STUDIES ON EXPONENTIAL CULTURES OF PLANT CELLS

Thesis by

Philip Filner

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This thesis is dedicated to my parents.

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ABSTRACT

The properties of tobacco cells growing exponentially in a chemically defined liquid medium were investigated for the purpose of characterizing the exponential plant cell culture and to explore its potential as a developmental model system.

The cells multiply exponentially with a generation time of 2 days, and exponential multiplication persists for 4.5 generations. The replication of DNA was studied by means of N^{15} density labeling. All the DNA in the cells replicates in about one generation, and replication proceeds by a semi-conservative mechanism. The average cell properties of the culture are not constant during the exponential phase, indicating that the population is not in a steady state condition. The number of cells per group, the water content per cell, the soluble protein content, and the rate of RNA synthesis vary. Enzyme levels of the soluble protein fraction do not vary in parallel with the protein content, but rather they vary in their own characteristic ways.

The changes in cell properties are related to changes in the chemical environment which the cells themselves bring about. The cells deplete the medium of phosphorus after three generations, and they deplete it of potassium and nitrogen by the end of the exponential phase. Nitrogen is

the growth limiting nutrient, and nitrogen may be supplied either as nitrate or as a complete amino acid mixture. The nitrate reductase (NR) level in the culture falls as the nitrate supply is depleted, so that the enzyme must be induced before the cells can grow when subcultured into a medium in which nitrate is the sole nitrogen source. There is a lag in the onset of mitosis which correlates with the time necessary for NR induction. The initial and terminal events of the exponential phase have been tentatively identified as the induction of NR and the depletion of nitrate, respectively.

The regulation of NR involves both substrate induction and end-product repression. NR is induced by nitrate and repressed by a complete amino acid mixture in proportion to the ability of the mixture to meet the nitrogen requirement of the cells. If amino acids are available at a sub-optimal level, and nitrate is also present, sufficient NR is induced and nitrate reduced to bring the nitrogen supply up to the optimal level.

The amino acids may be classed as repressors or derepressors, according to their action in the regulation of NR. The derepressors are arginine, lysine, cysteine and isoleucine. A single repressor inhibits the growth of the cells on a nitrate medium as a consequence of the condition of nitrogen starvation which results from NR repression. In the presence of a repressor and an appropriate derepressor,

NR is induced and the cells grow. Since the growth of the cells can be controlled through the NR regulatory mechanism, it has been suggested that this mechanism may be used by the whole organism to regulate the growth of its parts.

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I. INTRODUCTION

ORIGIN OF THE PROBLEM

The growing interest in problems of development among experimental biologists is typified by the series of lectures given on the subject by the faculty of the Division of Biology at the California Institute of Technology early in 1965. There is general agreement that we are on the threshold of a period of discovery in this exceedingly interesting, yet "underdeveloped" area of biology.

In order to study the mechanics of development, it is necessary to investigate systems that can undergo development, which ultimately means studying multicellular organisms. Among them, plants possess a characteristic that makes them particularly suitable for studies of development. That is the sensitivity of their developmental processes to the physical and chemical environment. Plants are responsive to gravity, light, temperature, inorganic nutrients and

organic compounds.

Presumably, the developmental responses to the environment have biochemical explanations. It seems clear that one way to reveal the biochemistry underlying developmental events in plants is to use their sensitivity to the environment as a tool for inducing controlled perturbations in their biochemistry, then to identify the perturbations and finally to trace them to their true cause in the cell.

Cell properties are more accessible in culture than in situ, and in the case of plant cells, it is well known that in culture they retain the ability to differentiate in response to the chemical environment(1, 2). Consequently, the plant cell culture system is very well suited in principle for the investigation of the cellular events that bring about differentiation and development.

The first step in the investigation of developmental biochemistry is to identify the relevant biochemical systems. Since plant development is sensitive to the environment, a search was conducted for properties of cultured cells that showed environment dependent variability. It became apparent in the course of the search that nitrogen utilization can be environmentally regulated in cultured plant cells, and furthermore, the regulation of nitrogen utilization is a major factor in the behavior of cultured plant cells. The potential importance of this regulatory mechanism in plant

development prompted a more detailed investigation of the mechanism.

BRIEF HISTORY OF CULTURED PLANT CELL RESEARCH

Plant cells began to be successfully cultured about thirty years ago, as a consequence of the development of suitable nutrient media (3, 4). The major difficulties with plant cell cultures have been their slow growth rates, which have tended to slow progress proportionately. Improved growth rates have been gradually obtained by adding specific growth substances to the medium (5); by adding complex medium supplements (6); by improving the balance of inorganic nutrients (7); and by the use of suspension culture methods (8).

Caplin and Steward (9) introduced to plant cell culture technique the use of liquid media and agitation for the purpose of aeration. Muir, Hildebrandt and Riker (10) appear to have been the first to grow true suspension cultures of higher plant cells. However, the potential of the suspension culture method only became apparent after the work of Tulecke and Nickell (8), who developed methods, media, and cell lines that enabled them to produce kilogram quantities of cultured cells in two weeks with little difficulty. Their methods made it feasible for the first

time to undertake biochemical investigations that require large quantities of rapidly multiplying plant cells.

Research on cultured plant cells has been largely concerned with nutrition (11), totipotency for development (12), tumorogenesis (13), virology (14), and the assay of plant growth substances (15). There has been relatively little biochemical research on cultured plant cells. Their ability to synthesize certain natural products has been studied (16, 17), and it has been compared with the biosynthetic activity of the plants from which they were derived (18, 19). The biosynthesis of amino acids has received some attention (20, 21). The biosynthesis of histone has been studied (22), and methods for preparation of RNA from cultured cells have been developed (23). Perhaps fifteen enzymes have been assayed in cultured plant cell extracts (24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34). There have also been some reports concerning the effects of ultraviolet and visible radiation on viability and growth (35).

II. THE CULTURE SYSTEM

INTRODUCTORY REMARKS

The essential components of any culture system are the culture conditions and handling procedures, the culture medium composition, and the cell line. Handling procedures and media are relatively uniform among cultured plant cell systems, largely due to the pioneer work of White(3). The more recently developed suspension culture systems generally follow the methodology of Tulecke and Nickell (8). Although plant cells have been studied in culture for thirty years, there have not developed any widely used standard cell lines comparable to the various strains of bacteria or animal tumor cell lines, with the result that there is a minimum of coherence in the work emanating from the many laboratories in which cultured plant cells are studied.

The culture system which has been developed for the present work employs media derived from that of White (3), the suspension culture methods of Tulecke and Nickell (8), and cell lines which were isolated from tobacco specifically

for this work. Tobacco was chosen as the cell source in order to gain the greatest benefit from the published findings of others. Tobacco cells have probably been studied in culture more extensively than any other kind of cell. They have been used in studies of nutrition (7), tumorogenesis (36), virus multiplication (14), differentiation (1), and biochemistry (17, 40, 41).

The cell lines are relatively homogeneous and relatively stable. They grow rapidly and reproducibly on a chemically defined medium. The medium lends itself to manipulation, and the cells are responsive to such manipulations. Handling procedures are both simple and convenient, and quantities of cells adequate for most preparative purposes can be produced with little difficulty. In general, the system is well suited for biochemical investigations.

CULTURE CONDITIONS

All cultures were grown in a room maintained at 27° C. The humidity was not controlled, and except when occupied, the room was kept dark.

Solid medium cultures were grown in 125 ml. Erlenmeyer flasks which were cotton stoppered and sealed with aluminum foil. Each flask contained 30 ml. of 0.8%

agar medium. Agar cultures were generally allowed to grow for one to two months.

Liquid medium cultures were grown in 500 ml. or 1 L. Erlenmeyer flasks containing 200 ml. or 500 ml. of medium respectively. These flasks were also cotton stoppered and sealed with aluminum foil. They were kept on a specially constructed horizontal platform reciprocal shaker. The shaker had five tiers which gave it a capacity of 200 one-L. flasks. It operated at 95 cycles per minute, with a horizontal displacement of 4.5 cm. Liquid cultures were usually grown for no more than two weeks.

Cell masses of 50-100 mg. fresh weight were used as initial inocula for agar cultures. Liquid cultures were started at an initial cell concentration of 4500 cells per ml., or about 1.3 mg. fresh weight per ml. Agar culture transfers were made with stainless steel spatulas, while liquid culture transfers were made with wide bore pipettes (Bellco). The overall contamination frequency during this work was between 1 and 5%.

PREPARATION OF MEDIA

All media used in this work contained the same basic mixture of inorganic salts, B-vitamins, sucrose, and 2,4-dichlorophenoxyacetic acid. The basal medium is called

M1D, while the basal medium plus a supplement of seventeen amino acids is called M2D, and the basal medium plus casein hydrolysate and malt extract, M31.

All media were prepared with glass distilled water. They were adjusted to pH 6.2-6.5 with NaOH before sterilization, which was accomplished by autoclaving at 20 lb. for 15 minutes.

The basal medium was prepared from six stock solutions and solid sucrose. The stock solutions were: WM-I: 20 g./L. $Ca(NO_3)_2 \cdot 4H_2O$, 8 g./L. KNO_3; WM-II: 1.65 g./L. NaH_2PO_4, 20 g./L. Na_2SO_4; WM-III: 36 g./L. $MgSO_4 \cdot 7H_2O$, 6.5 g./L. KCl; WM-IV: 2.0 g./L. $FeC_6H_5O_7 \cdot 3H_2O$ (ferric citrate), 4.5 g./L. $MnSO_4 \cdot 4H_2O$, 1.5 g./L. $ZnSO_4 \cdot 7H_2O$, 1.5 g./L. H_3BO_3, 0.75 g./L. KI; WM-V: 0.5 g./L. nicotinic acid, 0.1 g./L. pyridoxine hydrochloride, 0.1 g./L. thiamine hydrochloride; 2,4-D: 0.1 g./L. 2,4-dichlorophenoxyacetic acid.

One liter of M1D was prepared by mixing 10 ml. WM-I, 10 ml. WM-II, 10 ml. WM-III, 1 ml. WM-IV, 1 ml. WM-V, and 5 ml. 2,4-D, to which was added 20 grams of solid sucrose and 500 ml H_2O. The solution was adjusted with NaOH and more H_2O was added to bring the volume to 1 L. The medium was then distributed among culture flasks and sterilized immediately. The final concentrations of ingredients in M1D are given in Table I.

M2D was prepared similarly, except that 20 ml. of an amino acid stock solution was added before the pH was adjusted, so that M2D contained 212 mg. amino acids per

Table I

A. Composition of M1D

Constituent	Moles/L x 10^5
$Ca(NO_3)_2.4\ H_2O$	84.8
KNO_3	79.1
$NaH_2PO_4.H_2O$	11.9
Na_2SO_4	141.
$MgSO_4.7\ H_2O$	146.
KCl	87.1
$FeC_6H_5O_7.3\ H_2O$	0.669
$MnSO_4.4H_2O$	2.02
$ZnSO_4.7\ H_2O$	0.52
H_3BO_3	2.42
KI	0.451
Nicotinic acid	0.407
Pyridoxine HCl	0.0485
Thiamine HCl	0.0297
2,4-dichlorophenoxyacetic acid	0.226
Sucrose	5,840.

B. Inorganic Ions in M1D

Ion	Moles/L x 10^5
Na^+	294.
K^+	167.
Mg^{+2}	146.
Ca^{+2}	84.8
Mn^{+2}	2.02
Fe^{+3}	0.669
Zn^{+2}	0.520
SO_4^{-2}	290.
NO_3^-	249.
Cl^-	87.1
PO_4^{-3}	11.9
BO_3^{-3}	2.42
I^-	0.451

liter. The amino acid stock solution contained per liter: 2.5 g. L-glutamate, 1.6 g. L-phenylalanine, 0.9 g. L-aspartate, 0.8 g. L-proline, 0.65 g. L-lysine, 0.55 g. L-histidine, 0.5 g. L-serine, 0.4 g. L-alanine, 0.4 g. L-threonine, 0.4 g. L-valine, 0.35 g. L-cysteine, 0.35 g. L-methionine, 0.25 g. L-arginine, 0.25 g. glycine, 0.25 g. L-isoleucine, 0.25 g. L-leucine, 0.2 g. L-tryptophan.

M-31 was prepared in a manner identical to the preparation of M1D, except that 500 mg. of malt extract (Difco) and 200 mg. of casein hydrolysate (2.0 ml. of Nutritional Biochemicals Co. vitamin free 10% casein acid hydrolysate, or 200 mg. of Difco Casamino Acids) were added before adjusting the pH.

Agar media were prepared by making up the nutrient mixture for a given medium at two-fold concentration, then mixing it with an equal volume of melted 1.6% agar (Difco Bacto-Agar). The agar medium was then distributed among culture flasks and sterilized in the usual manner.

HISTORY OF THE CELL LINES

A derivative of Turkish tobacco, called Nicotiana tabacum var. Xanthi, was used as the cell source. The derivative gives a local lesion response to Tobacco Mosaic Virus (TMV) in contrast to the systemic response of Turkish.

The plants were grown in Campbell Plant Research Laboratory until they were about 120 cm. high, whereupon the stems were harvested and cut into 20 cm. sections. In a sterile transfer room, the stem sections were surface sterilized by immersion in 1% sodium hypochlorite (Purex bleach diluted 1 : 4.25 with H_2O) for five minutes, followed by two successive five minute rinses in sterile distilled water. Then with the aid of sterile forceps and scalpels, a region of each stem section was peeled down to the pith. The pith was cut into cubes of tissue weighing between 200 and 300 mg. fresh weight, and four cubes from each section were planted on agar M31.

Cell proliferation was apparent after 10 days. It occurred at both ends of each section, and no difference was encountered among stem sections from the various positions in the whole plant. The cell mass (callus) produced was soft and friable. Cells could be picked up by merely drawing a spatula over the surface of the callus. The friable growth characteristic of tobacco on M31 seems to be due to the combination of malt extract and 2,4-D, since Bellamy was able to induce friable growth from hard,woody callus of Nicotiana tabacum var. Wisconsin 38 which had been growing on Murashige and Skoog medium (7). Bellamy merely supplemented the Murashige and Skoog medium with malt extract and 2,4-D at the concentrations in M31 (23, 37). Both Turkish and Wisconsin 38 tobacco produced soft friable

callus when cultured on M31 in this laboratory.

After three serial subcultures on agar M31, the cells were introduced to liquid M31 by dispersing a one month old callus in the liquid form of the medium, with continuous shaking for aeration. The cells multiplied for one week, but then a large portion of the cells died. The survivors continued to multiply, and did not again go through the dying phase in subsequent subcultures in liquid M31. The cells were serially subcultured in M31 more than ninety times over a three year period.

At the eleventh subculture after isolation, some cells which had been growing in liquid M31 were subcultured into liquid M31 lacking malt extract. The cells grew as well in this simplified medium as they did on M31. After one more subculture in this simplified medium, which contained the basal medium plus casein hydrolysate, they were subcultured into M2D, the basal medium plus seventeen amino acids. The cells grew immediately on M2D and appeared normal in every way. They were not again examined closely until the twenty-fourth subculture after isolation, at which time it was discovered that the cells grown on M2D had developed a strikingly different growth morphology from cells grown on M31.

The cells from M2D cultures grew as filamentous chains of cylindrical cells, while the cells continuously grown on M31 grew as clusters of spherical cells, which was the appearance of the cells in the earliest liquid medium

cultures. The filamentous chain formers persisted in their growth pattern even when returned to M31 for many generations. Thus the morphological change was not a reversible effect of the chemical environment.

The parent line also underwent an irreversible change during the same time period, since it could no longer generate a filament forming cell line when cultured in M2D for many generations. Furthermore, the cluster formers at the thirteenth subculture grew equally rapidly on M2D and M31, but at the twenty-fourth subculture, they grew about one-third as rapidly on M2D as on M31. The cluster formers have been named the "X" line and the filament formers have been named the "XD" line.

After maintaining XD cells on M2D from the twenty-fourth through the forty-second subculture, it was discovered that they grow equally well on the basal medium M1D, which lacks the amino acid mixture present in M2D. The XD cells have been cultured on M1D and M2D in separate but parallel lines for more than forty subcultures, and in that time no known differences between them have developed.

The chronology of the development of the X and XD cell lines is summarized in figure 1.

MORPHOLOGICAL FEATURES OF THE CULTURES

Both X and XD cells grow on agar as an amorphous pile

Chronological Development of the Culture System

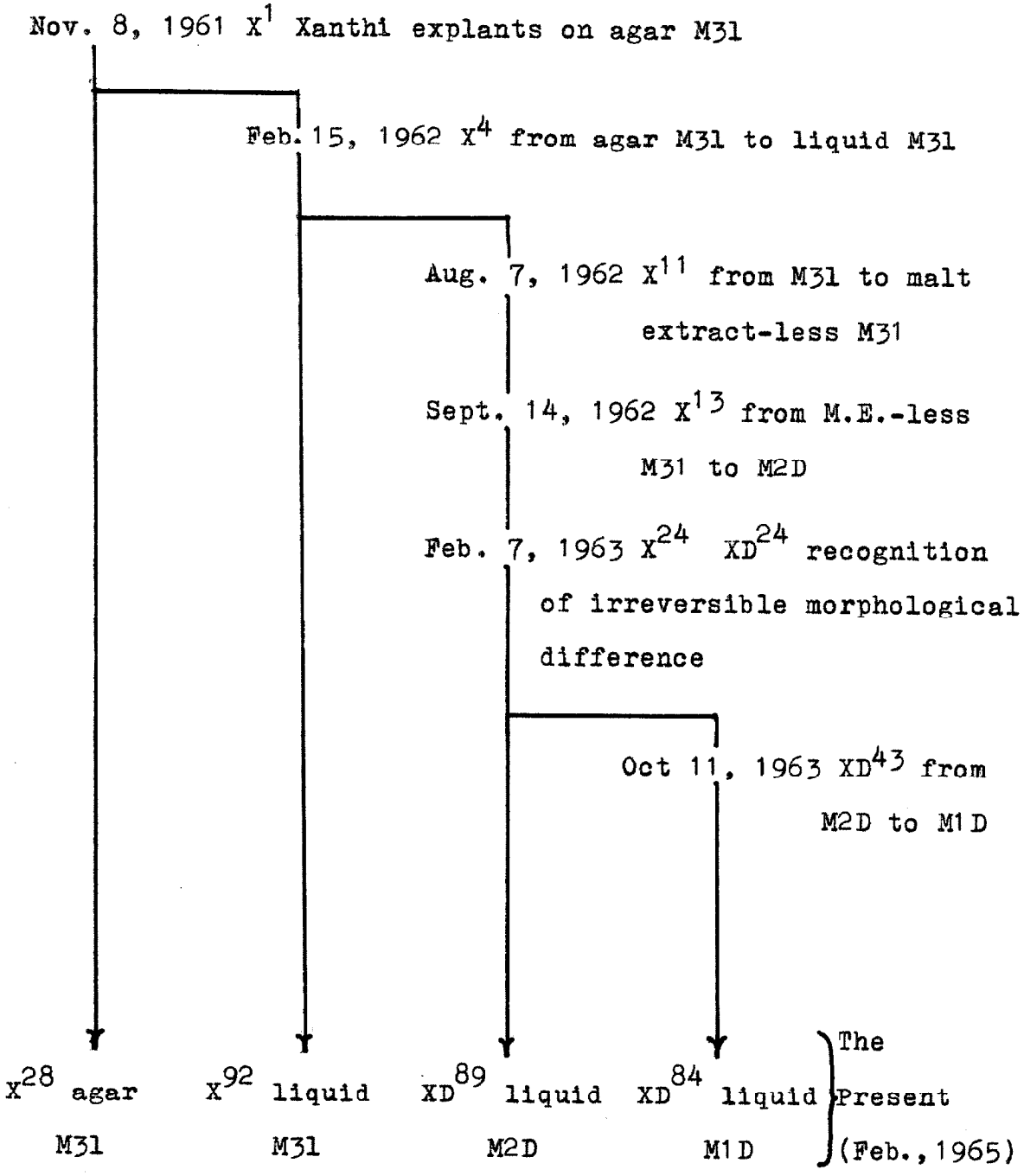


Figure 1

of loosely associated cells. Neither macroscopic nor microscopic organization has ever been seen in agar cultures of these cell lines. It seems fairly likely that intimate association of the cells in a mass is an essential prerequisite to organized growth. This kind of cell association occurs in hard, woody callus, and it is hard, woody callus which has been frequently observed to differentiate. Culture conditions and cells were selected in this system to promote the production of soft friable callus, and this was probably equivalent to selecting against cells and conditions which would lead to differentiation and organized growth.

Microscopic examination of X or XD cells grown on agar reveals that they have essentially identical cell morphology under these culture conditions, in contrast to the morphological difference seen in liquid cultures. On agar they are predominantly spherical, about 400 microns in diameter. They are highly vacuolated, almost completely transparent, and very weakly pigmented. The pigmentation is of a pale yellow-green color. The cells are mononucleate, and the nucleolus is usually prominent. The cells contain numerous amyloplasts, usually concentrated in the vicinity of the nucleus. The cytoplasm is distributed as a thin layer on the inner surface of the cell wall and a thicker layer around the nucleus. The two regions are connected by transvacuolar strands in which there is usually active cytoplasmic streaming. The nucleus appears to be suspended

in the middle of the vacuole by means of the transvacuolar strands. Some examples of typical agar grown tobacco cells can be seen in figure 2.

The description of cells grown on agar applies to cells in old liquid medium cultures, with the exception that in liquid medium the shapes of the cells are more irregular. The cells in young rapidly dividing cultures, however, are much smaller, their largest dimension being about 80 microns. They are also more intimately associated in their cell groups than the cells of old cultures. The X cells form large clusters of tightly packed spherical cells during the period of rapid multiplication, while the XD cells form long smooth-surfaced cylindrical filaments which are segmented into cells. As a culture of X cells ages, they become larger, less tightly packed in the clusters, and the number of cells per cluster decreases. As a culture of XD cells ages, a similar phenomenon occurs. The cell groups change from smooth-surfaced segmented filaments to chains resembling links of sausage. The cells also get larger, and the number of cells per chain decreases. Photographs of young and old X and XD cells from liquid medium cultures are presented in figure 3.

