AN INVESTIGATION INTO THE PHYSIOLOGICAL AND NUTRITIONAL REQUIREMENTS OF THE PROTOPLAST STAGE ON THE FUNGUS ENTOMOPHTHORA EGRESSA MacLEOD AND TYRRELL (PHYCOMYCETE: ENTOMOPHTHORALES)

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An Investigation into the Physiological and Nutritional Requirements of the Protoplast Stage of the Fungus *Entomophthora egressa* MacLeod and Tyrrell (Phycomycete: Entomophthorales)

By

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Abstract

The physiological and nutritional requirements of the entomogenous phycomycete Entomophthora egressa MacLeod and Tyrrell were determined using the wall-free protoplast stage.

Based on the uptake of ninhydrin-positive compounds the amino acids in the standard medium, Grace's modified insect tissue culture medium, were simplified to contain the amino acids methionine, asparagine and glutamine.

The buffer 2-(N-morpholino) ethanesulfonic acid afforded excellent buffering capacity. The protoplasts utilized glucose, were vitamin autotrophic in two different media and required protein for membrane integrity.

The regeneration patterns were studied throughout the experiments using various liquid and solid media. It was possible to induce mycelial and hyphal body development in the two simplified liquid media.

Biochemical analysis of the fungal host, Lambdina fiscellaria fiscellaria (Guenée) in terms of haemolymph amino acids resulted in the development of a medium superior to the standard medium. Fatty acid analysis and total lipid production for various stages of the fungal host and protoplasts were determined.

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Introduction

The eastern hemlock looper, Lambdina fiscellaria fiscellaria (Guenée) is a serious defoliator of balsam fir (Abies balsamea (L.) Miller) in Newfoundland. Outbreaks last from 5 to 7 years with 5 to 12 years between cycles. The 1966 outbreak covered about 2 million acres by 1971. Aerial spraying programmes using fenitrothion and phosphamidon in 1968 and 1969 were believed to be effective in terminating the outbreak in treated plots. Infection by two entomogenous fungi, Entomophthora egressa MacLeod and Tyrrell and Entomophthora sphaerosperma Fresenius also contributed to the collapse of several infestations (Otvos et al., 1971; Otvos et al., 1973).

The application of E. egressa and E. sphaerosperma in biological control programmes for the eastern hemlock looper appears promising based on a 5% to 95% incidence of infection in the field. Otvos et al. (1973) reported the transmission of E. egressa to healthy larvae in the field from larvae artificially infected with the wall-free protoplast stage. The fungus also has been reported in the spruce budworm, Choristoneura fumiferana Clem. (Vandenberg and Soper, 1975). It is the thick-walled, environmentally resistant, resting spore stage which appears to offer the greatest potential for field application (Tyrrell, 1970) but the practical utilization of Entomophthora species as

microbial pathogens has been hindered by the inability to mass produce resting spores (Matanmi and Libby, 1976).

Because of the potential of E. egressa the physiological and nutritional requirements as well as the morphological development of the fungus were examined. Biochemical analysis of the host larvae were also done.

Tyrrell and MacLeod (1972b) discovered the wall-free protoplast stage of E. egressa when conidia, obtained from infected looper larvae in Newfoundland, germinated in Grace's modified insect tissue culture medium (Grace, 1962). Serial propagation of the protoplasts was readily accomplished. It was this feature which made the protoplast stage the preferred stage for experimentation as opposed to the difficulty of maintaining the mycelial stage on Muller-Kogler's coagulated egg yolk medium (Muller-Kogler, 1959).

The composition of Grace's medium is fairly well defined, although composed of many components and having fetal calf serum as the major non-defined or complex component. The natural host was generally available for ninhydrin-positive analysis of the hemolymph as well as total lipid and fatty acid analysis of whole larvae. The pathogen is believed to grow in the haemolymph and destroy fatty tissues. The former determinations were instrumental in formulating media which reflected more closely the chemical environment provided by the host and allowed for reasoned modification of Grace's modified medium. The nutritional and morphological behaviour of the fungal cells on Grace's medium

fungal cell morphology constituted the second approac . to medium simplification.

Literature Review

The majority of protoplasts derived from fungal mycelia and yeasts have been produced by the use of enzymes such as the gastric enzymes, e.g. helicase of Helix pomatia L. combined with cellulase from Aspergillus niger Teigh and extracellular enzymes of species of Streptomyces (Villaneuva, 1966; Gabriel, 1968; Musilkova and Fencl, 1968; Peberdy and Gibson, 1971; Seitsma and De Boer, 1973; Kopecka, 1974; and Akimenko et al., 1975). Berliner and Reca (1970) reported the development of protoplasts from types A and B of the parasitic yeast phase of the human pathogen Histoplasma capsulatum Darling in 2 M magnesium sulfate. Tyrrell and MacLeod (1972b) described the development of Entomophthora egressa protoplasts from conidia in Grace's insect tissue culture medium (Grace, 1962) supplemented with heat inactivated fetal calf serum.

The removal of the protoplast forming enzymes and the transfer of protoplasts to a nutritive medium usually initiated cell-wall regeneration and mycelial development. Protoplasts of Rhizopus nigricans Ehrenberg (Zygomycete: Mucorales) (Gabriel, 1968) and a species of Pythium (Seitsma and De Boer, 1973) so treated did not exhibit the diversification of regenerative patterns reported for Aspergillus nidulans (Eidam) Winter (Peberdy and Gibson, 1971) and Fusarium culmorum (Smith) Sacc. (Garcia-Acha et al., 1966). Gabriel (1968) reported the development of a cell wall

around the larger protoplasts of R. nigricans followed by hyphal development. A. nidulans protoplasts revealed two patterns when grown in liquid media (Peberdy and Gibson, 1971). Many protoplasts gave rise to chains of yeast-like cells, the terminal one of which produced a true hypha. The second reversion process consisted of the formation of a shell into which the cytoplasm migrated. Sietsma and De Boer (1973) reported bud-like development from protoplasts of Pythium PRL 2142 using both solid and liquid media.

The study of nutritional and physiological requirements of protoplasts has been limited. Villaneuva (1966) reported on the vitamin requirements of the walled yeast Saccharomyces cerevisiae Hansen and the protoplast stage. The effects of osmolarity on respiration of the protoplasts from yeasts were also considered.

Temperature is one of the limiting factors for the growth and development of Entomophthora species in the natural environment (Hall and Bell, 1961). Vegetative growth has been reported over a temperature range of 15°C to 30°C. The optimum temperature for vegetative growth of E. ignoblis Hall and Dunn, E. obscura Hall and Dunn was 24°C (Hall and Bell, 1961). Gustafsson (1965a) reported the optimum temperatures for eight species of Entomophthora grown on both Sabauraud maltose broth and agar: E. exitalis Hall and Dunn, E. dipterigena (Thaxter) Gustafs, E. thaxteriana (Petch) Hall and Bell, E. aphidis Hoffman, E. ovispora Nowakowski and E. sphaerosperma Fres. grew and sporulated well over a

temperature range of 20°C to 25°C. The precise cardinal temperatures were species variable. E. virulenta Hall and Dunn when grown on Sabouraud dextrose agar possessed an optimum temperature of 30°C (Hall and Bell, 1961).

Sawyer (1929) reported the optimum pH for vegetative growth of E. sphaerosperma to be pH 6.2 with upper and lower cardinal points of pH 5.8 and 8.2, respectively. Wolf (1951) documented the optimum pH values for E. apiculata as pH 5.5 to 7.0. Gustafsson (1965a), on the basis of his data proposed a feature common to the Zygomycetes to be the wide pH range tolerated. Entomophthora species grew well over a pH range of 4.5 to 7.8. Entomophthora egressa exhibited a narrow tolerance range of pH 5.6 to pH 7.5 with an optimum pH of 6.8.

The obligate parasitism of many species of Entomophthora and their sparse growth on coagulated egg yolk implies the need for specific growth factors (Sawyer, 1929; Latge, 1975a). Sawyer (1929) successfully cultivated E. sphaerosperma and E. muscae using complex natural products as media.

The vitamin autotrophic nature of many species of the Order Entomophthorales has been well documented. Wolf (1951) demonstrated vitamin autotrophy for E. apiculata and E. coronata (Cost.) Kevorkian ((Delacroxia coronata (Sacc and Syd) Tyrrell and MacLeod (Tyrrell and MacLeod, 1972a))) with confirmation for the later species by Smith (1953). Latge (1975b) reported E. virulenta, E. destruens Weiser and Batko Basidiobolus ranarum Edam and Conidiobolous osmodes Drechsl

to be vitamin autotrophic.

Sawyer (1929), Wolf (1951), Gustafsson (1965a) and Latgé (1975b) reported that nitrates were not utilized by many species of the Order Entomophthorales, whereas ammonia was readily assimilated. Latgé (1975b) found that the anionic component of the ammonium salt influenced fungal development. The sulfate and acetate salts of ammonia supported growth and sporulation of B. ranarum, C. osmodes, E. destruens and E. nr thaxteriana, while ammonium nitrate did not. Gustafsson (1965a) reported ammonium nitrate uptake by eight species of Entomophthora tested.

In general, amino acids appear to be poor carbon sources for lower fungi (Gleason, 1968). Nolan (1970), Nolan and Lewis (1974), and Nolan (1975, 1976) followed amino acid and amino derivative uptake for representatives of the orders Blastocladiales and Saprolegniales grown in a liquid medium containing a mixture of amino acids with and without a readily usable carbon source throughout a growth curve. The effects of deleting a carbohydrate favoured increased ammonia production and a more rapid initial decrease in the levels of a few amino acids than when a carbohydrate was supplied. The rise in ammonia concentration was believed to be the result of oxidative deamination. Wolf (1951) and Gustafsson (1965a) detected the ability of E. apiculata and D. coronata to use amino acids as carbon sources. This was also true for E. culicis (Gustafsson, 1965a).

Wolf (1951) in his study on D. coronata compared the

ability of 24 amino acids to support vegetative growth and found that arginine was best. Latge (1975b) tested, in addition to individually added amino acids, an amino acid mixture and protein hydrolysate solution. The effects were considered in terms of vegetative growth, conidia and resting spore production. All organic nitrogen sources favoured extensive mycelial growth. Only protein hydrolysates yielded "good" to "excellent" resting spore production.

'In terms of sporulation and growth, the effects of individually added amino acids varied within a genus and between genera of the Entomophthorales. Basidiobolus ranarum was the most metabolically versatile in terms of sporulation and C. osmodes was second. Resting spore production was moderate among the species of Entomophthora tested (Latge, 1975b).

Pelletier and Keitt (1954), Cochrane (1958), Nicholas (1965), and Moore-Landecker (1972) stated that fungal development is usually better in a medium with a mixture of different amino acids than with any single amino acid. Latge (1975b) reported this for representatives of the Order Entomophthorales.

Gustafsson (1965a) reported utilization of the fatty acids C16:0, C18:0, and C18:2 by E. culicis, E. curvispora. E. ovispora, E. sphaerosperma and confirmed Wolf's (1951) findings for E. apiculata and D. coronata. Entomophthora virulenta and E. thaxteriana also used these fatty acids (Gustafsson, 1965a; Latge, 1975a).

Two patterns of lipid synthesis in the lower fungi

have been reported by Weete (1974). During the experimental growth phase of Rhizopus arrhizus Fischer there was a rapid synthesis and accumulation of lipid. Lipid utilization occurred during the stationary - reproductive period. The reduction of lipid may have been a reflection of nutrient exhaustion or metabolic changes inherent in reproduction. The Oomycete, Pythium ultimum Trow tended to accumulate fat as the mycelium aged.

One of the interesting features of the fatty acid profiles of the lower fungi is the formation of C18:3 $\Delta^{6,9,12}$ (γ - linolenic acid) (Shaw, 1965, 1966; Tyrrell, 1967; Weete, 1974). The fatty acid distribution in the Zygomycetes is less complex than other lower fungi. The Thamnidiaceae and Mucoraceae produce unsaturated fatty acids up to C18:3, the Entomophthoraceae up to C20:4. The Zygomycetes also contain a greater abundance of fatty acids of the C20 and C22 series than the other lower fungi (Weete, 1974). Tyrrell (1967) found that the most salient feature among 17 isolates of Entomophthora was the presence of high levels of C14:0 and C20:4 fatty acids. The fatty acids C16:0 and C18:3 were also abundant.

Materials and Methods

I. Protoplast preparation and maintenance

Entomophthora egressa (isolate 458) was obtained from Dr. D. M. MacLeod (Insect Pathology Research Institute, Canadian Forestry Service, Sault Ste. Marie, Ontario) on the mycelial stage on Muller-Kogler's coagulated egg yolk medium.

The outer most periphery of the mycelium was aseptically added to a clinical centrifuge tube, suspended in Grace's modified insect tissue culture medium (Table 1) and washed by agitation on a test tube vortex. The resulting suspension was centrifuged at 150 xg in a clinical centrifuge (Damon/IEC Division, Needham Heights, Massachusetts), the supernatant was decanted and the pellet re-suspended in fresh Grace's medium. This procedure was repeated until no trace of egg yolk remained. The final suspension was distributed into 30 ml tissue culture flasks containing 15 ml of the Grace's medium.

Incubation was initially at $21^0C \pm 1^0C$. Once protoplasts were detected they were subcultured in fresh media (either a commercial preparation obtained from Grand Island Biological Company, Grand Island, New York or a preparation produced in the laboratory) every two days, later every three days.

The cells grew in catenulate chains of varying length, although individual chains were also detected.

Table 1

Composition of Grace's Modified Insect Tissue

Culture Medium Supplemented with Fetal Calf Serum

Compound	Concentration[a]	Compound	Concentration
$NaH_2PO_4.H_2O$	1013.0	Sucrose	26680.0
KCl	2240.0	Fructose	400.0
$CaCl_2$	1000.0	Glucose	700.0
$MgCl_2.6H_2O$	2280.0		
$MgSO_4.7H_2O$	2780.0	Malic acid	670.0
$NaHCO_3$	350.0	α-Ketoglutaric acid	370.0
		D-succinic acid	60.0
L-Argmine HCl	700.0	Fumaric acid	55.0
L-Aspartic acid	350.0		
L-Asparagine	350.0	Thiamine HCl	0.020
L-Alanine	225.0	Riboflavin	0.020
B-Alanine	200.0	D-Capantothenate	0.020
		Pyridoxine HCl	0.020
L-Cystine	22.0	P-Aminobenzoic acid	0.020
L-Glutamic acid	600.0	Folic acid	0.020
L-Glutamine	600.0	Niacin	0.020
L-Glycine	650.0	i-Inositol	0.020
L-Histidine	2500.0	Biotin	0.010
		Choline Cl	0.200
L-Isoleucine	50.0		
L-Leucine	75.0	Fetal calf serum	20.0[b]
L-Lysine HCl	625.0		
L-Methionine	50.0		
L-Proline	350.0		
L-Phenylalanine	150.0		
DL-Serine	1100.0		
L-Tyrosine	50.0		
L-Tryptophan	100.0		
L-Threonine	175.0		
L-Valine	100.0		

a - in mgm/L

b - in ml added to one liter of media

II. Cleaning of glassware

All glassware for mycological and biochemical use was vigorously washed in hot soapy water and rinsed several times under hot running tap water. A solution of concentrated sulfuric acid (reagent grade) saturated with potassium dichromate was used to chemically clean the glassware. The glassware, after soaking for at least five hours in the acid bath, was rinsed several times with hot running tap water followed by double-distilled water. Pyrex glassware was used unless otherwise stated.

III. Chemicals, plastic equipment, media preparation, and osmotic pressure measurements

All chemicals were reagent grade from Sigma Chemical Company (St. Louis, Mo.) except for the heat inactivated fetal calf serum (Grand Island Biological Company, Grand Island, N.Y.) and agar (Oxoid Agar, No. 3, Oxoid Ltd., London).

Tissue culture flasks were obtained from Falcon Division, Becton Dickinson and Company (Oxnard, California) and Nalgene filters, 0.2 µm from Nalgene Labware Division, Sybron Corporation (Rochester, N.Y.).

All media were filter sterilized. Stock cultures were maintained on standard media filtered through 0.20 µm filters and experimental media through 0.45 µm Millipore membranes. The components of the Millipore membrane system were individually autoclaved and aseptically assembled.

The pH of the culture media was, if commerically prepared, checked prior to inoculation. Laboratory preparations were pH adjusted prior to filtration and checked before inoculation. Initially pH 6.15 ± 0.05 was used for the preliminary experiments and for the standard medium. Adjustments were made using 4 N KOH.

Generally 125 ml Bellco flasks (Bellco Glass, Inc., Vineland, New Jersey) with stainless steel caps were used. With the exception of the experiments dealing with the growth curve for stationary cultures of protoplasts in standard media, modified standard media, media based on haemolymph analysis and lacking serum and buffers, optimum temperature, the osmotic stabilizers, optimum osmotic pressure and buffer analysis 50 ml aliquots of media were used.

For the experiments dealing with the osmotic stabilizers, optimum osmotic pressure and buffer analysis 20 ml volumes of media in 30 ml tissue culture flasks were used and 5 ml for media based upon haemolymph analysis lacking serum and artificial buffers. The stationary culture growth curve and optimum temperature experiments used 30 ml of media in 125 ml Bellco flasks.

The osmotic pressure of modified media was determined using an Advanced Osmometer (Model 31, Needham Heights, Mass.) prior to inoculation. The osmotic pressure was also checked during most growth curve experiments. At least three replicates were used.

IV. Inoculum preparation and cell counts

The age of the cell cultures used as inocula varied from 24 to 58 hours old and were always in the exponential phase of growth. The volumes of inocula used varied from 0.1 ml to 0.3 ml, and the cell concentrations used varied from $1.1x10^5$ to $6.0x10^5$ cells per ml (for precise values refer to the text).

The procedure used for obtaining the inoculum for the stationary growth curve, experiments with modified media, the medium based on haemolymph analysis lacking serum and artificial buffers, and optimum temperature consisted of centrifugation of the cells at 150 xg for two minutes. Using a sucrose solution (330.6 mM) buffered by 2- (N-morpholino) ethanesulfonic acid (MES) at pH 6.2 the cells were washed and centrifuged three times. The fourth resuspension was used as the inoculum.

Later experiments omitted the centrifugation and washing, the cellular suspension being directly added to the experimental media. To determine the effect of omission of fetal calf serum on the growth rate of protoplasts in stationary culture a series of dilution transfers of cells from stock culture to medium M13Aa without fetal calf serum was used. After a $1.5x10^{70}$ fold dilution a growth curve was done using the non-protein containing M13Aa.

The same procedure of serial transfers was used for testing the vitamin requirements except that the medium M13Aa contained fetal calf serum and lacked vitamins. The culture

was incubated on the gyrotary shaker.

Cell counts were made on a haemocytometer (1/400 sq mm x 1/10 mm) (C.A. Hauser and Sons, Phil.). Usually five replicate flasks were sampled, each sample being counted at least twice.

Each swelling on a protoplast chain as well as each cell not in a chain was considered as one cell based on nucleic acid staining (Appendix I).

Morphological development was generally followed throughout most growth curves. The haemocytometer was used, for the most part, to ensure realistic spatial relationships, later samples which possessed a cell wall were mounted in lactophenol.

With the exception of the stationary growth curve, optimum temperature and buffer response experiments, all subsequent experiments were incubated on a horizontal gyrotary shaker (100 rpm, $20^0C \pm 1^0C$; Psychrotherm, Model G27, New Brunswick Scientific Co., New Brunswick, New Jersey) in darkness.

V. Analysis of the eastern hemlock looper larvae.

1. Rearing of larvae.

Eastern hemlock looper, Lambdina fiscellaria fiscellaria (Guenée) larvae for haemolymph analysis were reared from eclosion on the solid medium devised by McMorran (1965). When the larvae reached the third instar, they were placed on balsam fir foliage (Abies balsamea (L.) Mill.). Larvae for lipid and fatty acid analysis were field collected and reared in the laboratory on balsam fir.

The incubator (75% ± 5% RH) had a twelve hour photoperiod with a temperature regime of 20°C ± 0.5°C and 18°C ± 0.5°C for the light and dark periods, respectively. Larvae for haemolymph analysis were reared in groups of approximately 10, those for lipid analysis were reared in groups of 5 per cheesecloth covered glass container (8.0 cm high x 10.0 cm diameter). The insects were manipulated with alcohol sterilized wide forceps. Insects were checked twice daily to determine the instar and health of individual specimens. Those larvae exhibiting discoloration or abberant activity were discarded. Fresh foliage was added at three day intervals and the soiled cheesecloth was then replaced.

All glassware was vigorously washed in hot Tor disinfectant, rinsed with distilled water, and dried at 45°C for 72 hours.

The size of the samples was tested by the efficient sample size formula (Sokal and Rohlf, 1969) to determine that each sample was adequately large for testing the statistical significance of differences between the mean masses for the individual larvae of tested samples. The mean values were compared by the use of the t-test.

2. Bleeding procedure

Only larvae four days into the fourth instar and weighing between 90 to 157 mgm were used. To facilitate bleeding larvae were placed on an alcohol sterilized glass surface and gently restrained at both the anterior and posterior ends. The dorsal integument was punctured at the

anterior and posterior ends and the haemolymph was withdrawn using a sterile 100 µl pipette. The haemolymph was placed in a pre-chilled polyethylene vial containing a few crystals of phenylthiourea (BDH Chemicals Ltd., Poole, England). Two samples of pooled haemolymph were used, one from 117 larvae and the other from 43 larvae. Haemolymph samples were stored at -17°C (refer to Section V.4. for ninhydrin positive compounds analytical procedures).

3. Total lipid and fatty acid analysis

Chemicals and solvents were analytical grade and were used without further purification. The reference fatty acid methyl esters were obtained from Applied Science Laboratories (State College, Pennsylvania). Larvae 3 days into the third instar, 4 days into the fourth instar, 1 day old prepupae and pupae 5 days old were used.

The lipids were extracted by the method of Bligh and Dyer (1959). The total extractable lipid was determined by evaporating a known volume of the initial lipid extract in a pretared round-bottom flask under reduced pressure on a rotary vaporizer (Rinco Instrument Co. Ltd., Greenville, Illinois). The results for each stadium were expressed in mgm lipid per gm (wet mass) of insect.

Fatty acid esterification followed the method of Keough et al. (1972) except that the sealed tubes containing 6% sulfuric acid in methanol were heated at 80°C for 16 hours. The methyl esters were dissolved in carbon disulphide for chromatography on a 1.84 m (1.55 mm internal diameter) column packed with diatomaceous earth (Mesh size 100/120) coated

with 10% diethyleneglycol succinate (10% EGSS-Y, Lot SP-1178, Applied Science Laboratories). The column was designed to facilitate the separation of C18:0, C18:1, C18:2, C18:3, and C20:0 fatty acids. The Packard Model 7401 Gas Chromatograph (Technical Marketing Associates Ltd., Montreal) had input and output temperatures of 230°C. The column temperature was 130°C for elution of short chain fatty acids, 165°C for chain lengths ranging from C14 through C18, and 190°C for longer chain fatty acids. The runs consisted of two replicates at each temperature. Nitrogen with a flow rate of 40 ml per minute was the inert carrier gas. The effluent esters were detected by a B-ionization detector.

The unknown esters were tentatively identified by comparison with the retention times and elution positions of isothermally run standards for the three temperatures used.

Preliminary analysis revealed the absence of methyl-heptadeconate in all developmental stages studied. Therefore, this compound was included in all subsequent runs at a known concentration and served as a quantitative standard for the unknown methyl esters.

4. Ninhydrin-positive compounds and polyamine analysis

Each sample of haemolymph and growth medium was analyzed once for the concentrations of ninhydrin-positive compounds with a Beckman Spinco automatic amino acid analyser (Model 121, Spinco Division, Beckman Instruments, Inc., Palo Alto, California) equipped for physiological runs. Sodium citrate buffers were used. Prior to analysis, samples were

deproteinized with sulphosalicylic acid.

Polyamine analyses were made in an analogous manner except that a Beckman polystyrene resin (Beckman PA-35) and two citrate buffer concentrations (0.35 and 2.35 M) at pH 5.2 were used.

VI. Glucose determinations

Using a Glucostat Special enzyme kit (Worthington Biochemical Corp., Freehold, N.J.) glucose levels in Grace's modified insect tissue culture medium were determined.

The glucostat reagents of glucose oxidase and chromogen were dissolved in 50 ml of phosphate buffer, pH 6.9 (40 mM KH_2PO_4 and 49 mM K_2HPO_4).

Glucose standards of 100mgm/100ml, 200mgm/100ml and 300mgm/100ml were prepared. No benzoic acid was added to the standards.

A 0.1 ml volume of standards and unknowns was added to designated test tubes. To this 1.9 ml of distilled water was added and mixed on a test tube vortex. A reagent blank consisted of 2 ml of distilled water.

To each tube 2 ml of glucostat reagent was added at 15 second intervals followed by mixing. Ten minutes later 4 drops of 4N HCl were added, also in 15 second intervals, followed by agitation. The resulting suspension was allowed to settle for 5 minutes before reading absorbance at 420 nm on a Bausch and Lomb Spectronic 20 spectrophotometer. A 100 mgm % glucose standard was used to periodically check absorption.

VII. Protoplast regeneration on solidified media.

A 5 ml aliquot of 24 hour old protoplasts from the stock culture was dispensed onto a moderately dry coagulated egg yolk medium (Muller-Kogler, 1959) supplemented with tristearin (26 µgm/ml). The egg yolk medium had been previously exposed to 20°C for five days prior to inoculation to ensure rapid absorption of the liquid culture medium. A 5 ml sample was also added to a water agar plate (pH 6.1) treated the same as the coagulated egg yolk medium.

Initial observations were made by removing the surface layers of the coagulated egg yolk and washing the surface with 10 mM MES buffered sucrose solution (pH 6.15, final osmolarity 340 mM). A haemocytometer was used to view the stages of development.

VIII. Lipid production and fatty acid analysis of protoplasts of *E. egressa*.

Using the final simplified medium the protoplasts, grown under the shaken conditions previously described, were collected at 49.5 hours, 72.5 hours and 142 hours. Three replicates were used for each time.

Each sample was centrifuge washed in 175 mM sodium chloride five times. The separate samples were extracted three times by the method of Bligh and Dyer (1959) and the aqueous phase washed several times with 10 ml portions of chloroform. The organic phases for each sample were pooled. The cells were re-extracted by the method of Folch *et al.* (1957). All extracts for a given sample were pooled and

evaporated to dryness. The total lipid for each time of harvest was expressed as mgm lipid per gm of cells (dry mass).

The lipid samples were transesterified by the method of Morgan et al. (1963) and tentatively identified on the gas chromatograph previous described. A program run was used. The initial column temperature was 100°C and this was increased at a rate of 12°/min to 195°C followed by a 60 minute hold.

