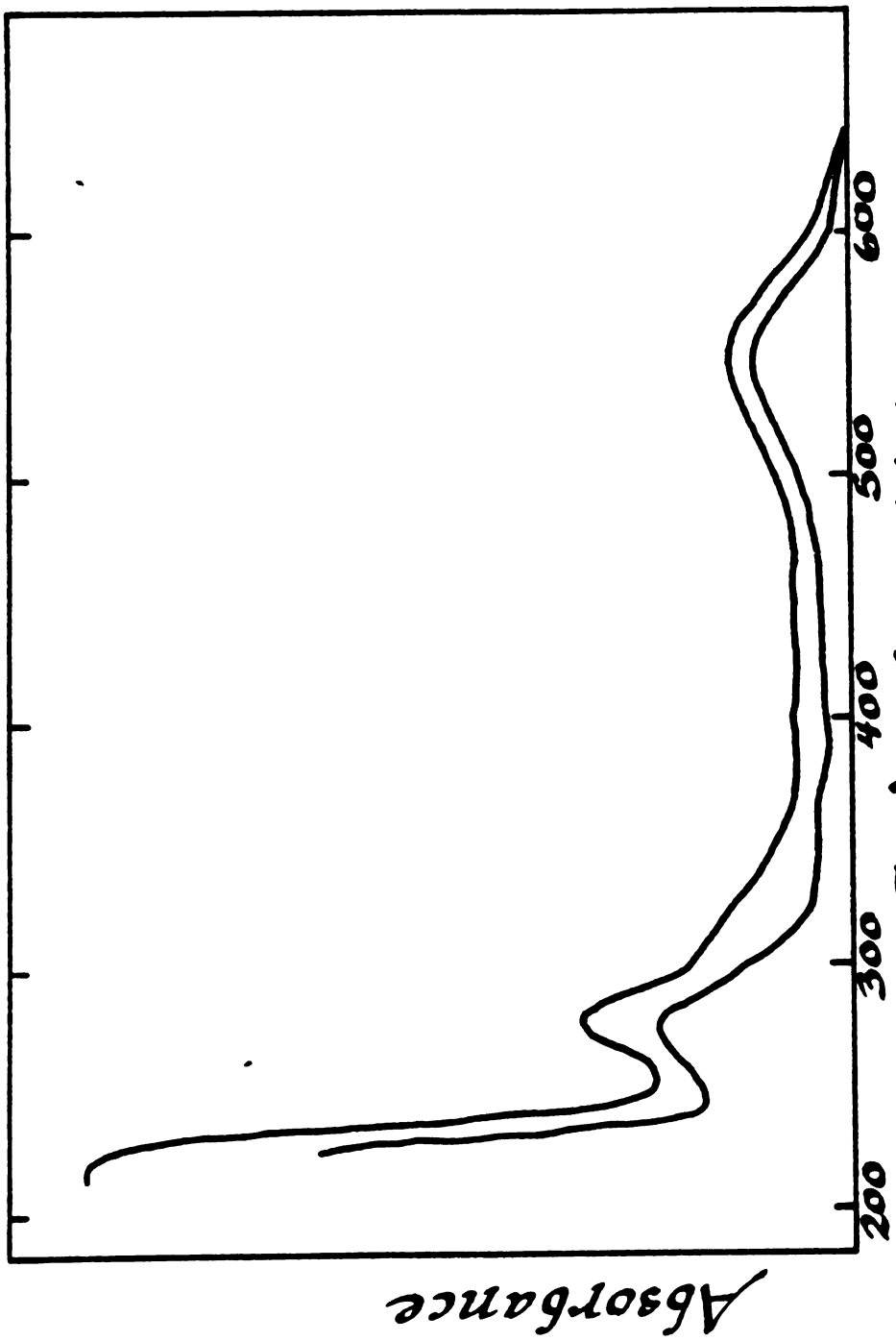


Figure 43: Absorption spectrum of Vr9a aglycone and malvidin standard. Solvent was 0.1% HCl in methanol. Upper curve is Vr9a; lower is malvidin standard.