The morphological cycle which is seen in suspension cultures can be considered to be a consequence of changes in the relative rates of three processes: cell expansion, cell division and daughter cell dissociation. When old cells are subcultured into fresh medium, there is a marked increase in

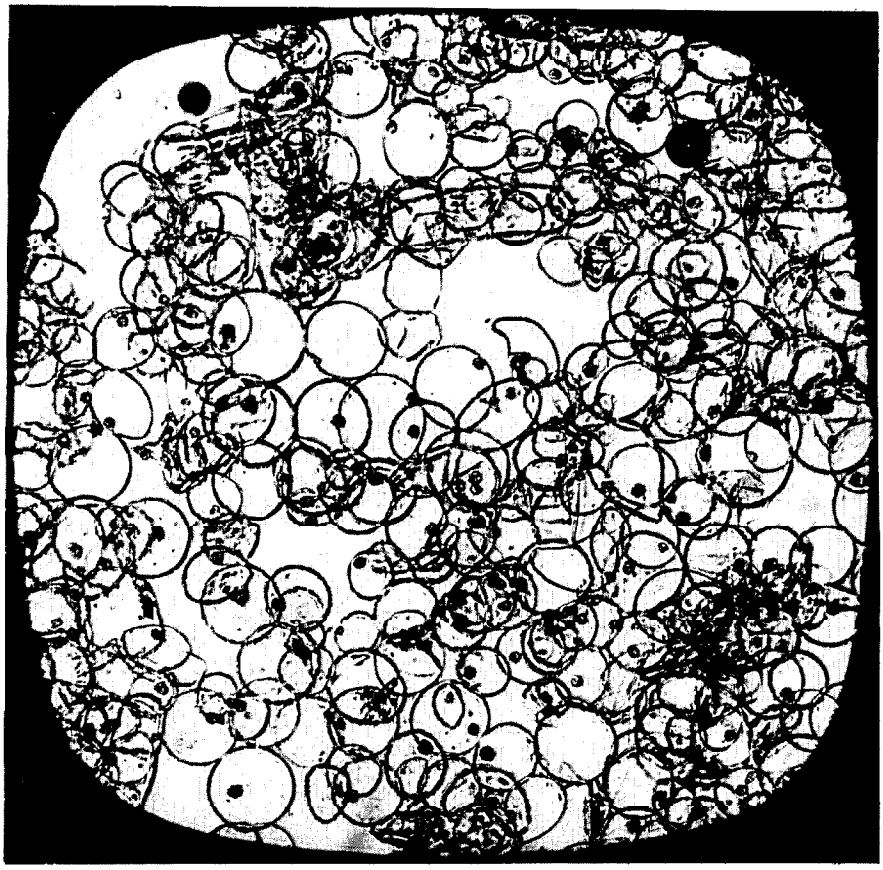


Figure 2

X Cells grown on agar M31. 50 X.

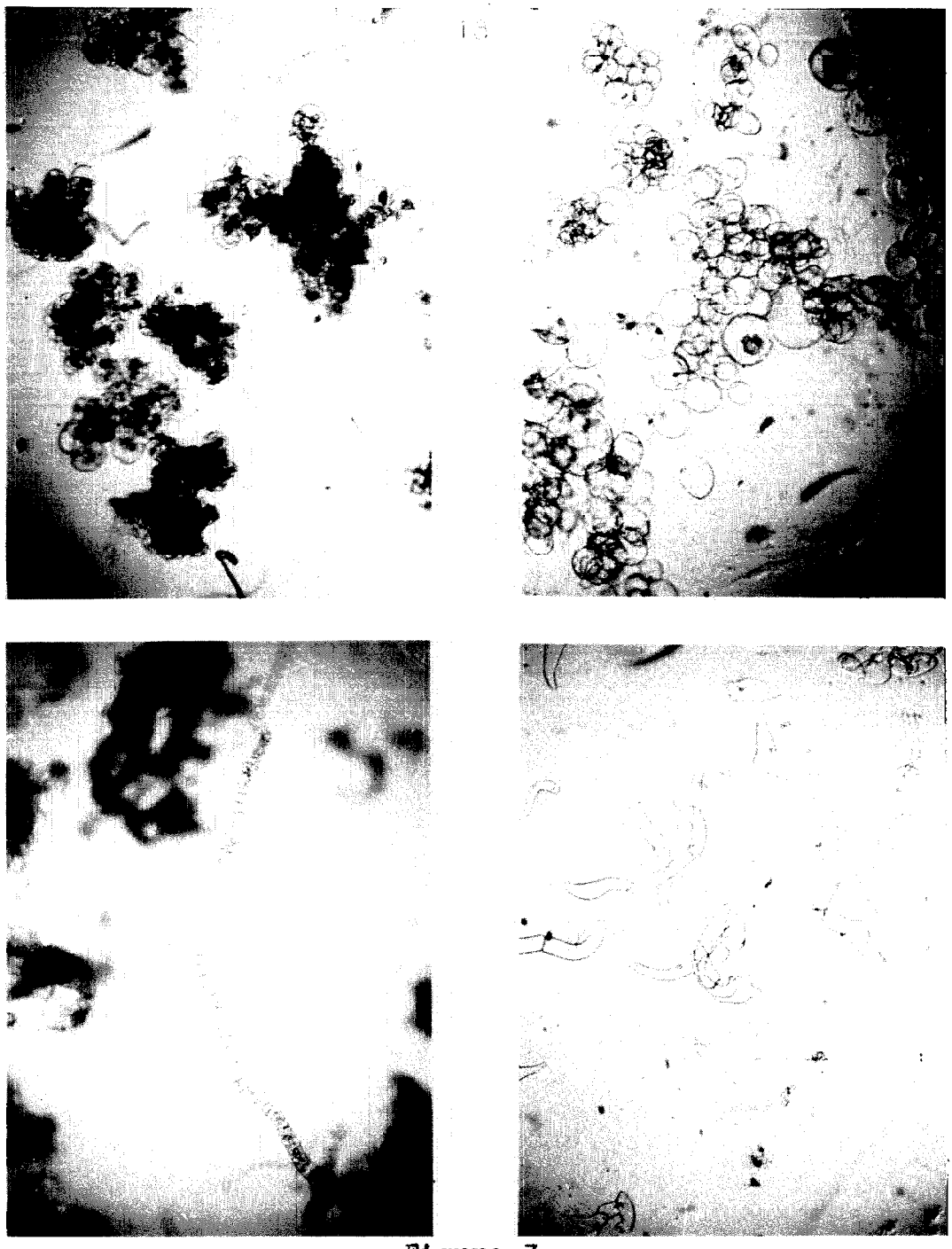


Figure 3

Top: X cells grown in liquid M31. Top left: Exponential Phase. Top right: Stationary phase. Bottom: XD cells grown in liquid M1D. Bottom left: Exponential Phase. Bottom right: Stationary Phase. 70 X.

the rate of cell division relative to the rates of expansion and dissociation, with the result that the cells generated are smaller and are associated in larger groups. When the culture begins to age, the rate of cell division decreases relative to the rates of expansion and dissociation, with the result that cells become large, and the number of cells per group decreases. A more quantitative treatment of these three processes will be presented in the section dealing with growth kinetics.

CONCLUDING REMARKS

Two cell lines derived from Nicotiana tabacum var. Xanthi have been developed. They can be grown on solid or liquid media under conditions quite similar to those employed in previously described systems (38, 39). Both cell lines grow as soft, friable, non-differentiating callus on agar, where their morphologies are virtually identical, in contrast to their distinct morphologies in liquid. The X line grows as clusters of spherical cells in liquid, and it grows better on the complex medium M31 than on the chemically defined basal medium M1D. The XD line grows as cylindrical filaments of cells in liquid, and grows equally well on M31 or M1D. Both cell lines undergo a transient morphological change in the size and shape of the average cell, as well as in the number of cells per group, and the change is related to the

age of the culture.

The basic value of this culture system lies in the opportunity it offers for studying the biochemistry of plant cells free from the constraints of the plant. The possibility of studying the response of plant cells to carefully controlled perturbatations of the chemical environment should prove particularly valuable, since it is the difficulty in performing such experiments in the whole organism which is the major block to an effective analysis of the behaviour of the cell in the midst of the organism.

In addition to offering active cells in a defined medium for study, the system includes two morphologically different (differentiated?) cell types. The two patterns of growth which they represent are encountered in the first steps in the development of the plant embryo, which first generates a filament of cells, and then differentiates at one end to form a cluster of cells. A comparative study of the biochemistry of the two cell types, or of the transition from one to the other, may provide clues to the basis of similar events in the developing embryo.

It has been pointed out by others (14) that the transient morphological change which depends on the culture age resembles the transition from meristematic to parenchymal cells in the growing tip of the plant. Not only is the morphology similar, but in both cases the transition parallels a change from rapid to slow cell multiplication.

It may be that a complete picture of the underlying events associated with this transition in culture will provide a basis for assembling a suitable picture of the events in the organism.

III. GROWTH KINETICS

INTRODUCTORY REMARKS

Before inquiring into how things are done by a cell, it is first necessary to form a clear idea of what things there are that the cell does. A study of the kinetics of culture growth can provide a base upon which can be constructed a useful quantitative description of some of the things a cell does in culture. The life of the culture can be divided into distinct phases, according to the kinetic behavior. The transition periods between phases are particularly interesting, because they are indicative of the occurrence of critical events within the cells, such that the conditions manifested in the phase before the transition cannot be maintained by the cells after the transition. It should be possible to deduce the critical events, and their relationship to the apparatus of the cell by studying the biochemistry of the transition period.

The major phase of any culture system is the period

of most rapid growth, and the most important transitions are those which mark the beginning and end of that period. It is obvious that the metabolism associated with cell multiplication must be switched on at the beginning of this period, and switched off at the end. In the present section, we shall be concerned with establishing the time of occurrence of these two transitions in standard tobacco cell suspension cultures. In later sections, the underlying biochemical events of the transition periods will be considered.

In addition to defining culture phases, kinetic analysis based on several parameters can provide information on the variability of the cells during a given phase. If a given set of parameters remain in fixed ratios to each other during a given culture phase, then it may be said that the cells are invariant by these criteria during this phase. If, on the other hand, the ratios of the given set of properties do not remain fixed, then the average cell in the culture will possess different characteristics at different times during the phase in question. The variability of some cellular characteristics during the period of most rapid growth of tobacco cells in suspension culture will also be considered in this section. These parameters provide a more refined analysis of the phases which occur in the life of a suspension culture.

METHODS

Cell titer growth curves were obtained from single cultures. The cultures were periodically sampled, and the sample was diluted so that the cell titer was reduced to between 5,000 and 10,000 cells/ml. A 0.2 ml. aliquot of the diluted suspension was placed on a slide and covered with a 22 x 50 mm coverglass. The area under the coverglass was systematically scanned and the cells in each cell group counted and recorded, according to the number of cells in the group. This counting method provides, in addition to the cell titer growth curve, a cell group growth curve and the distribution of cells according to the number of cells per group. The number of cells in large groups was estimated since they could not be counted accurately. Titers determined for replicate samples using this counting method and counting 1,000 to 2,000 cells per sample vary about $\pm$15% from the mean. The variation is due to the sampling problem rather than the counting. The cell groups are physically large and do not stay uniformly suspended in the liquid column of the pipette.

Fresh weight and dry weight growth curves were obtained from a set of cultures rather than a single culture. Thirty cultures were started simultaneously from one parent culture, and two cultures harvested daily. The cells were collected separately from both cultures by filtration

through Miracloth. The excess liquid was squeezed out by hand, the cells were scraped from the filter, and the fresh weight was determined in a tared beaker. Dry weight was determined after drying at 60° C. for 48 hours.

The "soluble" protein fraction was prepared for assay by homogenizing the cells in ice cold 0.1 M tris 0.001 M cysteine HCl, pH 7.5, 5.0 ml per gram fresh weight of cells. Homogenization was achieved by thirty strokes of a Thomas glass-teflon homogenizer with a motor driven pestle. The homogenate was centrifuged at 10,000 rpm for 20 minutes in a refrigerated Servall SS-1 rotor. Protein was determined on 1.0 ml of the supernatant. It was first precipitated with an equal volume of 20% trichloroacetic acid(TCA). After 12 hours the precipitate was collected by centrifugation for 10 minutes at 3,000 rpm, the pellet resuspended in 10% TCA and heated at 90° C. for 20 minutes. After cooling, the precipitate was again collected by centrifugation. It was resuspended in 10 ml of 90% ethanol, and once more collected by centrifugation. The ethanol-washed precipitate was air dried and dissolved in 1.0 ml 1 N NaOH. The protein content of a 0.1 ml aliquot of the NaOH solution was determined by the method of Lowry, Rosebrough, Farr and Randall (42). bovine serum albumin (Nutritional Biochemicals Co.) dissolved in 1 N NaOH was used as the standard.

Acid phosphatase activity in the soluble protein fraction was assayed in 0.1 M sodium acetate-acetic acid

buffer at pH 5.3, with p-nitrophenyl phosphate as the substrate. The reaction mixture contained 1.0 ml p-nitrophenyl phosphate, 0.8 ml buffer, and 0.2 ml enzyme. Two identical mixtures were used for each assay, one to serve as an unincubated blank, and the other to be incubated at 25° C. for 15 minutes. The reaction was stopped at zero time and 15 minutes, respectively, by the addition of 1.0 ml 60% perchloric acid and 5.0 ml H_2O. Phosphate was determined by a modification of the method of Sumner (43). One ml of $FeSO_4$ reducer (8 g $FeSO_4$ in 100 ml 0.3 N H_2SO_4) and 1.0 ml of molybdate reagent (8 grams ammonium molybdate in 100 ml 7.5 N H_2SO_4) were added and the A_{660} was measured after 30 minutes, using the unincubated reaction mixture as the blank. Phosphate was computed from the absorption produced by a standard amount of KH_2PO_4.

The fraction of cells in mitosis was determined on an aliquot of a culture which was fixed and stained with ferric-acetocarmine, prepared according to Sass (44). The cells were collected by centrifuging at low speed for a few seconds. The medium was decanted and a volume of stain equal to the packed cell volume was added. The cells were allowed to take up the stain for ten minutes, and then a portion of the dense suspension was transferred to a slide. The cells were dispersed with the aid of the flattened end of a glass rod. A coverglass was placed over the cells and excess stain drained off. The slide was passed quickly through a bunsen

burner flame several times, taking care not to boil the liquid. The cells were then squashed by pressing the slide uniformly. The preparations were sealed by painting the edge of the coverglass with a saturated solution of polyvinyl pyrrolidone. Mitotic figures were counted within twelve hours of making the preparations. Counts were made on randomly selected fields until 1500 cells had been examined. All cells which contained distinct mitotic figures from prophase through telophase were classed as being in mitosis. The per cent of cells in mitosis was computed from the count.

THE CULTURE PHASES

The growth curves of XD cells in M1D, as obtained by following cell titer, dry weight, group titer, fresh weight, and soluble protein are presented in figures 4 through 8. The data are plotted semi-logarithmically in order to show how nearly exponential the rate laws are which govern the increases of these parameters. All the parameters undergo a 25-fold increase during the life of the standard culture, which is consistent with the fact that new cultures are routinely started by diluting an aliquot of a twelve day old culture 25-fold into fresh medium. Not all the parameters increase according to a simple exponential rate law. In

fact, only the cell titer and dry weight conform to such a law. Both exhibit a generation time of two days, so dry mass per cell cannot vary greatly during the life of the culture. In a true exponential steady state population, this would hold for all cell parameters. The XD cells in a standard culture clearly do not maintain a steady state condition, and yet they multiply exponentially. This becomes evident as soon as the other growth curves are examined and compared with the cell titer and dry mass growth curves. The suspension culture cells must be able to maintain the processes required for exponential multiplication as long as the cell properties fall within rather flexible limits.

Under the standard culture conditions, the exponential growth period begins either with no lag, or at most with a lag of something less than one day. Progressively longer lags were obtained with progressively lower initial cell titers. The end of the exponential growth period falls on the ninth or the tenth day, which means that the cells multiply exponentially for between 4 and 5 generations.

The group titer growth curve indicates that during the first four days, i.e., the first two generations, there is no increase in the number of cell groups. This means that the daughter cells produced in the first two cell divisions do not dissociate, with the result that the average number of cells per group increases approximately 4-fold. The variation of the number of cells per group as a

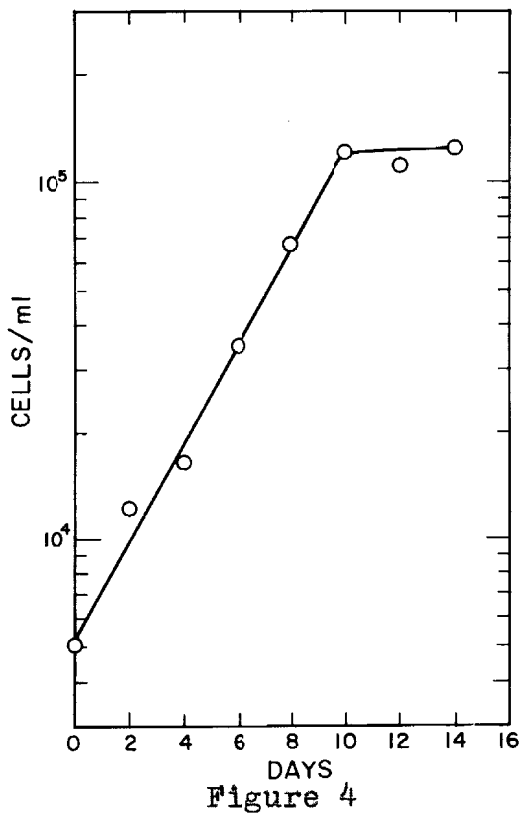


Figure 4

Growth Curve of XD Cells in MID:

Cell titer Assay.

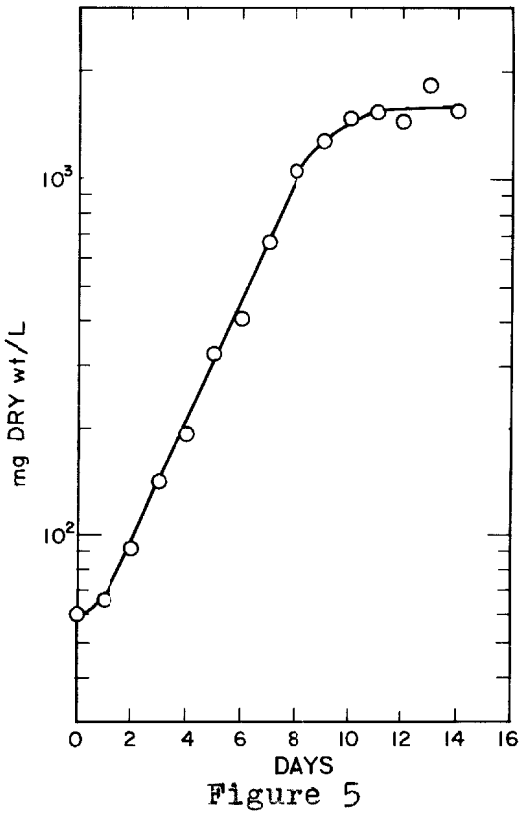


Figure 5

Growth Curve of XD Cells in MID:

Dry weight assay.

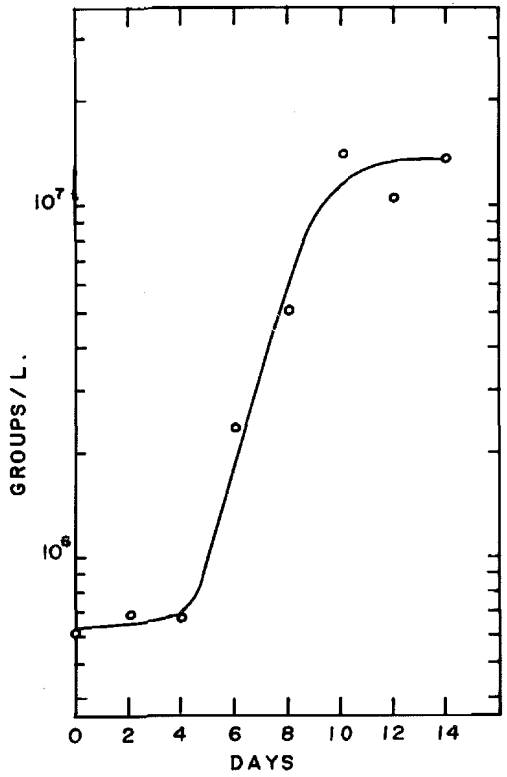


Figure 6

Growth Curve of XD Cells in MID: Group titer assay.

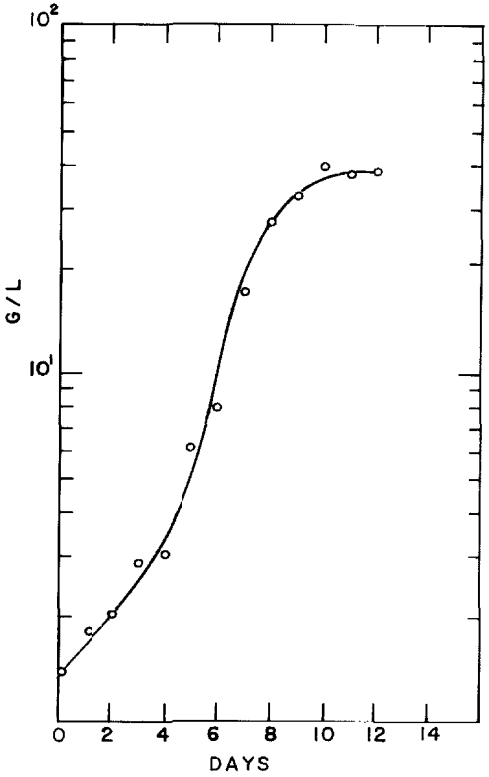


Figure 7

Growth Curve of XD Cells in MID: Fresh weight assay.

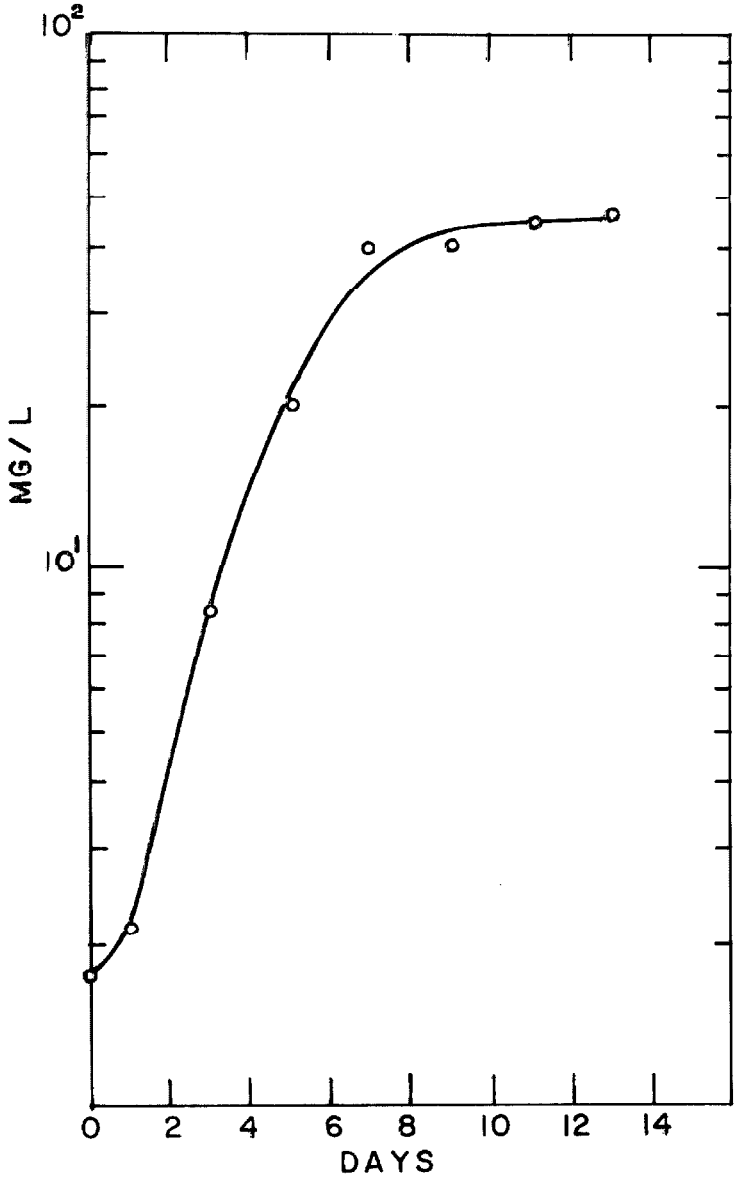


Figure 8

Growth Curve of XD Cells in MID:

Soluble protein assay.

function of culture age is plotted in figure 9. During the second half of the life of the culture, daughter cell dissociation must occur at a greater rate than cell division, since the average number of cells per group drops to the initial level. It may be concluded that on about the fourth day some critical change occurs in the culture which results in rapid dissociation of daughter cells. It is interesting to note that the dissociation process ceases or greatly slows down after the tenth day, indicating another change in the culture.