IX. Statistical analytical procedures

The following calculations were made for experimental data.

Mean (M) = $\frac{\Sigma Y}{N}$ for cell counts and all biochemical measurements,

Standard error (SE) = $\sqrt{\frac{\Sigma Y^2 - \frac{(\Sigma Y)^2}{N}}{N(N-1)}}$ where ΣY is the sum of data Y and N is the number of data for all biochemical measurements,

Standard error (SE) = $\sqrt{\frac{M}{N}}$ for protoplast counts,

95% Confidence Intervals: $M \pm t_{0.05,\, df} SE$ for all data where $df \neq n-1$

Statistical analysis was restricted to comparison of confidence intervals as opposed to nominal statistic tests (Natrella, 1972) except for the comparison of the effects of shaking cultures on cell yield in which case a t-test was used.

All graphs were recorded with 95% confidence intervals and all table with standard errors of the mean.

Results and Discussion

I. Effects of physical factors on protoplast growth

1. Shaken and stationary cultures

A stationary culture of 24 hour old protoplasts growing in chains had a mean nuclear count of 1.21 nuclei per swelling (S = 0.11, N = 111). This was felt sufficient evidence to warrant counting each swelling as one cell, a belief that was later supported by growth of shaken cultures.

Initially the protoplast cells did not tolerate shaking on the horizontal, gyrotary shaker at 50, 100 or 150 rpm. By considering a series of fetal calf serum increments of 2 ml to a liter of Grace's medium (standard medium) over the range of the shaking regime starting with 20 ml to a liter of standard medium it was found that 28 ml to a liter of standard medium supported spindle form and permitted growth in media shaken at 100 rpm. At levels of serum less than 28 ml the cells became spherical and revealed no signs of growth over the 72 hour test period. At 150 rpm the 28 ml volume of serum was not effective.

Using a 24 hour old culture of protoplasts a 0.1 ml inoculum consisting of 6.0×10^5 cells/ml was added to ten Bellco flasks containing the standard medium. One sample of five replicates was incubated as a stationary culture; the other, also of five replicates, was incubated on a gyrotary shaker at 100 rpm. Incubation temperature was 21°C. Cell concentrations were compared after 23 hours incubation.

The results (Table 2) revealed the shaken culture mean yield to be 1.6 times greater than that of the stationary culture. Smith (1953) reported that shaking D. coronata in liquid media resulted in greater yields. This was believed to be due to increased aeration (Smith, 1953; Cochrane, 1958; Rhodes and Fletcher, 1966) although nutritional homogeneity was also thought to have been a contributing element (Hawker and Linton, 1972). Shaking cultures was the preferred method for most of the experiments.

Although protoplasts in stationary culture generally grew in catenulate chains (Plate 1, Figure 1) protoplasts of shaken cultures grew as separate non-chain cells (Plate 1, Figure 2) for over five successive transfers. This was believed to be further support for the contention that each swelling on the protoplast chain was the equivalent of a cell and in view of the serial transfer results each cell was presumably capable of dividing.

2. Osmotic stabilizer analysis

Because of the absence of cell walls it was thought that the osmotic pressure of the medium would be critical to protoplast survival and growth. The osmolarity of the standard medium was 349 milliosmoles per liter (mOsm), and the calculated osmolarity was 350 mM. Thus, the osmolarity calculation was believed to be sufficiently close to the actual osmotic pressure to permit accurate determinations of the mass of an osmotic stabilizer required to sustain the necessary osmotic pressure of simplified culture media.

TABLE 2

Effects of shaken and stationary incubation of protoplast cells after 23 hours incubation

Incubation conditions	Cell yield (cells/ml)
Stationary culture	$1.67 \times 10^5 \pm 1.3 \times 10^4$
Shaken culture[a]	$2.72 \times 10^5 \pm 1.6 \times 10^4$

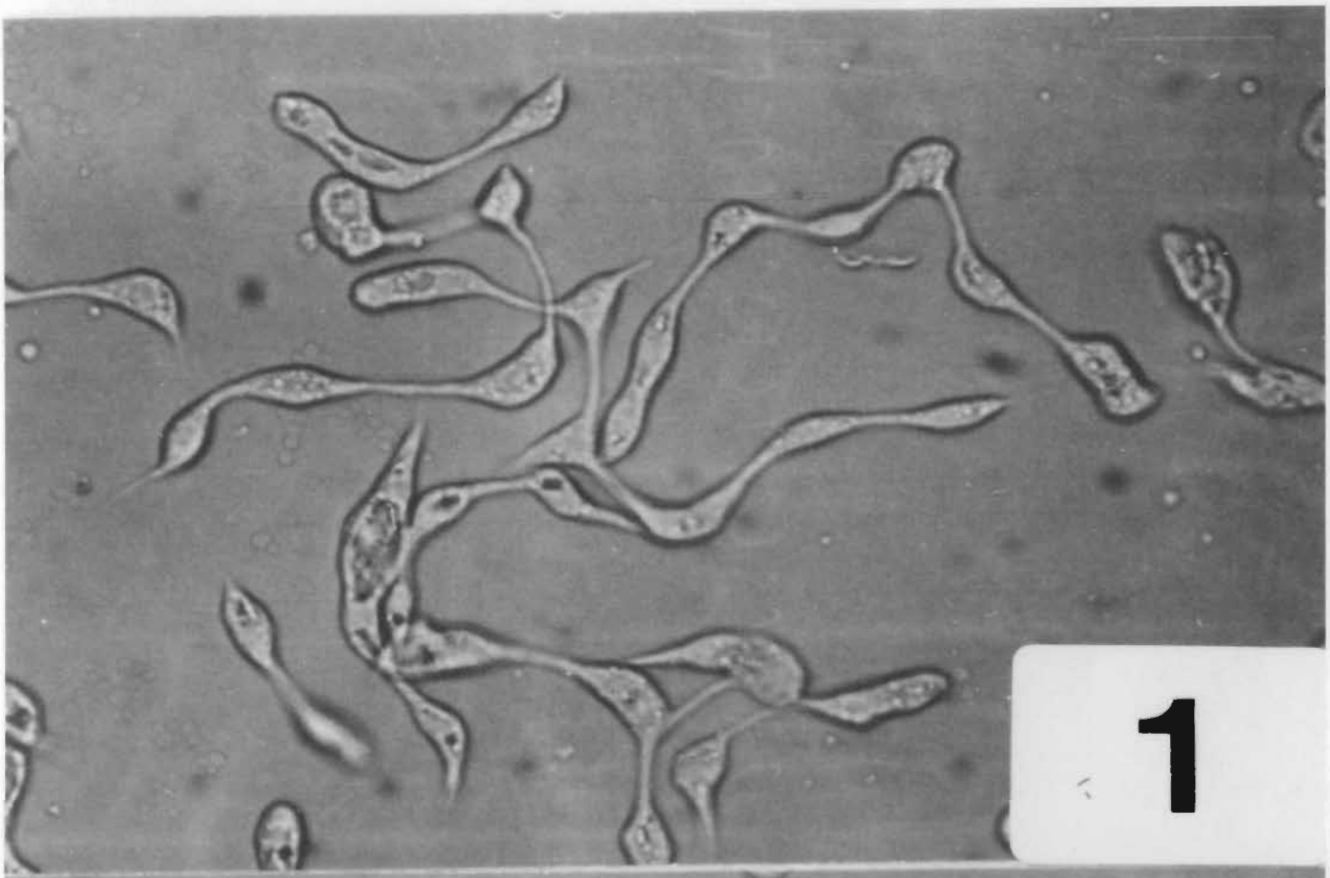
[a] Shaken at 100 rpm

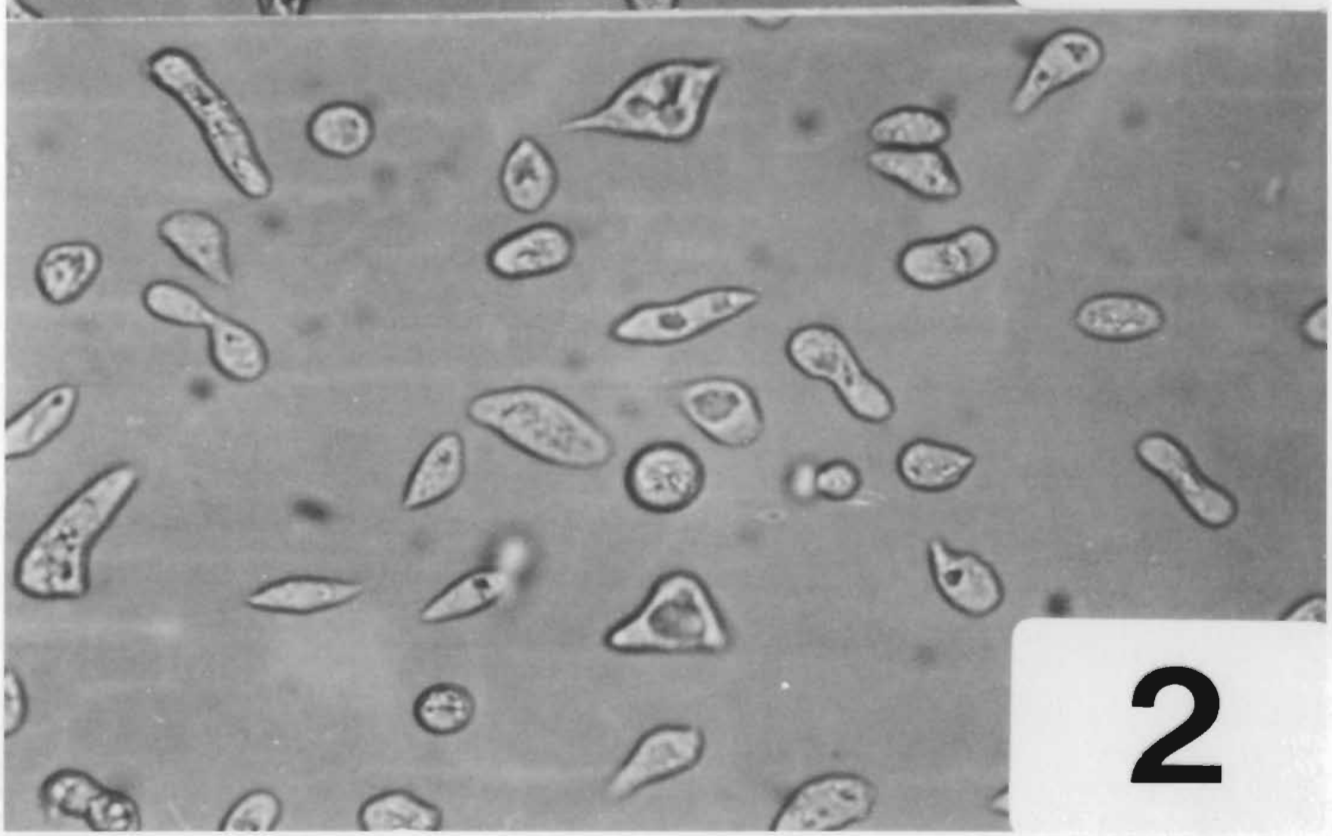
[b] n=10 for all values

[c] Probability of significance $P < 0.001$

Plate 1, Figure 1 Protoplasts growing in catenulate chains in stationary culture. X800

Figure 2 Protoplasts growing as non-chain cells in shaken culture. X800


1


2

To determine the preferred osmotic stabilizing agent those compounds present in the standard medium in high concentration were tested. The salts calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2.6H_2O$), magnesium sulphate ($MgSO_4.7H_2O$) and potassium chloride (KCl) and the sugars sucrose, fructose and glucose at the concentrations of 116.3 mM, 116.3 mM, 175.5 mM, 174.5 mM for the respective salts and 349 mM for all sugars were tested by comparing the effects of each individual compound on protoplast morphology and protoplast growth. All test solutions were at pH 6.15.

After 12 hours incubation in stationary cultures at $20^0C \pm 1^oC$, KCl, $MgSO_4.7H_2O$ and sucrose solutions yielded healthy looking spindle-shaped protoplasts with the greatest number of cells per chain (Table 3). In the $MgCl_2.6H_2O$ and fructose solutions the cells remained spherical for over 168 hours and later lysed. In the other test solutions the cells remained in catenulate chains up to this time. The pH of all test solutions remained constant.

A 0.1 ml inoculum from each test solution was added to fresh standard media and incubated as above. The growth rates and total cell numbers were compared.

Table 4 shows the cell concentrations in the treatments using the different salts to be comparable as were those among the sugars for a given time. However, the results obtained with the different salts revealed significantly greater cell concentrations than the cell levels for the sugars used.

It was of interest to note that the population doubling

Table 3

The morphological effects of various salts and sugars on protoplasts

Compound	% Spindles	Chain length (cells/chain)
KCl	72.5	2.8 ± 0.6
$CaCl_2$	2.0	1.0 ± 0.3
$MgCl_2$	0.0	-
$MgSO_4$	95.0	3.7 ± 0.6
Sucrose	81.4	2.1 ± 0.5
Glucose	3.4	1.5 ± 0.5
Fructose	0.0	-

[a] n=10 for all values

Table 4

Rate of growth of protoplasts previously incubated for 12 hours in various salts and sugars solutions

Incubation time (hours)	Compound	Cell Yield (cells/ml)
67.5	KCl	$1.24 \times 10^5 \pm 8.3 \times 10^3$
	$CaCl_2$	$1.56 \times 10^5 \pm 9.3 \times 10^3$
	$MgSO_4$	$6.31 \times 10^4 \pm 5.8 \times 10^3$
	Sucrose	$7.50 \times 10^4 \pm 6.8 \times 10^3$
	Glucose	$8.10 \times 10^4 \pm 6.5 \times 10^3$
69.5	KCl	$1.87 \times 10^5 \pm 1.1 \times 10^4$
	$CaCl_2$	$1.47 \times 10^5 \pm 9.6 \times 10^3$
	$MgSO_4$	$1.42 \times 10^5 \pm 8.9 \times 10^3$
	Sucrose	$8.60 \times 10^4 \pm 7.1 \times 10^3$
	Glucose	$7.10 \times 10^4 \pm 7.1 \times 10^3$
72.0	KCl	$2.52 \times 10^5 \pm 1.2 \times 10^4$
	$CaCl_2$	$2.76 \times 10^5 \pm 1.2 \times 10^4$
	$MgSO_4$	$2.48 \times 10^5 \pm 1.2 \times 10^4$
	Sucrose	$8.60 \times 10^4 \pm 7.8 \times 10^3$
	Glucose	$1.00 \times 10^5 \pm 8.8 \times 10^3$
76.0	KCl	$4.76 \times 10^5 \pm 1.6 \times 10^4$
	$CaCl_2$	$4.88 \times 10^5 \pm 1.7 \times 10^4$
	$MgSO_4$	$4.01 \times 10^5 \pm 1.5 \times 10^4$
	Sucrose	$2.07 \times 10^5 \pm 3.6 \times 10^3$
	Glucose	$1.8 \times 10^5 \pm 3.2 \times 10^3$

[a] n=10 for all values

times of the cells exposed to the salt solutions was significantly less than the rate of the cells exposed to the sugar solutions, approximately 3.8 hours per generation and 5.1 hours per generation, respectively.

The cells from the $MgCl_2.6H_2O$ and fructose solutions were swollen when in the solutions and shrank somewhat when added to the standard medium but never exhibited the characteristic morphology or growth.

Although $MgSO_4.7H_2O$ and sucrose were comparable in maintaining E. egressa protoplast spindle shape, the fact that sucrose favoured a significantly larger doubling time implied some toxicity by sucrose to the cells. This was further confirmed by replacing 10 mM sucrose with a 5 mM $MgSO_4.7H_2O$ supplement in the standard medium. Cells exposed to this medium immediately contracted and lysed within 96 hours. Thus sucrose was chosen as the osmotic stabilizer.

Fructose has rarely been used as an osmostabilizer although Bachmann and Bonner (1959) found it and 20% sucrose to be very effective for Neurospora protoplasts. Emerson and Emerson (1958) and Emerson (1963) found that a 2% sucrose - 20% sorbose mixture was an effective osmotic agent for Neurospora crassa.

A number of salts studied in protoplast stabilization experiments, Villaneuva (1966) reported that 600 to 800 mM KCl was effective in sustaining yeast protoplasts and $MgSO_4$ at 800 to 1000 mM good for many protoplast suspensions. Magnesium sulfate favoured protoplast formation. Berliner and

Reca (1970) followed the development of protoplasts of the yeast phase of H. capsulatum in 2 M $MgSO_4$ broth. Sietsma and De Boer (1973) reported that the inorganic salts $MgSO_4$, sodium chloride (NaCl), KCl and ammonium chloride (NH_4Cl), at equal osmolarity were very effective osmotic stabilizers and enhanced protoplast formation. Kopecka (1974) used 600 mM KCl and 10 mM $MgSO_4$ for osmotic stabilization of S. cerevisiae protoplasts.

The stability of a given type of protoplast is affected not only by the type of stabilizer but also by the pH of the medium. A pH range of 6 to 8 has been found to be useful (Villanueva, 1966).

3. Osmotic pressure (osmolarity) analysis

Because sucrose was found to be less toxic than most of the other salts or sugars tested, the optimum osmolarity using pure sucrose solutions over a concentration range of 150 mM to 410 mM (Table 5) at pH 6.2 was determined. An unwashed 24 hour old inoculum, 0.1 ml containing 1.10×10^5 cells/ml, was added to each tissue culture flask and the percentage of protoplasts in the spindle form after 60 minutes of incubation recorded.

Table 5 indicates that the optimum sucrose osmolarity was 350 mM. Removal of 0.5 ml of the cell suspension from each treatment and its addition to standard media of 349 mOsm produced growth, based on visual increase in turbidity, for only those cells exposed to sucrose solutions from 230 mM to 350 mM concentrations. Those cells from the 350 mM sucrose

Table 5

Effects of sucrose concentration on protoplast morphology

Concentration (mM)	Percent of cells in spindles
150	9.5
160	16.0
180	15.0
190	3.6
220	16.7
230	53.3
278	47.3
300	75.0
350	92.5
410	31.3

solution achieved greater turbidity sooner than cells exposed to the other osmolarity concentrations.

Although it would have been preferable to determine optimum sucrose osmolarity in the growth medium, the range of values permitted would be extremely low for values less than 350 mM, 78 mM being the maximum reduction possible achievable by removal of sucrose. Any attempt to exceed this lower limit would have introduced nutrient dilution effects.

4. Growth rate of stationary cultures

The rate of growth of Entomophthora egressa in stationary cultures incubated at 21°C ± 1°C was determined using 30 ml of standard medium. Cell yield at given time intervals was determined by removing 0.1 ml samples from five replicate flasks. The initial pH was 6.15 ± 0.05.

The results (Table 6 and Figure 1) revealed maximum growth by 30.5 hours and a stationary phase from 30.5 hours to 35.5 hours. The stationary phase was followed by a decline phase. The pH remained constant throughout the growth curve presumably because of the buffering effects of the phosphate and bicarbonate salts, amino acids, and proteins in the medium. It was noted that significant growth occurred by 13 hours. This incubation time could be used for growth measurements.

5. Effect of temperature on the growth of protoplast

Because of the lack of sufficient numbers of

Table 6

Growth of protoplasts in stationary culture in the standard medium

Hours Incubation	pH	Cell Yield (Cells/ml)
1.00	6.15 ± 0.00	$8.54 \times 10^4 \pm 4.1 \times 10^3$
6.00	6.15 ± 0.00	$5.60 \times 10^4 \pm 3.7 \times 10^3$
8.00	6.15 ± 0.00	$4.82 \times 10^4 \pm 3.1 \times 10^3$
10.00	6.15 ± 0.01	$1.33 \times 10^5 \pm 5.2 \times 10^3$
12.00	6.15 ± 0.00	$1.00 \times 10^5 \pm 4.5 \times 10^3$
18.50	6.15 ± 0.00	$1.22 \times 10^5 \pm 4.9 \times 10^3$
21.50	6.15 ± 0.00	$2.44 \times 10^5 \pm 7.0 \times 10^3$
24.00	6.15 ± 0.00	$2.70 \times 10^5 \pm 7.3 \times 10^3$
25.00	6.15 ± 0.02	$3.67 \times 10^5 \pm 8.6 \times 10^3$
26.00	6.15 ± 0.00	$3.51 \times 10^5 \pm 8.4 \times 10^3$
26.50	6.15 ± 0.00	$4.43 \times 10^5 \pm 9.4 \times 10^3$
29.00	6.15 ± 0.00	$4.25 \times 10^5 \pm 9.2 \times 10^3$
30.50	6.15 ± 0.00	$5.56 \times 10^5 \pm 1.05 \times 10^4$
32.00	6.15 ± 0.00	$6.08 \times 10^5 \pm 1.10 \times 10^4$
35.00	6.15 ± 0.00	$5.55 \times 10^5 \pm 1.05 \times 10^4$
35.50	6.15 ± 0.00	$5.37 \times 10^5 \pm 1.04 \times 10^4$
37.0	6.15 ± 0.00	$4.53 \times 10^5 \pm 9.5 \times 10^3$
38.5	6.15 ± 0.00	$4.41 \times 10^5 \pm 9.4 \times 10^3$

[a] n=10 for all values

Figure 1. Growth curve of protoplasts in stationary culture. Inoculum 0.3 ml, 1.8×10^5 cells/ml, 24 hours old.

LOG OF CELL CONCENTRATION
8
6
4
2
1
2
4
6
8
0
10
15
20
25
30
35
40
HOURS OF INCUBATION

environmental shakers, the study of the effects of temperature on the growth of cells in shaken cultures was not feasible. Small, stationary incubators were used to assess the effects of temperature on the growth of stationary cultures. The cells were tested over a temperature range of 10°C to 37°C in replicates of 5 flasks using methods and materials analogous to the experimental set up used in the study of the stationary growth curve.

The results (Table 7 and Figure 2) revealed an optimum temperature range of 17°C to 21°C. Temperatures in excess of 21°C produced decreased growth. The cells tended to become spherical at temperatures in excess of 21°C. Cells incubated at 24°C and 26°C for 48 hours resumed the protoplast shape and, based on visually observed turbidity increases, growth when subsequently incubated at 21°C. Those incubated initially at temperatures greater than 26°C failed to develop when returned to 21°C and were observed to become crenated at the edges.

The optimal temperature range for vegetative growth reported by Smith (1953) for E. coronata was 15°C to 30°C. This was confirmed by Gustafsson (1965a) who reported that E. thaxteriana, E. apiculata, E. culicis and E. aphidis had an optimum temperature of 21°C with decreasing yields at temperatures above 21°C.

Interestingly the optimum range for protoplast growth occurred over the same range for which the mycelial stage caused extensive mortality of the eastern hemlock looper at

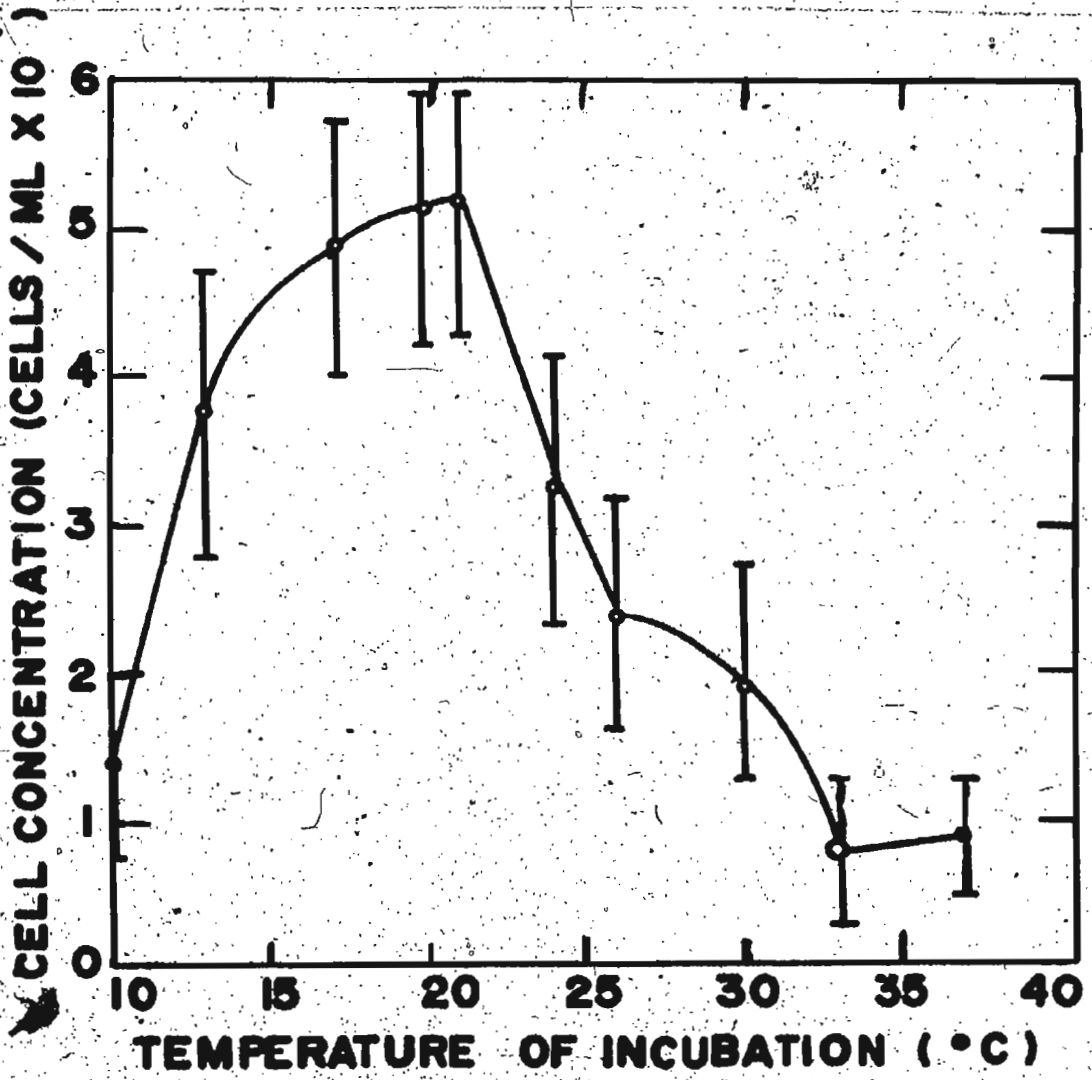
Table 7

The effects of temperature on protoplast growth

Temperature (^{o}C)	Cell yield (cells/ml)
10	$1.38x10^{5} \pm 9.3x10^{3}$
13	$3.75x10^{5} \pm 1.53x10^{4}$
17	$4.90x10^{5} \pm 3.13x10^{4}$
20	$5.10x10^{5} \pm 3.20x10^{4}$
21	$5.20x10^{5} \pm 3.20x10^{4}$
24	$3.25x10^{5} \pm 2.80x10^{4}$
26	$2.38x10^{5} \pm 2.40x10^{4}$
30	$2.00x10^{5} \pm 2.20x10^{4}$
33	$7.50x10^{4} \pm 1.40x10^{4}$
37	$8.80x10^{4} \pm 1.20x10^{4}$

[a] n=10 for all values

Figure 2. Effects of temperature on protoplast growth. Inoculum 0.3 ml, 1.8×10^5 cells/ml, 24 hours old.