The Vr9a pigment is a vacuolar anthocyanin. The aglycone portion of the pigment molecule has been tentatively identified as malvidin. Thin layer chromatography of the aglycone demonstrated that the pigment is neither delphinidin nor cyanidin (Figure 42). The absorption spectrum (Figure 43) of the pigment extracted into 0.1% HCl in methanol was identical to that similarly extracted from Lythrum salicaria (purple loosestrife, a standard source of malvidin). The absorption maxima at 275 and 545 nm are in good agreement with published spectral characteristics of malvidin (Harbourne, 1967). Although the A_{440}/A_{max} ratio of 0.2 of the Vr9a pigment agrees with the value of 0.19 published for malvidin (Harborne, 1967), the Lythrum standard exhibited a ratio of 0.14. In addition, the Forrestal R_f for the Vr9a pigment was 0.76, whereas the published R_f for malvidin is 0.60. The R_f's of delphinidin and cyanidin standards were also high compared to published values in the chromatogram shown in Figure 42. Anthocyanidin candidates with published Forrestal R_f's near 0.76 are apigeninidin, pelargonidin, rosinidin and hirsutidin (Harborne, 1967). However, the former two are not purple pigments and the latter two lack the UV absorbance peak clearly evident in Figure 43. Thin layer chromatography of the Vr9a pigment with the malvidin standard would settle this issue. Malvidin has recently been reported to occur in Petunia callus at similar levels to those occurring in whole plants (Colijn et al., 1981). Anthocyanins have not been determined in Vigna species (Harborne et al., 1971), although a red pigment does occur in the hypocotyl of seedlings of some species.

It was observed that the underside of Vr9a callus, cultured under fluorescent lights, was less pigmented than the upper surface. Light induced flavanoid biosynthesis has been reported in several plant cell systems (Zucker, 1965; Stickland and Sunderland, 1972; Hahlbrock and Griesbach, 1979). However, the amount of pigment per gram of fresh weight was found to decline over the

Figure 44: Growth cycle and light effect on pigment accumulation in Vr9a. Vr9a (triangles) and Vr9 (circles) were grown in darkness (filled symbols) and light (open symbols). Growth was measured by PCV_{20} (solid lines) and pigment accumulation (broken lines) was measured as described in the text and expressed as A_{512} per gram fresh weight. Each point represents the mean of 2 replicates.