The initiation of the dissociation process in XD cultures seems to be dependent on cell titer, rather than on age of the culture. In experiments with lower initial cell titers, the cells went through several more cell divisions before they attained the concentration of a 4-day old standard culture. These divisions occurred without dissociation and generated very large cell groups. As soon as the culture reached a high titer, these large groups dissociated.

The fresh weight growth curve deviates less drastically from an exponential rate law. As can be seen in figure 10, the fresh weight per cell passes through a minimum and a maximum. There is more than a 2-fold difference between the two extremes. The pattern suggests that there are changes in the water uptake process on days 4, 8 and 10.

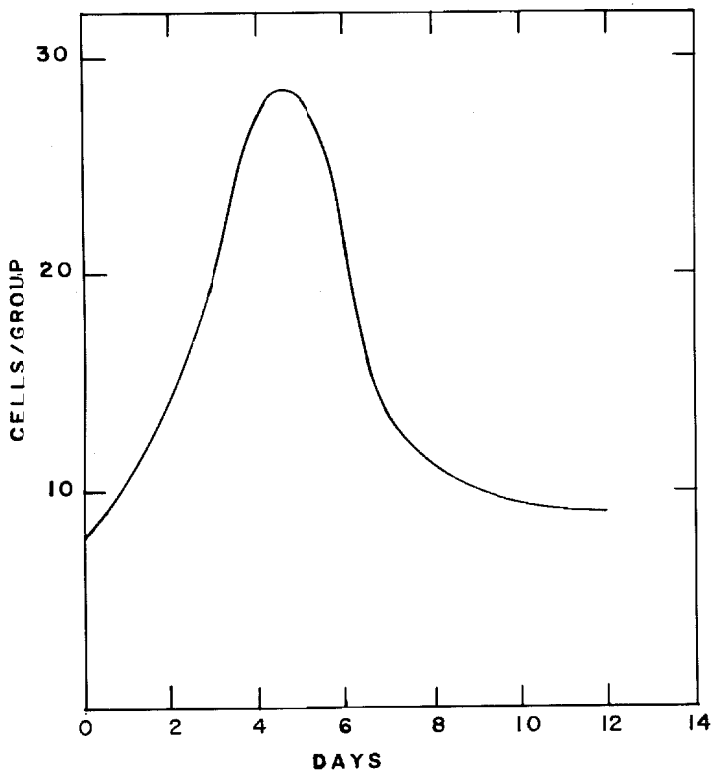


Figure 9

Variation of cells per group with XD culture age. Curve derived from figures 4 and 6.

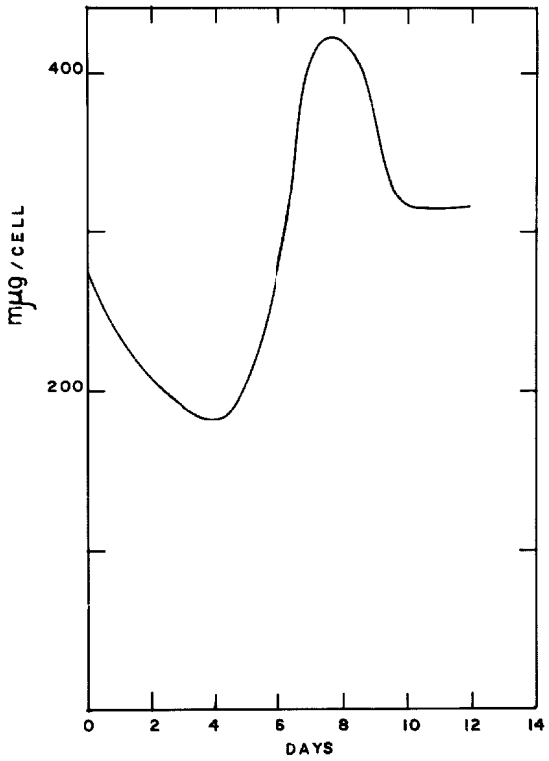


Figure 10

Variation of fresh weight (water content) per cell with XD cell culture age. Curve derived from figures 4 and 7.

The soluble protein growth curve has two unique features. The first is that soluble protein increases more rapidly than the cells multiply. The second is that the 25-fold increase in culture soluble protein is reached in 7 days, while all the other parameters require ten days. The net result of this phenomenon is illustrated in figure 11, which presents the variation of soluble protein per cell as a function of culture age. There is a symmetric increase and decrease, with the maximum being reached at about the fifth day. The soluble protein per cell at that point is 2.5 times the initial amount. Soluble protein accounts for about 40% of the non-wall-bound protein in cultured plant cells. Protein in the other cell fractions increases and decreases in a manner quite similar to that of the soluble protein fraction.

These very substantial increases in cell protein may be due to either a general increase in the levels of all proteins, or they may be due to regulated, selected increases in the levels of only certain proteins. This matter was resolved by determining the variation with culture age of acid phosphatase and nitrate reductase in the soluble protein fraction. The acid phosphatase curve is presented in figure 12. The nitrate reductase curve can be found in figure 39. It can be seen that acid phosphatase does not increase with increasing soluble protein. In fact, the enzyme's specific activity drops about 3-fold, which is

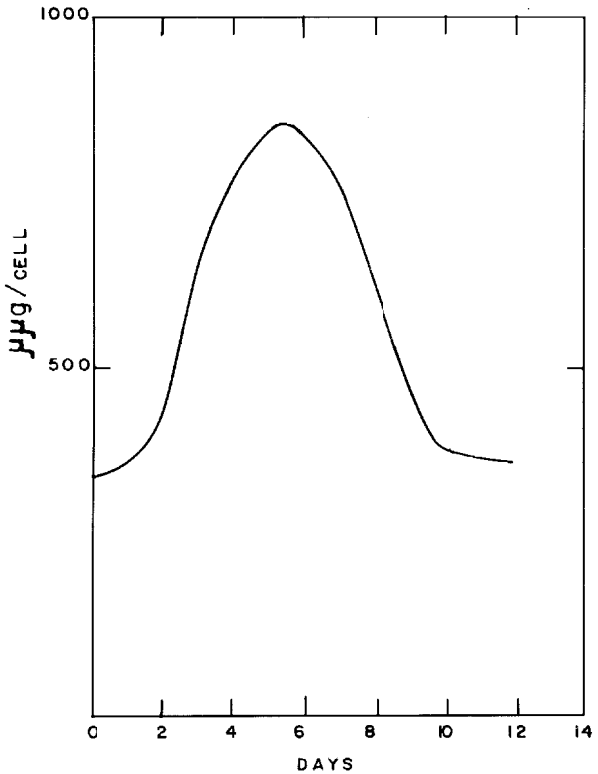


Figure 11

Variation of soluble protein with XD cell culture age. Curve derived from figures 4 and 8.

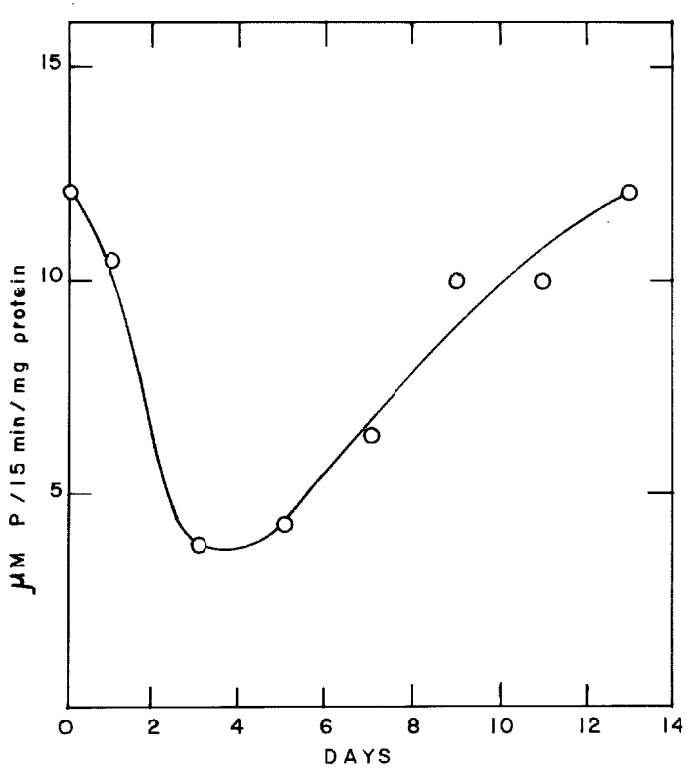


Figure 12

Variation of acid phosphatase specific activity in the soluble protein fraction with XD cell culture age.

just about the amount that protein increases. Nitrate reductase, on the other hand, increases many times more than soluble protein, so that its specific activity actually increases. The increases in soluble protein clearly are indicative of increases of selected proteins rather than all proteins, if the levels of the enzyme activities are proportional to the concentrations of the enzyme molecules.

The cell titer growth curve extrapolates to zero time, and thus suggests that there is no lag in cell division initiation when non-dividing cells are subcultured into fresh medium. This matter was re-examined by determining the per cent of cells in mitosis. It was found that there is a sixteen hour delay in the onset of mitosis (figure 13). This mitotic delay probably indicates the time necessary for non-dividing cells to modify their metabolism so that it is suitable for cell multiplication.

The picture which has been constructed for XD cells in M1D is generally applicable to X cells also, with the notable exception that the group titer increases exponentially with approximately the same generation time as the cell titer. This parallelism is illustrated in figure 14. As a consequence of this situation, and in contrast to XD cells, the average number of cells per group varies very little during the exponential phase of an X cell culture. Moreover, the frequency distribution of groups is approximately constant during the exponential phase. Group frequency falls off

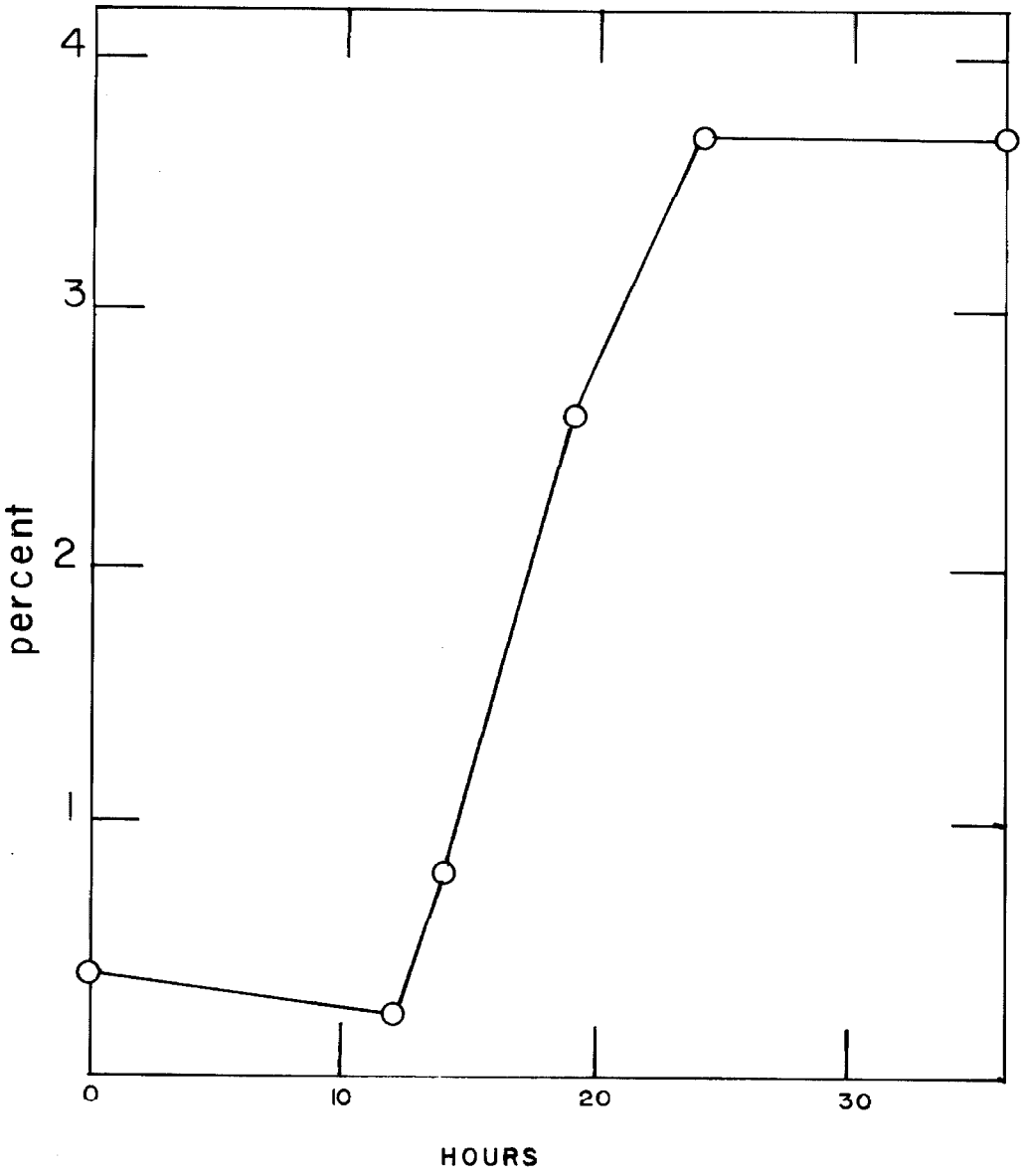


Figure 13

The percent of cells in mitosis as a function of time after inoculating MID with XD cells from a 12 day old culture.

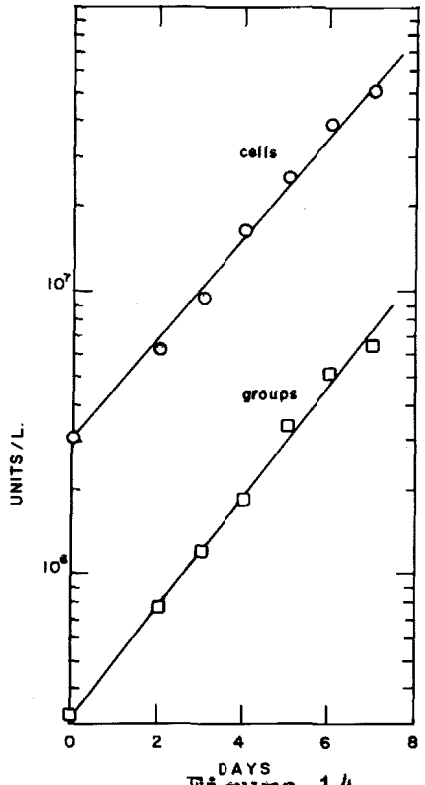


Figure 14

Growth Curves of X Cells in M31: Cell Titer and Group Titer assay.

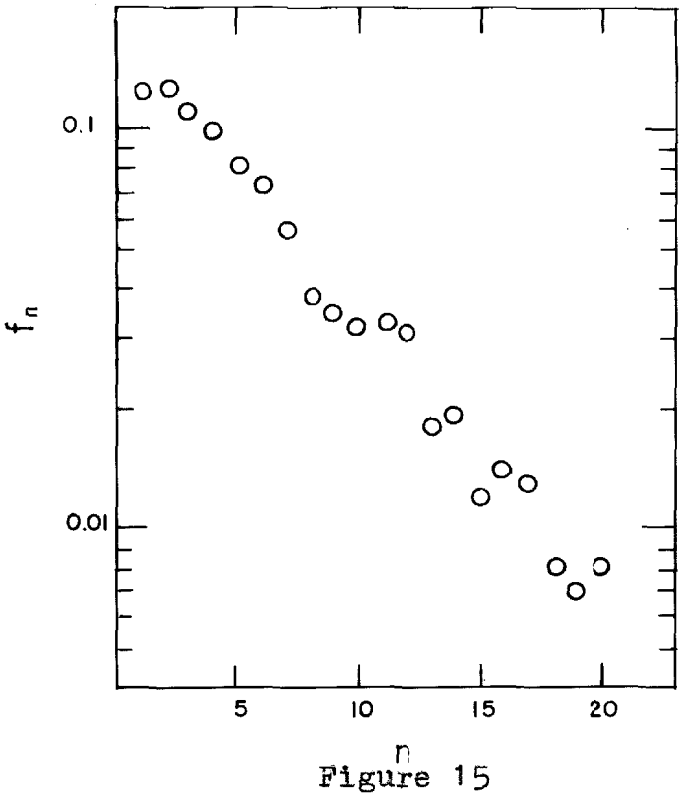


Figure 15

The frequency distribution of groups containing n cells per group in cultures of X cells in M31.

exponentially with n, the number of cells per group, according to the equation:

$$f_n = \frac{G_n}{\sum_n G_n} = \frac{1}{\bar{n}}\left(1 - \frac{1}{\bar{n}}\right)^{n-1}$$

$n = 1, 2, 3, \ldots$

G_n = concentration of Groups of class n

$$\bar{n} = \frac{\sum_n n G_n}{\sum_n G_n}$$

The observed frequency distribution is presented in figure 15. This is the result that would be expected if cell division and daughter cell dissociation were two independently occurring random events, with the rate constant for cell division greater than that for cell dissociation.

CONCLUDING REMARKS

The culture life cycle begins with a sixteen hour long period which precedes mitosis. The cells multiply exponentially both in number and mass with a doubling time of 2 days. Exponential growth persists for between 4 and 5 generations. Although the cells multiply exponentially,

their average properties are not constant during any part of the exponential phase. Daughter cell dissociation does not occur during the first two generations. During the next two generations it occurs at a rate which exceeds the cell multiplication rate. Daughter cell dissociation ceases at the tenth day. The average wet weight per cell drops during the first four days, rises during the next four days, and drops again to day ten. The soluble protein per cell increases until about the fifth day and then declines gradually to the initial level. The increase in soluble protein appears to be due to production of selected proteins rather than to a general increase in protein production.

The first phase in the culture is the sixteen hour lag. The second phase lasts through the fourth day. During this period, there is no cell dissociation, a reduced uptake of water per cell, and increased soluble protein per cell. The next phase lasts through the eighth day. It is characterized by a sharp rise in water uptake per cell, a sharp rise in the rate of daughter cell dissociation, and decreased soluble protein per cell. The fourth phase lasts from the eighth to the tenth day. Daughter cell dissociation ceases, cell division ceases, soluble protein falls, and water per cell falls. The last phase extends from the tenth day to the fourteenth. During this period, the cells are unchanged. The second, third, and fourth phases together comprise the phase of exponential growth.

This analysis of the culture life cycle indicates that the plant cell suspension culture system is not as simple as it could be. There are two alternatives: to attempt to simplify the system, or to study it as it is. The second alternative was chosen, in the belief that the variability of the cell properties with culture age could be used as a lever to get at some of the rules which govern the behavior of the plant cell. To put it another way, if the system could be simplified and the variability of the cell properties could be eliminated, the first thing which would be done in the course of studying the cells would be to perturb them and observe their response to the perturbation. The suspension culture system apparently comes with built-in self-generating perturbations, so it is sensible to first study them, rather than to arbitrarily generate some other perturbation and study it.

IV. REPLICATION OF DNA

INTRODUCTORY REMARKS

Once it was established that XD cells in M1D multiplied exponentially, it became feasible to study the mechanism of DNA replication in the cells. The elegant experiments of Taylor, Woods and Hughes (45), using thymidine-H^3 autoradiography, established that the DNA of a higher plant cell chromosome is equally distributed among two subunits of the chromosome, and that these two subunits extend the full length of the chromosome. The subunits segregate during chromosome duplication, one going to each daughter chromosome, and they are conserved through at least two rounds of chromosome duplication. The relationship of DNA structure to chromosomal subunit structure was not determined by these workers, so that the question of whether single whole DNA molecules, groups of whole DNA molecules, or parts of whole DNA molecules are conserved during chromosomal duplication remained unanswered.

According to the Watson-Crick hypothesis (46), the single strands of the double stranded DNA molecule should be conserved during replication of DNA, and should segregate among the daughter DNA molecules (47). To test this hypothesis, Meselson and Stahl (48) studied the replication of DNA in exponential cultures of Escherichia coli. They first density labeled the DNA by allowing the bacteria to multiply in medium containing inorganic forms of N^{15}. The labeled cells were then transferred to N^{14} medium, and after various amounts of cell multiplication in N^{14}, the density distribution of the DNA of the cells determined by cesium chloride density gradient equilibrium centrifugation (49).

If the DNA were replicated conservatively, they would have observed the persistence of the N^{15} DNA band, and the appearance of an N^{14} DNA band during the first replication. If the DNA were replicated non-conservatively, so that bits of the dense DNA were randomly incorporated into two equivalents of DNA, they would have observed a halving of the density difference between N^{15} DNA and N^{14} DNA during the first replication, and a similar halving of the difference between it and N^{14} DNA during the second replication and so on. If the DNA were replicated semi-conservatively, so that the single strands were conserved, they would have observed the disappearance of the N^{15} DNA band during the first replication, and the concomitant appearance of a DNA band with a density precisely intermediate between those of

N^{14} and N^{15} DNA. During the second replication, the hybrid band would be expected to persist and an N^{14} DNA band would be expected to appear.

Meselson and Stahl (48) concluded from their experiments that exponentially multiplying E. coli replicate their DNA by a semi-conservative mechanism.

The same experiment has been performed by Sueoka (50) on the alga, Chlamydomonas reinhardii. The results were similar to those obtained with E. coli, with the reservation that some heavy DNA persisted beyond one doubling of the cells, so that not all of the DNA in the culture had completed one replication before some of the DNA began a second replication. This was taken to indicate inhomogeneity in the cell population.

Mammalian cultured cell DNA has been shown to replicate semi-conservatively by using 5-bromouracil rather than N^{15} as the density label (51, 52, 53). Although 5BU is a superior density label, it has the drawback that it is toxic to the cells. It is not convenient to use N^{15} with cultured animal cells because of the presence of high levels of organic nitrogenous compounds in culture media used for animal cells.

The experiment of Meselson and Stahl (48) has been performed on XD cells multiplying exponentially in M1D. Since XD cells utilize nitrate as their sole source of nitrogen in M1D, it was possible to density label the DNA

with N^{15}-nitrate. The results indicate that exponentially multiplying cultured tobacco cells replicate their DNA by the same semi-conservative mechanism previously demonstrated in bacteria, algae and mammalian cells.