CELL CONCENTRATION (CELLS / ML X 10)
4
0
1
2
3
4
5
6
10
15
20
25
30
35
40
TEMPERATURE OF INCUBATION (°C)

Ming's Bight, Serptine Lake and McIvers, Newfoundland, i.e. 17°C to 21°C (Otvos, 1973; Otvos et al., 1973).

Because of the extreme sensitivity of the protoplasts to temperatures in excess of 21°C and the chances of temperature fluctuation within the incubator, the next lowest temperature giving comparable cell concentrations to that of 21°C was chosen as the incubation temperature, i.e. 20°C.

6. Buffer analysis

Because one of the objectives was to reduce the number of amino acids in the standard medium, the buffering capacity of the medium would be expected to diminish. To offset this effect a series of hopefully biologically inert buffers active in the pH 6.2 range was desired. Good and Izawa (1966) proposed three buffers of potential value with the buffering capacity such that the protonated: non-protonated ratio was contained within the 10 to 0.1 limits. The buffers tested were 2 -(N-morpholino) ethane sulfonic acid (MES, pKa 6.15), 3 -(N-morpholino) propane sulfonic acid (MOPS, pKa 7.15) and N -tris(hydroxymethyl) methylaminoethane sulfonic acid (TES, pKa 7.5).

The four buffer concentrations used were 1 mM, 10 mM, 25 mM and 40 mM. Each buffer was dissolved in distilled water and autoclaved. The volume of water used was such that the total osmolarity of the standard medium to which it was added remained constant at 350 mM. Control media consisted of standard media adjusted with a filter sterilized sucrose solution to give a constant osmolarity and equal the

dilution of nutrients that occurred by the addition of buffer to the test media.

The pH was aseptically adjusted using filter sterilized 4N potassium hydroxide (KOH).

Using a 0.1 ml inoculum, four replicates for each treatment and control were established and incubated at 20°C in a stationary incubator. The final cell yield and pH were determined after 36 hours of incubation.

Table 8 clearly shows that all three buffers at the levels tested were toxic because the 95% confidence intervals indicate the mean values to be significantly less than the corresponding control values. At the 25 and 40 mM concentrations, the cell yields for the buffers were comparable. The control values at these concentrations were significantly lower than those at 1 mM and the 10 mM levels reflecting the effects of dilution of the nutrients. The lower yields which occurred as the buffer concentration increased also reflected the dilution of nutrients. The lower yields for TES were significantly lower than those obtained with MOPS or MES and the yields obtained with MOPS were generally significantly lower than the MES values.

Comparison of pH data (Table 8) revealed that only the presence of the MES buffer resulted in pH values statistically comparable to the control pH values. Later experiments revealed the optimum pH for protoplast growth to be 6.2. Thus, in view of the need for pKa for a buffer to be equal to the pH of a solution (Good and Izawa, 1966; Lehniger, 1975)

Table 8

The effects of three types of buffers
at four concentrations on protoplast cell yield

Buffer type	concentration (mM)	Cell yield (cells/ml)$_b$	pH f
Control for	40 mM buffer$_a$	$2.03 \times 10^5 \pm 2.66 \times 10^4$	6.18 ± 0.01
Control for	25 mM buffer$_a$	$3.12 \times 10^5 \pm 3.95 \times 10^4$	6.15 ± 0.00
Control for	10 mM buffer$_a$	$8.20 \times 10^5 \pm 5.25 \times 10^4$	6.15 ± 0.00
Control for	1 mM buffer$_a$	$9.02 \times 10^5 \pm 5.48 \times 10^4$	6.14 ± 0.01
MES	40	$1.14 \times 10^5 \pm 1.94 \times 10^4$	6.15 ± 0.00
	25	$2.28 \times 10^5 \pm 2.76 \times 10^4$	6.17 ± 0.01
	10	$7.00 \times 10^5 \pm 4.80 \times 10^4$	6.15 ± 0.00
	1	$4.73 \times 10^5 \pm 3.97 \times 10^4$	6.15 ± 0.00
MOPS	40	$1.94 \times 10^5 \pm 2.54 \times 10^4$	6.27 ± 0.07
	25	$2.32 \times 10^5 \pm 2.78 \times 10^4$	6.20 ± 0.07
	10	$3.38 \times 10^5 \pm 3.35 \times 10^4$	6.21 ± 0.06
	1	$2.80 \times 10^5 \pm 3.05 \times 10^4$	6.20 ± 0.11
TES	40	$6.5 \times 10^4 \pm 1.47 \times 10^3$	6.33 ± 0.06
	25	$3.88 \times 10^4 \pm 1.14 \times 10^3$	6.27 ± 0.06
	10	$4.30 \times 10^4 \pm 7.55 \times 10^3$	6.25 ± 0.07
	1	$2.00 \times 10^4 \pm 8.16 \times 10^2$	6.25 ± 0.05

f - Final pH value based upon three replicates

a - Control represents the effects of dilution of nutrients on protoplast growth

b - n=10 for all values

and the toxicity of the three buffers, 10 mM MES was chosen to be the buffer added to all subsequent experimental media.

7. Growth curve for shaken cultures

Using a 24 hour old culture of cells grown in the standard medium, a 0.1 ml aliquot was added to each of 10 flasks containing the modified standard medium. The modification consisted of the addition of 10 mM MES buffer and the deletion of 20 mM sucrose to maintain the 350 mOsM osmolarity. The initial pH was 6.15. The medium, after the removal of cells at the same time as the cell counts were made, was stored for glucose and ninhydrin-positive compound analyses.

The data in Table 9 and Figure 3 indicate significant growth after 16 hours incubation. Growth occurred until at least 72 hours at a rate of 6.3 hours per generation. The cells entered the stationary phase at approximately 83.75 hours.

Throughout the entire growth cycle no pH change was noticed. There was no evidence of autolysis.

Because of differences in inoculum size and volumes of media used for stationary and shaken growth curves, comparisons of the growth rates and cell yields was not possible.

8. Optimum pH

After selection of the buffer, experiments were prepared to determine the optimum pH for protoplast growth. Flasks of MES buffered standard medium were adjusted with

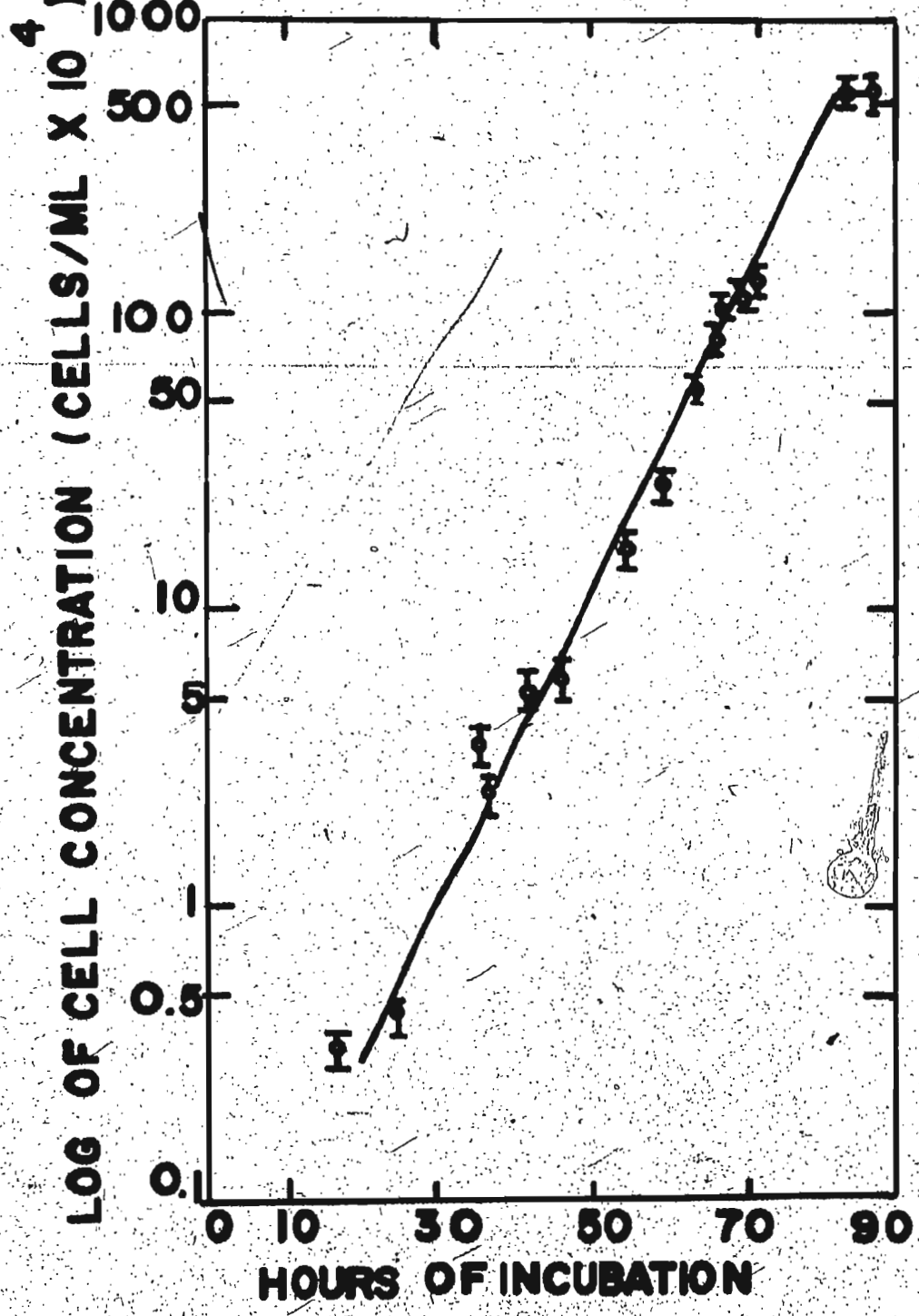
Table 9

Growth of protoplasts under shaken conditions and in modified standard medium

Incubation time (hours)	Cell yield (cells/ml)[a]	Incubation time (hours)	Cell yield (cells/ml)
0.0	0.00 ± 0.00 (pH 6.20 ± 0.00	46.0	$5.74 \times 10^4 \pm 4.88 \times 10^3$
10.5	0.00 ± 0.00	49.5	$9.83 \times 10^4 \pm 6.40 \times 10^3$
16.0	$3.30 \times 10^3 \pm 1.20 \times 10^2$	54.0	$1.56 \times 10^5 \pm 8.06 \times 10^3$
24.0	$4.20 \times 10^3 \pm 1.30 \times 10^2$ (pH 6.12 ± 0.01)	59.0	$2.55 \times 10^5 \pm 1.04 \times 10^4$ (pH 6.12 ± 0.01)
34.0	$3.50 \times 10^4 \pm 3.80 \times 10^2$	64.0	$5.54 \times 10^5 \pm 1.52 \times 10^4$
36.0	$2.46 \times 10^4 \pm 3.20 \times 10^3$	67.5	$9.68 \times 10^5 \pm 2.01 \times 10^4$
38.0	$2.50 \times 10^4 \pm 1.02 \times 10^4$	70.0	$1.22 \times 10^6 \pm 2.23 \times 10^4$ (pH 6.12 ± 0.01)
42.0	$5.12 \times 10^4 \pm 4.62 \times 10^3$	87.5	$6.14 \times 10^6 \pm 2.10 \times 10^5$ (pH 6.12 ± 0.01)

[a] n=10 for all values

Figure 3. Growth curve of protoplasts in shaken culture. Inoculum 0.1 ml, 2.3×10^5 cells/ml, 24 hours old.


LOG OF CELL CONCENTRATION (CELLS/ML X 10^4)
1000
500
100
50
10
5
1
0.5
0.1
0
10
30
50
70
90
HOURS OF INCUBATION

KOH to cover the pH range of 4.8 through 8.8. The incubation period was 45.5 hours.

The results (Table 10 and Figure 4) indicated that growth occurred over a pH range of 5.2 to 8.2 and no growth was detected at pH 4.8 or pH 8.8. Growth increased significantly up to pH 6.2 and decreased thereafter.

Because the salts in the medium precipitated out at pH values greater than and equal to 7.7, the effects on cell yield reflected the combined effects of a lower osmolarity as well as the effects of pH.

Early investigations into optimum pH consisted of Sawyer (1929) growing E. sphaerosperma on various foodstuffs of known pH. Haddock and yellow perch at pH 5.6 yielded best growth. The use of flasks of 1.5% proteose-peptone adjusted over a pH range of 5.0 to 8.6 revealed, on the basis of visual observation, that poor growth occurred at both pH extremes with maximum growth and conidia production occurring at pH 6.5 (Sawyer, 1929). Wolf (1951) reported pH 5.5 to pH 7.0 strongly favoured vegetative growth of D. coronata in an asparagine salts medium. Smith (1953) extended the observation on the effects of pH on growth yield and found pH 6.7 to be optimum from the range of pH 5.9 to pH 7.5. Gustafsson (1965a) concluded that Entomophthora species can be cultured on a neutral to slightly acidic medium, E. thaxteriana and E. excitalis grew well at pH 4.9 to pH 7.8 and poorly at pH 4.5. The two strains of E. aphidis used by Gustafsson (1965a) revealed sharp responses to pH gradients,

Table 10

The effects of hydrogen ion concentration on protoplast growth

pH	Cell yield (cells/ml)[a]
4.8	0.00 ± 0.00
5.2	$3.56 \times 10^4 \pm 4.44 \times 10^3$
5.6	$9.50 \times 10^4 \pm 8.90 \times 10^3$
6.2	$1.25 \times 10^5 \pm 1.02 \times 10^3$
6.6	$8.20 \times 10^4 \pm 7.65 \times 10^3$
7.2	$7.10 \times 10^4 \pm 8.43 \times 10^3$
7.7	$5.40 \times 10^4 \pm 5.20 \times 10^3$
8.2	$2.30 \times 10^4 \pm 3.57 \times 10^3$
8.8	0.00 ± 0.00

[a] n=12 for all values

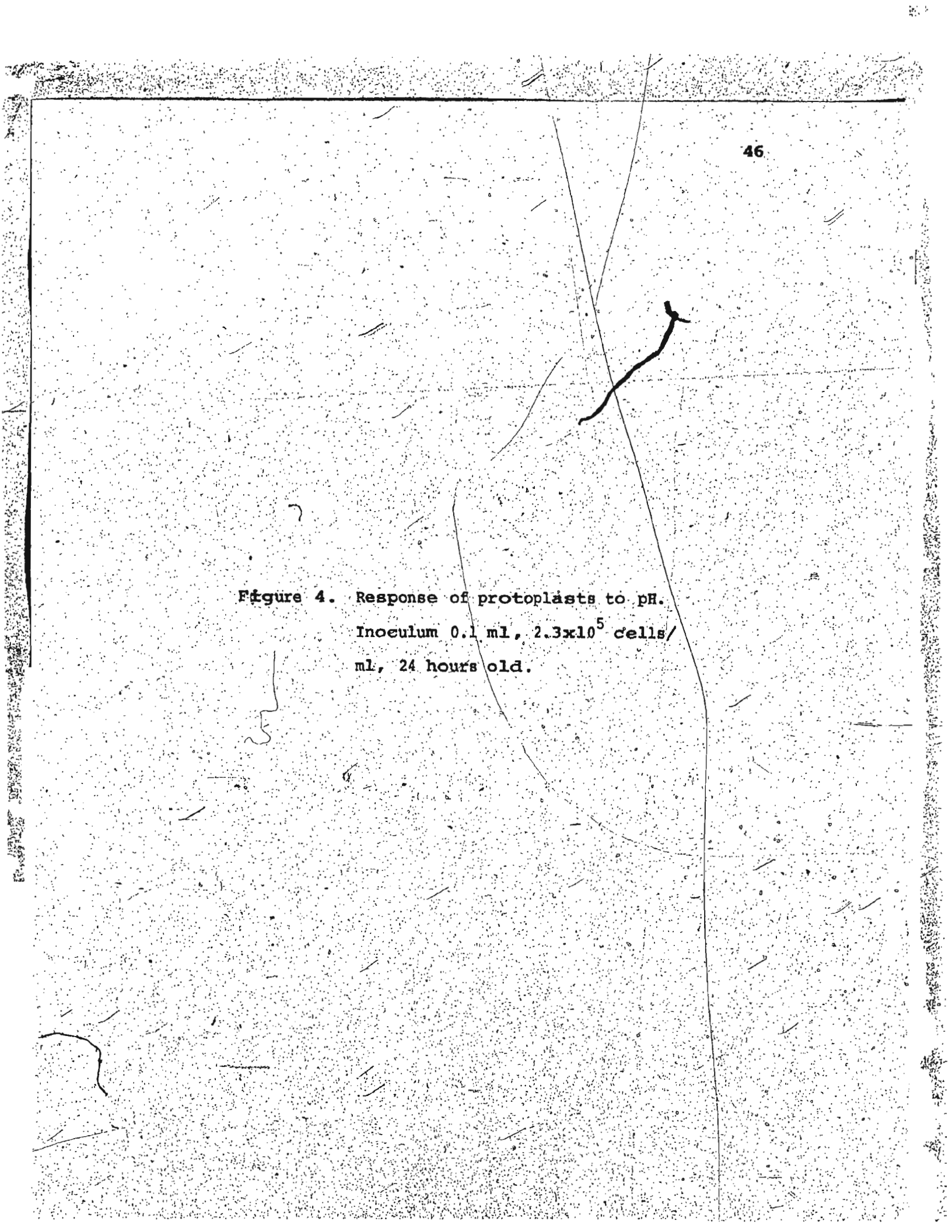
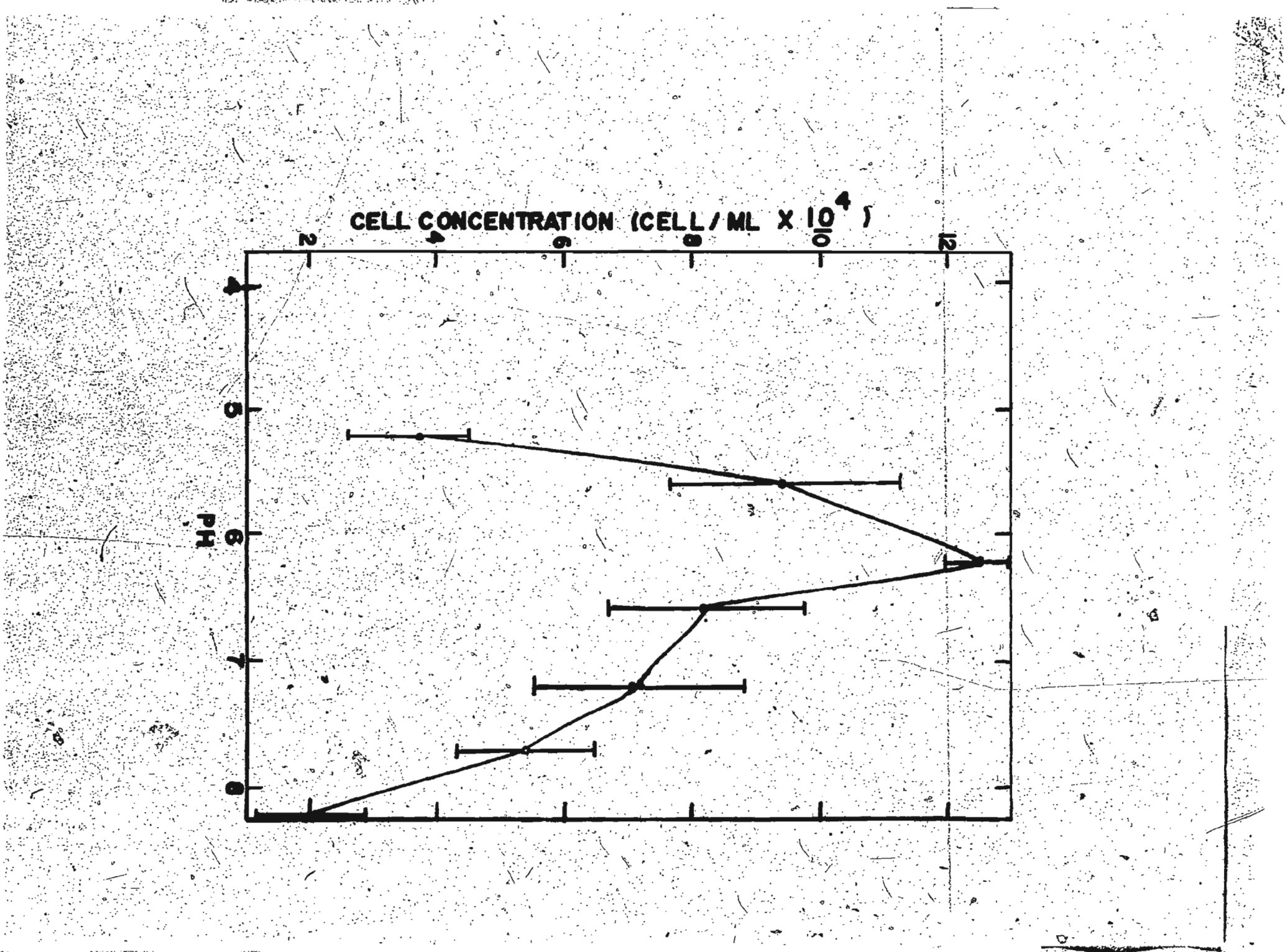


Figure 4. Response of protoplasts to pH. Inoculum 0.1 ml, 2.3×10^5 cells/ml, 24 hours old.


CELL CONCENTRATION (CELL / ML X 10^4)
2
4
6
8
10
12
4
5
6
7
8
PH

poor growth occurring at pH 5.5 and 7.6, good growth at pH 5.9 to 7.2.

Although the possibility of adaptation to the pH 6.2 condition exists, the fact that Vandenberg and Soper (1975) reported that E. egressa mycelium isolated from the spruce budworm, Choristoneura fumiferana Clems. on AK agar grew best throughout the pH range of 6.0 to 7.5 implied that the optimum pH for the protoplast may not be an artifact of culture. Possibly the optimum pH may reflect an adaptation to the acidic haemolymph reported for phytophagous Lepidoptera by Heimpel (1950).

II. Biochemical analysis of the eastern hemlock looper.

1. Analysis of ninhydrin-positive compounds in the haemolymph.

The mean masses for the individual larvae in the two larval samples were not significantly different ($P > 0.95$) implying, at least on a mass basis, that the rearing conditions for the two samples were comparable.

The analytical results (Table 11) indicated that of the ninhydrin-positive compounds for which the analyser was calibrated, at least 38 occurred in the fourth instar haemolymph. The compounds urea, glycerophos phoethanolamine, hydroxy-proline, half cystine, homocitrulline, glucosamine, galactosamine, DL-allo- hydroxyproline, ethanolamine, creatinine, reduced and oxidized glutathionine and uric acid were not detected. There were 13 unidentified peaks, ten of which were common to both samples.

Table 11

Ninhydrin-positive compound levels in the fourth instar haemolymph of Lambdina fiscellaria fiscellaria

Compound	Concentration (mM)	Compound	Concentration (mM)
L-Alanine	3.656 ± 0.168	L-Histidine	9.576 ± 1.535
Ammonia	4.015 ± 0.998	L-Isoleucine	2.320 ± 0.070
L-Anserine	unresolved	L-Leucine	2.258 ± 0.211
L-Arginine	1.688 ± 0.223	L-Lysine	5.918 ± 1.232
L-Aspartic acid	0.083 ± 0.048	L-Methionine	0.354 ± 0.033
B-Alanine	0.164	L-Methionine sulfoxide	0.113 ± 0.098
B-Aminobutyric acid	0.317 ± 0.099	L-1-Methylhistidine	trace
B-Aminoisobutyric acid	0.060b	L-3-Methylhistidine	0.021 b
γ-Aminobutyric acid	0.317 ± 0.099	L-Ornithine	0.764 ± 0.025
γ-Aminoadipic acid	trace b	L-Phenylalanine	1.011 ± 0.050
γ-Amino-n-butyric acid	0.009 b	O-Phosphoethanolamine	0.766 ± 0.317
L-Carnosine	trace b	DL-O-Phosphoserine	trace b
L-Citrulline	trace b	Sarcosine	0.146 b
L-Cystathionine	0.281 b	L-Serine	3.652 ± 0.111
Cysteic acid	trace b	Taurine	0.0176
Dihydroxyphenylalanine	0.114 b	L-Threonine	2.441 ± 0.064
L-Glutamine + L-Asparagine	14.875 ± 1.749	L-Tryptophan	trace
L-Glutamic acid	1.283 ± 0.449	L-Tyrosine	1.836 ± 0.070
Glycine	2.970 ± 0.088	L-Valine	2.314 ± 1.944
		L-Proline	0.874 ± 0.192

a - Means based on two replicates, unless otherwise stated

b - Value based on one determination

The results, in common with those obtained in studies with other Lepidoptera (Boctor and Salem, 1973; Florkin and Jeuniaux, 1974; Wyatt et al., 1956), showed a general tendency for glutamine and/or asparagine, histidine and lysine to occur at high concentrations (5.0 mM) and ammonia, arginine, threonine, serine, glutamic acid, glycine, alanine, valine, isoleucine, leucine, phenylalanine and tyrosine occurred at intermediate levels (1.0 - 5.0 mM).

The presence of tyrosine and dihydroxyphenylalanine would be expected because of the indirect role these compounds play in the tanning of cuticular proteins. As a result of the rapid transfer of the haemolymph to chilled vials containing phenylthiourea and subsequent storage at low temperature, the possible influence of tyrosinase activity was considered to be negligible.

Kawase (1966) reported the presence of B-alanine, ornithine, phosphoethanolamine and taurine in healthy larvae of Bombyx mori (L.); and in contrast to the present study he detected glycerophosphoethanolamine. Boctor and Salem (1973) reported that γ-aminobutyric acid occurred in Spodoptera littoralis Boisduval and that its concentration steadily increased during larval development. Chen (1966) reported that B-alanine, taurine, ornithine and γ-aminobutyric acid are found in a number of insects.

The majority of amino acids detected have been reported to be amino group donors to α-ketoglutarate in insects (Bheemeswar, 1958). Arginine metobolism and the

ornithine-citrulline cycle has been demonstrated in B. mori by Garcia et al. (as cited in Bheemeswar, 1958). The presence of high concentrations of ammonia as in the present study is not unusual (Chen, 1966). Levenbook (1950) reported that ammonia may be formed from unknown labile compounds in the haemolymph on exposure to air. Meister (1965) postulated on four possible sources of ammonia production including the release of ammonia from the amide groups of proteins. The methods of analysis used in this experiment may also reflect ammonia artifically introduced (Nolan, 1970).