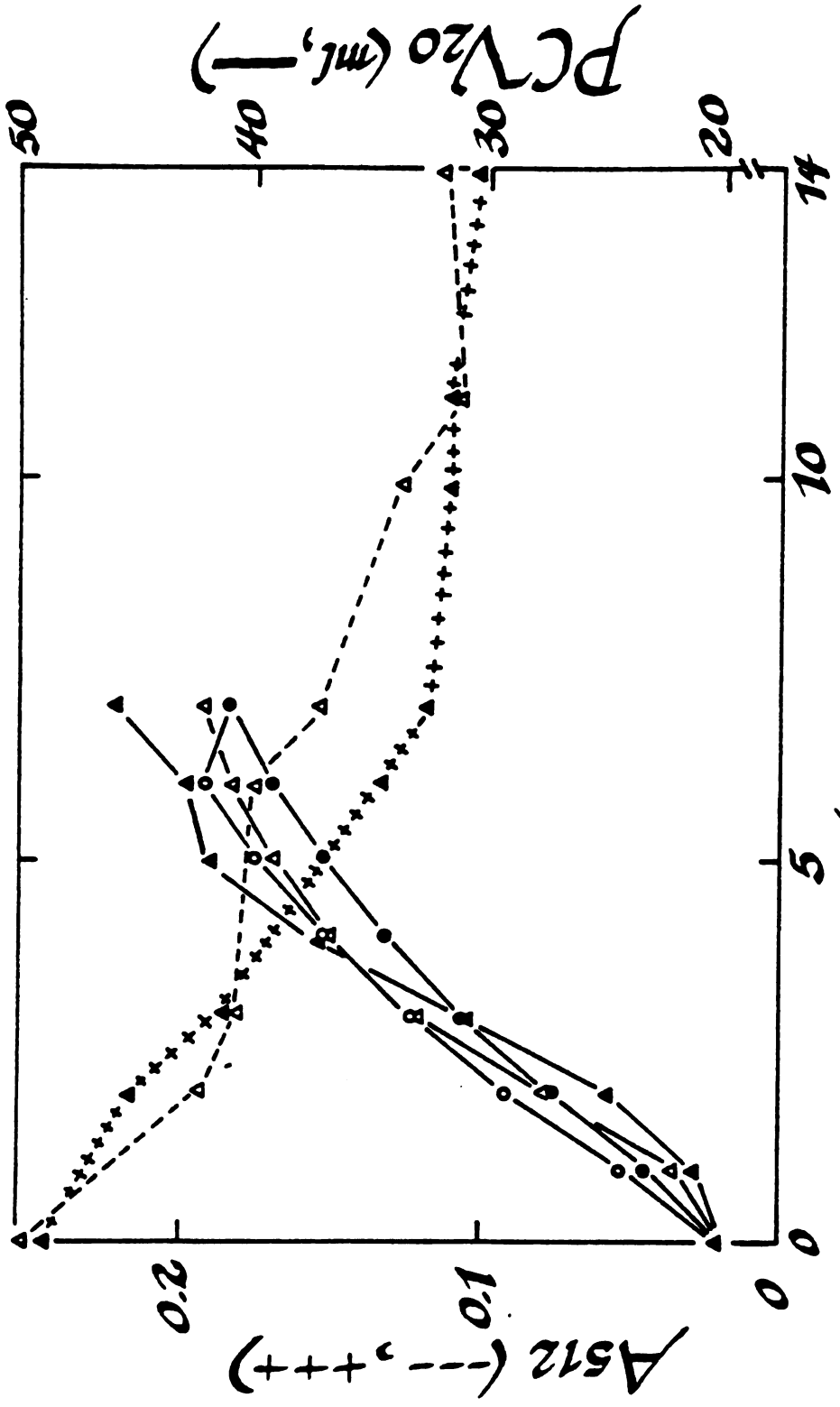


Figure 44: Growth cycle and light effect on pigment accumulation in Vr9a.

culture growth cycle in both light and dark grown Vr9a suspensions (Figure 44). Nor were the growth rates of Vr9 or Vr9a differentially affected by light or dark treatments (Figure 44). This result was unexpected but reproducible. As a consequence of this finding, Vr9a and Vr9 callus stocks were henceforth maintained in the dark, with no apparent decline in Vr9a pigmentation. In retrospect, this result is not surprising, since the original pigmented clone appeared in a Vr9 culture grown in the dark. This lack of the usual light mediated control of flavanoid biosynthesis in vitro supports the proposition that a genetic change has disrupted the normal regulation of flavanoid biosynthesis in Vr9a.

Anthocyanin pigmented maize cells lose their pigmentation when enzymatically relieved of their cell walls (S. McCormick, personal communication). Such phenotypic instability in Vr9a cells would seriously limit their utility in protoplast fusion hybridization. However, pigmentation is stable in Vr9a protoplasts (Figure 45). In fact, in two separate experiments, it appeared that protoplasts were more readily released from the pigmented than from the nonpigmented cells in the heterogeneous Vr9a suspensions. Observation under Nomarski optics revealed that isolated Vr9a tonoplasts stably retained pigmentation in the protoplast incubation medium (Figure 46). No attempt was made to regenerate callus clones from Vr9a protoplasts.

The infrequency of pigmented cells in Vr9a cultures suggested that they might be at a selective disadvantage compared to non-pigmented cells. phenylalanine is the first precursor from which anthocyanidins are built by the phenylpropanoid biosynthetic pathway. Complete anthocyanin molecules are formed by the addition of carbohydrate moieties onto the tricyclic anthocyanidin. Depletion of phenyalanine or carbohydrate pools via the metabolically useless pathway of flavanoid biosynthesis might put Vr9a's

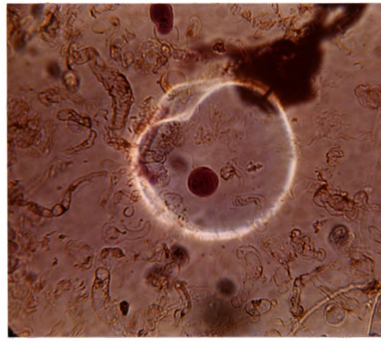


Figure 45: Vr9a protoplast under bright field.

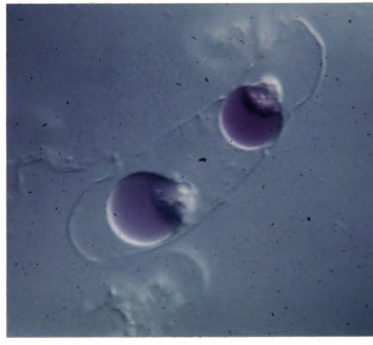


Figure 46: Vr9a tonoplasts under Nomarski optics.