METHODS

The labeling medium, N^{15}-MlD, was prepared with 98.4 atom % N^{15}-nitric acid purchased from Volk Chemical Co. Cells were grown in N^{15}-MlD for two subcultures, or 10 generations. Shortly before the end of the exponential phase of the second subculture in N^{15}-MlD, the cells were harvested by filtration on Miracloth, and then resuspended and diluted in N^{14}-MlD to a concentration of 15,000 cells per ml. The time of resuspension in N^{14}-MlD constitutes time 0 in the replication experiment.

Nucleic acid extractions were performed on a minimum of 5 grams fresh weight of cells. After harvesting on Miracloth, the cells were resuspended in ice cold 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0 (SSC) at 2 ml per gram fresh weight. The suspension was homogenized by 50 strokes of a motor driven Thomas teflon-glass homogenizer. Unbroken cells and wall debris were removed from the homogenate by filtration through two layers of Miracloth. The filtrate was centrifuged at 1,500 x G for 30 minutes, and the

supernatant discarded. The pellet was resuspended in 3.0 ml SSC, and then extracted by a procedure based on that of Marmur (54). To the suspension was added 0.3 ml 25% sodium dodecyl sulfate, and sufficient solid NaCl to bring the NaCl concentration in the aqueous phase to 1 M. An equal volume of 24:1 chloroform:isoamyl alcohol was added and the emulsion shaken for 30 minutes at 27° C. The emulsion was broken by centrifugation at 12,000 x G for 10 minutes. The aqueous supernatant was pipetted into two volumes of ice cold 95% ethanol. The fibrous, white nucleic acid precipitate was wound up on a glass rod and redissolved in 1.0 ml SSC. The solution was stored over chloroform at 2°C.

The crude nucleic acid solution was used without further purification for the density gradient runs. This preparation consisted of approximately 50% DNA and 50% RNA, so the DNA was purified further for physical characterization. RNA was removed by hydrolysis with RNAase which had been pretreated according to Marmur (54). Following the RNAase treatment the solution was re-extracted with 24:1 chloroform: isoamyl alcohol and precipitated with two volumes of ice cold 90% ethanol. The precipitate was collected, redissolved and stored in the same way as was the unpurified preparation.

Melting curves of the DNA were run in a Beckman Model DK-2 ratio-recording ultraviolet spectrophotometer equipped with a heating block. The sedimentation rate was determined in a Spinco Model E Analytical Ultracentrifuge

operating at 35,600 rpm and 25° C. SSC was used as the solvent in both cases. Cesium chloride density gradient equilibrium ultracentrifugation was also performed in the Model E, but at 44,700 rpm and 25°C. Approximately 2μg of crude nucleic acid was centrifuged in ρ= 1.711 CsCl buffered with 0.05 M tris pH 8.0 for 24 hours. Micrococcus lysodeikticus DNA, the gift of Dr. Jerome Vinograd, was included as a density reference.

RESULTS

The purified DNA from XD cells has a T_m=85.5° C. in SSC, with a hyperchromicity of 35%. The sedimentation constant, corrected for temperature and solvent, was $S_{20,w}$=21.9. The buoyant density of N^{14}DNA in CsCl was computed to be ρ=1.696, using the formula of Sueoka (55), and a value of ρ=1.731 for the reference DNA. The T_m and buoyant density both correspond to a G-C content of about 38% (56,57).

Since the generation time is 2 days, nucleic acid was prepared from cells harvested at 24 hour intervals following transfer from N^{15} to N^{14} medium. The results of density gradient equilibrium runs of such preparations are presented in figure 16. The fully N^{15} labeled DNA band disappears between days 2 and 3. There is a concomitant appearance of a hybrid density DNA band. Light N^{14} DNA only appears after

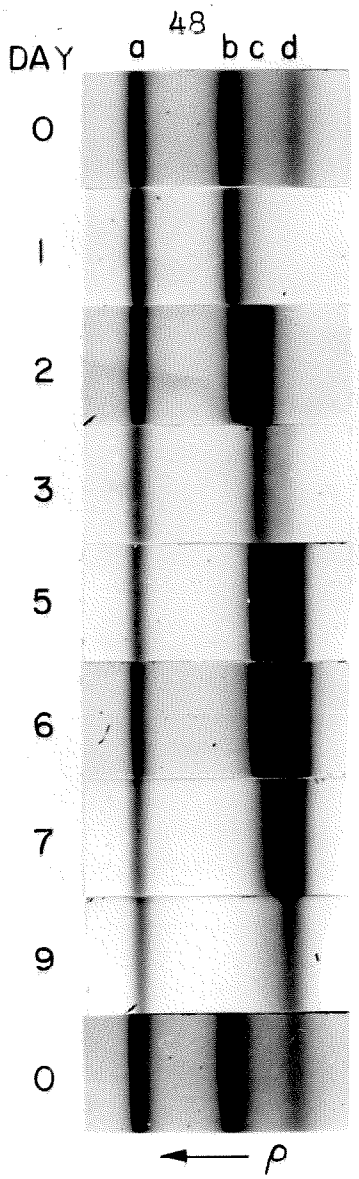


Figure 16

XD-DNA isolated at 24-hour intervals after transfer from N^{15} to N^{14}, and banded in cesium chloride. Band a: Reference (Micrococcus lysodeikticus) DNA; Band b: N^{15} DNA; Band c: Hybrid DNA; Band d: N^{14} DNA. Top and bottom frames show the resolution of a mixture of reference, N^{15} and N^{14} DNA.

the second replication has begun. The hybrid persists during the second and subsequent replications, but becomes a progressively smaller fraction of the total DNA as more N^{14} DNA is produced.

Attempts to demonstrate the presence of separable N^{15} and N^{14} strands in the hybrid were unsuccessful, since denatured N^{14} and denatured N^{15} DNA's yielded very broad unresolvable bands in the density gradient. It is not known whether this is primarily due to DNAase damage during isolation and storage, or to the inherent density heterogeneity of tobacco DNA.

CONCLUDING REMARKS

These experiments have shown that the cultured tobacco cell replicates all of its DNA, that the replication takes approximately one generation, and that the replication proceeds by a semi-conservative mechanism.

The completion of DNA replication in about one generation indicates that the rate of DNA synthesis is uniform both intercellularly and intracellularly. If there were a subpopulation of cells, or a subpopulation of DNA molecules within each cell that replicated their DNA more rapidly, the N^{14} band would have appeared sooner. Similarly, a slowly replicating subpopulation of cells or molecules

would have caused the N^{15} band to linger. The results of the present experiments indicate however that a cycle of DNA replication must be completed before the next replicate can begin to be synthesized. They also indicate that there is little, if any, non-replicating DNA within the cell. In intercellular terms, it means that the cell population is homogeneous with respect to the rate of DNA replication. This is particularly interesting in light of the variability of average cell properties during exponential multiplication. It means that the variability is not due to the prominence of one subpopulation of cells at one time, and another subpopulation at another time. The events which produce variation in the average cell properties must be occurring in all or nearly all the cells.

The persistence of the hybrid density DNA band indicates a semi-conservative mode of replication. Thus, higher plant cell DNA replication exhibits the same semi-conservative characteristics which are found in DNA replication of bacteria, algae and animal cells. This result brings into focus an important aspect of chromosome structure as well as the mechanism of synthesis. There must be single strand continuity in the DNA of individual plant chromosomes. According to the experiment presented above, the conserved subunit is a subunit of the DNA molecule, which is the single strand, and according to Taylor, Woods and Hughes (45), the subunit extends the full

length of the chromosome. It is not known whether the DNA of the plant chromosome consists of many molecules or one very large one. If it is one long molecule, strand continuity is implicit. If it is many small ones, then they must be linked so that subunits produced during a given round of replication are associated.

V. NUTRITION

INTRODUCTORY REMARKS

The transitions between the phases revealed by kinetic analysis must reflect critical events within the cells, and these events in turn may either cause or be caused by a change in the interaction of the cell with its environment. The interactions generally take the form of either depletion of essential nutrients or accumulation of compounds the cells themselves manufacture.

The virtue of a chemically defined medium such as M1D or M2D is that all the initial conditions are known, so that by systematic analysis it is possible to find out which if any medium constituents are depleted, as well as when this happens. The coincidence in time of the depletion of a constituent and transition to a new phase may be an indication of a relationship between the two processes.

The relationship between nutrient depletion and phase transition can be more firmly established by

demonstrating that artificial acceleration or deceleration of the depletion process results in a corresponding shift in the time of occurrence of the transition in question. Once the relationship is established, the metabolic fate of the nutrient may be explored in order to discover the biochemical events which result in the altered behavior of the cells.

METHODS

For the determinations of Na, K, Mg, Ca, Mn, Fe, and B in the medium as a function of culture age, 50 ml of medium was collected every 2 days from cultures of XD cells in M2D. The medium was evaporated to dryness on a hot plate in a porcelain crucible, and the residue was then ashed at 500° C. for 24 hours. The weight of ash was determined and then the samples were analyzed by quantitative emission spectroscopy. Mrs. Elisabeth Bingham of the Geology Department at the California Institute of Technology made the analyses.

Phosphate was determined on aliquots of the medium by the colorimetric method described on page 26.

Nitrate in the medium of XD cultures in M1D was determined indirectly. The Kjeldahl nitrogen in the cells was determined, and this amount subtracted from the amount of nitrogen originally added as nitrate. Each day the cells

of one 200 ml culture were harvested and hydrolyzed in 5.0 ml of concentrated H_2SO_4. The color was cleared with H_2O_2. Ammonia was assayed with Nessler reagent. To 0.5 ml of acid, sufficient H_2O to bring the volume to 35 ml was added, and then 15 ml of Nessler reagent. The absorption at 430 mμ was measured after 30 minutes. Ammonium sulfate was used as the standard.

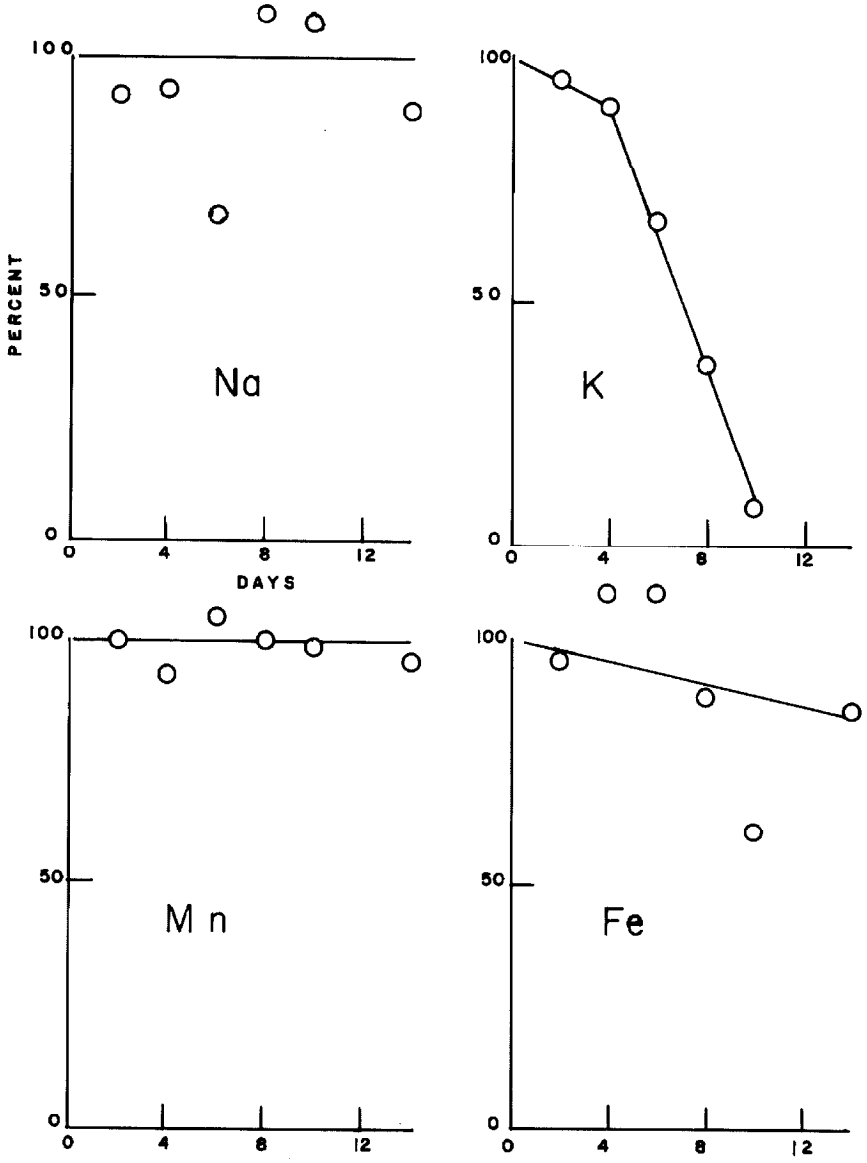
The apparent rate of RNA synthesis as a function of culture age was measured by incubating approximately 1.5 grams fresh weight of XD cells from cultures of various ages in 100 ml of the culture medium of that age plus 5 microcuries of orotic acid-C^{14}, 5 microcuries/micromole (New England Nuclear Corp.), for 2 hours, and then determining the radioactivity incorporated into RNA. The radioactivity incorporated into total nucleic acid was in fact measured, but other experiments had indicated that there is about 10 times as much RNA as DNA in the cells, and that the specific activity of the DNA is about one-fourth that of RNA in an orotic acid incorporation experiment. The correction for radioactivity in DNA was consequently neglected. After incubation, the culture was chilled in ice water and the cells were harvested. After washing with 0.25 M sucrose, 0.01 M tris pH 8, the cells were homogenized, the walls were filtered out, and the filtrate was precipitated with an equal volume of cold 40% TCA. The precipitate, collected by centrifugation, was processed according to

Smillie and Krotkov (124) to determine RNA-tides. The concentration was measured by means of A_{260}, and similarly processed N. B. Co. yeast RNA was used as a standard. The Smillie and Krotkov procedure was modified for determination of the radioactivity. After the precipitate was extracted and dried, it was redissolved in 1.0 ml of formic acid, and 2-0.1 ml aliquots plated on planchets and counted in a Nuclear Chicago Planchet Counter. The remaining formic acid solution was again evaporated to dryness and processed in the usual way for determining RNA-tides.

The fresh weight assay was routinely used for measuring growth in the nutritional studies. The smaller cultures, containing 200 ml of medium, were used.

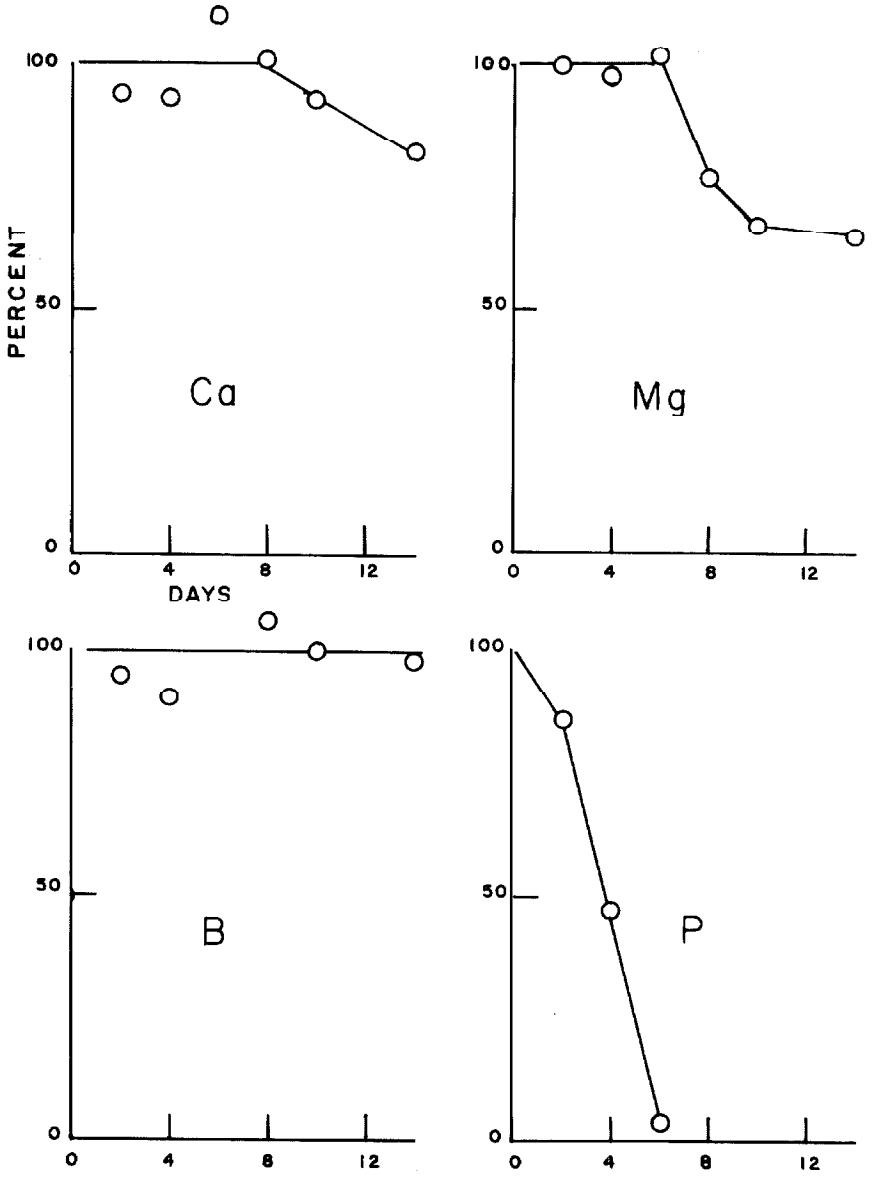
RESULTS

The variations with culture age of Na, K, Mg, Ca, Mn, Fe, B, and P in M2D is presented in figures 17 - 24. The variation of N in M1D is presented in figure 25. The data are plotted as the fraction of the total initially included in the medium. It can be seen that there is little or no change in the levels of Na, Ca, Mn, Fe, and B, while Mg decreases by 35%. Only P and K were completely removed from M2D by XD cells, P being depleted by day 6 and K by



Figures 17 - 20

Depletion with culture age of Na, K, Mn, and Fe from M2D by XD cells. Data are expressed as percent of initial concentration.



Figures 21 - 24

Depletion with culture age of Ca, Mg, B and P from M2D by XD cells. Data are expressed as percent of initial concentration.

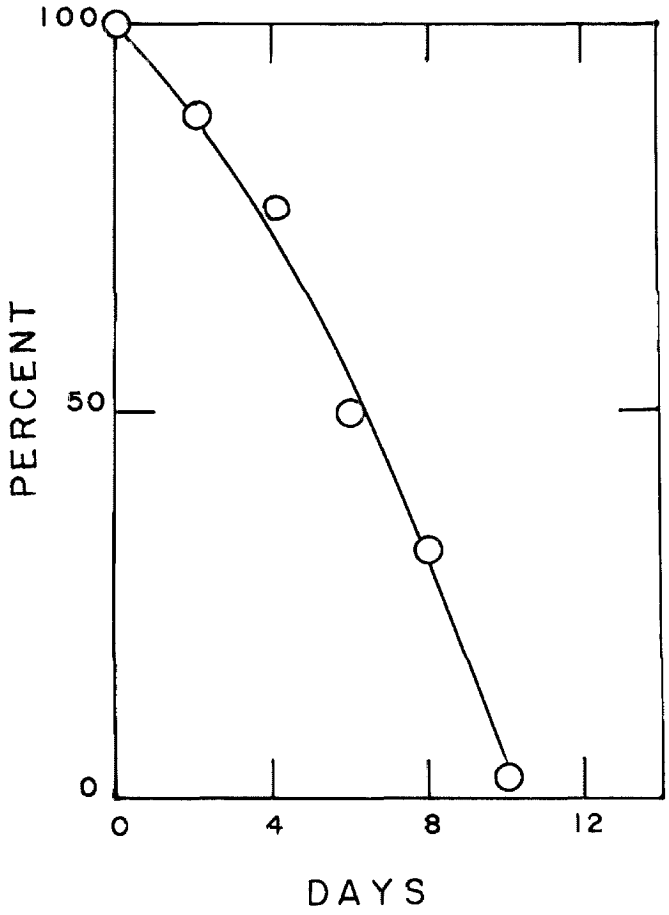


Figure 25

Depletion with culture age of Nitrate from M1D by XD cells. Data are expressed as percent of initial concentration.

day 10. In M1D, XD cells completely depleted N from the medium by day 10.

Four inorganic constituents, S, Cl, I, and Zn, were not assayed, nor were the organic constituents, nicotinic acid, thiamine, pyridoxine, 2,4-dichlorophenoxyacetic acid, and sucrose.

Besides removing constituents from the medium, the cells contribute constituents. During the life of a standard XD culture on M1D, the A_{275} of the medium increases linearly from 0.00 to 0.15. The ultraviolet absorbing material has not been characterized further.

The question of whether growth in the standard culture was limited by depletion of nutrients or accumulation of toxic substances was resolved by studying the growth of cells on media containing all the nutrients of M2D at 2 and 3 times the normal concentrations. The results are presented in figure 26. The first notable result was that the higher nutrient concentrations did not increase the rate of growth during the first week. In other words, the rate constant for exponential growth was unchanged. The higher concentrations of nutrients did prolong the growth period, however, and the maximum growth was proportional to the concentration of nutrients. This is a strong indication that growth of XD cells in M2D is limited by the nutrient supply.

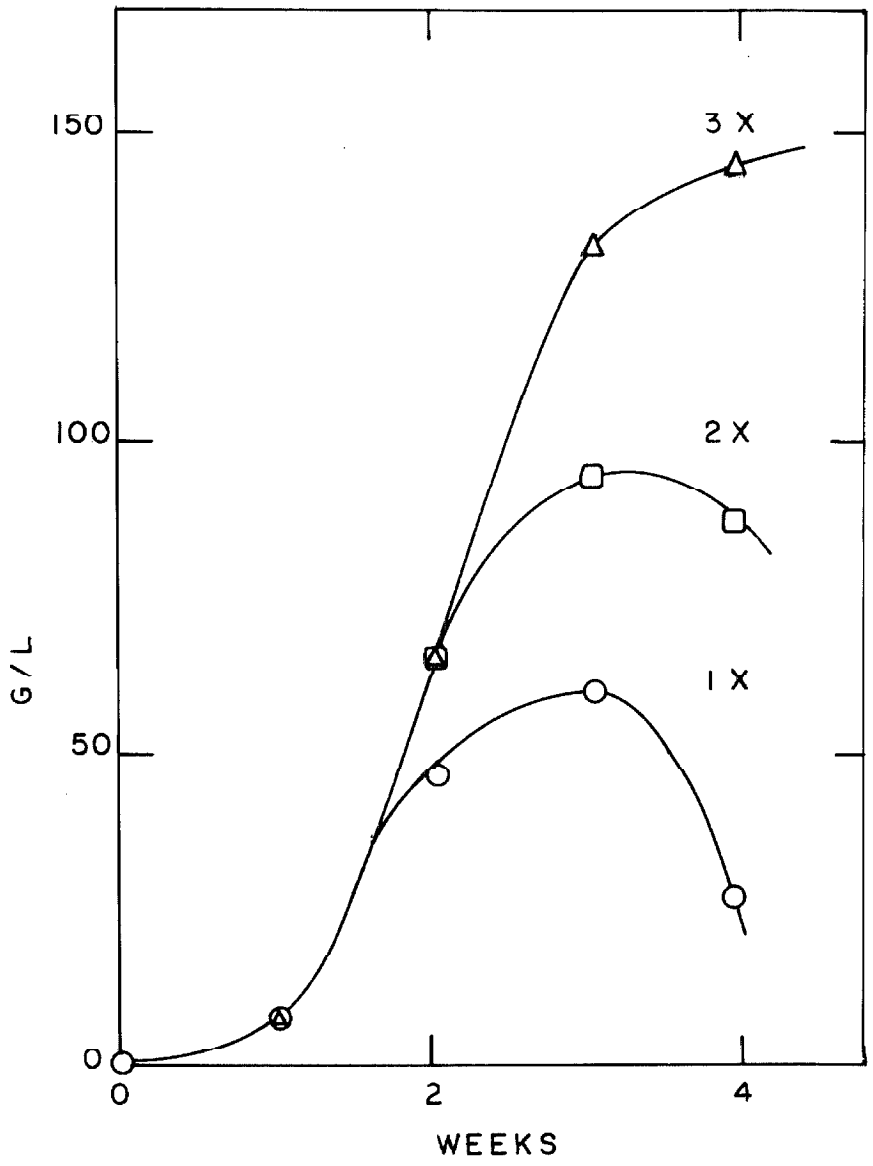


Figure 26

Fresh weight growth of XD cells in 1X, 2X, and 3X M2D.