The current study is the third report of 1-methylhistidine and/or 3-methylhistidine in the haemolymph of insects. The only previous report of the occurrence of a methylhistidine were those of Villeneuve (1962) from larvae of Agria affinis (Fallen) and Gordon and Bailey (1976) in blackflies. Methylhistidine was previously considered to be species specific and of unknown physiological significance (Chen, 1966). Lehninger (1975) reported that this acid was a component of muscle and other connective tissue.

The role of diet in determining the amino acid composition of the hemlock looper is unknown. Samokvalova and Zakhratkina (1970) found that silkworm larvae fed on young mulberry leaves (Morus species) had a higher free amino acid content than those fed on old foliage. Gagnon (1964) reported that alanine, γ-aminobutyric acid and tyrosine were the predominant amino acids in freshly fallen balsam fir

needles. The γ-aminobutyric acid and alanine may have been obtained from the balsam fir needles, however, the former can be produced by decarboxylation of glutamic acid and the latter by transamination of pyruvate with either aspartic acid or glutamic acid (Meister, 1965).

2. Medium based on haemolymph analysis

Based on the ninhydrin-positive compound analysis of the haemolymph of the eastern hemlock looper a medium (Table 12) was prepared reflecting modified Grace's medium in terms of non-amino acid components and pH. The rate of protoplast growth in this new medium was compared to that in Grace's medium supplemented with 28 ml of fetal calf serum to a liter of media. The medium based on haemolymph analysis lacked serum.

The data (Table 13 and Figure 5) revealed that the modified standard medium favoured continuous exponential growth with a uniform population doubling time of 6.2 hours. The medium based on haemolymph analysis allowed slow growth up to 36 hours with a doubling time of 25 hours but by 41 hours were growing logarthmically (doubling time of 4.7 hours). Because the 95% confidence intervals for all points along the growth curves, excluding zero hours, did not overlap with their respective counter points, the two curves, like the population doubling times, were significantly different. The haemolymph based medium was more favourable for protoplast growth than the modified standard medium.

Table 12

Medium based on hemlock looper haemolymph analysis

Compound	Concentration[a]	Compound	Concentration
$NaH2PO_4.H_2O$	1013.0	L-Phenylalanine	168.0
KCl	2240.0	O-Phosphoethanolamine	108.0
$CaCl_2$	1000.0	L-Proline	100.0
$MgCl_2.6H_2O$	2280.0	Sarcosine	12.0
$MgSo_4.7H_2O$	2780.0	L-Serine	384.0
$NaHCO_3$	350.0		
		Taurine	4.0
L-Alanine	324.0	L-Tyrosine	332.0
DL-Allo cystathionine	16.0	L-Threonine	292.0
L-Arginine HCl	356.0	L-Valine	272.0
L-Aspartic	196.0		
L-Aspartic acid	8.0	Sucrose	29800.0
		Fructose	400.0
B-Alanine	16.0	Glucose	700.0
DL-B-Aminobutyric acid	4.0	MES	1952.0
DL- -Aminobutyric acid	32.0		
Dihydroxyphenylalanine	24.0	Malic acid	670.0
Ethionine sulfone	152.0	α-Ketoglutaric acid	370.0
		D-Succinic acid	60.0
L-Glutamic acid	188.0	Fumaric acid	55.0
L-Glutamine	1956.0		
Glycine	224.0	Thiamine HCl	0.020
L-Histidine	1488.0	Riboflavin	
L-Isoleucine	304.0	D-Ca pantothenate	0.020
		Pyridoxine HCl	0.020
L-Leucine	296.0	P-Aminobenzoic acid	0.020
L-Lysine HCl	864.0	Folic acid	0.020
L-Methionine sulfoxide	20.0	Niacin	0.020
Ornithine HCl	80.0	i-Inositol	0.020
		Biotin	0.010
		Choline Cl	0.200

a - in mgm/L

Table 13

Comparative effects on two media on the growth of protoplasts[a]

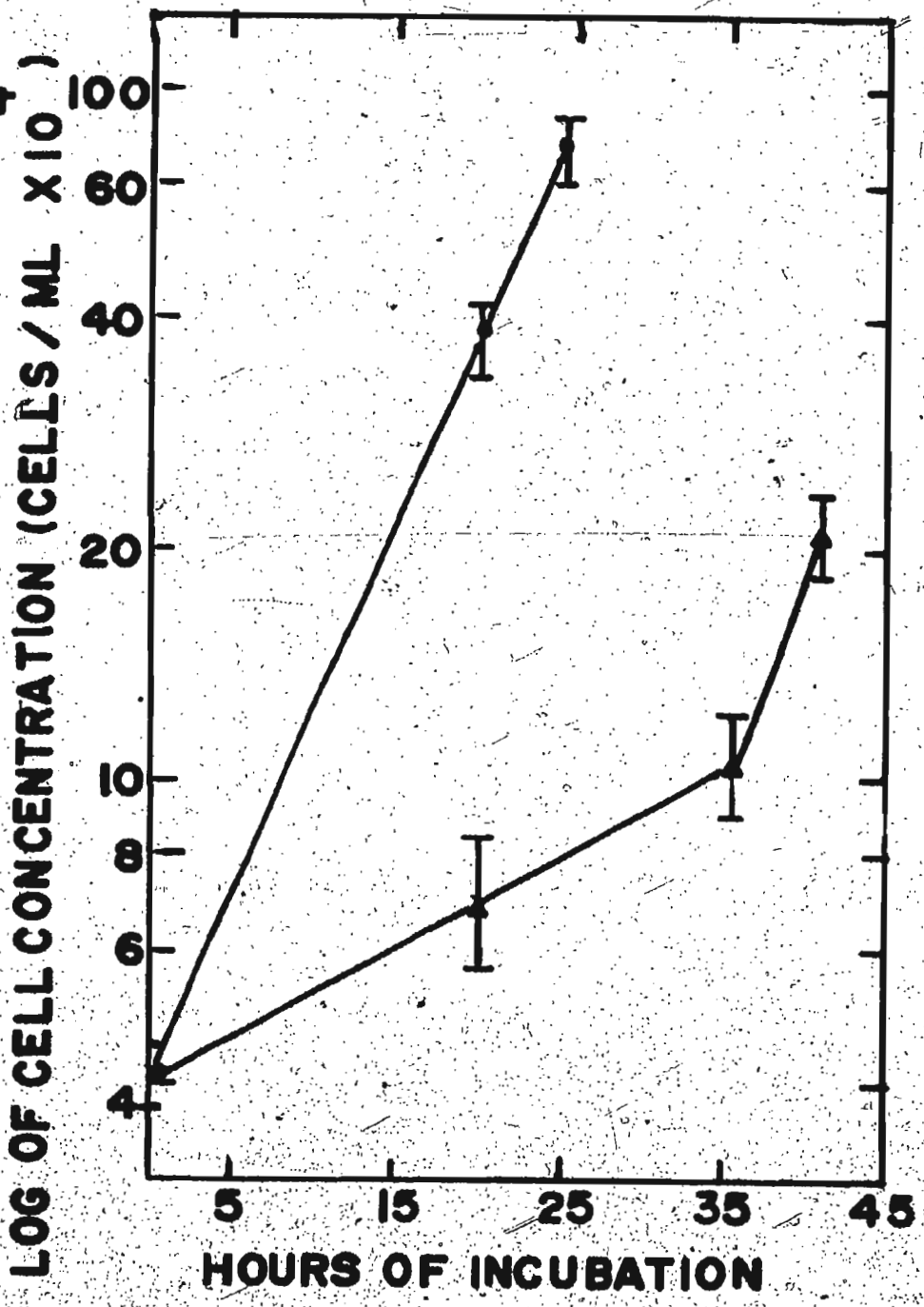
Medium	Incubation time (hours)	Cell yield (cells/ml)	pH
Medium based on haemolymph analysis	0	$4.08 \times 10^4 \pm 9.03 \times 10^3$	6.15 ± 0.00
	20	$6.53 \times 10^4 \pm 1.14 \times 10^4$	6.15 ± 0.00
	36	$1.04 \times 10^5 \pm 4.56 \times 10^4$	6.16 ± 0.01
	41	$2.17 \times 10^5 \pm 6.59 \times 10^4$	6.15 ± 0.00
Modified standard medium	0	$4.08 \times 10^4 \pm 9.03 \times 10^3$	6.15 ± 0.00
	20	$3.87 \times 10^5 \pm 8.80 \times 10^4$	6.15 ± 0.00
	36	$6.55 \times 10^5 \pm 1.14 \times 10^4$	6.15 ± 0.00

a - pH mitially 6.15

Figure 5. Growth curves of protoplasts in two media. Inoculum 0.1 ml, 20×10^5 cells/ml, 53 hours old.

0-0 Grace's MES-buffered medium

▲-▲ Medium based on haemolymph analysis


LOG OF CELL CONCENTRATION (CELLS / ML X 10^4)
100
60
40
20
10
8
6
4
5
15
25
35
45
HOURS OF INCUBATION

The modified standard medium was readily utilized by the cells since no adaptation time was exhibited. This was anticipated since the stock cultures were maintained in this medium. The more rapid protoplast growth after adaptation in the haemolymph based medium could be due to two factors, (i) increasing the amino acid complexity would reduce the metabolic demands on the cells and/or (ii) the presence of compounds more readily utilized than those in the modified standard medium. In addition to having a shorter doubling time on the haemolymph based medium, the protoplasts formed chains of five or more spindle-shaped cells. In the standard medium chains of three to six cells were common.

3. Total lipid and fatty acid analysis

The results (Table 14) indicated that C8:0, C10:0, C22:1 and C24:0 fatty acids and an unknown fatty acid were absent from the third instar larvae, the earliest stage examined. The fatty acids C8:0 and C10:0 and the unknown fatty acid were also absent in the fourth instar. The unknown fatty acid had a retention time 3.9 times that of C18:0 and based upon carbon number versus log retention time plots it was not believed to be a saturated fatty acid. The unknown eluted after C22:1. The C24:0 fatty acid was absent from all but the fourth instar. The C22:1 fatty acid was not detected in either the third instar or in the prepupae. With the above exceptions developmental stages contained C9:0, C10:0, C14:0, C15:0, C16:0, C18:0, C18:1, C18:2, C18:3, C20:0, C20:1, C22:0, C22:1 and C24:0 fatty acids and the

Table 14

Fatty acid analysis of stages of Lambdina fiscellaria fiscellaria (µg/g insect)

Fatty acid[a]	Developmental stage[b]				
	Third Instar	Fourth Instar	Prepupa	Pupa	Pupa
8:0	0 ± 0	0.05[c]	0.23[c]	0.29[c]	-[d]
10:0	0 ± 0	0.16[c]	0.39[c]	0.49[c]	-
11:0	59.8 ± 6.1	1.01 ± 1.00	5.55 ± 2.19	0.87 ± 1.00	2.75 ± 0.87
15:0	35.9 ± 9.2	0.47 ± 0.09	8.46 ± 2.73	0.47 ± 0.31	0.45 ± 0.51
16:0	18.0 ± 0.6	1.05 ± 1.81	3.03 ± 2.10	0.83[c]	0.18 ± 0.71
18:0	53.9 ± 12.1	15.2 ± 3.0	30.6 ± 4.63	9.90 ± 1.18	11.1 ± 1.07
18:1	551. ± 121	9.52 ± 2.91	18.3 ± 3.7	1.30 ± 0.09	1.02 ± 1.00
18:2	75.4 ± 9	19.8 ± 5.9	15.8 ± 3.2	2.89 ± 0.91	3.04 ± 1.21
18:3	39.4 ± 5.5	60.3 ± 11.8	trace	trace	trace
20:0	390. ± 78	77.6 ± 8.6	195. ± 18.7	33.3 ± 1.2	26.0 ± 2.1
20:1	221. ± 44	82.3 ± 14.46	85.5 ± 5.7	10.6 ± 0.1	11.7 ± 1.8
22:0	275. ± 82	63.0 ± 12.6	108 ± 11.2	16.7 ± 3.2	20.3 ± 1.9
22:1	0 ± 0	5.57 ± 4.10	0 ± 0	0.70[c]	1.28 ± 0.00
24:0	0 ± 0	8.09 ± 2.0	0 ± 0	0 ± 0	0 ± 0
Unknown	0 ± 0	0 ± 0	29.6 ± 7.1	5.59 ± 1.19	6.49 ± 0.95
Totals	1719.4	344.12	500.46	83.93	84.31 ± 7.21

[a] First number represents the number of carbon atoms; second designates the number of double bonds.

[b] All values, excluding totals, are based upon two replicates, unless otherwise stated and are rounded off to three significant figures.

[c] One determination

[d] Not determined

unknown fatty acid.

The C18:3 fatty acid level determinations revealed two interesting phenomena (i) the increased levels by the fourth instar and (ii) the rapid depletion in the non-feeding instars. The increase at the time of the fourth instar may reflect either increased incorporation for the synthesis of phospholipids and/or an increase in the level for use as an energy source during the non-feeding stages. Turunen (1973) reported an increase in the C18:3 content of phospholipids prior to pupation of Pieris brassicae Edward. The fatty acid is essential in the diet of P. brassicae and is a constituent of egg lipids and also must have an important role in adult flight muscles (Turunen, 1974b). The decreased C18:3 levels in the eastern hemlock looper during the non-feeding stages suggests a key metabolic role for this fatty acid. In general, the level of a given fatty acid decreased with succeeding developmental stages.

The most abundant fatty acids in the third instar larvae and subsequent stages were C18:1, C20:0, C20:1, and C22:0. The major fatty acids reported herein are in contrast with the fatty acid determinations made using other Lepidoptera in which C16:0, C16:1, C18:0, C18:2 and C18:3 predominated (Lindsay and Barlow, 1970; Turunen, 1974a). A high level of C18:1 has been reported for Galleria mellonella (L.) where it comprised 49.8% of the total fatty acids (Thompson and Barlow, 1971). The presence of high levels of C18:1 and polyunsaturated fatty acids is characteristic of

phytophagous Lepidoptera (Turunen, 1974a). Terriere and Grau (1972) reported that the addition of C18:1 to diets enhanced the larval growth of Trichoplusia ni (Hubner) and Heliothis zea (Boddie). The levels of C20:0, C20:1 and C22:0 were comparable to that of C18:0 in the male and female pupae. The metabolic requirement for these four fatty acids appears to be relatively low in the pupal stages compared to that for the C18:1 fatty acid. Little work has been done on the longer chain fatty acids in insects. However, C20:0, C20:1 and C22:0 have been found in larvae of Tribolium confusum Duval; and C20:0, C20:1, C21:0, C22:0 and C24:0 have been detected in larvae of Tribolium castaneum (Herbst) (Worthington and Payne, 1974). The fatty acids C20:0 and C20:1 have been reported in G. mellonella (Thompson and Barlow, 1971), and low levels of C20:0 were found in the adults or mature larvae of the Lepidoptera: Archips cerasivoranus Fitch, Paleacrita vernata Peck and Datana integerrima Groted and Robinson (Fast, 1966).

The lipid level, on a g lipid per g insect basis, was highest for the prepupae (0.114 ± 0.000, n=2) and approximately equal but lower than the female and male pupae (0.039 ± 0.000, n=2 and 0.041 ± 0.000, n=2, respectively). The drop in lipid level during prepupal development was similar to that shown to occur during prepupal development of the lepidopteran Hyalophora cecropia (L.) (Bade and Wyatt, 1962). This phenomenon is, in general, a characteristic of holometabolous insects where the energy for pupal transformation

is derived from lipid, especially reserves of triacylglyceride accumulated during the larval stages (Fast, 1970). The author's results did not indicate sexual dimorphism with regard to the total fatty acids, most individual fatty acid or the total lipids at the pupal stage.

The two fatty acids C8:0 and C10:0, which were absent in the third instar but progressively increased in concentration with succeeding stages, are of special interest because of their antifungal activity against Aspergillus flavus Link. These fatty acids were the most active constitutents of cuticular lipids of Bombyx mori (L.) and Chilo simplex (Butler) (Koidsumi, 1957).

III. Regeneration of E. egressa protoplasts on solid media and in liquid media.

Initially, the protoplasts underwent increased vacuole formation in all media tested (Plate 2, Figure 1) followed by dismembering of the regular chain series or coalescence of the chain members into spheres (Plate 2, Figure 2). The latter was more common in the liquid media.

Within most of the liquid media individual cells, not in a chain sequence, developed a denser cytoplasm and increased from 14.5 µm (s=17.0 µm) to 25.5 µm (s=5.2 µm) in diameter. The time sequence for the formation of these prohyphal spheres was not synchronous. By 26 to 40 hours, protrusions from the prohyphal spheres emerged and developed into hyphae (12.2 µm diameter, s=0.0 µm) (Plate 2, Figure 3).

With increasing age the hyphae branched (4.9 µm to 12.2 µm diameter). Occasionally (<1%), the prohyphal spheres developed a bud from which a hypha developed (not shown).

By 36 hours many of the chains of vaculated protoplasts developed into spheres with the cytoplasmic beads in strands located on the outside of the spheres (>80%). During the 40 to 48 hour period the beads fused with juxtapositioned members (Plate 2, Figure 2) forming fusion spheres (32.7 µm diameter, s=7.8 µm). During the 36 to 48 hour period many of the fusion spheres developed thick walls (3.8 µm thick, s=2.2 µm) (Plate 2, Figure 4, arrows). Although many of these spheres were single or multivacuolated (Plate 3, Figure 5, arrows), nonvacuolated spheres occurred frequently (47% incidence). During the 144 hours of observation no further development for the thick-walled spheres was detected in liquid media.

The patterns of development were essentially the same in liquid shaken and stationary cultures. Both patterns included small mycelial tufts (72 to 96 hours). In no instances were conidia present.

On the coagulated egg yolk medium, with a 5 ml overlay of protoplasts in Grace's modified medium, the formation of fusion spheres (≤10% incidence) and prohyphal spheres (incidence ≤30%) was usually superceded by the aggregation of individual, non-chain, protoplasts within 3 hours. These coalesced (Plate 3, Figure 6, single arrows) with the resulting protoplast often possessing two or more protoplasmic

Plate 2. Figure 1. Catenulate series of protoplasts showing increased vacuole formation. X1400.

Figure 2. Coalescence of chain-member protoplasts into a fusion sphere. X1400.

Figure 3. Hypha (arrow) developing from prohyphal sphere. X800.

Figure 4. Thick-walled fusion spheres (arrows) X800.

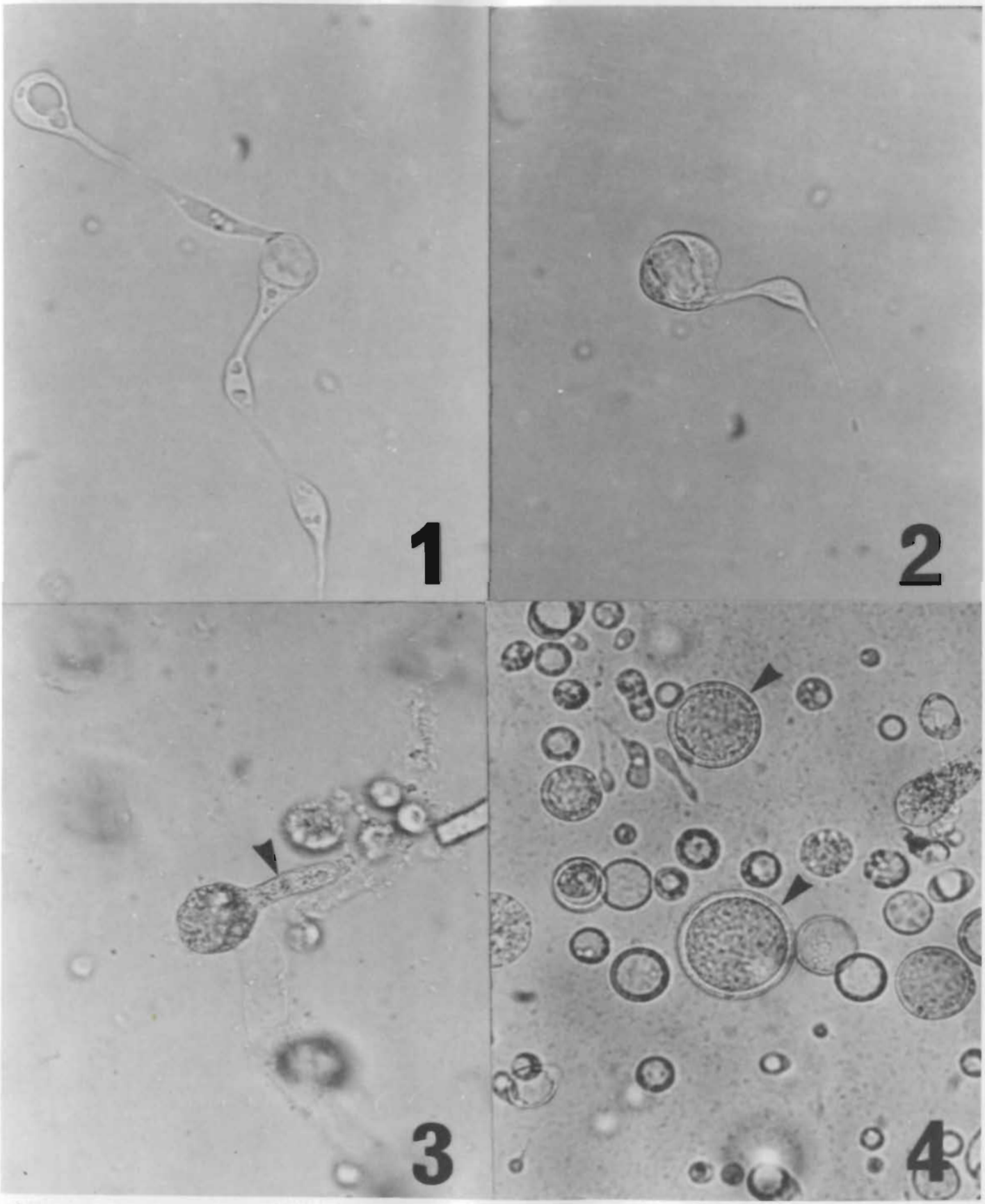
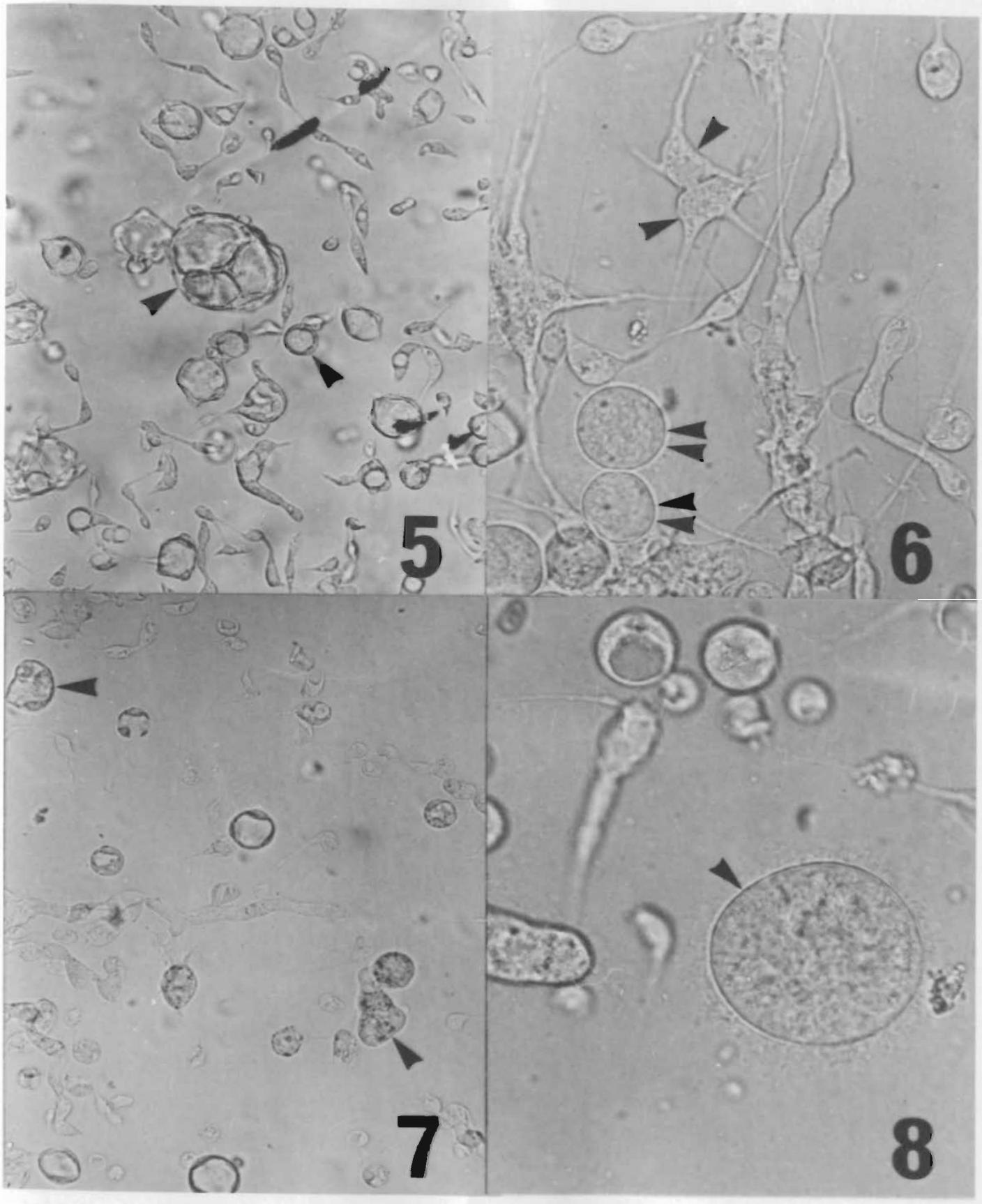

1
2
3
4

Plate 3. Figure 5. Single and multivacuolate fusion spheres (arrows). X450.

Figure 6. Fusion of two protoplasts (single arrows) with extensions. Post-protoplasts (double arrows). X560.

Figure 7. Cells formed by coalescence and exhibiting pleomorphism (arrows). X450.

Figure 8. Post-protoplast (arrow) with peripheral protrusions. X1100.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8

extensions. By 25 to 49 hours many of the coalesced cells exhibited pleomorphism (Plate 3, Figure 7, arrows). Later the pleomorphic forms assumed a general oval shape with fine protrusions around the periphery (Plate 3, Figure 6, double arrows, on Figure 8, arrow).