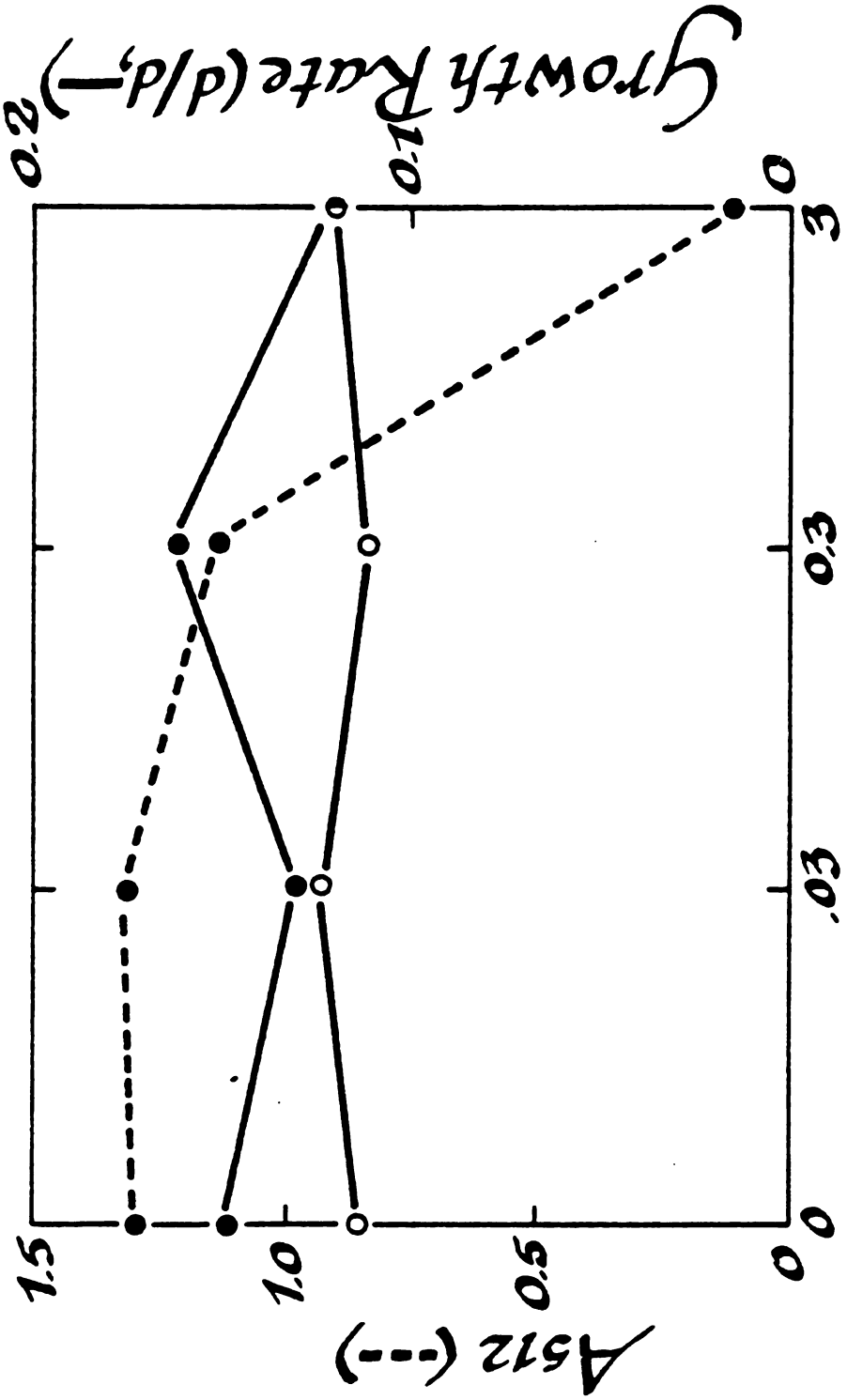


Figure 47: Effect of phenylalanine on growth and pigmentation of Vr9a. Growth rates (solid lines) and pigmentation (dashed line) of Vr9a (●) and Vr9 (O) were measured as described in Figure 43. Each point represents the mean of 2 replicates.