In order to discover which nutrient or nutrients were essential for the extension of the growth period, media were tested which contained 3 times the standard amount of one of the components used in preparing M2D: WM-I to WM-V, 2,4-D, amino acids, or sucrose. Only the amino acid stock solution extended the growth period. It was as effective as 3 times M2D for the second week of growth, but could not replace it during the third week of growth. It was concluded that the growth of XD cells on M2D is partly limited by the amino acid supply. The effects of the individual amino acids in the mixture, as well as simpler amino acid mixtures were explored at length, and the results will be presented in a separate section.

Evidence regarding the non-critical nature of the concentration of some of the medium components was gathered by studying the effect of omitting them completely. Omission of WM-IV, which supplies Fe, Mn, Zn, B, and I, resulted in only a 35% reduction in growth during the first subculture, and omission of WM-V, the B-vitamin source, also produced a 35% reduction. Omission of 2,4-D reduced growth by 60%. None of these omissions prevented growth, but rather they all allowed more than 3 generations of growth to occur, which means that the exogenous supplies of these factors is not growth limiting under the standard culture conditions.

It should also be noted that adding excess amounts of 2 nutrients which are depleted from M2D, namely K and P, does

not affect growth as measured by fresh weight. The metabolic importance of P would lead one to expect that an inadequate supply of P would quickly be detrimental to growth, since the synthesis of P-rich compounds would be inhibited. The apparent rate of RNA synthesis was studied as a function of culture age to see if in fact RNA synthesis was unaffected by the depletion of P from the medium, since this depletion had no marked effect on growth. The data are presented in figure 27, and shows a sharp drop in the rate of RNA synthesis immediately after the time when P is completely depleted from the medium by day 6. Replenishing the exogenous supply of P on day 9 did not restore the rate of RNA synthesis to the earlier high level, while complete replenishment of the medium did (see day 0). Thus it appears that although there is a coincidence in time of the depletion of P from the medium and the decrease in apparent rate of RNA synthesis, P depletion is not the sole cause of the decrease.

The discovery that XD cells could be grown in M1D raised the question of the growth limiting factor in this medium compared to the growth limitations on XD in M2D. In the latter case, it was known to depend on the amino acid supply. This, plus the facts that the growth curves in M1D and M2D were superimposable, and that XD cells depleted M1D of its nitrogen by day 10, made it seem very likely that growth of XD cells on M1D was limited by the sole available

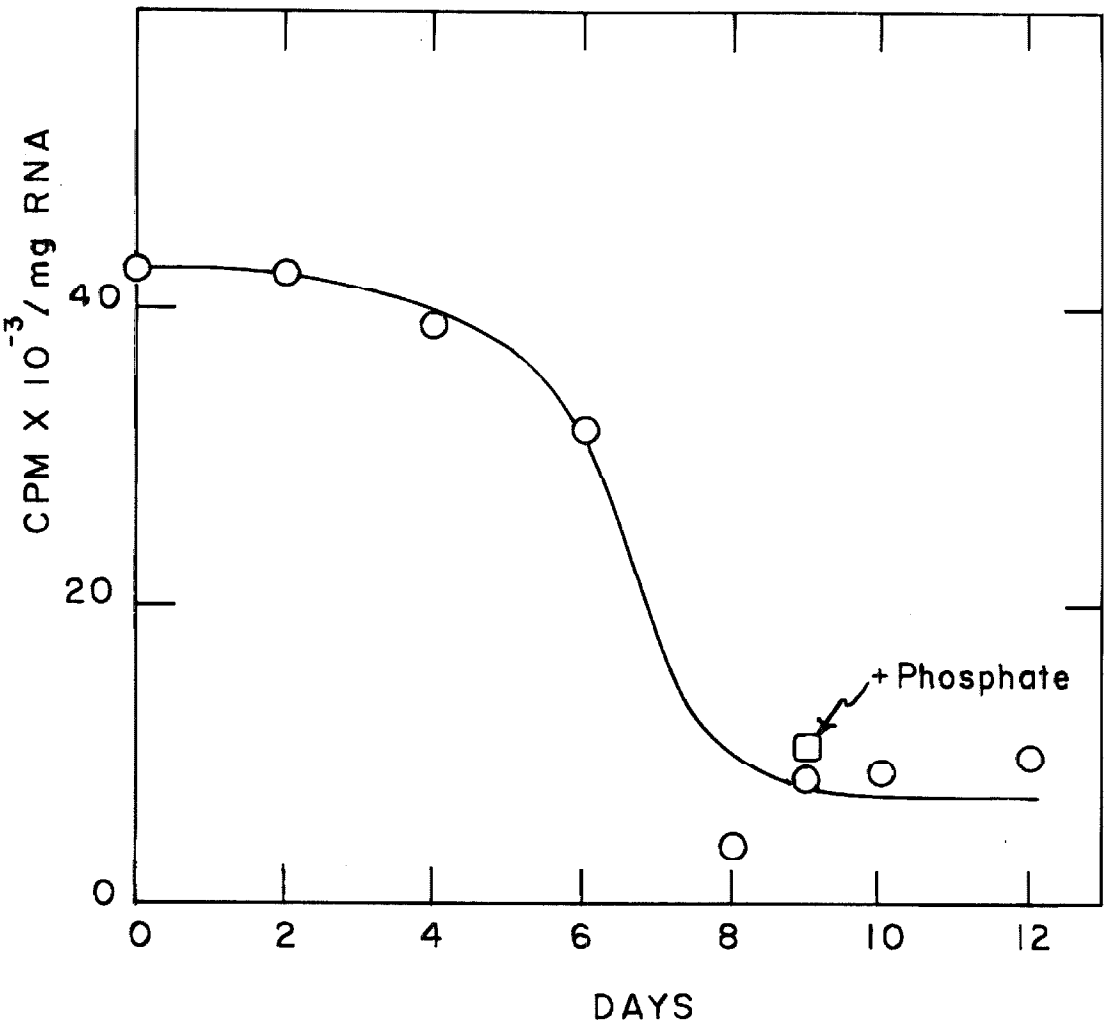


Figure 27

Variation with culture age in the rate of RNA synthesis in XD cells on M1D. The rate of RNA synthesis was measured as the incorporation of orotic acid - C^{14} into RNA during a 2 hour incubation.

precursor for amino acid nitrogen: nitrate. The dependence of growth on the initial nitrate concentration is illustrated in figure 28. It can be seen that growth is strictly proportional to the nitrate concentration, and is therefore limited by the nitrate.

In order to see if amino acids could fully replace nitrate as a nitrogen source for XD cells, and to see if both amino acids and nitrate can be used as growth limiting factors, media were prepared in which chloride was first substituted for nitrate in M1D, and then varying amounts of nitrogen as either amino acids (casein hydrolysate) or nitrate were added. The ability of the cells to grow on such media is illustrated in figure 29, which shows that either casein hydrolysate or nitrate can be growth limiting, and that both nitrogen sources support about the same maximum growth rate, since they plateau at about the same level of growth. Since growth was measured after ten days, this means that both support the same exponential growth rate. The amount of nitrogen necessary to support the maximum growth rate differs for the two sources, however, with nitrate nitrogen being about five times as efficient as amino acid nitrogen.

No other nitrogen source has been found which can support this high growth rate. A few amino acids, notably glutamate and arginine, can support growth as the sole nitrogen source, but the growth is at a greatly reduced rate.

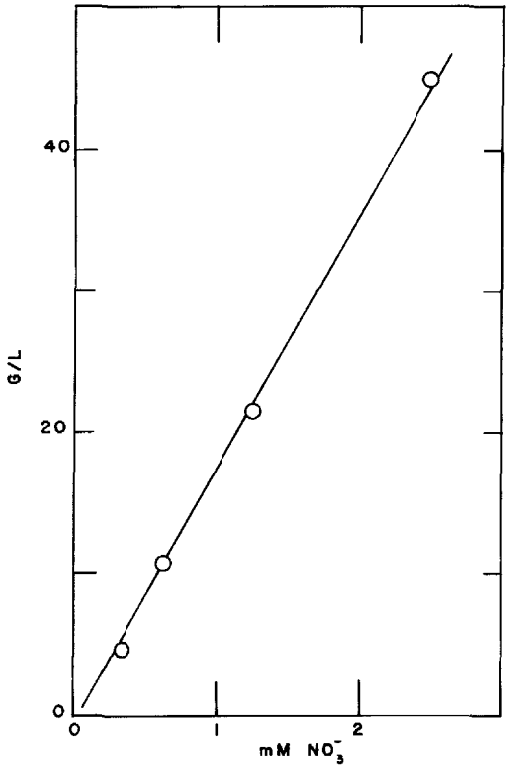


Figure 28

Dependence of Fresh Weight Growth of XD cells in M1D on the amount of nitrate added. Growth period: 21 days.

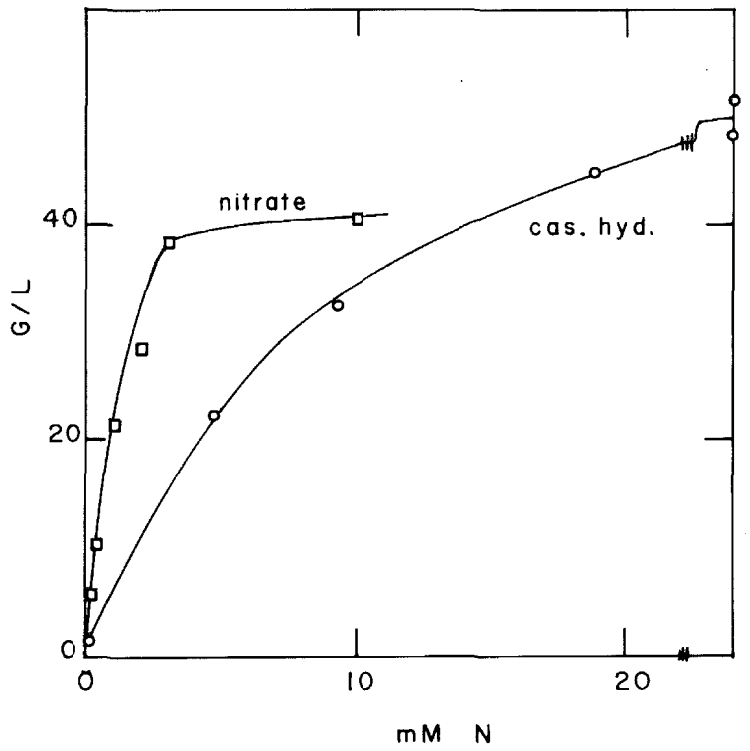


Figure 29

Dependence of Fresh Weight Growth of XD cells in nitrate-less M1D on the amount of nitrogen added as nitrate or as casein hydrolysate. Growth period: 10 days.

No culture conditions have been found under which the cells can utilize ammonia as the sole source of nitrogen.

CONCLUDING REMARKS

A study of the depletion of inorganic nutrients from the culture medium revealed that P is removed by the sixth day, while K and N are removed by the tenth day. Varying the levels of P and K does not affect growth, while varying N does affect growth.

Growth of XD cells on M2D was found to be limited by the supply of nutrients. Among the medium components, the amino acids accounted for part of the limitation, but not all of it. Growth of XD cells in M1D was completely limited by the nitrate supply. XD cells could also be grown under nitrogen-limiting conditions in a medium containing only casein hydrolysate as a nitrogen source.

The results strongly indicate that limitation in available nitrogen is responsible for the termination of the exponential phase. Presumably removal of this limitation is responsible for reinitiation of growth upon subculturing. The ability of the cells to grow equally well on nitrate, amino acids, or nitrate plus amino acids, raises the problem of the choice the cell must make when offered both nutrients. It may use one in preference to the other, or it

may use them simultaneously. The resolution of this problem is the subject of the next two sections.

VI. AMINO ACID EFFECTS

INTRODUCTORY REMARKS

It was observed that although the XD cells grew equally well on nitrate, or a complete mixture of amino acids, or both nitrate and a complete amino acid mixture, the cells could not grow on nitrate plus an incomplete mixture. This meant that in some way the amino acids of the incomplete mixture inhibited growth, and furthermore, the inhibition was prevented by other amino acids in the complete mixture. This property of the cells was examined in detail.

The basic question was the nature of the complete amino acid mixture. This was approached by studying the effects on growth of various amino acid combinations, consisting of one to seventeen amino acids in M2D. The fresh weight growth assay was used.

RESULTS

Any one of the seventeen amino acids in M2D can be omitted with little or no effect on growth.

In order to test the effects of omission of groups or combinations of groups of amino acids, the seventeen amino acids were divided into six groups: I (aspartate, glutamate), II (arginine, lysine, serine, threonine), III (cysteine, methionine), IV (phenylalanine, tryptophan, histidine), V (glycine, alanine, valine, leucine, isoleucine), VI (proline). The results of a number of group omission experiments are summarized in figure 30, where the growth is plotted against the fraction of total M2D amino acid nitrogen remaining after the omission. It can be seen that the experiments fall into two classes: those in which group II was omitted, and those in which group II was not omitted. Omission of group II has a totally inhibitory effect, while omission of other groups has a negligible effect unless more than 50% of M2D amino acid nitrogen is omitted. This result clearly indicates that the presence of group II or part of group II is essential for a "complete" amino acid mixture. The inhibition due to deletion of group II has to be due to more than one amino acid, since any single amino acid can be omitted without ill effect.

The effects of omission of pairs of group II amino

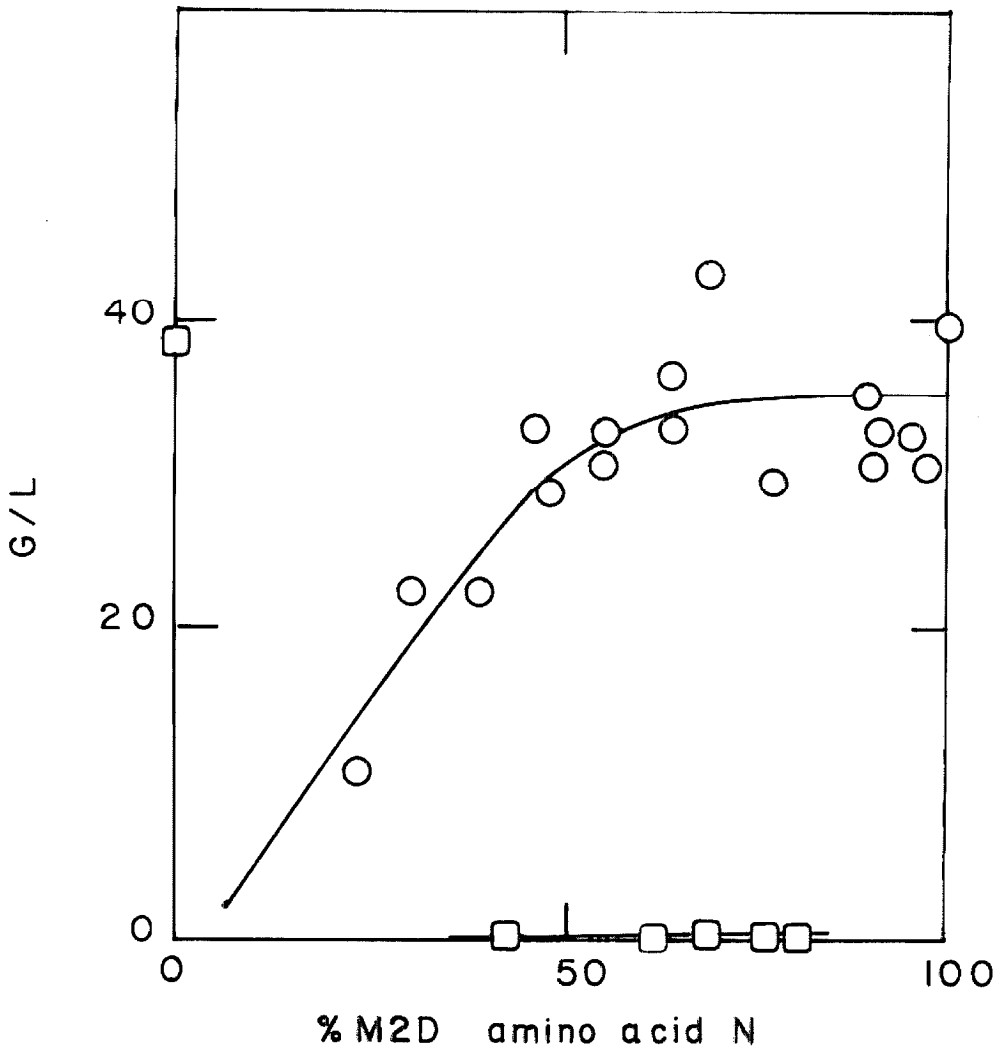


Figure 30

Dependence of Fresh Weight Growth of XD cells in modified M2D media on the presence of group II amino acids. Group II present: ○. Group II absent: □. Growth period: 10 days.

acids were next examined. The results are presented in table II. They indicate that the effect of omitting group II is due to the simultaneous omission of arginine and lysine. Thus either arginine or lysine can complete an amino acid mixture, and enable XD cells to grow on nitrate in the presence of amino acids.

The data in table III indicate that most amino acids at 10^{-4} M are quite inhibitory when individually added to M1D. The outstanding exceptions are arginine and lysine. Cysteine behaves erratically from experiment to experiment, but it too is non-inhibitory. It is noteworthy that the two amino acids which can prevent inhibition by all the others in a mixture are themselves non-inhibitory. One might expect that arginine and lysine can prevent inhibition by single amino acids as well as by mixtures. This was spot-checked and found to be true. Glycine inhibition was chosen as a representative case for further study.

The concentration dependence of glycine inhibition is illustrated in figure 31. It can be seen that inhibition is complete by 4×10^{-5} M glycine. The curves for other amino acids have similar shapes, but the concentrations necessary for complete inhibition vary

The specificity of relief of inhibition is demonstrated by the data of table IV. Only the non-inhibiting amino acids, arginine, lysine and cysteine, can

Table II

Deletion from M2D	Relative growth[a]
none	100
group II (arg, lys, ser, thr)	2
arg	86
lys	93
ser	102
thr	107
arg, lys	2
arg, ser	62
arg, thr	50
lys, ser	86
lys, thr	71
ser, thr	75

a) 10 day growth period, fresh weight assay

Table III

Supplement to M1D	Relative Growth[a]
None	100
10^{-4} M ala	9
10^{-4} M asp	11
10^{-4} M glu	16
10^{-4} M gly	1
10^{-4} M his	14
10^{-4} M ileu	14
10^{-4} M leu	1
10^{-4} M met	11
10^{-4} M phe	51
10^{-4} M pro	32
10^{-4} M ser	1
10^{-4} M thr	1
10^{-4} M tryp	24
10^{-4} M tyr	25
10^{-4} M val	1
10^{-4} M arg	118
10^{-4} M cys	73
10^{-4} M lys	118

a) 10 day growth period, fresh weight assay

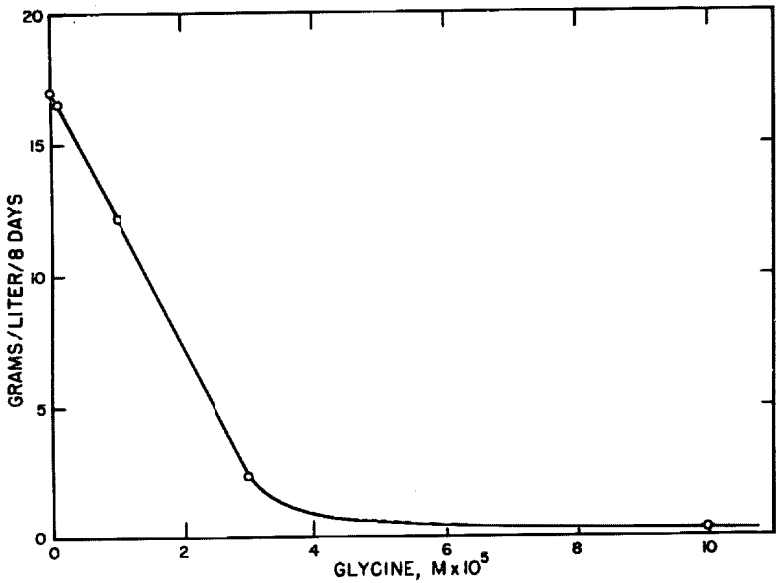


Figure 31

Concentration dependence of glycine inhibition of XD cell growth on M1D. Growth period: 10 days.

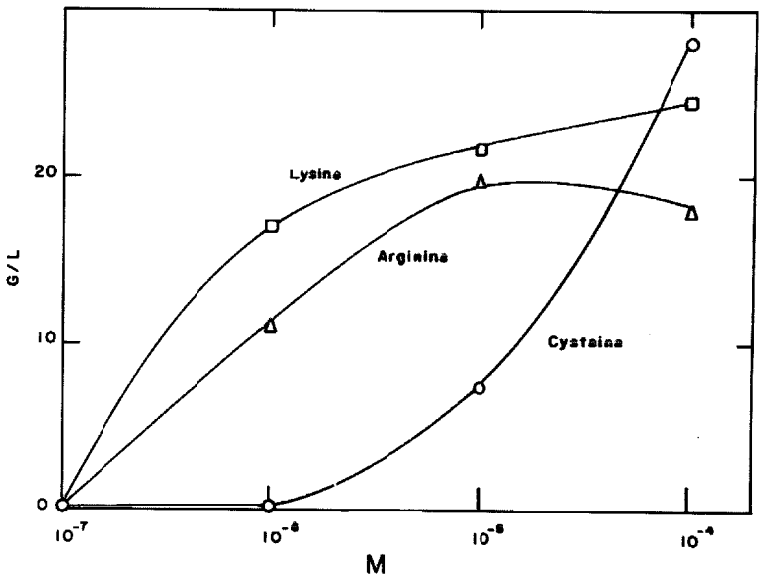


Figure 32

Concentration dependence of relief of glycine inhibition by arginine, lysine or cysteine. XD cells were grown for 10 days on M1D + 10^{-4} M glycine + relieving amino acid.