This stage was particularly sensitive to light, rapidly becoming spherical when so stimulated. Because this structure exhibited the first signs of osmotic shock resistance in the presence of distilled water, it was designated the post-protoplast stage. Between 49 to 69 hours the post-protoplast developed hyphal extensions (14.7 µm diameter) (Plate 4, Figure 9, arrow) and osmotic shock resistance usually associated with walled fungal structures. By this time the prohyphal spheres had given rise to hyphae directly or after forming a bud.

In the period from 76 to 96 hours the clavate hyphal tips developed into single conidia [(60.2 µm (s=13.5 µm) x 42.6 µm (s=8.1 µm), Plate 4, Figure 10)] with the columella being visible in many instances. Often, a large oval structure, believed to be an oil droplet, was detected in the conidia (Plate 4, Figure 11). No mucilaginous caps were noticed. The conidia discharged after 96 hours and exhibited either lateral (Plate 4, Figure 11) or apical germination. Some hyphae possessed mucilaginous plugs, creating the appearance of septation.

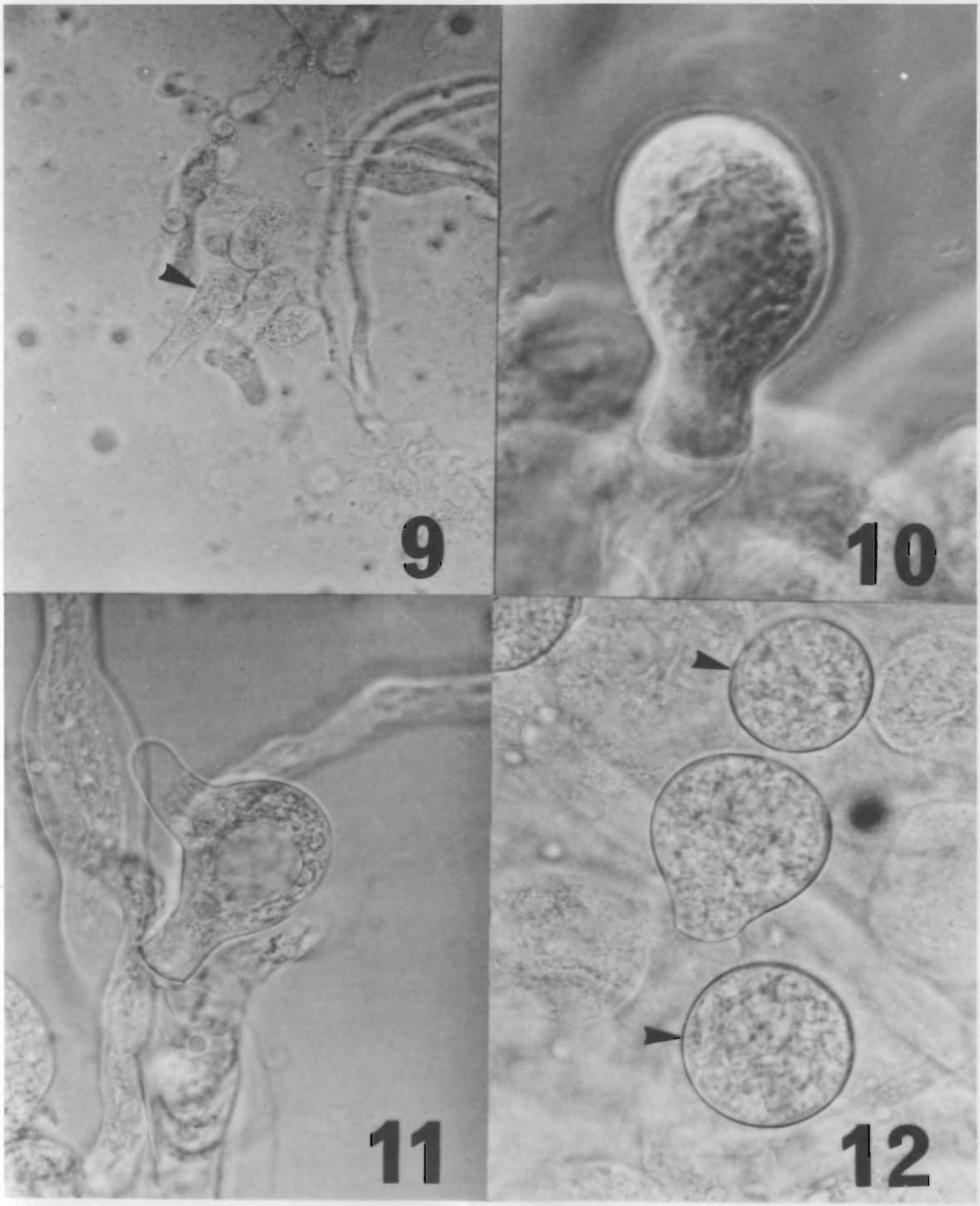
After 146 to 150 hours, large, densely cytoplasmic spheres (42.7 µm diameter, s=5.0 µm; Plate 4, Figure 12,

Plate 4. Figure 9. Post-protoplast (arrow) with emerging hypha. X450.

Figure 10. Conidium produced from clavate tip of hypha. X1700.

Figure 11. Conidium showing lateral germination and internal oil droplet. X1100.

Figure 12. Immature resting spores lacking thick cell walls (arrows). X1100.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11
12

arrows) were detected. Some immediately gave rise to hyphae (14.7 µm diameter, s=0.9 µm); whereas, others developed extremely thick walls (14.2 µm to 19.3 µm) by 216 hours. Both types germinated to form germ conidia as in Nolan et al. (1976). On this basis these structures were regarded as resting spores.

Mycelial development was observed on the water agar, but the hyphae were narrow and very fragile as compared to the type of mycelial development on the coagulated egg yolk medium.

The mycelial mat produced on the egg yolk medium from protoplasts was successfully subcultured twice. The last subculture was used to produce protoplasts in Grace's modified medium supplemented with fetal calf serum. These protoplasts were morphologically indistinguishable from stock cells.

Regeneration could be avoided by adding fresh Grace's modified medium, either with or without fetal calf serum, to the liquid media used or to coagulated egg yolk plates. By transferring E. egressa protoplasts, it has been possible to sustain the spindle morphology of the transferred cells for over 200 transfers. Cells subcultured over 200 times and those subcultured only 10 times readily regenerated in the fashion described for those placed on the egg yolk medium. Cells over 192 hours old and in the fusion sphere stage in liquid media readily developed into protoplasts upon the addition of fresh Grace's modified medium.

Pythium protoplasts (Sietsma and De Boer, 1973) and E. egressa protoplasts exhibited more than one type of development with one method predominating. Results for E. egressa reveal that the physical nature of the medium appeared to influence the predominant type of reversion pattern. Mycelial development never achieved appreciable status in the liquid media as it did on either water agar or coagulated egg yolk. Thus, a solid substrate appeared to favor continued development towards a mycelial stage. Reproduction via conidia also occurred on the egg yolk medium. Necas (1965) found protoplast cell wall development in Saccharomyces cerevisia Meyer strongly enhanced by solidification of the liquid culture medium by 15% gelatin.

The present results most closely approximated the regeneration of mycelia from protoplasts of Fusarium culmorum Smith (Garcia-Acha et al., 1966). The initial development consisted of an aggregation of cells, many of which were vacuolated. Many of the protoplasts produced a spherical osmotic shock resistant, structure from which hyphae developed. Garcia-Acha et al. (1966) also documented protoplasts giving rise directly and indirectly by "budding" to hyphae. These observations seem to parallel the formation of prohyphal spheres and post-protoplasts in the present study. This type of regeneration was noted to occur for eight species of Fusarium (Garcia-Acha et al., 1966). Conidial production was reported for the mycelia regenerated from protoplasts of Fusarium culmorum (Villaneuva, 1966) and Aspergillus nidulans

wild-type and strain P76 (Peberdy and Gibson, 1971).

The morphology and dimensions of the resulting E. egressa reproductive structures and hyphae formed upon the initial transfer of protoplasts to Müller-Kogler's medium were indistinguishable from mycelial cultures routinely maintained on Müller-Kogler's medium. Temporal comparisons between the regenerative patterns on the coagulated egg yolk and liquid media were not feasible because inoculum size, the volume of liquid media used, and agitation affect growth rate and, consequently, regeneration rate.

Tyrrell and MacLeod (1972b) reported the formation and development of E. egressa protoplasts from conidia in Grace's modified insect tissue culture medium supplemented with serum. The ameboid structures of Tyrrell and MacLeod (1972b) were not detected in the present study, however, the large spherical structures (Figure 16 in Tyrrell and MacLeod, 1972b) were occasionally observed. These spheres may have been formed by the method proposed for fusion sphere formation. Tyrrell and MacLeod (1972b) did not record the common fusion spheres. The nature of the fusion spheres is not presently known.

The reasons for the variety of regenerative patterns, even within the same culture, remains unknown. Peberdy and Gibson (1971) believed the origin of the protoplasts may be a factor. The differences between the present results and those of Tyrrell and MacLeod (1972b) may reflect differences due to the origin of protoplasts (i.e. mycelial versus

conidial) and/or differences in culture media. The temporal heterogeneity of development reported here and in other studies may also be a consequence of genetic differences.

Necas (1971) thought that prevention of protoplast regeneration for extended periods of time would result in the loss of regenerative ability. Entomophthora egressa protoplasts transferred over a period of ten months (over 200 transfers) still retained the ability to revert to the mycelial stage. Thus, the ability of protoplasts to regenerate cell walls on coagulated egg yolk medium and the ability to revert back to protoplasts in Grace's modified insect tissue culture media appeared to be a fixed property for E. egressa.

IV. Protoplast growth on MES-buffer standard medium

1. Glucose uptake

No detectable glucose utilization occurred until after 70 hours incubation. Over the next 17 hours the glucose concentration decreased by 55% (Table 15, Figure 6). Prior to the period of utilization of glucose the protoplasts were growing exponentially (Figure 3) and after 70 hours the cells entered the stationary phase. The rate of glucose utilization was greatest during the stationary phase.

Utilization of glucose as an energy-carbon source is a common feature among species of Entomophthora (Wolf, 1951; Gustafsson, 1965b; Krejzova, 1970 and Latge, 1975a). Gustafsson (1965a) and Latge (1975a) reported excellent vegetative growth on glucose for the 17 species tested.

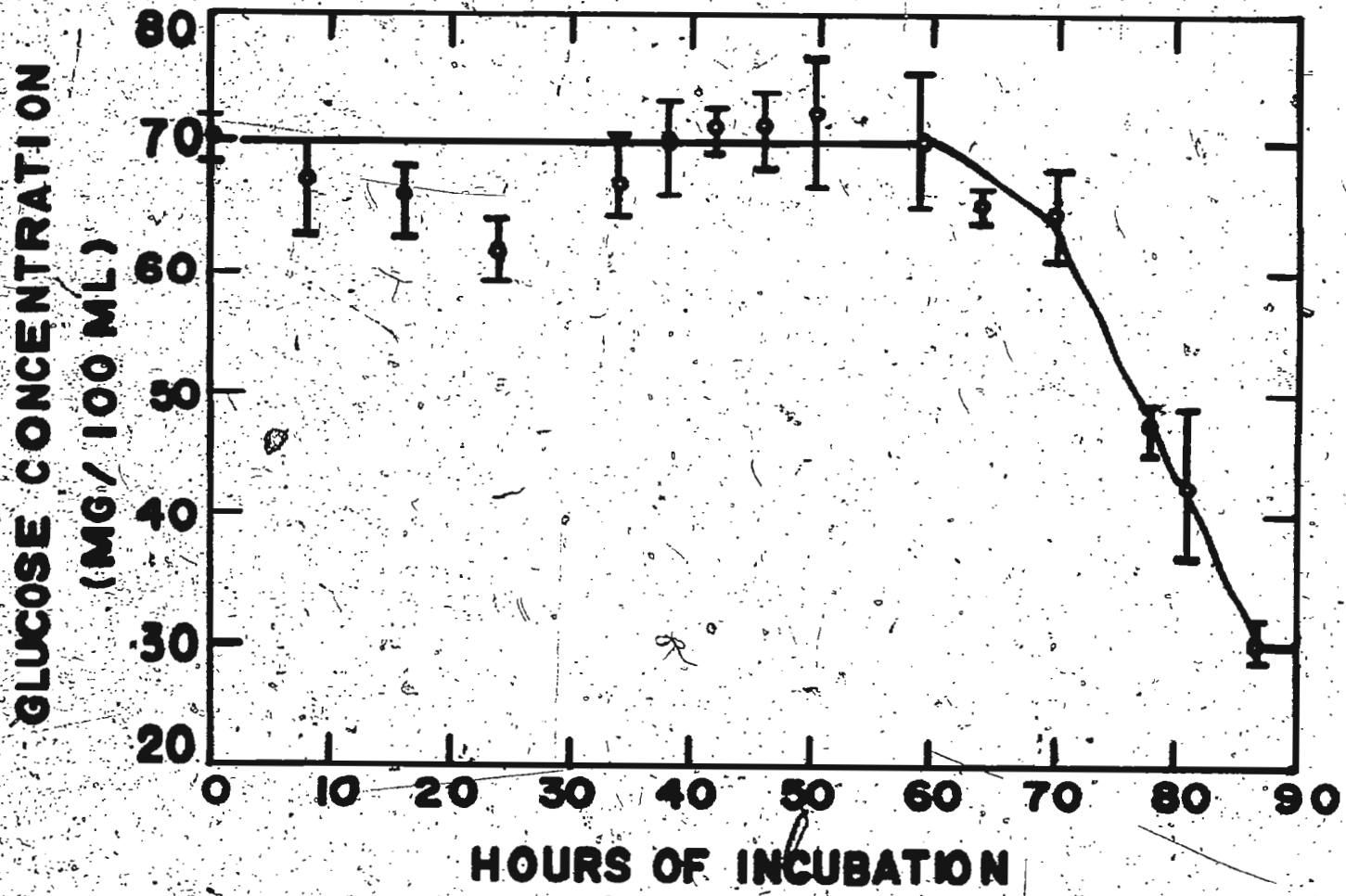
Table 15

Glucose uptake by protoplasts in MES-buffered medium

Incubation time (hours)	Concentration (mgm/100ml)	Incubation time (hours)	Concentration (mgm/100ml)
0	70.4 ± 1.9	50.5	72.4 ± 5.1
8	67.8 ± 2.5	55	72.0 ± 0.0
16	72.8 ± 7.8	59	71.2 ± 4.0
24	61.4 ± 2.0	64	64.8 ± 1.3
34	68.8 ± 1.8	70	64.4 ± 2.5
38	69.6 ± 3.3	78	46.6 ± 1.0
42	68.8 ± 1.8	81	42.8 ± 4.8
46	71.6 ± 2.2	87	30.8 ± 0.7

[a] n=5 for all values

Figure 6. Glucose utilization by protoplasts grown in the standard medium.


GLUCOSE CONCENTRATION
(MG/100 ML)
80
70
60
50
40
30
20
0
10
20
30
40
50
60
70
80
90
HOURS OF INCUBATION

The non-utilization of glucose prior to 70 hours may have represented the period of utilization of fructose and/or the Kreb's cycle acids present in the medium.

Further discussion on carbon sources has been considered later in the text.

2. Changes in ninhydrin-positive compound concentrations

The results of the ninhydrin-positive compounds analysis revealed changes in the amino acid concentrations by 16 hours (Table 16). Relative to the initial values cysteic acid, isoleucine, leucine, methionine, proline, serine, threonine and valine decreased in concentration.

Generally, many of the amino acids exhibited variation in concentration over the 46 to 64 hour incubation period while the levels of methionine and glycine remained essentially constant.

Prior to the stationary phase and during the stationary phase marked reductions in the concentrations of asparagine and/or glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine and valine occurred. Proline, methionine and glutamine and/or asparagine exhibited the greatest reduction rate.

The levels of ammonia, ornithine, glutamic acid and alanine increased throughout the growth period. The rate of ammonia production was greater in the late exponential phase and early stationary phase than in the early exponential phase and greatest during the late stationary phase. This pattern also existed for ornithine and alanine.

Table 16

Per cent change in concentration of ninhydrin-positive compounds of MES buffered Grace's modified medium[a]

Compound	Incubation time (hours)							
	16	36	46	54	64	70	83.75	87.5
Cysteic acid	-7.9[b]	-2.2	-16.8	55.7[c]	272.2	43.6	- 1.5	3.1
L-Aspartic acid	2.0[c]	9.5	- 0.1	1.4	3.6	1.9	- 0.4	1.2
L-Threonine	-1.6	8.0	- 1.2	3.4	0.9	- 0.7	-12.1	-12.8
L-Serine	-1.6	8.4	-1.2	3.7	2.2	0.1	- 7.1	0.0
L-Glutamine + Asparagine	-3.9	8.9	- 5.9	-0.8	-13.2	-15.1	-28.0	-32.9
L-Proline	-3.0	6.4	- 3.2	-1.1	- 0.8	-30.1	-70.3	-73.8
L-Glutamic acid	0.7	9.1	- 0.2	2.0	5.6	4.7	3.8	6.1
Glycine	40.2	51.4	39.1	44.9	49.5	40.2	34.3	38.6
L-Alanine	4.9	13.2	3.6	13.6	20.9	42.9	88.3	108.4
Halfcystine	-4.1	106.7	46.8	68.0	17.4	23.56	6.9	6.9
L-Valine	-3.3	10.0	- 2.9	1.2	-12.6	-10.6	-29.0	-28.9
L-Methionine	-10.6	0.7	-10.4	4.2	-10.6	-15.3	-71.4	-67.0
L-Isoleucine	-4.0	7.2	3.5	5.0	-10.9	-10.8	-44.8	-30.7
L-Leucine	-5.6	8.0	- 6.3	6.0	-11.4	-16.8	-11.4	-24.0
L-Tyrosine	0.1	11.7	2.3	5.3	- 0.8	- 1.9	-11.0	- 9.7
L-Phenylalanine	0.9	9.7	0.6	3.6	- 2.5	- 6.1	-27.9	-29.0
β-Alanine	1.3	8.7	1.1	4.5	-13.9	5.1	4.1	0.9
Ornithine	-6.2	28.6	35.7	162.2	5.5	30.2	50.0	167.5
Ammonia	14.8	76.8	125.4	123.7	186.2	184.1	304.5	320.7
L-Lysine	1.0	8.9	- 0.4	1.4	- 2.2	- 3.5	- 7.9	- 7.9
L-Histidine	1.6	10.7	0.6	3.4	2.4	- 4.5	- 5.0	- 3.8
L-Tryptophan	27.3	24.6	8.6	27.9	24.8	23.9	2.3	14.9
L-Arginine	0.9	11.5	0.0	5.4	2.1	- 1.0	- 3.5	- 2.3

[a] based on the change in concentration of mean, noncoded date of all values relative to the initial value

[b] per cent decrease

[c] per cent increase

In addition to the above compounds other ninhydrin-positive compounds were detected. Cystathionine was present in trace amounts at 34 hours and from 67.5 to 87.5 hours, methionine sulfoxide was noticed in trace amounts at 38 and 42 hours and from 59 to 87.5 hours; whereas, ethanolamine was detected during the entire stationary phase. Since these compounds were initially not present in the medium, they may have been released from the protoplasts; however, the possibility of exogenous enzymatic production could not be ignored.

The amines spermidine, agmatine, S-adenosyl methionine, histamine, tyramine and tryptamine and the polyamines putrescine, cadaverine and spermine were not detected. Since the cells did not lyse the above observation supported the hypothesis that the protoplasts did not release amine producing extracellular decarboxylases.

It should be noted that a reduced concentration of an amino acid does not necessarily mean that it serves a nutritional role. Halvorson and Corvice (see Nolan, 1970) reported the uptake of amino acid isomers which were not utilized by yeast. Patterson (1965) observed active uptake of some amino acids which may have reflected increased storage of non-essential amino acids in intracellular amino acid pools when these amino acids were included in a medium containing essential amino acids.

The precise role of the amino acids in the medium was not determined. Undoubtedly some of the amino acids served

as precursors in protein synthesis and other nitrogen requiring reactions (Nicholas, 1965). Since Nicholas (1965) proposed that amino acids in fungi can be directly incorporated into proteins without the production of ammonia, the continuously increasing concentration of ammonia may have stemmed from several sources. Meister (1965) and Lehninger (1975) reported that oxidative deamination of amino acids for carbon-energy metabolism will elicit ammonia production. Meister (1965) also stated that the spontaneous release of ammonia from amide groups of proteins was a contributing factor. Patterson (1965) reported that extracellular degradation of glutamine in tissue culture media often raises external ammonia levels. Nolan (1975, 1976) reported increased ammonia production and amino acid uptake by Saprolegnia ferax (Gruith.) Thuret. and Saprolegnia megasperma Coker in the absence of glucose, some of the amino acids presumably served as carbon sources under these conditions. The ammonia levels of the present experiment may also have reflected environmentally introduced ammonia (Nolan, 1970).

Based on the increased uptake of glucose and ammonia production by protoplasts of E. egressa it is postulated that the cells exhibited a concomitant carbon requirement for both glucose and some of the amino acids.

The pronounced amino acid uptake in the present experiment as the culture approached stationary phase is a common feature of fungal growth. Nolan and Lewis (1974) and Nolan (1975, 1976) reported the increased uptake of amino

acids by 3 species of Oomycetes as growth approached maximum yield.

Wolf (1951) and Gustafsson (1965a) detected the ability of E. corohata to use threonine, lysine and alanine for both carbon and nitrogen requirements while glutamic acid, asparagine and aspartic acid served solely as nitrogen sources. Gustafsson (1965a) and Latgé (1975b) reported assimilation of serine, threonine, cysteine, glutamine, asparagine, aspartic acid, glutamic acid, glycine, valine, isoleucine, methionine, tyrosine and phenylalanine by E. virulenta. Latgé (1975b) recorded the uptake of the above nitrogen sources by E. destruens and E. near thaxteriana when they were supplied individually; glutamine acid, asparagine and aspartic acid yielded excellent vegetative growth and resting spore production.

E. egressa continually removed glutamine and/or asparagine from the experimental medium. Glutamic acid generally increased in level and aspartic acid was marginally utilized during the later part of the growth cycle. The reduction of glutamine and/or asparagine may be more apparent than real. Patterson (1965) reported that a decrease in glutamine concentration in tissue culture media often represented the combined action of nonenzymatic degradation of glutamine catalyzed by phosphate and bicarbonate ions to pyrolidone carboxylic acid and ammonia and/or extracellular enzymatic breakdown.

Preferential amide uptake may have reflected the facile utilization of the amide nitrogen reported to occur for some Basidiomycetes (Nicholas, 1965). One function of amides in fungi is the provision of amino groups for glucosamine (Cochrane, 1958) the fundamental component of chitin in the Entomophthorales (Hoddinott and Olsen, 1972).

The continuously low level of proline in the MES-buffered standard medium was expected because Cochrane (1958) found it to be an excellent nitrogen source presumably because of its ready degradation to glutamic acid. The moderate to high levels of proline in the host looper haemolymph may have favoured an adaptation of E. egressa to utilize the amino acid.

The overall release of ornithine into the medium and the general decrease in arginine concentration implicates the presence of an ornithine cycle proposed for most fungi by Nicholas (1965).

V. Simplification of the standard medium.

1. Medium based on amino acid uptake from the standard medium

A. Growth rate analysis

On the assumption that a decrease in amino acid concentration represented utilization a medium composed of only those 12 amino acid used up to 16 hours incubation was made. The salts, sugars (except sucrose), vitamins, organic acids, and fetal calf serum were maintained at the initial levels present in Grace's medium. The sucrose concentration

was adjusted to compensate for the loss of the osmotic contribution of the deleted amino acids. Although the osmotic pressure and millimolarity were 350 mOsm and 350 mM respectively, the protoplasts failed to grow. The cells became constricted with a crenate periphery and later lysed.

Latge (1975b) reported tyrosine utilization by 3 species of Entomphthora and although tyrosine was not assimilated by E. egressa protoplasts in the standard medium the possibility of it being required in the simplified medium was considered. With the addition of tyrosine at the same concentration as initially present in Grace's medium and correcting the sucrose level, growth occurred.

Because the medium contained 13 amino acids it was designated as M13Aa (Table 17). In this medium the protoplasts grew well by 14 hours of incubation (Table 18, Figure 7). A generation time of 4.2 hours was maintained until 57.5 hours resulting in a maximum cell yield of 1.25×10^6 cells/ml followed by a rapid, apparent, decline in cell number (Figure 7). There was no stationary phase per se and the decline phase was the result of cellular aggregation and precipitation from the medium. Eventually the medium was devoid of suspended cells.

The osmotic pressure and pH of the medium remained constant.