Figure 48: Effect of sucrose on growth and pigmentation of Vr9a. Growth (●) was measured by weighing calluses after 3 weeks. Pigmentation (O) was measured as described in the text. Each point represents the mean of 3 replicate determinations.

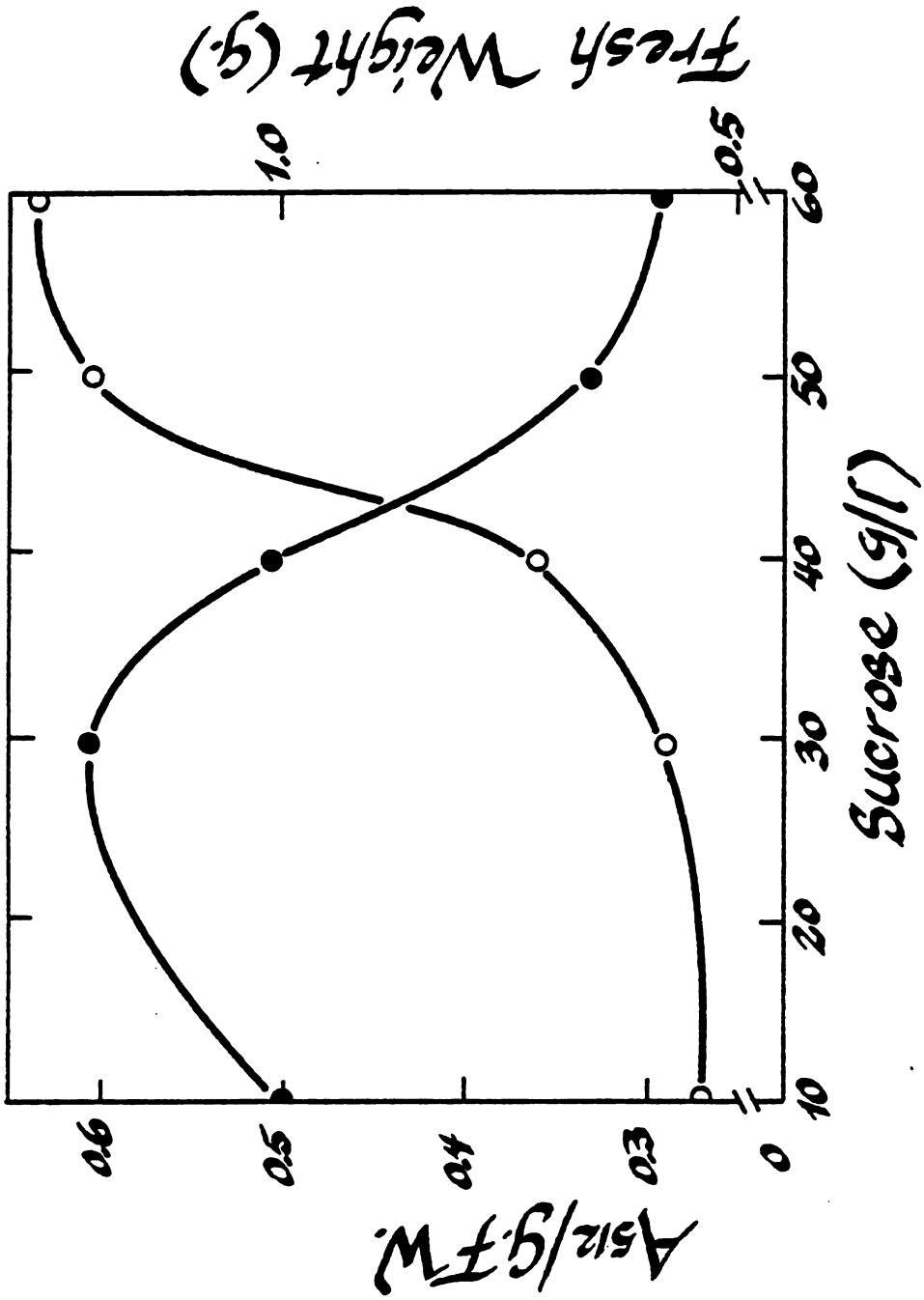


Figure 48: Effect of sucrose on growth and pigmentation of Vr9a.