Table IV

Supplement to M1D	Relative Growth[a]
None	100
10^{-4} M gly	2
+ 10^{-4} M ala	2
+ 10^{-4} M asp	3
+ 10^{-4} M glu	3
+ 10^{-4} M his	23
+ 10^{-4} M ileu	65
+ 10^{-4} M leu	9
+ 10^{-4} M met	6
+ 10^{-4} M phe	6
+ 10^{-4} M pro	3
+ 10^{-4} M ser	1
+ 10^{-4} M thr	4
+ 10^{-4} M tryp	37
+ 10^{-4} M tyr	49
+ 10^{-4} M val	4
+ 10^{-4} M arg	109
+ 10^{-4} M cys	116
+ 10^{-4} M lys	114

a) 10 day growth period, fresh weight assay

completely prevent the inhibition by glycine. Isoleucine also gives substantial relief. It may be concluded that there are three classes of inhibition-preventing amino acids: those which do not themselves inhibit, and which can prevent inhibition by the inhibitors singly or together; those which do not themselves inhibit, but which can prevent inhibition by only some inhibitors; those which themselves inhibit, but which can prevent inhibition by some of the inhibitors.

The dependence of the relief of inhibition on the concentration of non-inhibitor is illustrated in figure 32. The results of this experiment indicate again that arginine and lysine form one class of relievers, while cysteine is in another class. This conclusion can be drawn from the similarities between the concentration curves for arginine and lysine, and their difference from the concentration curve for cysteine.

CONCLUDING REMARKS

Experiments on the effects of amino acids on the growth of XD cells have indicated that the amino acids fall into two classes: inhibitory and non-inhibitory. The non-inhibitory amino acids are arginine, lysine and cysteine. Inhibitory amino acids cannot inhibit in the presence of either arginine or lysine. Some of them cannot inhibit in

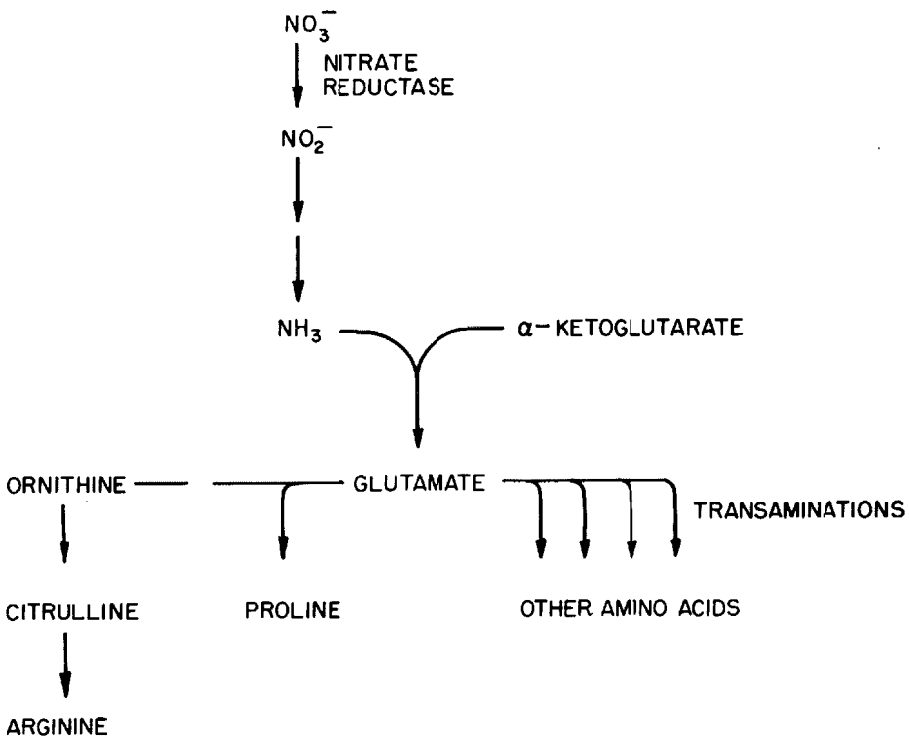
the presence of cysteine. The inhibitory amino acids are effective at concentrations in the region of 10^{-4} M. Arginine or lysine at concentrations of ca. 10^{-6} M relieve the inhibition, while cysteine must be at concentrations of ca. 10^{-4} M to be effective in the cases in which it can relieve inhibition.

The published accounts of effects of amino acids on the growth or morphogenesis of whole plants (58, 59), on excised embryos (60, 61, 62), on seedlings (63, 64), on cultured roots (65, 66, 67), and on cultured cells (68, 69) possess no universal pattern, but there are two generalizations which can be made from these studies. First, single amino acids are generally inhibitory, while mixtures are less inhibitory the more complete they are. Second, the nitrogen rich amino acids, particularly arginine, are non-inhibitory in many systems. It is tempting to speculate that some of these reports may be concerned with the same phenomenon which has been encountered in XD cells. The similarity of some of the findings of Skinner and Street (66), Riker and Gutsche (68), and of Harris (64) to the observations on XD cells is particularly striking, and suggests that a thorough dissection of the XD cell phenomenon will provide the basis for understanding the findings of others.

Among the hypotheses which were considered in trying to account for the amino acid effects was the idea that they

inhibit growth by inhibiting the biosynthesis of all the other amino acids. Since cells growing on M1D must utilize the nitrogen from nitrate to synthesize all the amino acids, the pathway from nitrate to glutamate (see p. 79) would seem a likely place for one amino acid to prevent the synthesis of all others. The first enzyme of the pathway, nitrate reductase, was therefore studied.

NITROGEN METABOLISM IN HIGHER PLANT CELLS



VII. NITRATE REDUCTASE

INTRODUCTORY REMARKS

The importance of nitrate reduction in plant nitrogen metabolism was first suspected many years ago when it was discovered that nitrate was the most common source of plant nitrogen, and that under certain circumstances, nitrite could be detected in plants (70). There were early reports of cell free systems which could reduce nitrate to nitrite (70). However, the validity of these reports is questionable in the light of later work which has shown that cysteine usually must be present if active enzyme is to be extracted (74), and reduced pyridine nucleotide must be added to the extract before nitrate reduction can occur at a measurable rate (71).

The enzyme or enzyme system which catalyzes the reduction of nitrate to nitrite is called nitrate reductase (NR). It was first unequivocally demonstrated in cell free extracts of a higher plant by Evans and Nason (71).

They prepared their enzyme from soybean leaves in the absence of cysteine. It consumed one mole of reduced pyridine nucleotide per mole of nitrate reduced. Either NADH or NADPH could be used, and the pH optimum for the reaction was in the neighborhood of 6. The reaction rate was somewhat increased by FAD. Nicholas and Nason (72) further demonstrated that soybean NR was a molybdenum requiring enzyme.

More recently, Beevers, Flesher and Hageman (73) have shown that soybean NR is atypical. They found that NR from 15 other plant species preferred or were specific for NADH, and possessed pH optima close to 7.5. They were able to show that soybean NR prepared in the presence of cysteine has the same pH6 optimum as NR prepared in the absence of cysteine. Extraction in the presence of cysteine yields an enzyme that prefers NADH, while extraction in the absence of cysteine yields enzyme that prefers NADPH. Extraction in the presence of cysteine yields about five times as much enzyme activity from soybean. The stabilizing action of cysteine on plant NR was noted by Sanderson and Cocking (74). Cysteine was also included in the homogenizing medium used by Nicholas and Nason (72) in their work on the role of molybdenum in soybean NR, but they did not mention why it was included.

Perhaps the most interesting property of NR is its variability in response to the environment. The enzyme is

induced by its substrate, nitrate, in a number of plant systems. These include rice seedlings (75), oat seedlings (76), and mustard and cauliflower leaves (77). NR is also substrate inducible in bacteria (78) and in fungi (79).

Candella, Fisher and Hewitt (80) found that dark treatment causes a loss of NR activity in cauliflower, but the activity can be restored in vivo by a light treatment. These workers also observed that a nitrogen atmosphere results in a loss of NR activity, but that the activity is regained upon exposure to an atmosphere of air. Hageman and Flesher (81) found similar light and dark effects on NR in corn seedlings. Zieserl and Hageman (82) observed seasonal variations in corn NR activity, and they also found a five-fold variation in NR level among inbred strains of corn.

Thus the literature offers abundant evidence that NR is quite variable in vivo. The NR level appears to be subject to genetic factors, and it also may depend upon the developmental stage of the plant. Four environmental factors are known to influence NR activity: nitrate, molybdenum, visible light, and the atmosphere.

PREPARATION AND ASSAY

NR was prepared and assayed by methods based on those

of Sanderson and Cocking (74). The soluble protein fraction was prepared as described in section III. This fraction was used as crude enzyme. Partial purification was achieved by ammonium sulfate precipitation. To the crude enzyme was added 400 mg ammonium sulfate per ml. After standing for 30 minutes at 3°C., the precipitate was collected by centrifugation at 10,000 rpm for 10 minutes. All of the activity precipitates. The pellet was redissolved in ice-cold 0.1 M tris, 0.001 M cysteine HCl pH 7.5, using only one-fifth the original volume. To this solution was added 200 mg ammonium sulfate per ml, and the precipitate again allowed to develop for 30 minutes before collecting. The pellet was redissolved in the same volume of the same buffer, and quick-frozen in a dry ice-acetone bath. The partially purified enzyme was stored at -70° C., and it was stable indefinitely under these conditions.

The enzyme was assayed in 1.0 ml of reaction mixture made up with 0.5 ml 0.1 M KPO_4, pH 7.5; 0.1 ml 0.1 M KNO_3; 0.1 ml 10^{-3} M NADH, and 0.3 ml enzyme plus water. The reaction was started by transferring the reaction mixture from an ice-water bath to a 25° C. water bath. After 30 minutes, the reaction was stopped by the addition of 1.0 ml 1% sulfanilamide in 3 N HCl, and 1.0 ml of 0.02% N-(1-naphthyl)-ethylenediamine dihydrochloride. After 15 minutes, A_{540} was measured against a zero time control. The nitrite concentration was computed from the value given

by a standard amount of nitrite in the presence of the reaction mixture less enzyme.

One unit of enzyme is defined as that amount which reduces 1 mμ mole of nitrate per hour, under the conditions of assay.

No NR activity could be extracted from XD cells in the absence of cysteine, in agreement with the observations of Sanderson and Cocking (74) on tobacco. The amount of NR which is extractable in the presence of cysteine depends on the volume of homogenizing medium used, and increases as the latter increases up to about 5 ml medium per gram fresh weight (figure 33). Almost all the activity extractable from XD cells is in the soluble protein fraction. The maximum yields of activity from XD cells have been around 1500 units per gram fresh weight with a specific activity of 1200 units per mg protein. These figures may be compared with approximate yields of 4000 units per gram fresh weight and 375 units per mg protein from tobacco leaves, and 600 units per gram fresh weight and 100 units per mg protein from tobacco roots (74).

The enzyme is unstable at 3° C., and so it is necessary to perform the assay at a consistent time after preparation of crude enzyme in order to compare the results of assays made on different preparations on different days. The assay was routinely performed 90 minutes after the beginning of homogenization, and this procedure gave

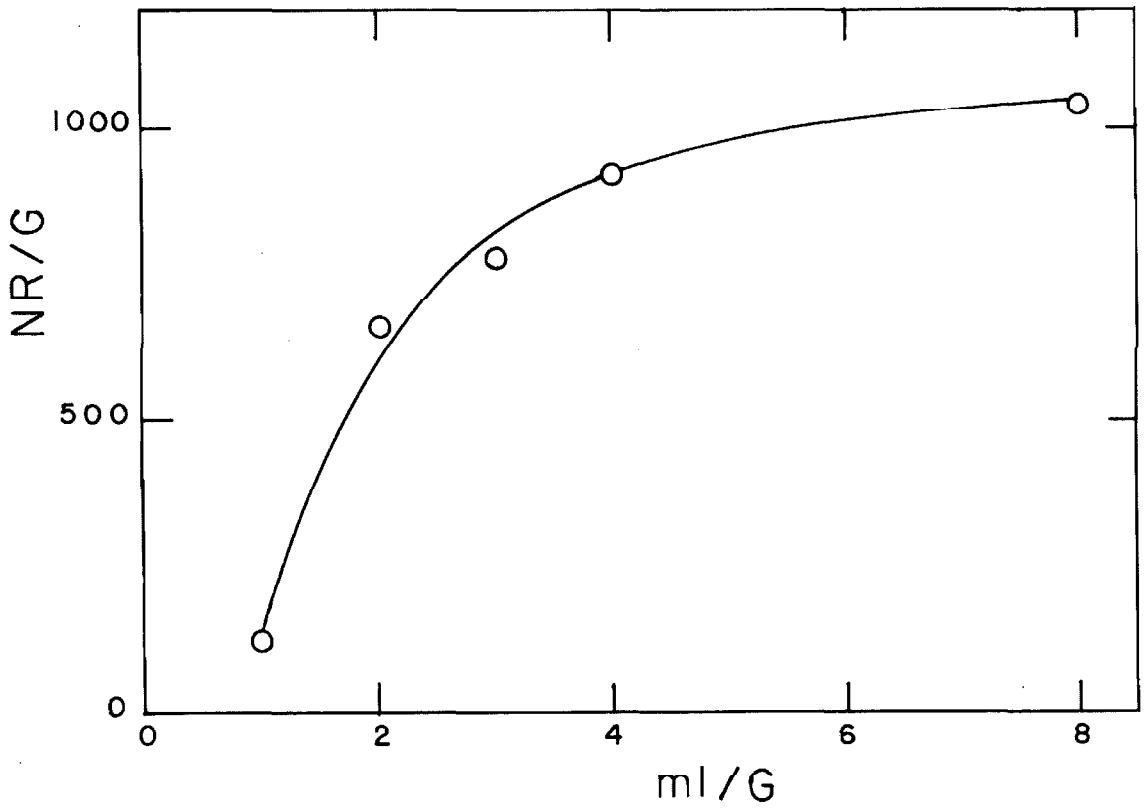


Figure 33

Dependence of Nitrate Reductase Units in the soluble protein fraction on the volume of extracting medium per gram fresh weight of XD cells.

tolerable reproducibility from day to day. The reproducibility of data gathered on one day was excellent, with duplicate parallel preparations being indistinguishable in their activities.

The dependence of the reaction on time and enzyme concentration is shown in figures 34-37 for both crude and partially purified enzyme. It is apparent that there is a slight deviation from linearity with time for crude enzyme.

The partially purified NR of XD cells is absolutely dependent on the presence of NADH and nitrate, and is more active in phosphate than in tris buffer (74). The reaction rate is not increased by either FAD or molybdenum, nor is it affected by the presence of hydroxylamine or ammonia. There is no detectable reduction of nitrite under the conditions of assay for NR. The presence of single amino acids or mixtures of amino acids also does not affect the reaction.

In summary, the methods of preparing and assaying NR which have been devised provide a satisfactory estimate of the NR activity in XD cells and should make it possible to directly compare the NR levels in cells subjected to various culture conditions; in particular, the presence of amino acids in the culture medium.

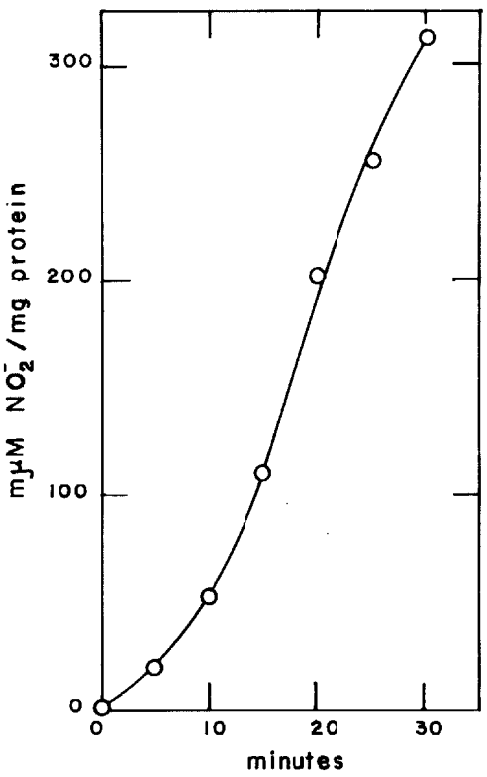


Figure 34

Kinetics of the Nitrate Reductase reaction using crude enzyme from XD cells.

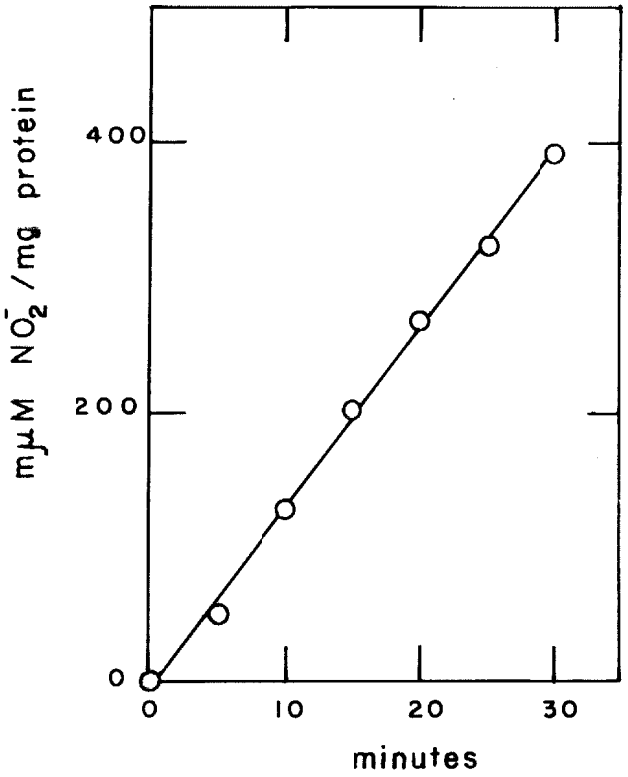


Figure 35

Kinetics of the Nitrate Reductase reaction using partially purified enzyme from XD cells.

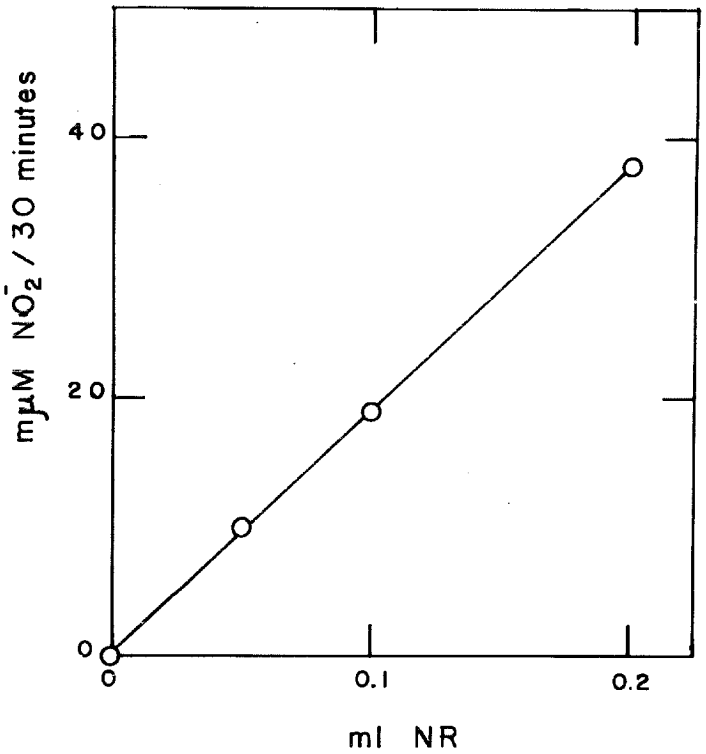


Figure 36

Concentration dependence of the activity of crude Nitrate Reductase from XD cells.

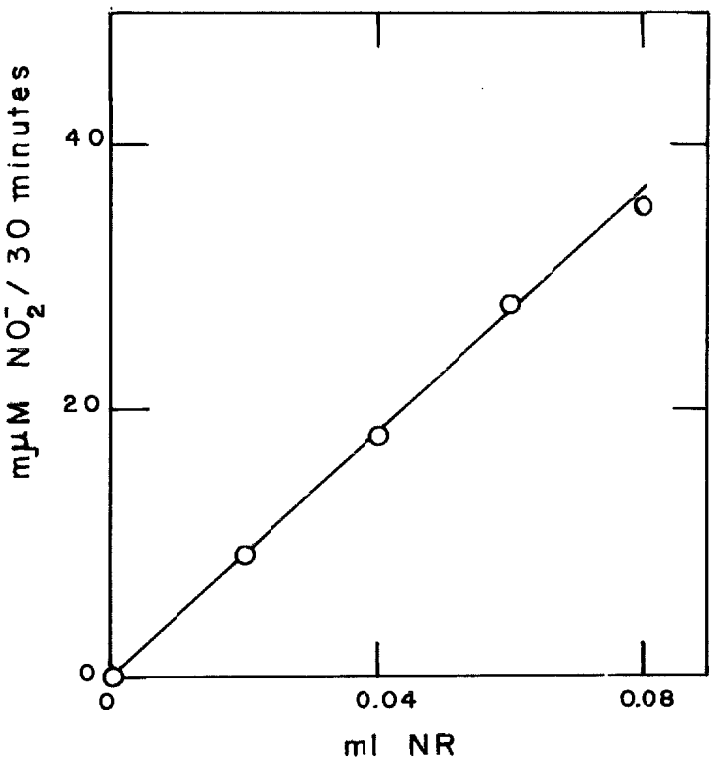


Figure 37

Concentration dependence of the activity of partially purified Nitrate Reductase from XD cells.

DEPENDENCE OF NR ACTIVITY ON CULTURE AGE

NR was determined in extracts of XD cells from M1D cultures 0 to 12 days old. The dependence of total culture NR on culture age is plotted in figure 38. The NR level is initially zero, but rises to about 1000 units per liter during the first 24 hours. It then continues to increase to a maximum of about 6500 units per liter, which is attained between days 5 and 6. There then follows a steady decline to zero again.

The specific activity of NR as a function of culture age is plotted in figure 39. This representation of the data emphasizes the fact that the enzyme is almost entirely induced on the first day. The subsequent increase in enzyme activity per culture roughly parallels the increase in soluble protein per culture, with the result that there is a relatively small change in specific activity between day 1 and day 5.