B. Morphological development of the cells in medium M13Aa

This medium favoured the development of a variety

Table 17

Simplified growth medium M13Aa

Compound	Concentration (mgm/L)	Compound	Concentration (mgm/L)
$NaH_2PO_4.H_2O$	1014	Sucrose	32828
KCl	2240	Fructose	400
$CaCl_2$	1000	Glucose	700
$MgCl_2.6H_2O$	2280	Malic acid	670
$MgSO_4.7H_2O$	2780	Fumaric acid	56
$NaHCO_3$	350	α-Ketoglutaric acid	370
L-Asparagine	350	Succinic acid	60
L-Aspartic acid	350	MES[b]	1952
L-Cystine	22	Thiamin.HCl	0.02
L-Glutamine	600	Riboflavin	0.02
L-Glutamic acid	600	D-Ca pantothenate	0.02
L-Leucine	76	Pyridoxine.HCl	0.02
L-Lysine.HCl	624	Para-amino benzoic acid	0.02
L-Methionine	50	Folic acid	0.02
L-Proline	350	Niacin	0.02
DL-Serine	1100	i-Inositol	0.02
L-Threonine	175	Biotin	0.01
L-Tyrosine	50	Choline Cl	0.02
L-Valine	100	Fetal Calf Serum[c]	28

[b] 2 - (N-morpholino) ethanesulfonic acid

[c] volume in ml of fetal calf serum added to one liter of media

Table 18

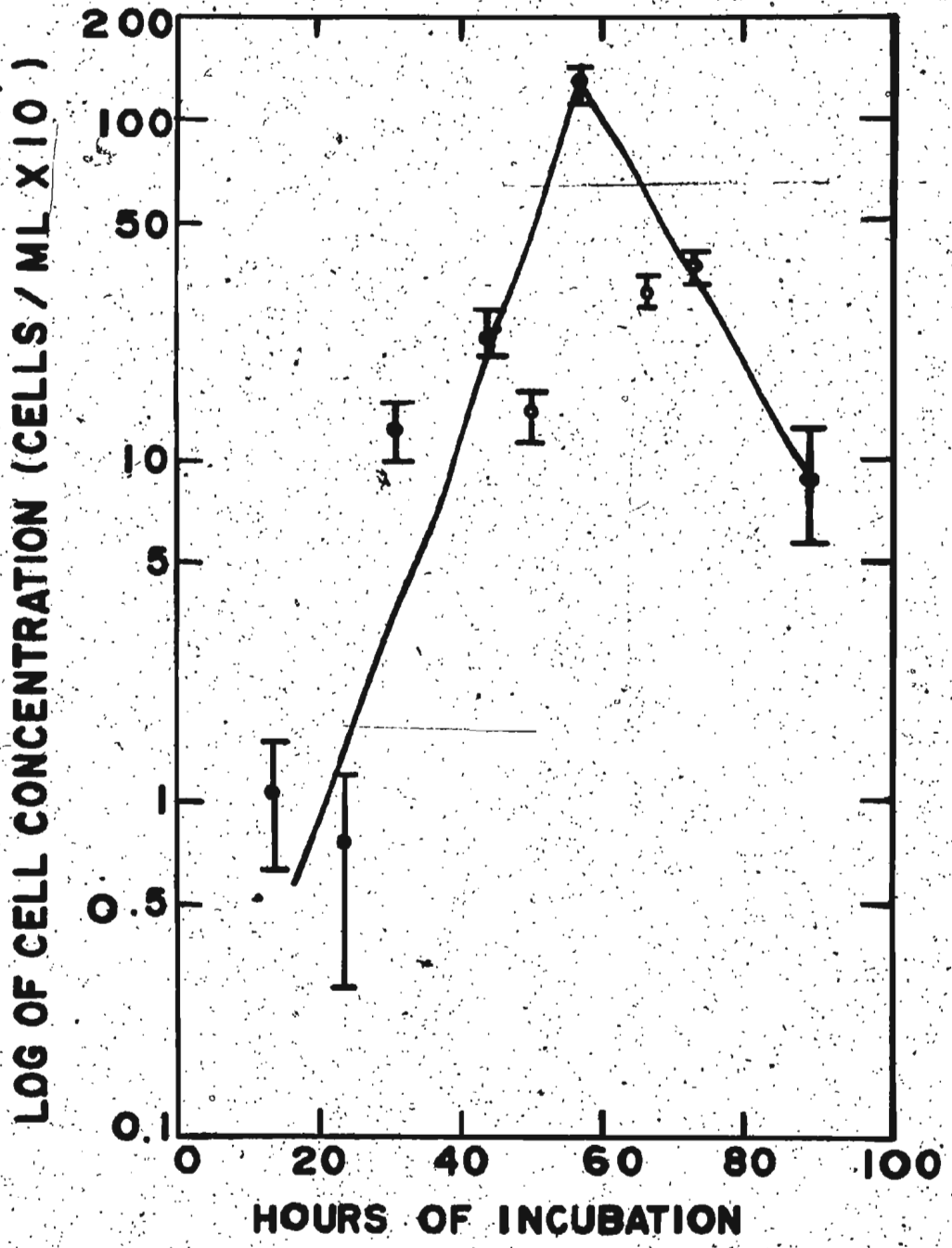
Growth of protoplasts in medium M13Aa plus fetal calf serum

Incubation time (hours)	Yield (cells/ml)[b]	pH
0.0	0.0 ± 0.0	6.10 ± 0.00 OP[a] = 349.0 ± 0.1 mOsm
14.0	$1.06 \times 10^4 \pm 2.6 \times 10^3$	6.10 ± 0.03
24.5	$7.50 \times 10^3 \pm 2.2 \times 10^3$	6.10 ± 0.00
31.5	$1.22 \times 10^5 \pm 1.1 \times 10^4$	6.10 ± 0.00
44.0	$1.33 \times 10^5 \pm 8.6 \times 10^4$	6.11 ± 0.01 OP = 350.2 ± 0.8 mOsm
49.5	$1.36 \times 10^5 \pm 1.1 \times 10^4$	6.11 ± 0.01
57.5	$1.26 \times 10^5 \pm 4.6 \times 10^4$	6.10 ± 0.01 OP = 347.1 ± 1.0 mOsm
66.0	$3.12 \times 10^5 \pm 1.4 \times 10^4$	6.17 ± 0.05
73.0	$3.68 \times 10^5 \pm 1.7 \times 10^4$	6.11 ± 0.07
89.0	$8.83 \times 10^4 \pm 1.2 \times 10^4$	5.98 ± 0.02 OP = 347.2 ± 0.5 mOsm

[a] Osmotic pressure in milliosmolar

[b] n = 15 for all values

Figure 7. Growth curve of protoplasts in medium M13Aa. Inoculum 0.1 ml, 2.3×10^5 cells/ml, 24 hours old.


LOG OF CELL CONCENTRATION (CELLS / ML X 10 4)
200
100
50
10
5
1
0.5
0.1
0
20
40
60
80
100
HOURS OF INCUBATION

of morphological types with varying degrees of temporal overlap (Table 19).

Plate 5, Figure 1 reveals the presence of a spherical structure (36.4 µm diameter, s=15.9 um) bearing active cytoplasmic projections. These projections, usually being symmetrically positioned about the sphere, exhibited several types of activity. The longer filaments revealed sinusoidal wave motion and the shorter projections possessed asynchronous ciliary-like motion. Both types of filaments contained highly refractile particles (Plate 5, Figure 1, 2, 3, 4) which moved distally and proximally to the main cytoplasmic mass. This structure, a mesoprotoplast, was light sensitive. The filaments increased waving motion as the light intensity increased. The mesoprotoplast stage was most abundant by 24.5 hours and decreased thereafter (Table 19). As the level of the mesoprotoplast decreased that of an elliptical mesoprotoplast (Plate 5, Figure 2) increased to a maximum by 43 hours. This light sensitive structure was of comparable size to the mesoprotoplast and often possessed one long filament (Plate 5, Figure 3) and/or shorter filaments around the remainder of the cell (Plate 5, Figure 2). Often the largest filaments possessed refractile granules which moved toward the termination of the filament and into the growth medium. Cytoplasmic undulations consisting of cytoplasm flowing in and out of the proximal portion of the filament occurred for all long filaments, even those lacking refractile particles.

The abundant elliptical mesoprotoplast stage was followed by regular spindle shaped protoplasts (Table 19) and a few aberrant forms (Plate 5, Figure 4).

By 57.5 hours several types of development were detected (Table 19) including the mesoprotoplast, elliptical mesoprotoplasts and protoplasts stages (Plate 6, Figure 5). The major types of cells consisted of rod shaped cells and spherical cells. Two sizes of rod-shaped hyphal bodies were present, one being 18.6 µm (s=1.2 µm) x 1.2 µm (s=0.9 µm), the other being 39.8 µm (s=21.6 µm) x 3.2 µm (s=1.1 µm). Extensive variation in form was recorded, many of the rods being sausage shaped (Plate 5, Figure 6) and later more rod shaped.

The peak of the growth curve and the decline phase represented cellular aggregation by the rod-shaped hyphal bodies and spherical cells (Plate 6, Figure 7). It was during this phase that the abundance of spherical hyphal bodies (Table 19) (20.6 µm diameter, s=1.4 µm) with large vacuoles (9.9 µm diameter, s=4.2 µm) increased (Plate 6, Figure 8). The cytoplasm of the spherical hyphal bodies become more granulated with age and exhibited extensive cytoplasmic flowing in the ectoplasm region.

Both types of hyphal bodies exhibited a refractile periphery and osmotic stability, a cell wall was believed to be present.

A mycelial mat was macroscopically visible by 89 hours. Hyphae (8.0 µm diameter, s=1.4 µm) emerged from

Table 19

Per cent abundance of various morphological types of E. egressa grown in medium M13Aa plus fetal calf serum

Time of incubation (hours)	Structure	Abundance (%)
14.0	mesoprotoplasts	0.9
24.5	mesoprotoplasts	32.1
31.5	mesoprotoplasts	9.7
	elliptical mesoprotoplasts	18.3
43.0	elliptical mesoprotoplasts	43.0
49.5	elliptical mesoprotoplasts	25.7
57.5	rod-shaped hyphal bodies	9.8
66.0	rod-shaped hyphal bodies	15.6
	spherical hyphal bodies	7.9
73.0	all structures	0.0

Plate 5. Figure 1. Mesoprotoplast stage showing filaments and refractile granules (arrow). X1280

Figure 2. Elliptical mesoprotoplast stage. X1280

Figure 3. Elliptical mesoprotoplasts with long filaments and refractile granules (arrow). X510

Figure 4. Aberrant protoplast. X510

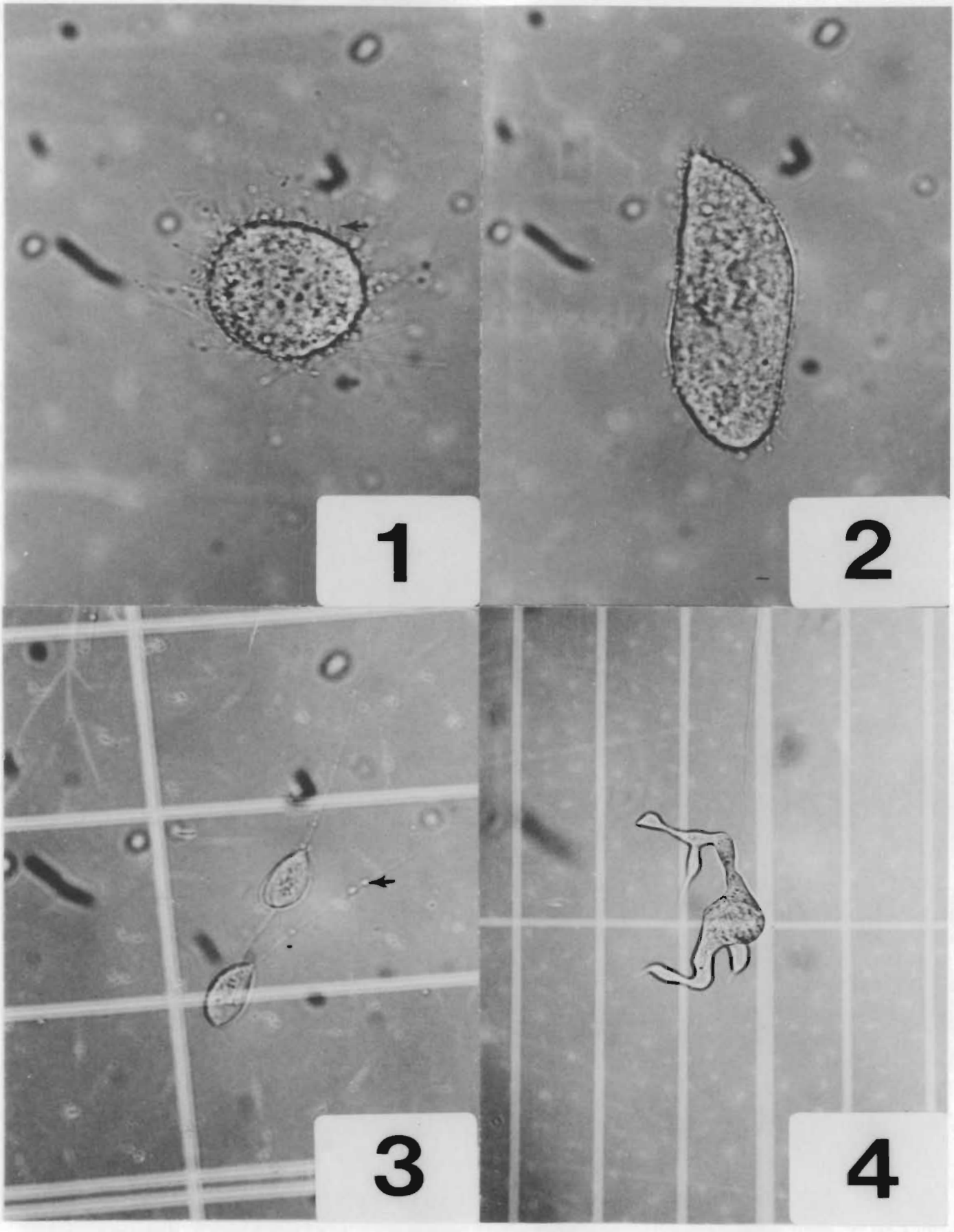
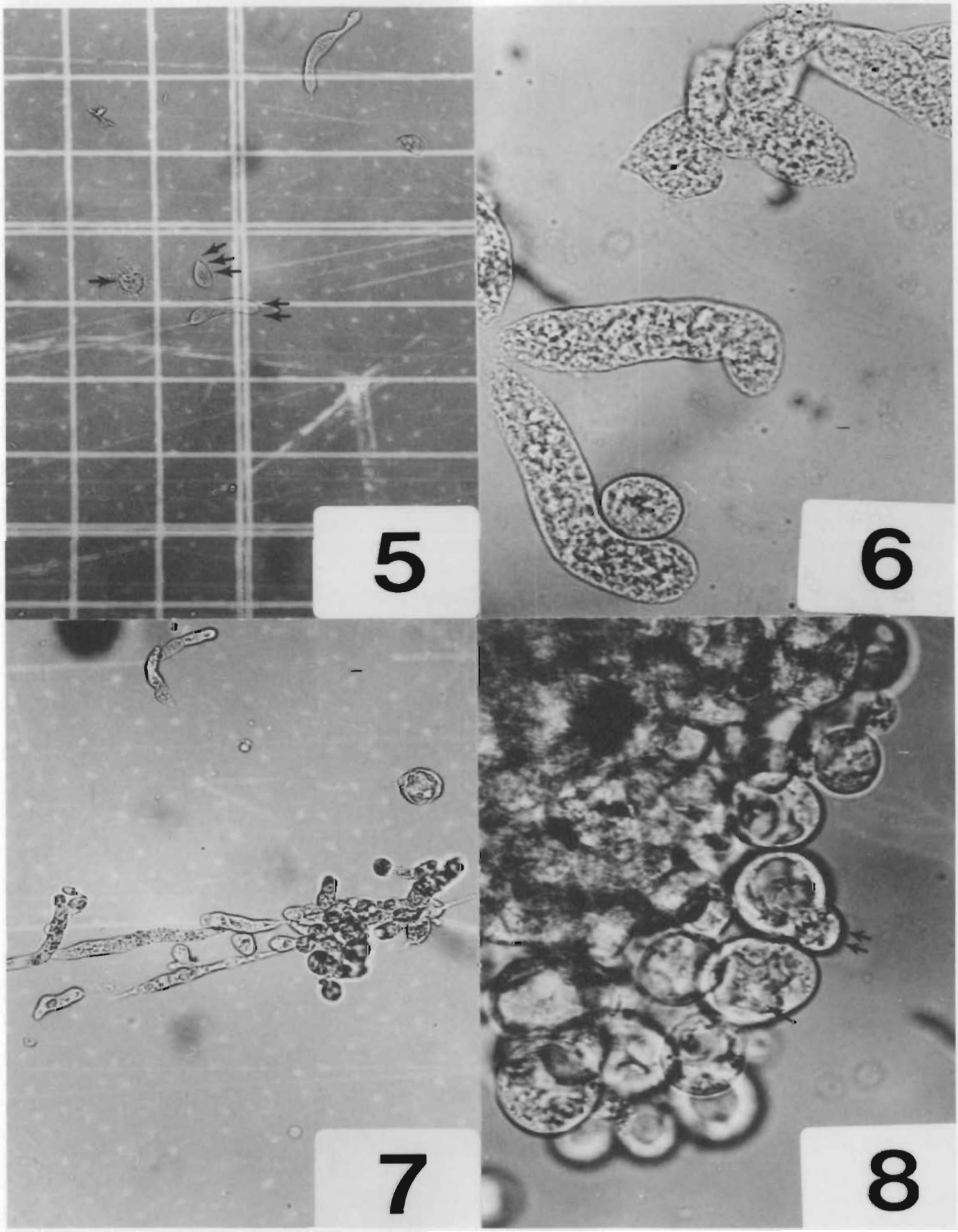

1
2
3
4

Plate 6. Figure 5. Mesoprotoplasts (arrow), elliptical mesoprotoplast (double arrows) and protoplasts (triple arrows) occurring together. X510.

Figure 6. Sausage shaped hyphal bodies. X1280.

Figure 7. Cellular aggregation of rod shaped and spherical hyphal bodies. X510.

Figure 8. Spherical hyphal body with vacuole (arrow). Germinating spherical hyphal body with germ tube (double arrows). X1280.


5
6
7
8

the spherical hyphal bodies (Plate 6, Figure 8 and Plate 7, Figure 9). Thin tube-like structures began to develop from the ends of the rod-shaped hyphal bodies (Plate 7, Figure 10) implying a role in hyphal development and mycelial mat formation.

A distinct-cream-white ball was formed by 131 hours. The hyphae were, however, not extensively interwoven (Plate 7, Figure 11). A fine mesh of hyphae formed on the outer surface of the ball (5.5 µm, s=0.9 µm). After 182 hours, the hyphae began to branch (Plate 7, Figure 12). During this time granular spheres with cell walls (18 µm diameter, s=2.1 µm) were observed to be of low abundance. Lysis and fragmentation of the hyphae occurred by 290 hours. No conidia were observed. The pH and osmotic pressure had changed to 7.2 $\pm$ 0.01 and 205.3 $\pm$ 0.9 mOsM, respectively.

It was possible to use any stage of development reported above to regenerate protoplasts in fresh M13Aa medium.

Inoculation of medium previously containing protoplasts after 57.5 hours incubation with a 24 hour old stock protoplast culture always accelerated the developmental phases and resulted in rapid mycelial ball formation.

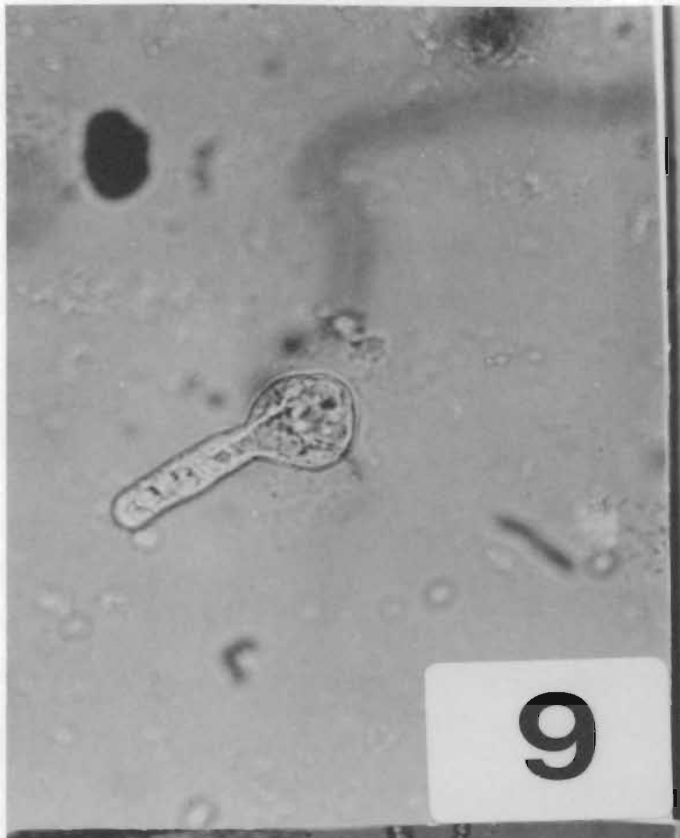
In terms of maximum cell yield the M13Aa medium was found to be comparable to the standard medium, both media produced cell yields of 1.2×10^6 cells/ml using the same size inoculum. In terms of growth rate medium M13Aa was superior to the standard medium, the former favouring significantly

Plate 7. Figure 9. Germinating spherical hyphal body with pronounced hypha. X510.

Figure 10. Germinating rod-shaped hyphal bodies. Hypha (arrow). X510.

Figure 11. Hyphae during mycelial ball development. X510.

Figure 12. Branching hypha. X1280.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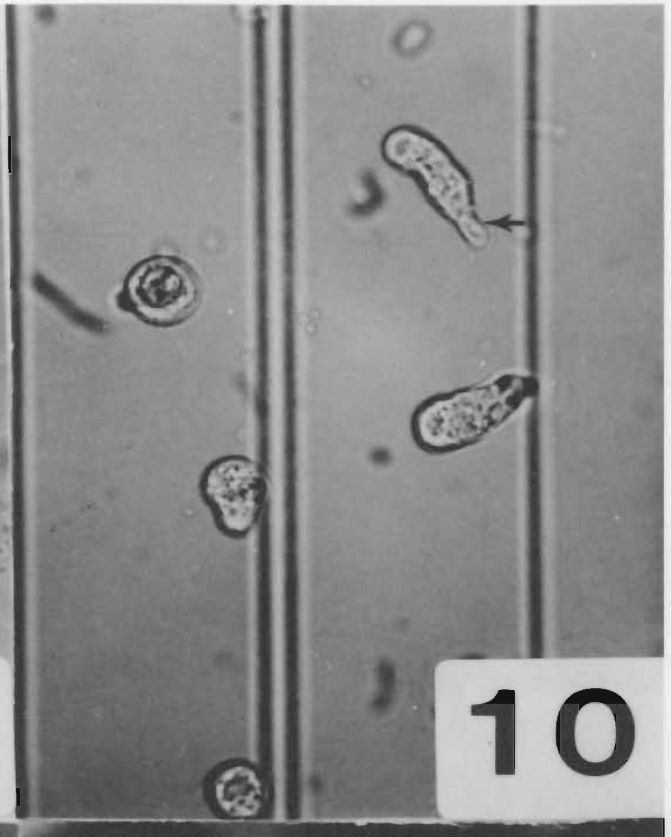
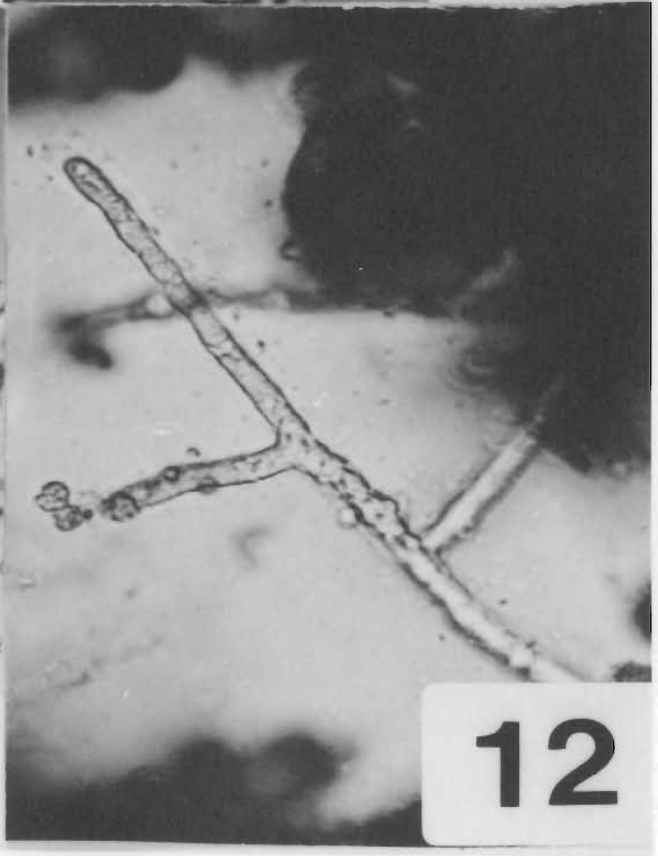

10


11


12

faster growth than the latter.

Because the amino acid uptake analysis revealed that many amino acids were not utilized, their removal from the medium would not have been expected to influence the maximum cellular yields as the amount of utilizable nitrogen would remain constant. By simplifying the medium the extent of amino acid antagonism may have been reduced with the consequential increase in growth rate.

The mesoprotoplast stage, because of its ability to readily revert to protoplasts and the distinct absence of a cell wall, was not believed to be the same structure as the postprotoplast. It is thought that the mesoprotoplast stage gives rise to the elliptical mesoprotoplast which in turn reverts back to the normal protoplast stage.

For the most part the refractile granules and filaments may have reflected a feeding activity comparable to that found with microvilli. Although Bangham and Pethica (see Ambrose and Easty, 1970) believed that cells in culture adhered to surfaces via fine projections on their surfaces, this was not believed to be the case for E. egressa mesoprotoplasts because these structures were found suspended in a liquid medium (M13Aa). Oberling and Bernhard (1961) reported the presence of microvilli varying in number and length on the membranes of cancer cells and Ambrose and Easty (1970) confirmed the presence of microvilli on Hela cells. Fawcett (1966) and Giese (1968) reported large numbers of microvilli only on those cells whose only function

was the exchange of materials with the environment.

Although the release of granules from the longer filaments may have been an excretory process, MacLeod (1976) postulated that the granules may be a form of reproduction analogous to microbody formation.

Villaneuva (1966) reported the occurrence of cytoplasmic threads connecting protoplasts of Polystictus versicolor. The threads were believed to serve as an "attraction focus" between connected protoplasts which later fused, a belief shared by Peberdy and Gibson (1971) for the formation of pleomorphic protoplasts of the wild type A. nidulans. Although there was no evidence of protoplast fusion by E. egressa protoplasts the pleomorphic forms detected may have been the result of fusion.

Rod-shaped hyphal bodies have been reported by Gustafsson (1965b) for 17 species of Entomophthora Prasertphon and Tanada (1968) reported that the rod-shaped hyphal bodies found in Galleria mellonella L. and Carner (1976) and the two spotted mite, Tetranychus urticae Hast. reproduced by binary fission. The ratio of the large to small rod-shaped hyphal bodies formed by E. egressa protoplasts in medium M13Aa was two implying multiplication by binary fission. Carner (1976) also reported early formed hyphal bodies to be somewhat irregular in shape, becoming more rod-shaped with time. These events seem to parallel those reported here for E. egressa.

Cohn (see Prasertphon and Tanada, 1968) reported the

presence of ovalular hyphal bodies for Entomophthora muscae (Cohn) Fresenius. The spherical hyphal bodies of E. egressa readily germinated forming dichotomously and asymmetrically branched hyphae which assisted in holding the mycelial ball together.

The formation of conidia and/or resting spores from hyphal bodies has been well documented (Alexopolous, 1962; Gustafsson, 1965b; Prasertphon and Tanada, 1968) but was not observed in this experiment. The granular spheres with walls failed to develop further and were not identified. The author is not aware of any report documenting the production of two types hyphal bodies by a given species of Entomophthora.

Emerson and Emerson (1958) and Hamilton and Calvet (1964) reported osmotic pressure to be the main factor inhibiting cell wall synthesis of N. crassa resulting in the formation of protoplasts. Because the osmotic pressure of medium M13Aa remained constant, it was believed that osmotic pressure alone, in the presence of constant pH, could not account for either the formation of the protoplasts nor cell wall regeneration.

The factors eliciting protoplast regeneration and hyphal body formation for E. egressa were not determined.