pigmented subpopulation at a competitive disadvantage. In two experiments, the effects of medium enrichment for these hypothetically depleted metabolites were examined. Supplementation of the medium with phenylalanine had no effect on growth rate of either Vr9 or Vr9a suspension cultures, and only served to depress pigment synthesis in Vr9a (Figure 47). However, supplementation of solid media with supranormal levels of sucrose enhanced pigment production in Vr9a callus on a per gram fresh weight basis (Figure 48). Growth and pigment formation displayed reciprocal effects as a function of medium sucrose concentration. The calluses grown on 50 or 60 g/l sucrose were compact and intensely pigmented, with no sign of nonpigmented subclones emerging on the callus surface (as is common on Vr9a cultured on 30 g/l sucrose). Shepard (personal communication) has reported that some potato cultivars elaborate a red pigment in response to "sucrose stress". Whether or not the enhanced pigmentation is a generalized osmotic stress response in Vr9a might be tested by supplementing normal sucrose levels with a non-metabolizable osmoticum such as mannitol or sorbitol. Colijn et al. (1981) observed that sucrose concentrations above 30 g/l strongly depressed pigment synthesis in Petunia callus.

A number of further observations have been made concerning the Vr9a cell line. It would be interesting to know which differences in flavanoid biosynthetic activities between Vr9 and Vr9a are responsible for the presence of pigment in the latter. Has a series of biosynthetic functions been derepressed in this variant, or is a single enzyme responsible? The classic approach to such a problem is to feed precursors to the null phenotype and thereby bypass its enzymatic block (McCormick, 1978). Vr9 suspensions were fed the relatively ubiquitous flavanoid precursors naringinin or dehydroquercitin, with no concomitant measurable pigment synthesis.

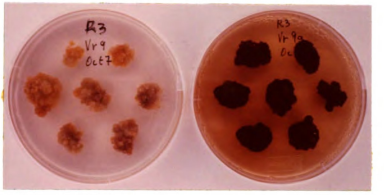


Figure 49: Vr9 and Vr9a calluses under visible light.

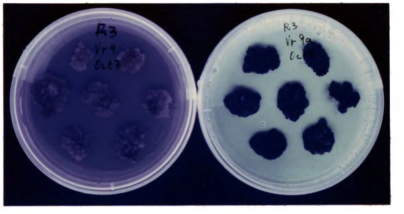


Figure 50: Vr9 and Vr9a calluses under long wave UV light.

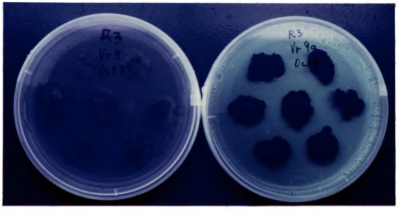


Figure 51: Vr9 and Vr9a calluses under short wave UV light.

After Vr9a calluses have grown on solid R3 medium for 3 weeks or more, an orange pigment accumulates in the medium (Figure 49). This pigment fluoresces yellow-green under ultraviolet light. Fluorescence intensity is greater under longwave UV (375 nm, Figure 50) than under short wave UV (254 nm, Figure 51). No attempt has been made to identify the fluorescent metabolite. No visibly fluorescent compounds are apparent in Vr9a cells, in Vr9 cells or in the media of the latter.

SDS polyacrylamide gel electrophoresis of whole cell protein extracts of Vr9 and Vr9a revealed no striking differences between the two cell lines. A faint band of ~20,000 daltons appeared to be present in the Vr9 pattern but absent from Vr9a. The resolution of the gel was insufficient to draw any firm conclusions.

Finally, on several occasions it was observed that pigment synthesis was diminished in Vr9a cultures grown at slightly elevated temperatures. Growth of the cell population appeared to be at worst unaffected and possibly enhanced at higher temperature. This effect was not studied quantitatively. Anthocyanin formation as a seedling or senescence stress response has been widely observed. In fact, summer's parting chill is believed to be at least partially responsible for the autumn colors of temperate forests (Harborne, 1967).

Conclusions

Two different selective schemes have failed to yield biochemical variants of Phaseolus vulgaris. The Pv25 cell line's strict minimum population density requirement may have reduced plating efficiency to such an extent that too few cells were challenged in these experiments to recover a variant. Nevertheless,

some aspects of Pv25's metabolism have been revealed that may be pertinent to future selections.