Integration of the curve drawn in figure 38 reveals that the extractable, assayable NR is sufficient to account for the reduction of only about 35% of the total nitrate consumed during the life of the culture. The fate of the remaining 65% of essential enzyme activity is unknown. It may be that all the enzyme was isolated, but that it functions more efficiently in vivo. On the other hand, the missing activity may have been lost as a consequence of

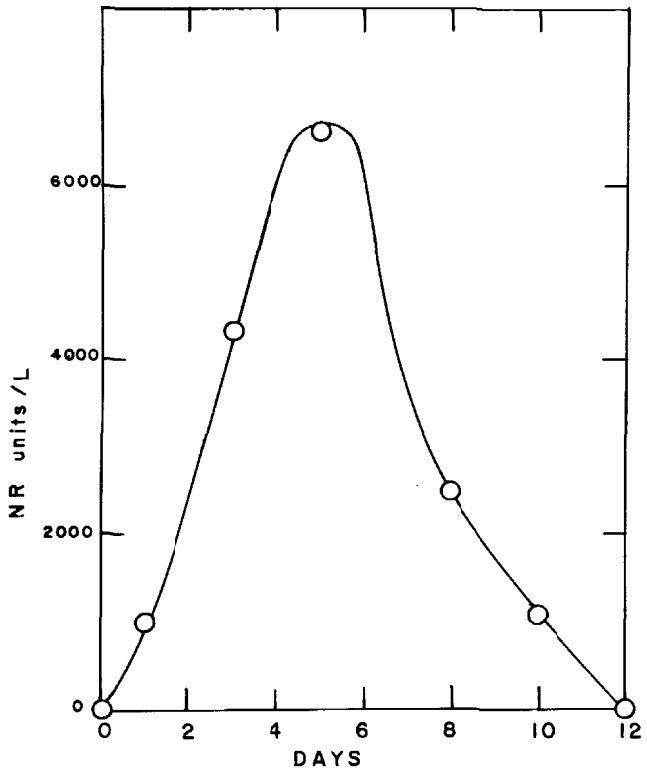


Figure 38

Variation of total Nitrate Reductase per liter of XD cell culture with culture age.

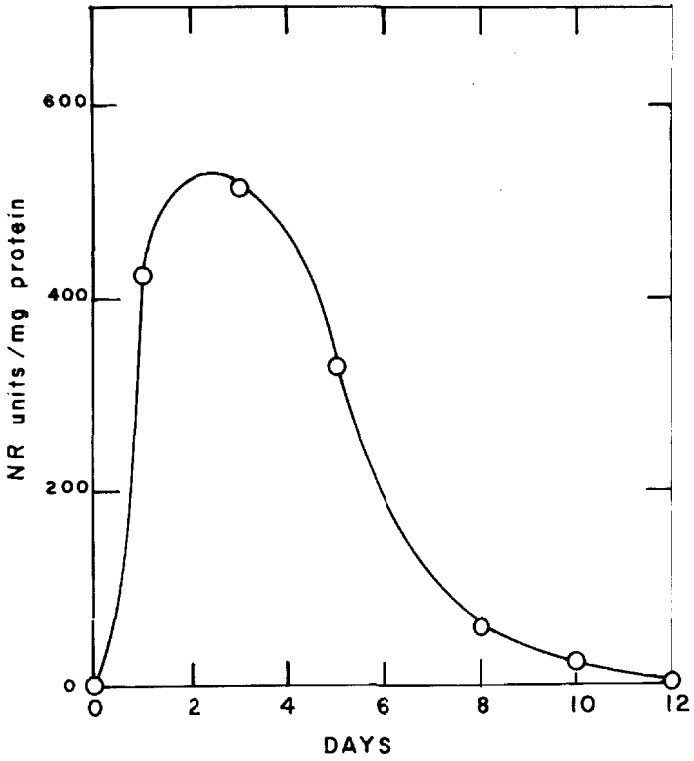


Figure 39

Variation of Nitrate Reductase specific activity with XD cell culture age.

inactivation during isolation.

The variation of NR activity with culture age fits quite well with the previous kinetic studies. The lag in the onset of mitosis occurs during the time that NR is being induced. The maximum total culture NR occurs at about the same time as maximum protein per cell, maximum number of cells per group, and minimum water content. As the amount of NR in the culture declines, so does soluble protein per cell and hence, it may be deduced, the rate of soluble protein synthesis also declines. The rate of RNA synthesis declines at about this time, and the amount of nitrate in the medium begins to be appreciably diminished.

These results suggest the following picture. When a 12 day old culture is subcultured into fresh M1D, the cells lack NR and so cannot synthesize the organic nitrogenous compounds such as protein and nucleic acids which are essential for cell multiplication. NR is induced by its substrate, nitrate, and after something less than a day there is sufficient NR to support the nitrogen requirements of the cells. The necessary compounds are then synthesized and after 16 - 18 hours the cells have enough of the appropriate materials to begin dividing. They then proceed through 2 cell divisions during which large quantities of protein are synthesized because of the abundant supply of nitrate and of NR to reduce it. During the third round of

cell division some critical event occurs in the culture which reduces the rates of NR production, protein synthesis, and RNA synthesis, but does not prevent the cells from dividing twice more. During these latter two divisions the supply of nitrate is depleted. NR not only depends on nitrate to be induced, but in addition, the enzyme decays in the absence of nitrate. The cells cease growing and dividing at about the time that nitrate is completely depleted and NR has almost completely decayed.

The two critical periods in the culture seem to be the first day and the fifth day. At least one critical event, the induction of NR, occurs during the first day. It is clear that for XD cells growing on M1D, prevention of the induction of NR would be a sufficient condition to block all growth. The nature of the critical event or events that occur at about the fifth day is unknown, but its effects are numerous, and they should eventually supply a clue to its nature.

INDUCTION

The kinetics of NR induction in a standard XD culture, and the dependence of the induction on nitrate, are illustrated in figure 40. It can be seen that there is about a six hour lag before activity appears, and that it

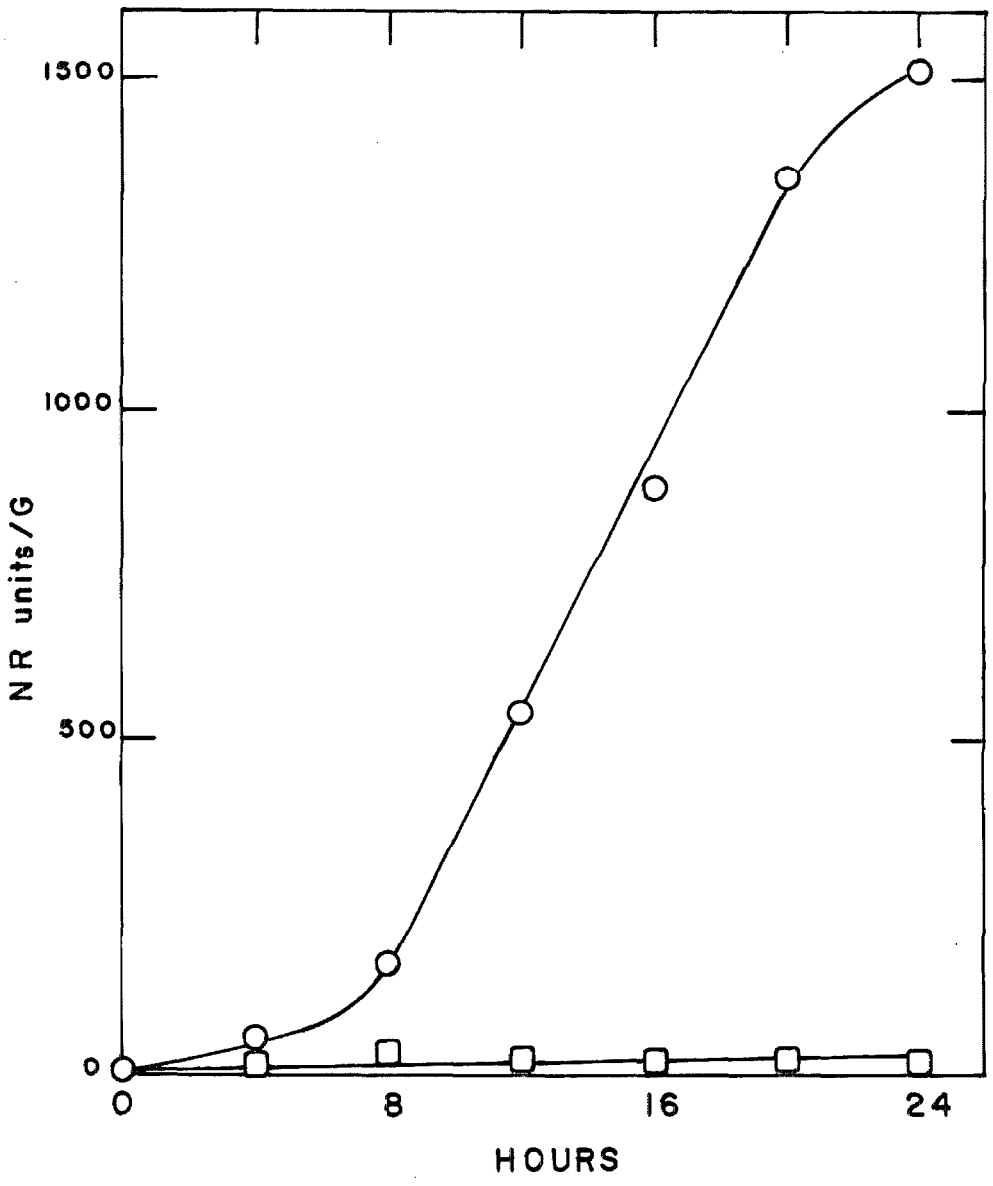


Figure 40

Kinetics of Induction of Nitrate Reductase upon subculturing 12 day old XD cells into fresh M1D, and dependence of the induction upon nitrate.
M1D: ○ ; nitrate-less M1D: □

then increases linearly for about 14 hours. In this particular experiment NR activity increased 150-fold during the first 24 hours. Over this same period there was only a 25% increase in soluble protein. The shape of the induction curve supports the hypothesis that the onset of mitosis is delayed until NR is induced to an extent adequate to permit the cell to synthesize organic nitrogenous compounds.

The dependence of induction on the presence of nitrate is absolute for XD cells on M1D. The nitrate-less medium was prepared by substituting the chlorides of calcium and potassium for the nitrate salts in M1D. The cells are not altered in their potential for NR induction after 24 hours in nitrate-less M1D, since the addition of nitrate after 24 hours results in normal induction during the subsequent 24 hours.

The possibility that NR induction is a consequence of cell multiplication rather than a specific effect of nitrate may be eliminated by growing the cells on some other nitrogen source which cannot be converted to nitrate, and then determining the NR level under these culture conditions. The possible nitrogen sources which come to mind are nitrite, hydroxylamine, ammonia, and amino acids. The first two are toxic to XD cells in suspension culture, and conditions have not been found under which the XD cells can utilize ammonia as their sole nitrogen source. The only nitrogen source which has been found to be capable of supporting growth as

well as does nitrate is casein hydrolysate. Arginine or glutamate can also serve as sole nitrogen source, but only at a low growth rate. This suggests that XD cells are not well equipped for interconversion of amino acids. A poor ability to interconvert is also indicated by the inhibitory action of single amino acids, since the cells merely have to manufacture arginine or lysine to prevent such inhibition. This is particularly surprising in the case of glutamate inhibition, since it is a precursor of arginine.

The level of NR in XD cells grown on various amounts of casein hydrolysate in the presence or absence of nitrate is shown in figure 41. The casein hydrolysate levels are growth limiting at the lower concentrations used, but less so at the upper levels (see figure 29). The data clearly indicate that there is no NR activity in cells multiplying on casein hydrolysate in the absence of nitrate, while there is NR in cells grown on the same level of casein hydrolysate, but in the presence of nitrate. This result definitely establishes that nitrate is required for NR induction.

REPRESSION

The data in figure 41 show that NR is progressively less induced as the level of casein hydrolysate in the medium is increased. That is, casein hydrolysate represses

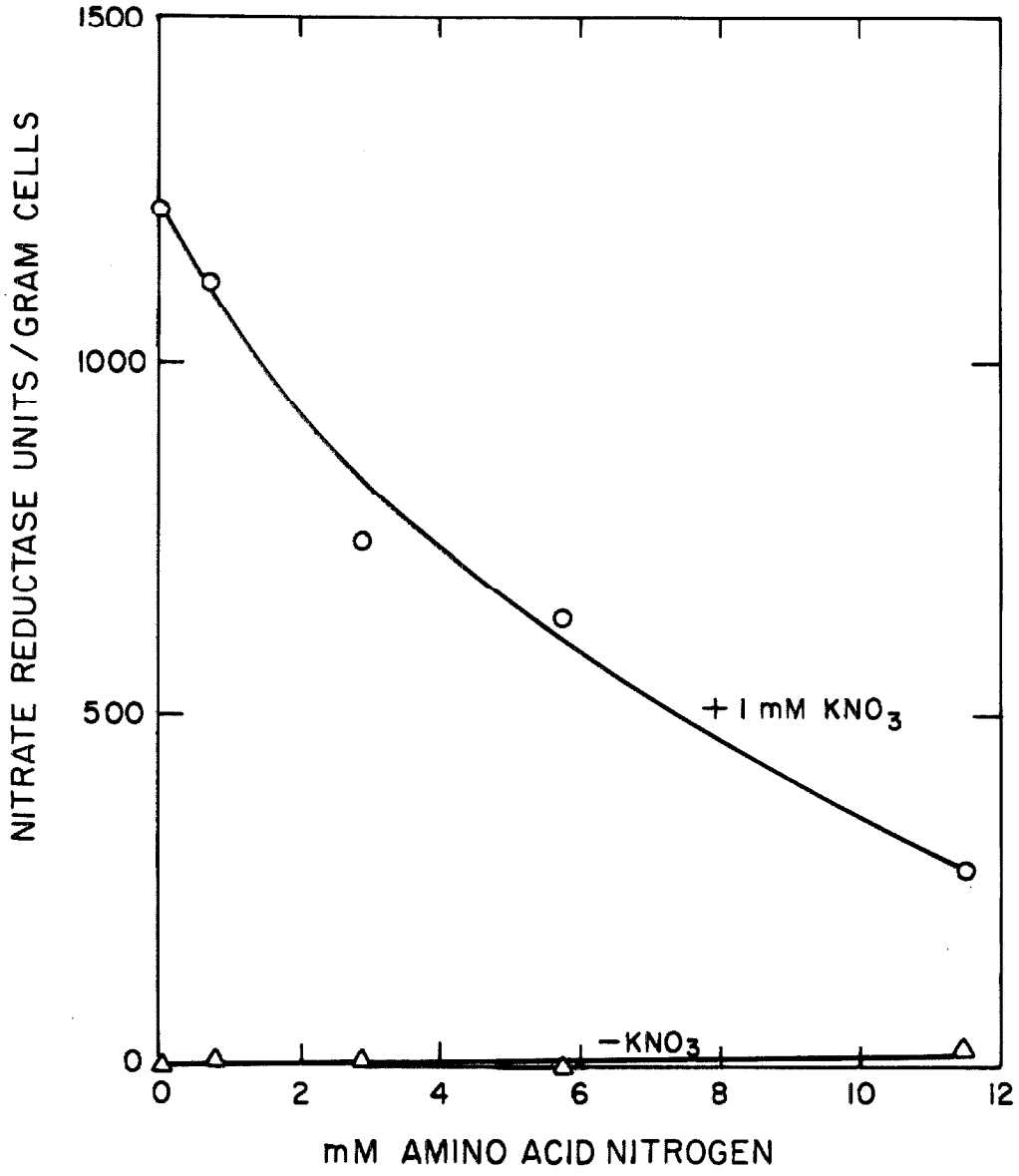


Figure 41

Dependence of repression of Nitrate Reductase in XD cells upon the concentration of casein hydrolysate amino acid nitrogen.

the development of NR activity.

The degree of repression by a given casein hydrolysate level is quantitatively related to its ability to support growth in the absence of nitrate. In table V, the repression curve of figure 41 is compared with the casein hydrolysate growth curve of figure 29. It is clear that repression of NR is strictly proportional to the growth supporting ability of each level of casein hydrolysate.

The presence or absence of nitrate in an amino acid-containing medium also influences the soluble protein content of the cells (figure 42). In the presence of nitrate, a high constant level of soluble protein is found over a wide range of amino acid concentrations. In the absence of nitrate there is a gradual rise in soluble protein content as amino acid concentration is increased. Amino acid concentrations sufficient to support the maximum growth rate are required to bring the soluble protein content up to the maximum level in the absence of nitrate.

These results are interpreted to mean that XD cells have a regulated maximum growth rate which requires a certain minimum endogenous level of organic nitrogen. The organic nitrogen requirements may be satisfied by either amino acids or nitrate, but the cells are so constructed that they prefer amino acids to nitrate. If there is sufficient amino acid nitrogen available, NR is fully repressed even in the presence of nitrate. If there is less

Table V

Amino Acids mM N	%NR Repression[a]	%Maximum Growth on Amino Acids[b]
0	0	0
2	23	25
4	40	40
6	52	53
8	62	64
10	71	70

a) 3 - day growth period - see fig. 41.

b) 10 - day growth period, fresh weight assay - see fig. 29.

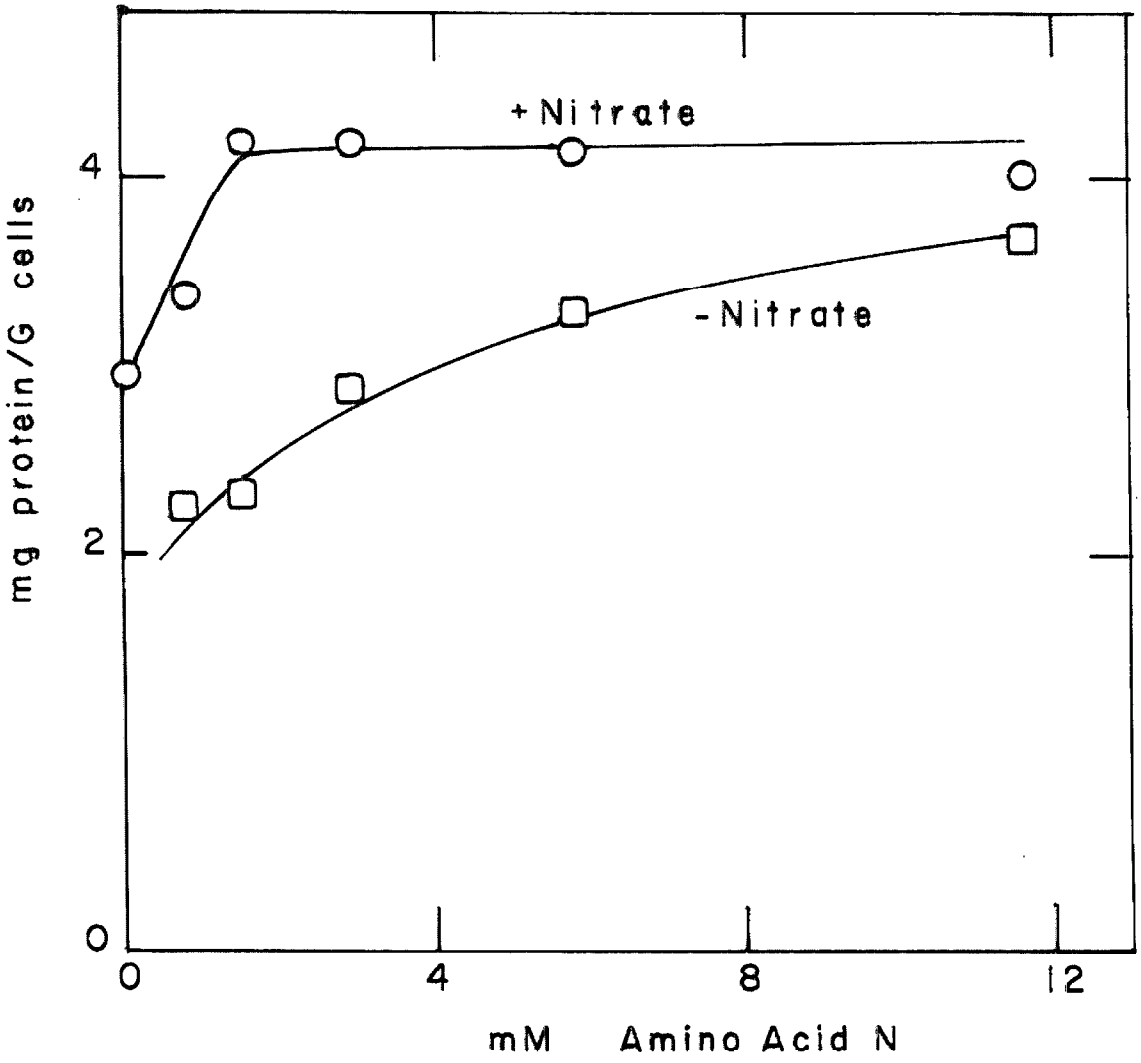


Figure 42

Dependence of the soluble protein content of XD cells on the concentration of casein hydrolysate, and on nitrate. Growth period: 3 days.

than sufficient amino acid nitrogen available, NR is only partly repressed so that the cell can supplement its organic nitrogen supply and establish the regulated maximum endogenous level. If there is no supply of amino acid nitrogen, then NR is not repressed. Full induction of NR in XD cells is sufficient to maintain the maximum growth rate of the cells if nitrate is the sole nitrogen source.

The cellular device which regulates NR induction-repression is a classic example of a negative feedback control system. In effect, it measures the difference between the endogenous amino acid concentration and the optimal concentration for the cell, then translates this into equivalents of NR activity, and finally adjusts the NR level accordingly. If the endogenous amino acid level is too low, NR activity goes up and more amino acids are made. If the endogenous level is too high, NR is repressed until the amino acid excess has been consumed.

The action of this regulatory mechanism may account for the difference in the abilities of X and XD cells to grow on M1D. X cells grow very poorly on M1D. When they are grown in M1D, they only develop about one-fifth the NR activity that is found in comparable cultures of XD cells on M1D. If, as has been suggested, the maximum level of NR is closely related to the maximum growth rate, then the low maximum in X cells could be the cause of the slow growth rate. The low maximum in turn is presumably due to a

difference in the NR regulatory mechanism.

The repressor activity of single amino acids at 10^{-4} M is summarized in table VI. The amino acids were added to M1D before inoculation, from sterile 100-fold stock solutions at pH 6.5. NR activity was determined after 24 hours. It is clear from the table that there is a high correlation between amino acids which inhibit growth (see table III) and amino acids which repress NR. It should be emphasized that the growth assay was performed after 10 days, while the NR assay was performed after only one day. In the presence of inhibitory amino acids, the NR levels are much too low to support cell growth, and hence the inhibitory effect on growth may be completely attributed to the repression of NR. This is valid for all of the inhibitory amino acids except isoleucine. It can be seen in table VI that isoleucine is a non-repressor. The repressors may be subdivided into strong and weak repressors. The weak repressor group includes aspartate, glutamate, histidine, and leucine.

The three non-inhibitory amino acids, arginine, lysine and cysteine are non-repressors. Isoleucine belongs to this group also. The most notable feature of the non-repressors is that they actually augment the induction of NR above that found in the control. This suggests that they may prevent repression by endogenous repressor amino acids.

The repressor does not have to be added before

Table VI

Medium	NR Units/gram cells
M1D	1320
+ 10^{-4} M ala	88
asp	325
asp-NH_2	45
glu	440
gly	20
his	291
leu	308
met	76
pro	48
thr	66
val	88
arg	1600
lys	1620
cys	2640
ileu	1410

induction in order to work. If glycine is added to cells which are fully induced after 24 hours in M1D, then during the next 24 hours all of the induced activity is lost. It thus appears clear that NR is a rapidly turning over enzyme, and that therefore the level in the cell is probably a steady state concentration. It is conceivable that repressors act by accelerating the rate of destruction of NR, rather than by preventing the formation of the active enzyme.