Garcia-Acha et al. (1966) reported that the amino acids glycine, glutamate, and asparagine had no effect on the regeneration rate of E. culmorum. However, regeneration of the phycomycete, Pythium species was stimulated by asparagine (Sietsma and DeBoer, 1973).

Aoki (1966) reported that glutamic acid, aspartic acid, histidine, arginine, serine, alanine, glycine, lysine, tyrosine, and phenylalanine favoured hyphal body formation in Beauveria bassiana (Balsamo) Viullemin and Isaria fumosarosea (Wize) Aoki.

Because any stage of the stated regeneration pattern could be used to form protoplasts in fresh medium and the addition of young protoplasts to the stated test medium resulted in the acceleration of regeneration, a decrease in a metabolite(s) concentration and/or production of excretory products may have determined the regeneration process.

2. Further modification of medium M13Aa.

In view of the metabolic versatility of glutamine and asparagine in amino acid catabolism and anabolism (Meister, 1965; Nicholas, 1965; Neiderpruem, 1965; and Lehninger, 1975) medium M13Aa was further simplified to contain these two amino acids and methionine (Table 20). Because the medium contained three amino acids it was designated M3Aa.

The growth curve (Table 21 and Figure 8) revealed measurable growth by 24 hours with a generation time of 13.4 hours. Maximum protoplast growth of 1.34×10^5 cells/ml was achieved by 89 hours after which a 9.8 hour stationary phase occurred. After 99.2 hours the number of cells suspended in medium M3Aa decreased in connection with the morphological events previously described for cells in medium M13Aa.

Relative to the results in medium M13Aa the increase in generation time and significantly lower maximum in medium

Table 20

Growth Medium M3Aa

Compound	Concentration (mgm/L)	Compound	Concentration (mgm/L)
$NaH_2PO_4.H_2O$	1014	α-Ketoglutaric acid	370
KCl	2240	Succinic acid	60
$CaCl_2$	1000	MES	1952
$MgCl_2.6H_2O$	2280	Thiamin.HCl	0.02
$MgSO_4.7H_2O$	350	Riboflavin	0.02
L-Asparagine	350	D-Ca pantothenate	0.02
L-Glutamine	600	Pyridoxine.HCl	0.02
L-Methionine	50	Para-amino benzoic acid	0.02
Sucrose	56172	Folic acid	0.02
Fructose	400	Niacin	0.02
Glucose	700	i-Inositol	0.02
Malic acid	670	Biotin	0.01
Fumaric acid	56	Choline Cl	0.02
		Fetal calf serum	28

[a] Volume in ml of fetal calf serum added to one liter of media

Table 21

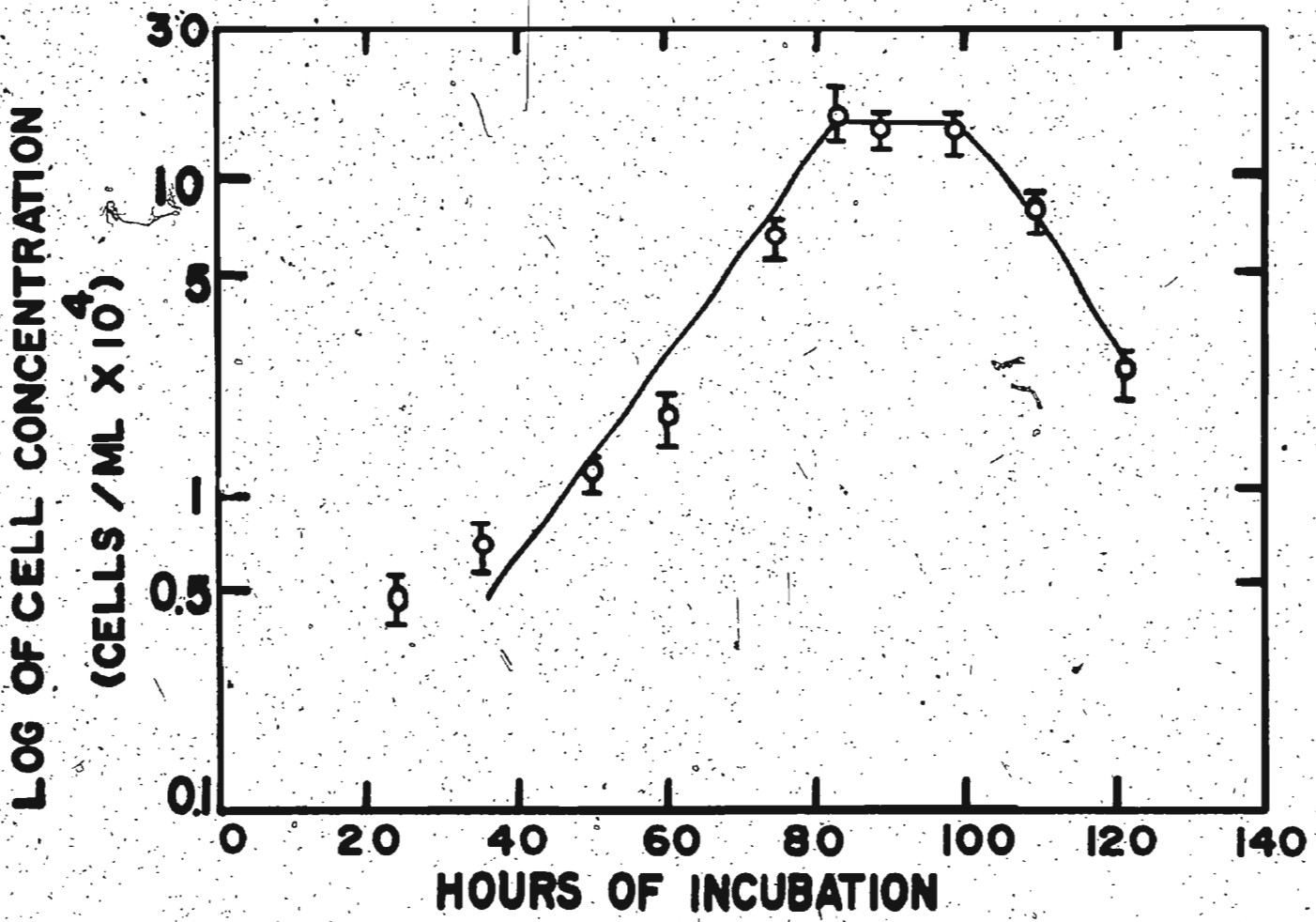
Growth of protoplasts in M3Aa

Incubation time (hours)	Yield (cells/ml)	pH
0.0	0.0 ± 0.0	6.09 ± 0.01 OP_a = 349.0 ± 0.2 mOsm
12.0	0.0 ± 0.0	6.10 ± 0.01
24.0	$5.0 \times 10^3 \pm 5.0 \times 10^2$	6.10 ± 0.01
36.0	$6.88 \times 10^3 \pm 6.6 \times 10^2$	6.10 ± 0.01
48.0	$1.06 \times 10^4 \pm 2.6 \times 10^3$	5.98 ± 0.02
60.0	$2.08 \times 10^4 \pm 4.2 \times 10^3$	6.09 ± 0.01 OP = 348.2 ± 0.1 mOsm
74.5	$6.65 \times 10^4 \pm 5.8 \times 10^3$	6.10 ± 0.01
83.0	$1.65 \times 10^5 \pm 1.2 \times 10^4$	6.10 ± 0.01
89.0	$1.34 \times 10^5 \pm 8.6 \times 10^3$	6.10 ± 0.01 OP = 348.8 ± 0.2 mOsm
99.2	$1.44 \times 10^5 \pm 7.3 \times 10^3$	6.10 ± 0.01
110.1	$8.44 \times 10^4 \pm 5.6 \times 10^3$	6.09 ± 0.01
121.1	$2.10 \times 10^4 \pm 3.2 \times 10^3$	6.09 ± 0.01
140.0	0.0 ± 0.0	6.09 ± 0.01 OP = 349.2 ± 0.3 mOsm

[a] Osmotic pressure in millosmolar

[b] n=15 for all values

Figure 8. Growth curve of protoplasts in medium M3Aa. Inoculum 0.1 ml, 2.3×10^5 cells/ml, 24 hours old.


LOG OF CELL CONCENTRATION
(CELLS / ML X 10⁴)
30
10
5
0.5
0.1
0
20
40
60
80
100
120
140
HOURS OF INCUBATION

M3Aa was expected. By decreasing the amino acid complexity one would anticipate a corresponding increase on the metabolic work load of the protoplasts and consequently a slower rate of growth. Because of the reduced availability of nitrogen a lower maximum yield would result.

The results (Table 22) revealed higher values for the mesoprotoplast and elliptical mesoprotoplast stages than those of medium M13Aa. The morphological types of cells were also present over a longer period of time than their counterparts of medium M13Aa.

The morphological patterns were the same as those of medium M13Aa. The dimensions of the same stage in both types of media were not statistically significant. Possibly the type of medium does not influence either the regeneration pattern or the dimensions of the various stages. Because the osmotic pressure and pH remained constant throughout the growth cycle, they did not appear to influence cell wall regeneration.

3. Effects of protein and serum components on protoplast growth and development.

A. Effects of the absence of serum and presence of serum components on protoplast growth in shaken culture.

The components of most sera include vitamins, free amino acids, lipids, organic acids, and a multitude of different types of proteins (Temim et al., 1972; and

Table 22

Relative per cent abundance of various morphological types of *E. egressa* grown in M3Aa

Incubation time (hours)	Structure	Abundance
24.0	mesoprotoplasts	64.5
36.0	mesoprotoplasts	76.5
	elliptical mesoprotoplasts	12.5
48.0	elliptical mesoprotoplasts	36.2
60.0	elliptical mesoprotoplasts	25.5
74.5	elliptical mesoprotoplasts	11.9
83.0	elliptical mesoprotoplasts	0.9
110.5	elliptical mesoprotoplasts	2.0
	rod hyphal bodies	34.7
	spherical hyphal bodies	15.0

Lehninger, 1975). Removal of serum from either medium M13Aa or M3Aa would produce a wholly defined medium.

In section I.1 the need for fetal calf serum in the standard medium when cultures were shaken was discussed. Removal of the serum from both medium M13Aa and M3Aa, with osmotic pressure compensation using sucrose, did not result in appreciable growth. The yields obtained with medium M13Aa lacking serum, using the same size inoculum as in section V.1, are given in Table 23. The results mirror those obtained in medium M3Aa.

Attempts to grow the protoplasts in medium M13Aa at osmolarities of 300, 330 and 360 mOsM were not successful. In the former two treatments the cells rapidly lysed, whereas, in the latter medium, the cells become deformed and reduced in volume.

Media composed of double concentrations of amino acids, vitamins and organic acids present in medium M13Aa and M3Aa individually and in combination failed to support growth.

A lipid extract of the serum was added to Bellco flasks such that the amount of lipid introduced by fetal calf serum in 50 ml of media was obtained. No growth was obtained in either medium M13Aa or M3Aa containing the extract. Lipid extracts in conjunction with 100% increased concentration of amino acids and/or organic acids and/or vitamins in modified medium M13Aa also did not support growth.

Growth in all media tested was sporadic with lysis occurring frequently. The cells did form a minute fungal

Table 23

Growth of protoplasts in medium Ml3Aa deficient in fetal calf serum

Incubation time (hours)	Cell yield (cells/ml) [a]	pH
0.0	0.0	6.09 ± 0.00
19.5	$4.28 \times 10^3 \pm 1.8 \times 10^3$	6.10 ± 0.00
24.5	$5.55 \times 10^2 \pm 5.5 \times 10^2$	6.10 ± 0.00
31.5	$1.12 \times 10^4 \pm 2.2 \times 10^3$	5.98 ± 0.03
44.0	$1.88 \times 10^3 \pm 1.1 \times 10^3$	6.08 ± 0.00
49.5	0.0	6.10 ± 0.00
54.5	0.0	6.10 ± 0.00
68.0	$3.64 \times 10^3 \pm 1.3 \times 10^2$	6.10 ± 0.00
100.5	$9.17 \times 10^3 \pm 2.9 \times 10^3$	6.10 ± 0.00

[a] n=10 for all values

pellet in all media tested but only after 400 hours of incubation. Melanism was detected by 300 hours, possibly the consequence of phenol oxidases released during cell lysis.

Lysis in 349 mOsM implied a change in membrane integrity and a protective function by the serum. Meister (1965) reported that the presence of serum in mammalian cell cultures was essential for maintaining form. The manipulation of some of the non-proteinaceous components of serum in the present work implicated a protein component favouring protoplast growth under shaken conditions. Maddy (1969) proposed that proteins confer stability and strength to membranes either through the direct interaction at the membrane exterior or indirectly by the binding of sialic acid to the membrane.

B. Effects of the presence and absence of fetal calf serum on protoplasts grown in medium 13Aa in stationary culture.

Using medium M13Aa with and without fetal calf serum at 349 mOsM osmotic pressure, growth rates of the protoplasts in stationary cultures were compared.

The results (Table 24) reveal exponential growth for cells in both types of media. The 95% confidence intervals revealed no significant differences among the treatment means for a given time interval. The population doubling times of medium M13Aa with fetal calf serum and medium M13Aa without the serum were 7.5 hours and 7.6 hours, respectively.

Table 24

Growth of protoplasts in medium M13Aa with and without fetal calf serum

Incubation time (hours)	Yield (cells/ml)[a]	
	Fetal calf serum	No fetal calf serum
0	0.0	0.0
24	$1.17 \times 10^4 \pm 2.5 \times 10^3$	$1.23 \times 10^4 \pm 2.6 \times 10^3$
48	$3.64 \times 10^5 \pm 2.1 \times 10^4$	$3.72 \times 10^5 \pm 2.2 \times 10^3$
72	$1.01 \times 10^6 \pm 5.9 \times 10^2$	$9.89 \times 10^5 \pm 4.6 \times 10^2$

[a] n=15 for all values

The generation times were not significantly different.

It was, thus, apparent that fetal calf serum did not confer a nutritional benefit to the media used since growth readily occurred in the absence of serum. Serial transferring of the culture in the medium lacking serum for over 20 transfers was additional evidence that the protoplasts did not require serum for growth in stationary cultures.

C. Bovine albumin as a protein source for protoplasts under shaken conditions.

Albumin has been reported to be the major protein in many types of sera (Temin et al., 1972; and Lehninger, 1975). The bovine albumin (Cohn, Fraction V) was reported to be 96.99% albumin, the remaining 3.01% being globulins (Sigma Chemical Company, see Materials and Methods).

The addition of albumin to medium M13Aa lacking serum to give an osmotic pressure of 349 mOsM failed to support growth. The cells revealed extensive shrinking. Using a concentration series of albumin ranging from 2 gm to 10 gms of albumin per liter of medium, in 2 gm increments, growth was detected by visual inspection for increased turbidity in the medium corresponding to 4 gms of albumin per liter. The osmotic pressure at which growth occurred was 334 mOsM. This level of albumin was used for physiological studies.

The results (Table 25 and Figure 9) showed initial growth by 14 hours, the cells achieving a maximum concentration of 2.64×10^5 cells/ml by 57.5 hours. The doubling time was

Table 25

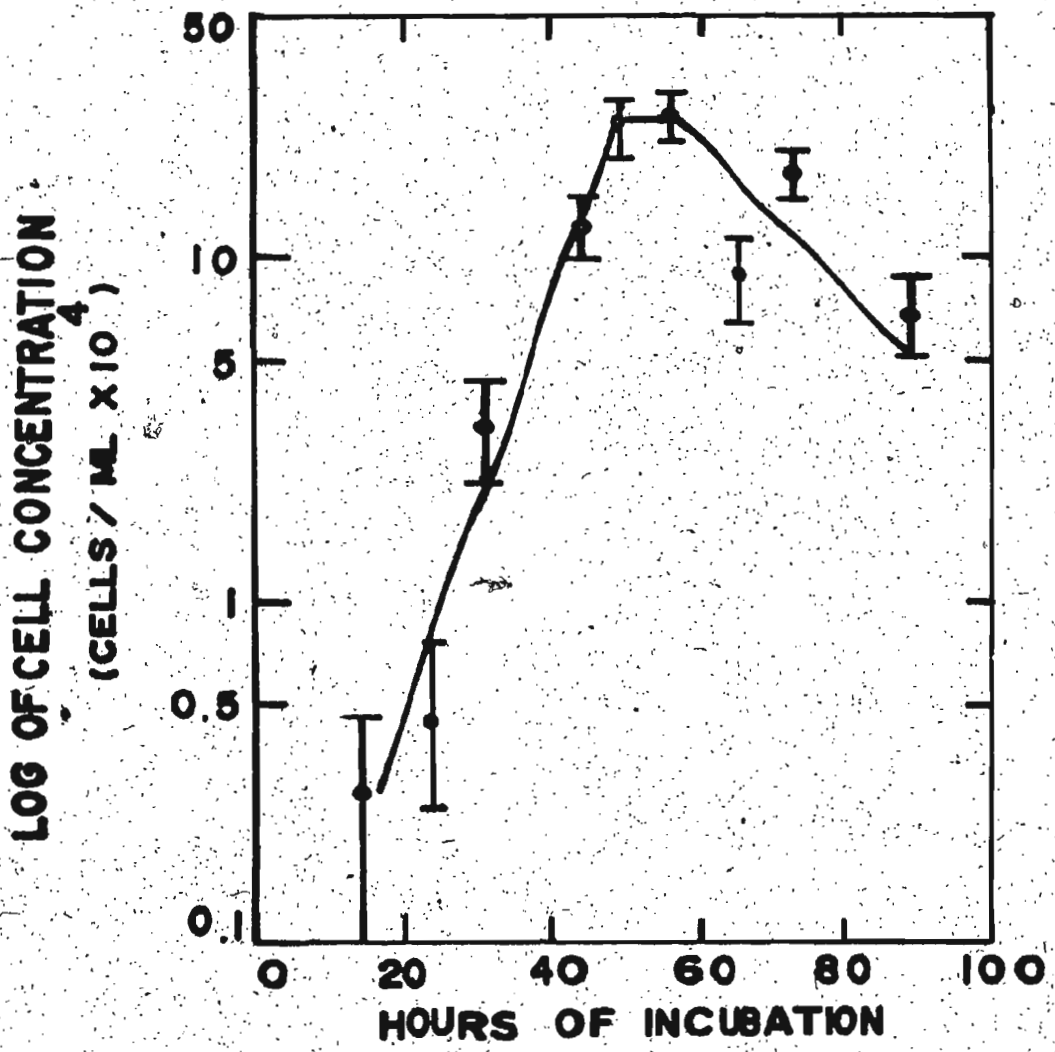
Growth of protoplasts in medium M13Aa with bovine albumin

Incubation time (hours)	Yield (cells/ml)[b]	pH
0.0	0.0 ± 0.0	5.98 ± 0.07 OP_a = 334.5 ± 0.5 $mOsm_a$
14.0	$2.78 \times 10^3 \pm 1.2 \times 10^3$	6.09 ± 0.00
24.5	$4.38 \times 10^3 \pm 1.6 \times 10^3$	6.10 ± 0.01
31.5	$3.25 \times 10^4 \pm 5.2 \times 10^3$	6.01 ± 0.01
44.0	$1.24 \times 10^5 \pm 1.0 \times 10^4$	6.10 ± 0.01 OP = 334.0 ± 0.7 mOsm
49.5	$2.48 \times 10^5 \pm 2.0 \times 10^4$	6.00 ± 0.00
57.5	$2.64 \times 10^5 \pm 1.8 \times 10^4$	5.98 ± 0.06 OP = 336.7 ± 0.4 mOsm
66.0	$8.88 \times 10^4 \pm 1.1 \times 10^4$	5.99 ± 0.03
73.0	$1.79 \times 10^5 \pm 1.3 \times 10^4$	6.07 ± 0.10
89.0	$6.78 \times 10^4 \pm 8.7 \times 10^3$	6.09 ± 0.02 OP = 336.2 ± 0.2 mOsm

[a] OP osmotic pressure in milliosmolar

[b] n=10 for all values

Figure 9. Growth curve of protoplasts in medium M13Aa with bovine albumin. Inoculum 0.1 ml, 2.3×10^5 cells/ml, 24 hours old.


LOG OF CELL CONCENTRATION
(CELLS / ML X 10⁴)
50
10
5
1
0.5
0.1
0
20
40
60
80
100
HOURS OF INCUBATION

6.6 hours. This pattern was comparable to that obtained from growing protoplasts in medium M13Aa with fetal calf serum. Since the inoculum and incubation conditions were the same as those used in all the analysis of medium M13Aa with serum (section V) statistical comparisons were valid. Statistical analysis revealed the cell yield at a given time and the generation time for medium M13Aa plus albumin was significantly different from medium M13Aa plus serum. The former medium supported a lower yield and slower growth rate.

The morphological developmental patterns (Table 26) were comparable to those in the serum based medium M13Aa. In terms of relative abundance only the hyphal bodies at 66 hours growth were greater than those of medium M13Aa plus serum. All other stages were generally less abundant.

The singificantly poorer results in terms of growth rate and morphology may represent the effects of the lower osmotic pressure and/or the albumin protein. The lower osmotic pressure required was possibly indicative of a form of membrane-albumin interaction.

4. The effects of vitamins on protoplasts.

A. Growth in medium M13Aa without vitamins.

Protoplasts in medium M13Aa without deliberately added vitamins revealed maximum growth by 44 hours (Table 27 and Figure 10) with a generation time of 8.1 hours. The doubling time was significantly greater than the generation

Table 26

Per cent abundance of various morphological types of *E. egressa* in medium M13Aa plus bovine albumin

Incubation time (hours)	Structure	Abundance
14.0	mesoprotoplasts	3.6
24.5	mesoprotoplasts	28.9
31.5	mesoprotoplasts	9.8
	elliptical mesoprotoplasts	15.4
43.0	elliptical mesoprotoplasts	35.8
49.5	elliptical mesoprotoplasts	0.0
57.5	rod-shaped hyphal bodies	7.8
66.0	rod-shaped hyphal bodies	34.4
	spherical hyphal bodies	24.1
73.0	all structures	0.0

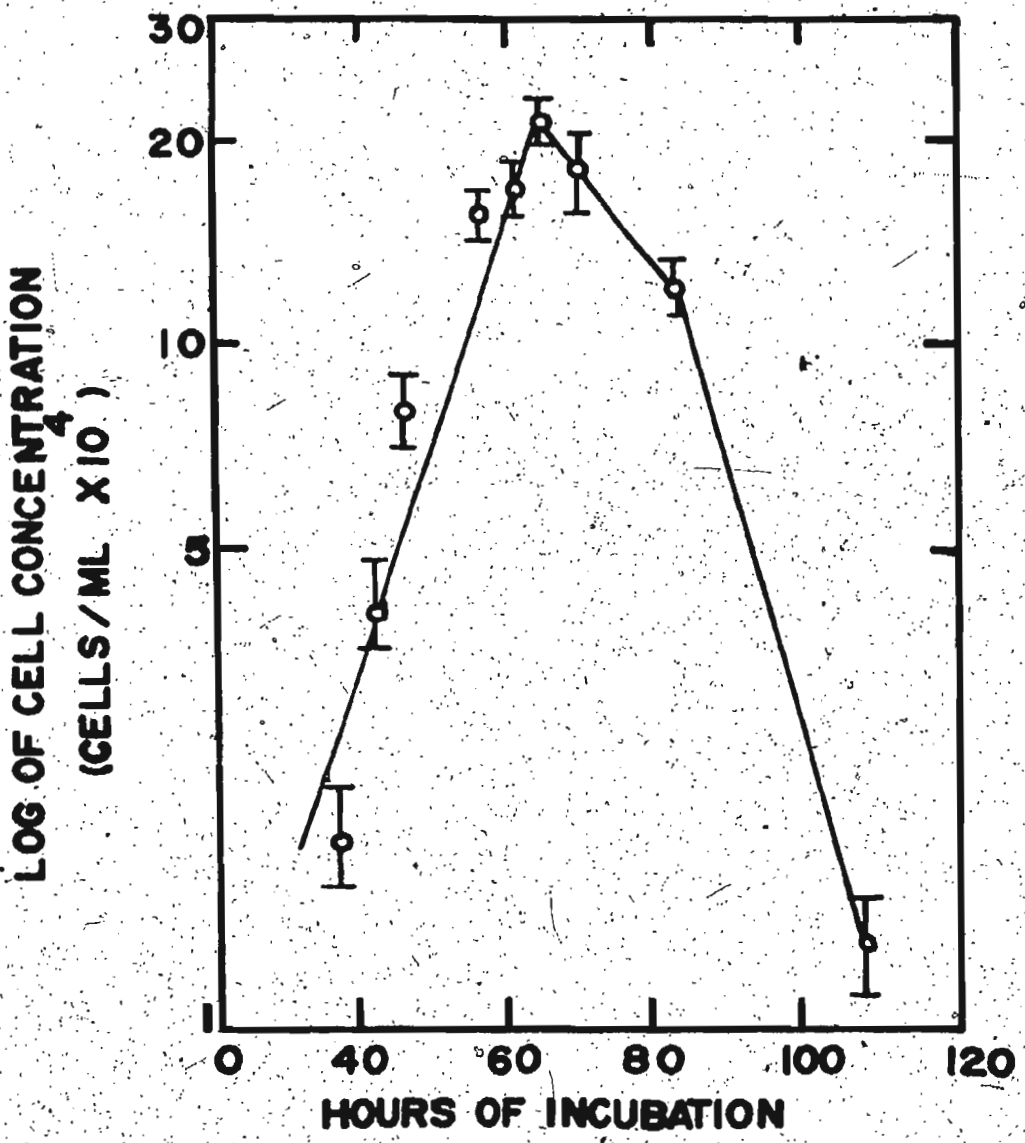
Table 27

Growth of protoplasts in medium M13Aa minus vitamins

Incubation time (hours)	Yield (cells/ml)[a]	pH
0	0.0	6.10 ± 0.00
19.5	$1.86 \times 10^4 \pm 3.6 \times 10^3$	6.10 ± 0.00
24.5	$4.00 \times 10^4 \pm 4.5 \times 10^3$	6.10 ± 0.00
31.5	$7.88 \times 10^4 \pm 5.7 \times 10^3$	6.10 ± 0.00
44.0	$1.51 \times 10^5 \pm 8.5 \times 10^3$	6.10 ± 0.00
49.5	$1.65 \times 10^5 \pm 9.3 \times 10^3$	6.10 ± 0.00
54.5	$2.08 \times 10^5 \pm 1.0 \times 10^4$	5.98 ± 0.05
59.5	$1.73 \times 10^5 \pm 1.1 \times 10^4$	6.10 ± 0.00
73.0	$1.19 \times 10^5 \pm 6.9 \times 10^3$	6.10 ± 0.00
100.5	$1.33 \times 10^4 \pm 6.0 \times 10^3$	6.10 ± 0.00

[a] n=15 for all values

Figure 10. Growth curve of protoplasts in medium M13Aa without vitamins. Inoculum 0.1 ml, 2.3×10^5 cells/ml, 24 hours old.