Pv25 cells grow in suspension with a reproducible doubling time of 5 to 5.5 days. Aggregation may be responsible for its relatively high ultraviolet light D_{37} of 204 J/m^2. The presence of DNA damage in UV survivors was not demonstrated. The methionine analog ethionine inhibited Pv25 lawn growth by 50 percent at a concentration of 20 μM. Methionine was not found to reverse growth inhibition caused by 1 mM ethionine. The natural metabolite's antidotal capacity was not tested at lower analog concentrations. However, it is significant that a combination of methionine, lysine and threonine, in the molar ratio of 1:4:2, could measureably rescue Pv25 from 1 mM ethionine toxicity. A more thorough optimization of the conditions for reversal of ethionine toxicity would better indicate potential selective systems than the relatively superficial treatment given to this question here. The relief conferred by the aspartate derived amino acids lysine and threonine indicates that ethionine is probably a false feedback inhibitor of an enzyme in the aspartate pathway of amino acid biosynthesis (Gengenbach et al., 1978). Reports of ethionine resistant variants (Widholm, 1976; Reisch et al., 1981) in other plant systems could be reconciled with the present data if the gratuitous allosterism proposed here were operative only at high (1 mM) ethionine concentrations. The apparently effective rescue of Pv25 by casein hydrolysate is of less theoretical than practical importance, although a quantitative analysis of the constituent amino acids in the commercial hydrolysate preparation might accelerate progress in optimizing defined selection conditions. It should be noted that the quantitative reversal by methionine of ethionine + lysine + threonine inhibition was never explicitly demonstrated in this system. Such a demonstration is, in the end, the best indication of the feasiblity of the selection attempted here.

Pv25 cells were found to grow on raffinose, glucose, fructose and sucrose as sole carbon sources. Growth tests using selected concentrations and combinations of the constituents of raffinose revealed that 1) galactose does not support the growth of Pv25 cells; 2) galactose is toxic to Pv25 at 10 g/l; 3) galactose toxicity can be partially relieved by the addition of a metabolizable sugar; 4) melibiose is not efficiently utilized by Pv25 but may be slowly cleaved to yield metabolizable glucose and subtoxic levels of galactose. Attempts to select a galactose-adapted cell line were unsuccessful. The proven scheme of Maretzki and Thom (1978) employed a shorter exposure of cells to galactose in suspension culture prior to plating on solid galactose medium. Such a protocol might be profitably followed here. The observation that galactose is both non-metabolizable and toxic in wildtype cells calls for a less stringent selection, perhaps based on the ability of variant cells to more efficiently utilize a growth limiting supply of raffinose.

A deeply pigmented cell line, Vr9a, originating from seedling tissue of a complex Vigna species hybrid has been isolated and partially characterized. The anthocyanidin pigment has been tentatively identified as malvidin by spectroscopic methods, but further chromatography will be necessary for positive identification. Pigmented cells do not grow in the absence of nonpigmented cells of the same cell line; consequently, all Vr9a cultures are mixtures of pigmented and colorless phenotypes. Pigment formation is not light inducible, though it is enhanced when elevated sucrose levels are supplied to callus cultures. The flavanoid precursors L-phenylalanine, or naringinin and dehydroquercitin did neither enhance pigment production in Vr9a nor elicit it in Vr9, respectively. This finding suggests that the activities responsible for pigmentation in Vr9a are downstream from the flavanone step in anthocyanin biosynthesis. The pigment was shown to be stably maintained in naked

protoplasts and tonoplasts made from suspension cultured Vr9a cells. A number of interesting lines of inquiry are presented by this cell line: 1) How do the activities of the enzymes of phenylpropanoid metabolism compare between Vr9 and Vr9a? 2) What is the fluorescent compound elaborated by Vr9a and how does it relate to the vacuolar pigment? 3) Can pigment formation be elicited in whole plants of the species and cultivars from which Vr9a originated? 4) Can a treatment be found which elicits pigment production in Vr9, the parent clone? 5) By what mechanism is pigment formation depressed at high temperature? Is one of the phenylpropanoid biosynthetic enzymes naturally temperature sensitive? 6) Can the pigmentation phenotype be introduced into heterologous cells by transformation or protoplast fusion? 7) What is the nature of the regulatory events, at the DNA level, which brought about this phenotype? and 8) Can pigmented cells divide, or is the pigmentation phenotype functionally lethal?

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