DEREPRESSION

In table VII is presented typical data from an experiment in which a pair of amino acids was tested for its effect on NR induction. The controls indicate that induction does not occur in the absence of nitrate. Repression by glycine is largely prevented by the presence of arginine. Arginine is not an alternative inducer, since it does not induce in the absence of nitrate. It may therefore be concluded that arginine is a derepressor with respect to repression by glycine.

The results of many such pair tests are summarized in figure 43. It is apparent that arginine and lysine are both derepressors with respect to all the amino acids in the table. Cysteine and isoleucine also have extensive

Table VII

Medium	NR Units/gram cells
M1D	857
M1D - nitrate	20
M1D - nitrate + 10^{-4} M arginine	0
M1D + 10^{-4} M glycine	30
M1D + 10^{-4} M glycine + 10^{-4} M arginine	581

Effects of Amino Acid Pairs on Nitrate Reductase Induction

	ALA	ARG	ASP	CYS	GLU	GLY	ILEU	LYS	MET	PRO	THR	VAL
ALA		+	−	−	−	−	−	+	−	−	−	−
$ASPNH_2$	−	+	−	+	−	−	+	+	−	−	−	−
ASP	−	+		+	−	−	+	+	−	−	−	−
GLU	−	+	−	+		−	+	+	−	−	−	−
GLY	−	+	−	+	−		+	+	−	−	−	−
MET	−	+	−	−	−	−	−	+	−	−	−	−
PRO	−	+	−	+	−	−	+	+	−		−	−
THR	−	+	−	+	−	−	+	+	−	−		−
VAL	−	+	−	+	−	−	+	+	−	−	−	

Figure 43

12 day old XD cells were subcultured into M1D containing a pair of amino acids, each at 10^{-4}M, and Nitrate Reductase was determined after 24 hours. Repression: − ; Derepression: +.

derepressor activity, and they share the inability to derepress alanine or methionine repression. The derepressors thus fall into two classes: those which are non-repressors and universal derepressors; those which are non-repressors and limited derepressors.

The data in figure 43 suggest a test of the apparently causal relationship of NR repression by amino acids to growth inhibition by amino acids. The data in table VI predict that a mixture of aspartate, asparagine, glutamate, glycine, proline, threonine and valine should be inhibitory, while figure 43 indicates that isoleucine can derepress each of these repressors individually, and hence should prevent the growth inhibition of the mixture. Furthermore, the addition of methionine to the mixture should make it impossible for isoleucine to prevent the inhibition, because isoleucine cannot derepress methionine repression. Finally, arginine should be able to derepress the whole mixture and therefore prevent growth inhibition. Table VIII shows that all of these predictions are borne out by experiment.

CONCLUDING REMARKS

A procedure for extraction and assay of NR from XD cells has been developed. It is believed that this procedure yields an accurate index of endogenous NR activity.

Table VIII

Supplement to M1D	Relative Growth[a]
None	100
Mix (10^{-4} M each: asp, asp-NH_2, gly, pro, thr, val)	2
Mix + 10^{-4} M ileu	66
Mix + 10^{-4} M met, 10^{-4} M ileu	11
Mix + 10^{-4} M met, 10^{-4} M arg	56

a) 10 day growth period, fresh weight assay.

The level of NR in XD cells on M1D varies with culture age in a way which can be correlated with the available nitrate in the medium. The complete loss of NR activity follows the complete disappearance of nitrate from the medium. When the nitrate supply is replenished by subculturing into fresh medium, NR is induced.

The amino acids of casein hydrolysate repress NR in proportion to their ability to support growth, and thus regulate their own biosynthesis by feedback repression of the first reaction in the pathway from nitrate to amino acids. The regulatory mechanism seems to be part of the cellular apparatus for maintaining a fixed maximum growth rate.

The amino acids fall into two classes: repressors and derepressors of NR induction. The repressors may be further subdivided into weak and strong, while the derepressors may be subdivided into universal and limited. The growth inhibitory effects of amino acid mixtures can be predicted on the basis of the repressor and derepressor activities of the amino acids in the mixture, indicating that NR repression is probably the primary cause of growth inhibition by amino acids. The inhibitory action of a single amino acid may be thought of as a pathological response of the plant cell which is a consequence of a misreading of the information available to it about its

environment. It mistakes a high level of one amino acid as an indication of a high level of all of them and therefore shuts down amino acid synthesis by repressing the induction of NR.

The inability of the cells to use single amino acids as the nitrogen source when they are supplied at levels sufficient to meet the nitrogen requirements of the cells indicates that the cells do not readily interconvert amino acids. However, they can grow on arginine or glutamate at a low rate. This low growth rate indicates that high levels of amino acids inhibit at least one other process, since glutamate is a key intermediate in amino acid biosynthesis. In the absence of a second mode of growth inhibition, glutamate would be expected to function as well or better as a nitrogen source than nitrate.

Inducer, repressor, and derepressor may all affect a single step in the formation of active NR. Alternatively there may be two such steps, one which involves the inducer, nitrate, and a second which involves the derepressor, let us say arginine. The second step would be the one which is sensitive to repressor.

Increases in enzyme activity may be due to increases in cofactor levels which activate preformed protein molecules; destruction of inhibitors; activation of zymogen molecules; or to de novo synthesis of enzyme. Similarly, decreases in enzyme activity may be due to destruction of cofactors;

production of inhibitors; a limited modification of the structure of the enzyme molecule; or complete degradation of the enzyme. The existence of both increases and decreases in NR activity in cultured tobacco cells has definitely been established, but the ways in which these changes are brought about remain to be discovered.

VIII. CONCLUSION

CONCLUDING SUMMARY

1. Two cell lines with distinguishable morphologies and distinguishable responses to nitrogenous nutrients have been isolated from tobacco. The X line grows as clusters of spherical cells, and it grows well on casein hydrolysate but poorly on nitrate. The XD line grows as cylindrical filaments of cells and it grows well on both casein hydrolysate and nitrate.

2. The growth of XD cells on the defined medium M1D was studied. The XD cells multiply exponentially with a generation time of 2 days, and they do so for 4.5 generations. The cell population does not possess constant properties during exponential growth. The rates of daughter cell dissociation, of water uptake, of RNA synthesis, and of soluble protein synthesis vary with culture age. The variation in the rate of soluble protein synthesis does not affect the levels of all enzymes equally.

There are three critical events in the life of an XD culture. The first is the initial reaction of the cells to fresh medium, and it occurs during the first day. The second is the beginning of the reversal of the trends that started with the first event, and it occurs during the fourth through sixth days. The third event occurs about the tenth day, and terminates the growth period.

3. The DNA in exponential XD cells is replicated semiconservatively. All the DNA in the culture is replicated, and it is all replicated in about one generation time. By the criterion of the DNA replication rate, the cell population is homogeneous.

4. During exponential growth, XD cells do not deplete the medium of Na, Mg, Mn, Ca, Fe, or B. They deplete the medium of P by day 6, and of K and N by day 10. Replenishing the supply of P or of K has no known effect on the cells. The growth of XD cells in M1D is strictly proportional to the amount of N supplied as nitrate, so that depletion of nitrate appears to be the growth terminating event on day 10.

5. Single amino acids fall into two classes on the basis of their effects on the growth of XD cells on nitrate. One group is inhibitory, while the other is not. The only non-inhibitors are arginine, lysine and cysteine. The presence of arginine or lysine prevents the inhibition caused by any one of the other amino acids. Cysteine has a similar but limited ability to prevent the inhibitory

action of other amino acids.

6. The effects of amino acids on growth can be explained by their action in the regulation of NR. In XD cells, NR is inducible by nitrate, repressible by inhibiting amino acids, with the exception of isoleucine, and derepressible by non-inhibiting amino acids and isoleucine. Arginine and lysine are universal derepressors, while cysteine and isoleucine have limited derepressor activity. A complete mixture of amino acids (casein hydrolysate) represses NR in proportion to the ability of the amino acid mixture to support growth. Therefore, the inhibition of growth and repression of NR by single amino acids may be thought of as a misinterpretation of the environment by the NR regulatory mechanism. It acts as if a high level of a single amino acid were indicative of a high level of all amino acids.

The growth initiating event which occurs during the first day of the culture cycle has tentatively been identified as NR induction.

CONCLUDING DISCUSSION

Exponential growth of cultured plant cells was first reported by Steward, Caplin and Millar (83), who used carrot root explants in a coconut milk medium. More recently,

Lamport (39), Dougall (84), and Becker, Hui and Albersheim (85) have reported exponential growth of plant cells on complex liquid media. They observed generation times of 2-3 days and exponential growth periods of 3.5 - 4.5 generations. Gadgil and Das (86) measured a generation time of 6 days which lasted for almost 5 generations in a defined liquid medium.

Rapid growth on complex liquid media has also been reported by Tulecke and Nickell (8), Ralph and Bellamy (23), and Flamm, Birnstiel and Filner (87). On defined liquid media, Tulecke (88) has reported a high growth rate, while Torrey and Reinert (38) found an intermediate rate and Staba and Lamba (89) a low rate.

It is clear that both the generation times and periods of exponential growth of the most active systems thus far reported that use suspension culture methods are very similar to those found for XD cells in M1D. Unlike the latter system, however, most others employ complex media. The distinctly advantageous combination of a rapidly multiplying cell line and a chemically defined medium is rare. One very noteworthy system with this combination of properties, but which employs agar medium methods, is that of Murashige and Skoog (7). Their yields per volume of medium were as much as four times the best yields that have been obtained on liquid media. The Murashige and Skoog medium is characterised by high salt concentrations.

The changes in cell properties during the exponential phase have received relatively little attention in the past. Wu, Hildebrandt and Riker (14) described the morphological change seen in suspension cultures as they age. It is characterized by cell enlargement, daughter cell dissociation and a reduced level of mitotic activity. The pattern of changes is strongly reminiscent of the transition from meristematic to parenchymal cells in the growing regions of whole plants. Perhaps the underlying causes of the transition in culture and the transition in the organism are the same. The properties of the transition are much more accessible in cultured cells, since the whole population undergoes the change, and it can be induced or reversed by altering the medium.

Torrey, Reinert and Merkel (90) studied the mitotic activity as a function of culture age. They observed an early increase to a maximum of 3-4% of all cells in mitosis, followed by a gradual decrease. Steward (91) has reported that there is a five-fold increase in RNA per cell, followed by an equal decrease during the exponential growth of carrot root explants. Lippincott and Lippincott (92) reported variations in enzyme activities with culture age for cells grown on agar media. Flamm and Birnstiel (22), working with X cells in M31, observed a three-fold increase followed by an equal decrease in the rate of histone synthesis relative to the rate of nuclear non-histone

synthesis. The maximum level of histone synthesis occurred after about three days of exponential growth.

Although the evidence is extremely limited, it may be tentatively concluded that events which occur in the middle and the end of exponential growth of XD cells also occur in other suspension culture systems. The first 24 hours has not been studied in other systems, so it cannot be said at this time whether the mitotic delay seen in XD cells is a common occurrence.

The changes in cell properties with time during exponential growth are surprising, since a randomly multiplying cell population would be expected to have constant average properties. The changes must be a result of changes in the environment which the cells themselves bring about. It can be concluded from the observations that the requisite cellular conditions for exponential multiplication of plant cells are quite flexible. This property of the cells is quite useful, since at different times during the exponential growth, the cell properties are more or less favorable for a particular study. For instance, the cells have approximately the same content of acid phosphatase throughout the exponential period, while the soluble protein varies three-fold, with the maximum in the middle of the period. The highest specific activities of acid phosphatase may be prepared from either very early or very late exponential cells. Similarly, DNA synthesis may be selectively studied

in late exponential growth, since the rate of RNA synthesis is greatly lowered at that time. The most active period of protein synthesis, on the other hand, occurs during the second and third days. If in fact NR induction involves de novo synthesis of protein, this may be studied with very high selectivity during the first eighteen hours, since NR activity increases at a rate about 400 times that at which general soluble protein increases during this time, while in the subsequent three days, NR activity and soluble protein increase at very similar rates.

Two of the environmental changes that bring about major alterations in cell behavior are the depletion of nitrogen from the medium, and the replenishment of the nitrogen upon subculturing. This is consistent with the findings of others for cultured plant cells. Harris (64) found that the growth of cultured oat embryos was nitrogen limited. Murashige and Skoog (7) found that the nitrogen supply was the major growth limiting factor for tobacco cells on a defined agar medium. Murashige and Skoog also noted interactions of N, P, and K in growth limitation. The growth stimulating activity of amino acid mixtures has been frequently reported for cultured plant material (7, 62, 64, 65, 93, 94).

The fact that growth of cultured plant cells may be experimentally "regulated" by the exogenous nitrogen supply, plus the fact that the endogenous organic nitrogen level is

regulated through feedback control of NR, suggests the possibility that the regulation of endogenous nitrogen may be used by the plant as a device for regulating and coordinating growth. That is, the endogenous nitrogen supply may not be regulated to support the maximum growth rate in all cells. If in the course of development, one cell of the organism must make twice as much protein as another, this could be achieved by giving the one cell access to twice as much nitrogen as the other.

The hypothetical developmental situation could be brought about by a differentiation that results in an altered sensitivity of the feedback mechanisms to amino acids. There is a substantial amount of evidence that amino acids can upset the normal development of a plant, and that NR level is related to developmental processes. The experiments presented herein suggest that amino acid effects on development may in part be due to their action in NR regulation, or perhaps in similarly amino acid sensitive regulatory mechanisms which have not yet been discovered.

Steinberg (58) found that tobacco plant morphology was greatly altered by individual amino acids. Waris (59) induced such totally different plant morphologies with amino acids that he called them neomorphs. The neomorphs retained their abnormal growth patterns for some time after removal of amino acids from their environment. Sanders and Burkholder (62) observed altered morphogenesis in Datura

embryos cultured in the presence of amino acids. Barnes and Naylor (67) found that pine root development was altered by amino acids. LaMotte and Skoog (95) reported an effect of tyrosine on tobacco pith differentiation in culture. Nakashima (96) found that amino acids inhibited flower formation in Lemna.

NR level has been correlated with differentiation and development in several cases. Candella, Fisher and Hewitt (80) found that the NR level in leaves depended on the position of the leaf on the plant. Zieserl and Hageman (82) found that NR in maize depended upon the developmental stage of the plant. Sanderson and Cocking (74) noted a marked difference between the NR levels in the leaves and roots of six species. Raghaven and Torrey (97) found that orchid embryos in culture could not produce NR activity until a certain stage of growth. Champigny (98) reported the presence of NR in maize seedlings independent of exogenous nitrate. The level of NR increased sharply on the fifth day of growth. Oaks and Beevers (99) found that this was about the age at which cultured maize embryos no longer required nutrients from the endosperm for growth on a nitrate medium.

The role of amino acids in the regulation of NR has not been previously documented, although it was suspected by Riker and Gutsche (68), and Afridi and Hewitt (100) tested the effects of some amino acids on NR induction. The effects of amino acids on plant cells in other systems

suggest that in a few cases the regulation of NR is the most likely cause of the observations.

Riker and Gutche (68) studied the effects of amino acids on the growth of sunflower crown gall tissue on a nitrate medium. They found that arginine and glutamate inhibited only at relatively high concentrations, while some amino acids inhibited at low concentrations and high concentrations, such as glycine. Still others inhibited at low concentrations, but inhibited less at high concentrations. Alanine behaved this way. The initial inhibition was probably due to NR repression, while growth at the higher concentration was probably due to the utilization of the amino acid as a nitrogen source.

Harris (64) encountered amino acid interactions when pairs of amino acids were added to a nitrate medium in which oat embryos were grown. Individual amino acids with the exception of arginine were inhibitory to root growth. Various pairs were less inhibitory than either amino acid alone.

Skinner and Street (66) reported an amino acid interaction which is very similar to what has been observed in XD cells. They found that cultured groundsel roots were inhibited by glycine, but that either arginine or lysine could overcome the inhibition due to glycine. Actually, the combination of glycine and either arginine or lysine resulted in more growth than arginine alone.

In other studies of amino acid effects on plant material, arginine has been frequently found to be atypical. This is true in the work of Spoerl (60), Bonner (63), Fries (101), Nakashima (96), and Tulecke (102). The ubiquitousness of arginine effects is perhaps an indication of some fundamental underlying phenomenon involving arginine that has many manifestations.

A common occurrence in systems which possess arginine effects is either the equivalence of arginine and lysine, or a competition between them. The biochemical basis for the arginine-lysine relationship is obscure, since no well known metabolic pathway links them closely. Two suggestive reports can be cited, however. Arginase is competetively inhibited by lysine (103), and both arginine and lysine compete with canavanine for the same sites of entry into Neurospora (104). These two observations suggest that lysine may act like arginine by preventing the destruction of endogenous arginine, or by preventing its accumulation in some intracellular compartment other than the one in which it acts, and thereby bringing about an elevation in the endogenous arginine concentration. When lysine competes with arginine, it may be assumed that lysine is either preventing arginine from getting to the site of action, or that lysine is preventing the formation of arginine derivatives that are the true effector substances.

A regulatory role for arginine was suggested by

Pharis, Barnes and Naylor (105), on the basis of observations of seasonal fluctuations in the xylem sap arginine of trees, and the differences seen in free arginine of pine seedlings grown on nitrate or ammonia. The seasonal fluctuations can be correlated with growth periods. Pine seedlings grow equally well on nitrate or ammonia, yet they have thirty times more arginine when grown on ammonia, indicating that arginine synthesis is excessive on ammonia. The regulation of arginine synthesis must depend on one of the reactions in the path between nitrate and ammonia. Since the arginine level increases greatly on ammonia while most other amino acid levels do not, it seems more likely that some product of the nitrate to ammonia pathway regulates arginine synthesis specifically, rather than that arginine acts as a feedback inhibitor of some step between nitrate and ammonia. The regulation of arginine synthesis and destruction in plant cells should prove to be as interesting as the regulation of nitrate reduction, with which it is connected.

It is also noteworthy that arginine accumulates to very high levels in certain circumstances, accounting for as much as 50% of the free amino acid nitrogen. It accumulates normally in the developing pine seed (106), in artichoke tubers (107), and tulip bulbs (108). Accumulation can be induced by cold treatment (109), iron deficiency (110), and sulfur deficiency (111).

The implication of the work which has been presented is that arginine has a regulatory role in addition to its well known role as a metabolic intermediate in plants. The regulatory function must be considered when arginine effects are investigated. In the past, the arginine effects have usually been assumed to be related to the better known metabolic function.

In whole plants, there are two occasions when NR regulation clearly must determine the behavior of the plant. The first of these occurs during seedling development. When the resevoir of seed protein nitrogen is exhausted, the embryo must have NR activity if it is to grow, since its nitrogen source will thereafter be nitrate. Champigny (98) found that there is NR in maize seedlings even if nitrate is not added, and the level of enzyme activity rises abruptly at about the time that the seed protein nitrogen is exhausted. In contrast, Rijven (76) and Tang and Wu (75) found that NR was induced by nitrate in wheat embryos and rice embryos,respectively. The differences may be due to the presence of endogenous nitrate in one case, but not the others. The rise seen on the fifth day in maize could be due to the cessation of repression as the seed protein nitrogen is depleted. In either case, the regulation of NR in developing seedlings appears to be a good place in which to evaluate the importance of the regulatory system first recognized in XD

cells.

The other time that NR regulation is important is when nitrate passes into the roots of a mature plant. Some plants transport nitrate to the leaves before it is reduced, while others reduce it in the roots and transport organic forms. The regulatory situation must be different in the two cases. In plants where nitrate is transported through the roots to the leaves, root NR must be well repressed. The low levels found in the roots relative to the leaves by Sanderson and Cocking (74) indicate this. The repression may be due to either a higher level of repressor in the roots compared to the leaves, or to a regulatory mechanism which is more sensitive to a given concentration of repressor in the roots than in the leaves. The latter seems the more likely situation, in the light of the low levels of amino acids found in the roots relative to the leaves. In contrast to the regulation of NR in the embryo, where it might be expected to be adjusted for the support of a maximum growth rate, NR regulation may be adjusted differently in roots and leaves. The hypothesized difference in root and leaf NR regulation would be an example of regulatory differentiation, and consequently of great theoretical interest to students of development.

The XD plus M1D culture system offers the intriguing possibility of selecting NR regulatory variants for use in the study of the mechanism. Amino acid resistance would be

the selective agent. The XD line itself may be a regulatory variant of the X line, since both grow equally well under optimal conditions, but the X line grows poorly on nitrate and has a low NR level compared to XD cells. One fundamental question which can be answered with such variants is whether all the repressors act in the same way. If they do, a variant resistant to one repressor should be resistant to all. The relationships among the derepressors could also be analysed with the aid of properly selected variants.

Permanent variants have frequently been observed in plant cell cultures. Limasset and Gautheret (112) reported the transformation of normal auxin requiring tobacco cells to crown gall tumor-like cells in culture. Fox (113) isolated two variants of tobacco cells, one requiring neither an auxin nor a kinin, and another requiring only an auxin, while the parent strain required both. Hildebrandt and Riker (114) found that clones of tobacco cells varied in their susceptibility to Tobacco Mosaic Virus. Blakely and Steward (115) used acriflavine resistance as a selection tool for isolating variants. Straus (116) isolated cultured maize endosperm variants that differed in their pigmentation. He observed a frequency of one unpigmented culture for every four thousand pigmented ones. Tulecke (117) reported the occurrence of a green variant in a colorless strain of Taxus pollen cells. Torrey and Shigemura (118) have studied a friable variant of pea root callus.

The many variants which have been observed are permanent in the sense that it is not known how to reverse them, and they have heritable characteristics, in the sense that they carry them for an indefinite number of cell generations. It is not known, however, whether these variants are a result of a permanent change in the genetic material, or merely a change in the expression of the information in the genetic material. That is, the selective procedures may in some cases yield differentiated rather than mutated cells.

Very few inducible enzymes have been described in plants. Tolbert and Cohan (119) reported induction of glycolic acid oxidase by glycolic acid in etiolated wheat seedlings. Galston and Dahlberg (120) found that indole-3-acetic acid (IAA) oxidase was induced by IAA in etiolated pea seedlings. The first report of NR induction was that of Tang and Wu (75), who found that nitrate induced NR in rice embryos. Venis (121) has reported that IAA also induces in pea seedlings an activity that synthesizes benzoyl aspartate. The induction is sensitive to actinomycin D, an inhibitor of DNA dependent RNA synthesis, and puromycin, an inhibitor of protein synthesis. Paleg (122) found that gibberellin induced alpha-amylase in barley aleurone. Varner (123), showed that alpha-amylase induction was due to de novo synthesis of enzyme.

If induction and repression are important in plants,

it is most likely in connection with differentiation and development. Cells inside a non-developing organism are surrounded by a stable environment, and should therefore have very little need for elaborate devices for dealing with transient environmental perturbations. The critical role of NR regulation in the growth of cultured plant cells strongly suggests that the induction and repression of this enzyme are important factors in the development of plants.

The techniques which were described and the results which were presented serve to illustrate how the properties of rapidly multiplying plant cells may be approached in suspension cultures. The suspension culture system bridges the experimental gap between plant cells and microorganisms, and allows the validity of hypotheses such as the semi-conservative mechanism of DNA replication, and the feedback control of amino acid biosynthesis through regulation of nitrate utilization, to be directly tested. The potential usefulness of plant cell suspension cultures as a tool for elucidating basic phenomena of higher organisms has only begun to be realized.

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