30
20
10
5
1
0
40
60
80
100
120
HOURS OF INCUBATION
LOG OF CELL CONCENTRATION
(CELLS/ML X10⁴)

time of medium M13Aa with vitamins. By 59.5 hours cellular aggregation started and mycelial ball formation occurred by 140 hours.

Vitamin auxoautotrophy has been reported for numerous members of the Entomophthorales. Wolf (1951) and Smith (1953) reported vitamin autotrophy for E. coronata grown in semi-synthetic and synthetic media. Latgé (1975b) reported that B. ranarum, C. osmodes, E. destruens, E. virulenta and E. near thaxteriana were vitamin autotrophic. In the present experiment, because the generation time in the "vitamin-free" medium was greater than that in the vitamin containing medium M13Aa, the protoplasts were believed to exhibit a partial vitamin autotrophy.

Using stationary cultures of medium M13Aa without added vitamins or fetal calf serum, it was possible to successfully subculture the protoplasts every 2 days for over 35 transfers representing thousands of generations and a dilution factor of 1.5×10^{70} for possible serum and vitamin carryover. The growth rate was not determined, but because of the duration of subculturing, it is proposed that vitamins and fetal calf serum were not essential for protoplasts in stationary cultures. The presence of Kreb's cycle acids and/or amino acids have been reported to by-pass vitamin requirements of numerous higher fungi (Fries, 1965). The effects of the tricarboxylic acid cycle intermediates in medium M13Aa were not determined.

B. Growth in medium M3Aa without vitamins

The pattern reported in the previous section was consistent with the present data (Table 28 and Figure 11). Interestingly the generation time for protoplasts in medium M3Aa plus vitamins (13.5 hours) was significantly greater than that in M3Aa without vitamins (6.9 hours). The result may have represented a vitamin growth inhibiting effect. Fries (1965) reported growth inhibition of Rhizopus species by thiamine.

The protoplasts were subcultured in stationary cultures in medium M3Aa without vitamin or fetal calf serum in the same fasion as in section V.4A. The cells were shown to be capable of growth in a vitamin free and serum free medium.

5. Effects of increasing glutamine concentration

The decrease in maximum cell yield obtained as the medium M13Aa was simplified to medium M3Aa may have reflected, in part, a reduced amount of nitrogen. Because the level of glutamine in medium M3Aa was 8.2 mM, a series of glutamine-nitrogen levels above and below this value (Table 29) was used to provide evidence for glutamine utilization.

The results, after 48 hours incubation, revealed increasing protoplast yield with increased glutamine-nitrogen levels (Table 29). Although the 4 mM and 8.2 mM concentration of nitrogen gave statistically comparable yields, they were significantly lower than the yields of the comparable

Table 28

Growth of protoplasts in medium M3Aa minus vitamins

Incubation time (hours)	Yield (cells/ml)[a]	pH
21.0	$7.14 \times 10^3 \pm 7.1 \times 10^2$	6.15 ± 0.00
32.5	$2.36 \times 10^4 \pm 4.1 \times 10^3$	6.15 ± 0.00
46.0	$9.20 \times 10^4 \pm 7.8 \times 10^3$	6.15 ± 0.01
59.5	$4.00 \times 10^5 \pm 1.1 \times 10^4$	6.15 ± 0.03
72.5	$5.34 \times 10^5 \pm 1.8 \times 10^4$	6.15 ± 0.01
84.0	$1.62 \times 10^4 \pm 3.5 \times 10^3$	6.15 ± 0.00
87.5	$3.33 \times 10^4 \pm 1.7 \times 10^2$	6.15 ± 0.00

[a] n=12 for all values

Figure 11. Growth curve of protoplasts in medium M3Aa without vitamins. Inoculum 0.1 ml, 2.3×10^5 cells/ml, 24 hours old.

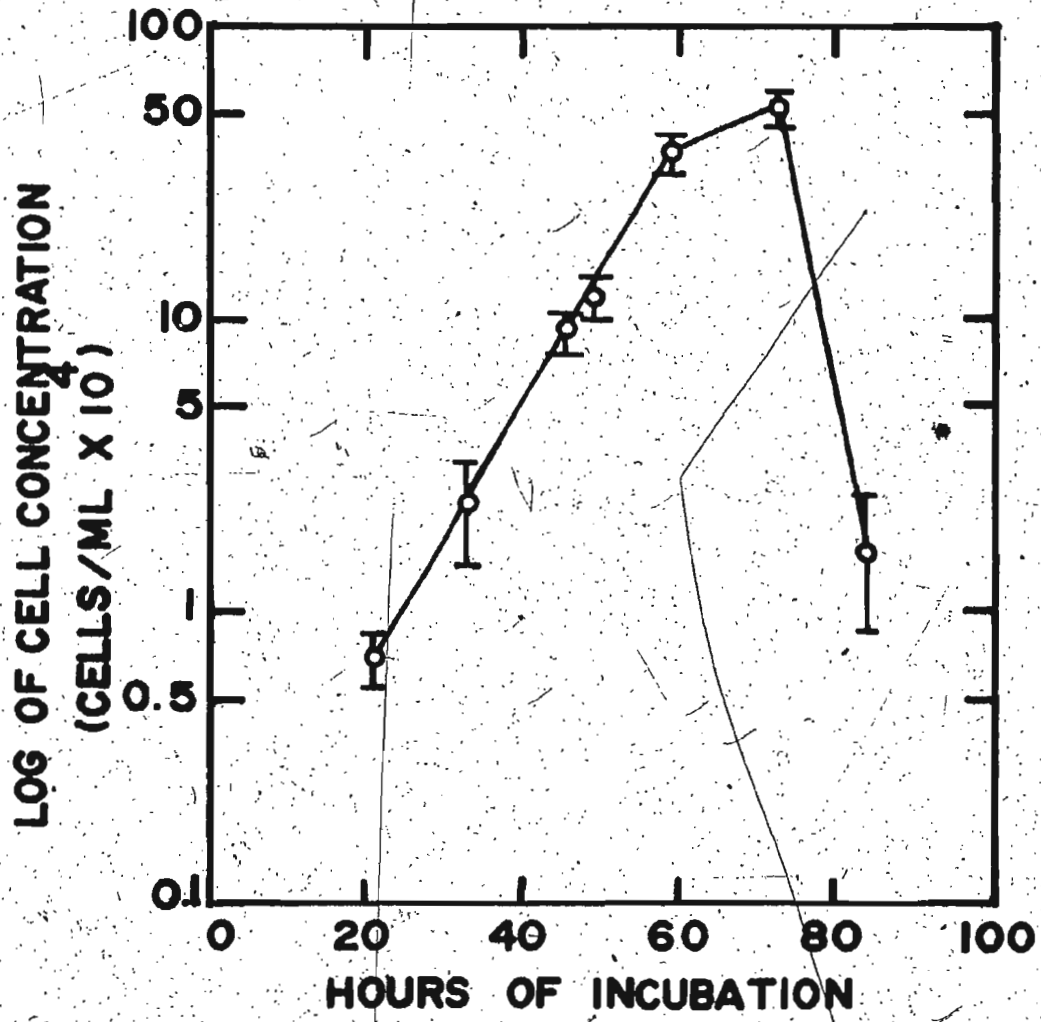

100
50
10
5
1
0.5
0.1
LOG OF CELL CONCENTRATION
(CELLS/ML X 10⁴)
0
20
40
60
80
100
HOURS OF INCUBATION

Table 29

The effects of glutamine concentration on cell yield and protoplast number per chain

Nitrogen level (mm)	Yield (cells/ml)	pH	Protoplasts per chain
4	$2.85 \times 10^5 \pm 1.7 \times 10^4$	6.10 ± 0.00	1.56 ± 0.26
8.2	$2.96 \times 10^5 \pm 1.7 \times 10^4$	6.10 ± 0.01	2.05 ± 0.31
16.0	$4.04 \times 10^5 \pm 2.0 \times 10^4$	6.10 ± 0.01	2.83 ± 0.40
20.0	$3.72 \times 10^4 \pm 1.9 \times 10^4$	6.10 ± 0.01	2.10 ± 0.32

[a] n=15 for all values

pair at 16 and 20 mM nitrogen concentration. The pH remained constant in all test media.

Counts of the number of cells per protoplast chain revealed that the level of glutamine in media of the same osmotic pressure strongly influenced the chain cell number (Table 29). The number of cells per chain increased significantly as the glutamine concentration increased achieving maximum length at 8.0 mM glutamine (16.0 mM nitrogen). Thereafter, the count decreased significantly.

Thus, in keeping with the initial objective to simplify Grace's modified insect tissue culture medium and sustain cell growth, the glutamine level was raised to 8.0 mM (Table 30).

VI. Further experiments with the medium based on the hemlock looper haemolymph analysis.

1. Rate of growth of protoplasts

Because it was desirable to compare the effects of the medium based on haemolymph analysis with those of the standard medium for shaking cultures 28 ml of fetal calf serum was added to the former medium.

Growth occurred as early as 12 hours (Table 31 and Figure 12). With a population doubling time of 4.4 hours a maximum yield of 1.43×10^6 protoplasts/ml was obtained by 64 hours. The decline phase represented cellular aggregation. The osmotic pressure and pH checked at 0, 36, 64, and 77 hours were found to remain constant at 349 mOsM and 6.0, respectively.

Table 30

Final simplified growth medium for protoplast cells grown under shaking conditions[a]

Compound	Concentration[b]	Compound	Concentration
$NaH_2PO_4.H_2O$	1013.0	Sucrose	53433.0
Kcl	2240.0	Fructose	400.0
$CaCl_2$	1000.0	Glucose	700.0
$MgCl_2.6H_2O$	2280.0	Malic acid	670.0
$MgSO_4.7H_2O$	2780.0	α-Ketoglutaric acid	370.0
$NaHCO_3$	350.0	D-succinic acid	60.0
L-Glutamine	1169.2	Fumaric acid	56.0
L-Asparagine	350.0	MES	1952.0
L-Methionine	50.0	Fetal calf serum	28.0[c]

[a] inoculum of 0.5 ml standard medium, 2.7×10^5 cells/ml

[b] in mgm/L

[c] ml added to one liter of medium

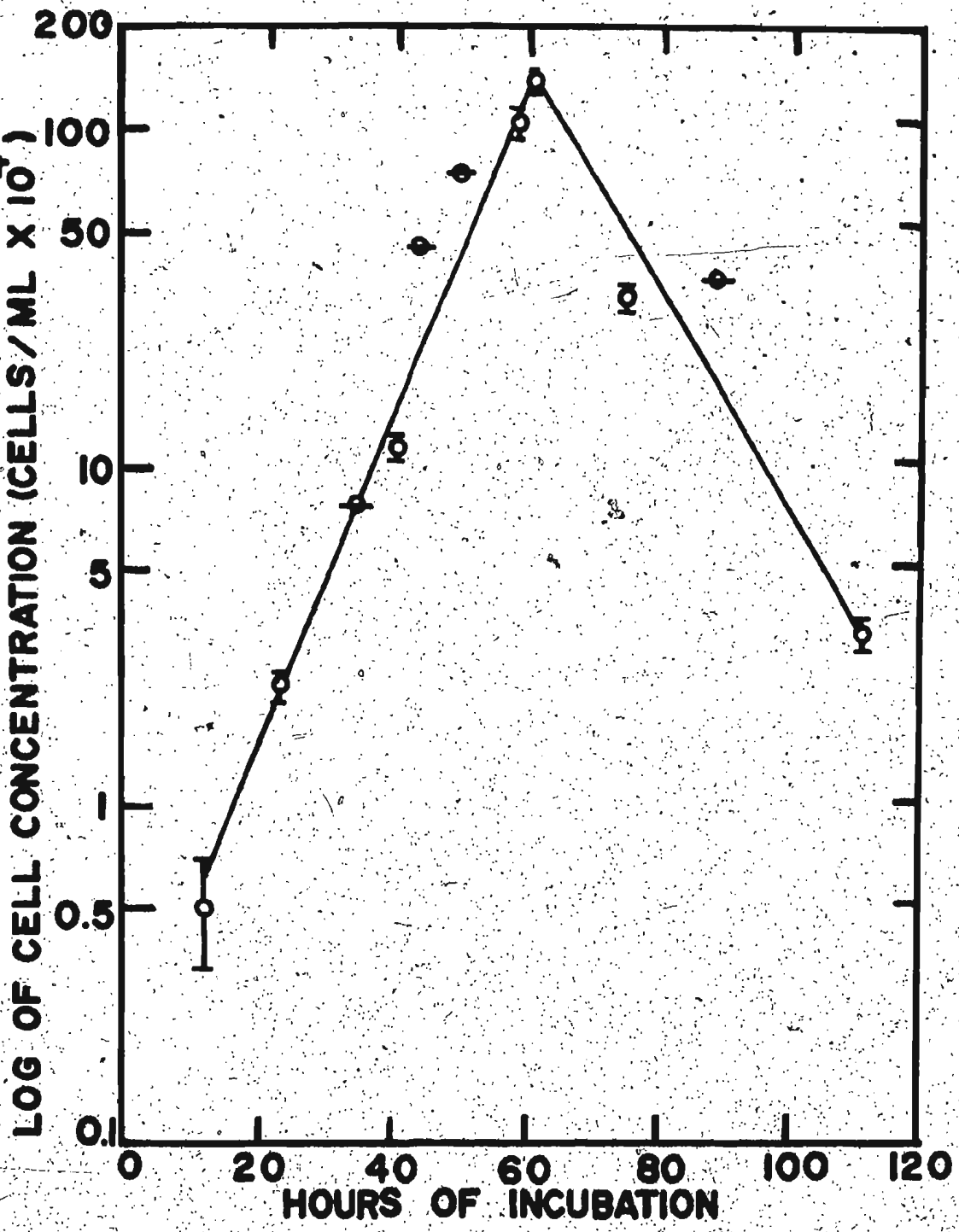
Table 31

Growth of protoplasts in the medium based on looper haemolymph analysis with fetal calf serum

Incubation time (hours)	Yield (cells/ml)[a]	pH
0.0	0.0 ± 0.0	6.10 ± 0.00
12.0	$5.00 \times 10^3 \pm 1.7 \times 10^3$	6.10 ± 0.00
24.0	$2.32 \times 10^4 \pm 3.2 \times 10^3$	6.10 ± 0.00
36.0	$7.76 \times 10^4 \pm 6.0 \times 10^3$	6.10 ± 0.00
42.0	$1.14 \times 10^5 \pm 8.0 \times 10^3$	6.10 ± 0.00
46.0	$4.87 \times 10^5 \pm 1.4 \times 10^4$	6.10 ± 0.00
52.0	$7.21 \times 10^5 \pm 1.9 \times 10^4$	5.97 ± 0.03
61.0	$1.08 \times 10^6 \pm 5.4 \times 10^4$	6.10 ± 0.00
64.0	$1.43 \times 10^6 \pm 3.1 \times 10^4$	6.09 ± 0.00
71.5	$7.44 \times 10^5 \pm 4.6 \times 10^4$	6.09 ± 0.00
77.0	$3.23 \times 10^5 \pm 1.5 \times 10^4$	5.98 ± 0.03
85.25	$3.72 \times 10^5 \pm 1.30 \times 10^4$	6.09 ± 0.00
112.75	$3.28 \times 10^4 \pm 4.84 \times 10^3$	6.09 ± 0.00

[a] n=10 for all values

Figure 12. Growth curve of protoplasts in the medium based on haemolymph analysis with fetal calf serum. Inoculum 0.1 ml, 2.3×10^5 cells/ml, 24 hours old.


200
100
50
10
5
1
0.5
0.1
LOG OF CELL CONCENTRATION (CELLS/ML X 10^4)
0
20
40
60
80
100
120
HOURS OF INCUBATION

The superiority of the medium based on haemolymph analysis has been previously documented (section II.2) when the medium lacked fetal calf serum. The present medium with the serum supported greater cell yields than the standard medium or either of the simplified media (M13Aa or M3Aa). The generation time was significantly less than most of the other types of media tested and comparable to that of medium M13Aa.

The increased values obtained in this medium may have stemmed from two sources acting alone or in concerted fashion. The medium, because of its closer similarity to the larval haemolymph, may have supplied more necessary amino acids and their derivatives than did the standard medium. The higher concentrations of amino acids and, therefore, nitrogen levels may also have favoured the increased growth rates and cell yield.

2. Morphological development of protoplasts

The morphological development of the cells in the medium bore similarities to media M13Aa and M3Aa but with interesting differences (Table 32).

At all times the relative abundance of the mesoprotoplasts, elliptical mesoprotoplasts, and hyphal bodies were greater than those of medium M13Aa and M3Aa. The stages also occurred sooner than in the two simplified media. Development in the pres ent medium was characterized by a low frequency of fusion spheres from 46 to 52 hours. The greatest

Table 32

Relative per cent abundance of various morphological types of *E. egressa* in the medium based on looper haemolymph analysis after varying periods of incubation

Incubation time (hours)	Structure	Abundance
12	mesoprotoplasts	63.5
24	mesoprotoplasts	86.2
	elliptical mesoprotoplasts	12.0
36	mesoprotoplasts	26.5
	elliptical mesoprotoplasts	28.8
42	mesoprotoplasts	0.5
	elliptical mesoprotoplasts	14.2
46	elliptical mesoprotoplasts	12.8
52	elliptical mesoprotoplasts	31.1
61	mesoprotoplasts	4.6
	elliptical mesoprotoplasts	7.0
	rod-shaped hyphal bodies	9.0
64	elliptical mesoprotoplasts	10.9
	rod-shaped hyphal bodies	9.0
71.5	rod-shaped mesoprotoplasts	9.0
	spherical hyphal bodies	60.0

occurrence of regular protoplasts occurred from 46 to 64 hours.

The dimensions of rod-shaped hyphal bodies (22.3 µm, s=1.5 µm x 1.4 µm, s=1.1 µm) and spherical hyphal bodies (24.3 µm, s=4.4 µm) were found to be significantly greater than those of medium M13Aa. The abundance of the rod-shaped hyphal bodies remained essentially constant over a 61 to 71.5 hour period; whereas, the level of the spherical hyphal bodies increased to 60% by 71.5 hours. By 112 hours the spherical hyphal bodies had a thick cell wall (6.1 µm, s=0.9 µm).

In contrast to development in the other media tested, conidia were detected by 245 hours. Based on cursory examination of selected samples mycelia on the surface had more conidia than those in submerged culture. The conidia were observed in different stages of development from conidial initials to the mature globose form with a central oil globule (43.2 µm, s=5.8 µm x 34.0 µm, s=8.5 µm). The more mature conidium was found to be statistically and morphologically comparable to the description by Otvos et al. (1973) of conidia isolated from the larval stages of E. fiscellaria fiscellaria. The conidia often gave rise to primary, secondary, tertiary and quatenary conidia. A septum was usually produced across the germ tube. Behind each septum the tube was devoid of cytoplasm whereas in front of the cross wall granular cytoplasm was observed flowing into the new conidium. Often a conidium gave rise to another conidium by budding, the bud usually forming near the apex of the columella. The

pH of the medium by this time was 7.4.

The formation of conidia and their resemblance to those isolated from the host was considered as further support for the superiority of this medium relative to the standard medium and simplified media.

The failure of the conidia to initiate vegetative growth was probably due to staling effects which resulted from the growth prior to conidia formation. The fungal balls had ceased spherical growth and were forming sheets of mycelia as the balls "broke down". The stratification of conidia formation implied a possible oxygen requirement, the amount of dissolved oxygen in the medium being less, under the present culture conditions, the further from the medium's surface. Krejzova (1970) reported better conidia production by E. virulenta in flasks that were aerated. The effects of aeration are not only the introduction of oxygen, but also the reduction of carbon dioxide and/or reduction in the accumulation of free ammonia (Hawker, 1966) and a better circulation of media components. Media rich in amino acids and proteins have been reported to favour conidia production (Krejzova, 1970; Latgé, 1975b). Hawker (1966) reported that the hexoses glucose and fructose strongly favour conidia formation.

Hutchison (1962) reported spherical hyphal bodies of Entomophthora kansana Hutchison in the haemocoel of calyptrate flies. These hyphal bodies were observed giving rise to conidiophores.

3. Changes in the concentration of ninhydrin-positive compounds throughout the growth curve.

Throughout the growth curve the concentrations of glycine, alanine, ammonia, ornithine and B-alanine increased continuously. After 112 hours of incubation the levels of alanine, β-alanine and γ-aminobutyric acid decreased (Table 33).

By 12 hours the concentrations of phosphoethanolamine, taurine, threonine and/or an unknown believed to be methionine sulfoxide, glutamine, asparagine, glutamic acid, valine, methionine, isoleucine, ornithine, lysine and/or a unknown decreased in concentration (Table 33). Thereafter, the levels of threonine and/or an unknown, asparagine, glutamic acid, valine, methionine, isoleucine, ornithine, lysine and/or an unknown increased in concentration while taurine decreased. Taurine utilization peaked at the time of maximum mesoprotoplast level, 36 hours. Phosphoethanolamine decreased continuously throughout the growth curve.

During the regular protoplast stage those ninhydrin-positive compounds which previously decreased in concentration increased in concentration (Table 33). The individual concentrations usually achieved a maximum level by either 46 or 64 hours after which significant decreases occurred.

Sarcosine was maximally released into the medium until 46 hours incubation. From 64 to 77 hours, the amino acid was utilized and after 77 hours was released into the medium. Proline was utilized until 77 hours after which it was also

Table 33

Per cent change in concentration of ninhydrin-positive compounds of the medium based on looper haemolymph analysis[a]

Compound	Incubation time (hours)							
	12	36	46	64	77	85	112	116
Phosphoethanolamine	-17.1[b]	-19.5	-16.4	-21.8	-23.2	-18.8	-84.2	-100.0
Taurine	-23.2[b]	-41.1	-10.7	- 7.1	- 7.1	17.8	17.8	28.6
L-Aspartic acid	5.8[c]	4.1	-19.0	-10.1	-26.4	141.3	495.0	6.6
L-Threonine + unknown	- 2.2	2.1	15.5	11.2	8.4	17.3	7.6	22.1
L-Serine + unknown	0.9	3.8	21.9	15.7	13.4	24.1	19.5	2.7
L-Glutamine	- 5.5	-15.0	27.4	- 2.3	-14.8	38.2	37.2	21.8
L-Asparagine	- 1.4	- 0.5	6.7	6.7	- 1.2	20.2	19.3	25.2
Sarcosine	0.0	0.0	185.5	-44.0	-58.5	-23.0	- 6.5	- 14.0
L-Proline	1.4	- 7.1	-33.1	-80.3	-83.1	-64.7	-44.4	- 47.9
L-Glutamic acid	- 0.1	6.2	30.9	77.2	47.7	57.0	15.9	- 50.0
Glycine	7.6	14.5	36.74	45.8	36.3	49.7	42.8	70.9
L-Alanine	1.7	12.5	37.2	57.4	74.1	60.9	61.3	19.8
L-Valine	- 2.4	7.1	20.1	12.7	14.8	20.8	7.7	- 2.7
L-Methionine	- 2.5	6.5	17.0	-27.8	-42.2	-67.7	-77.0	107.3
L-Isoleucine	- 2.1	7.9	21.7	17.1	10.6	-20.2	-30.5	- 47.5
L-Leucine	2.6	4.2	22.0	20.0	1.4	-20.8	-54.0	- 47.4
L-Tyrosine	3.3	2.7	26.6	22.1	19.1	3.9	-34.7	- 99.0
B-Alanine	0	0	86.3	146.6	121.5	57.4	69.6	- 11.1
γ-Aminobutyrate	3.4	7.3	25.1	24.0	5.0	59.2	62.5	34.6
Ornithine	- 1.9	1.0	24.8	12.0	25.6	54.0	70.2	132.1
Ammonia	74.4	217.4	153.6	255.6	564.4	340.9	353.5	319.6
L-Lysine + unknown	- 0.7	1.4	25.4	22.2	19.8	18.6	10.9	4.8
L-Histidine	0.8	0.7	20.5	19.9	13.1	63.6	80.9	79.3
L-Arginine	5.4	4.5	26.8	16.7	16.8	16.9	2.70	6.6
L-Phenylalanine	1.5	2.6	21.3	-19.2	-47.4	-82.4	-94.4	- 78.1

[a] based on the change in concentration of means of all values relative to the initial value

[b] per cent decrease

[c] per cent increase

realeased into the medium. Phenylalanine levels decreased continuously after 64 hours while tyrosine decreased after 112 hours. The reduced levels in phenylalanine and later tyrosine may account for the melanism in the cells detected by 85 hours and later in the medium by 112 hours.

During the cellular aggregation, hyphal body and cell wall formation stages of 77 to 85 hours the concentrations of methionine, leucine, isoleucine, and phenylalanine decreased.

By 116 hours most of the amino acids and their derivatives revealed pronounced decreases in concentration, the compounds exempt from this being those previously stated to continuously increase in level. The marked reductions occurred when the mycelial stage was fully developed. Patterson (1965) reported changes in amino acid concentrations in cell culture media reflecting differences due to the proliferation phase. In view of this the ninhydrin-positive compounds analysis results of the growth of protoplasts in Grace's medium and the present medium were not rigorously discussed due to pronounced differences in morphological development.

From both studies it was apparent that, depending on the physiological stage, glutamine, asparagine, proline, leucine, isoleucine, phenylalanine and tyrosine were utilized by E. egressa. The extent of amino acid useage also depended on the type of amino acids present (Meister, 1965) and the concentration of these amino acids (Cochrane, 1958).

Diamines and polyamines were not detected in either the present medium or in the Grace's medium substantiating the belief that no decarboxylases were released and no cell lysis occurred.

The increased level of γ-aminobutyric acid may have been the result of degradation of arginine, decarboxylation of glutamic acid and/or the oxidation of succinic acid (Bender, 1975). The continuous utilization of phosphoethanolamine was probably the consequence of incorporation of the lipid into the cell membranes.

V. Total lipid and fatty acid analysis of E. egressa.

1. Lipid production by E. egressa

The total lipid analysis (Table 34 and Figure 13) of cells grown in medium M3Aa without vitamins (section V.4.B) revealed a significant increase in lipid production during the exponential growth phase. Lipid production was maximum at 72.5 hours, the time of maximum cell yield (Figure 11). Thereafter the lipid content decreased substantially.

This general pattern of lipid turnover has been reported for two other Zygomycetes, Phycomyces blakesleeanus and R. arrhizus (Weete, 1974). Weete et al. (1973) postulated that the decrease in lipid may be associated with metabolic alterations which occur during the transition from vegetative to reproductive growth or to a depletion of certain nutrients in the medium. The decrease reported in the present experiment might reflect lipid utilization for carbon and energy

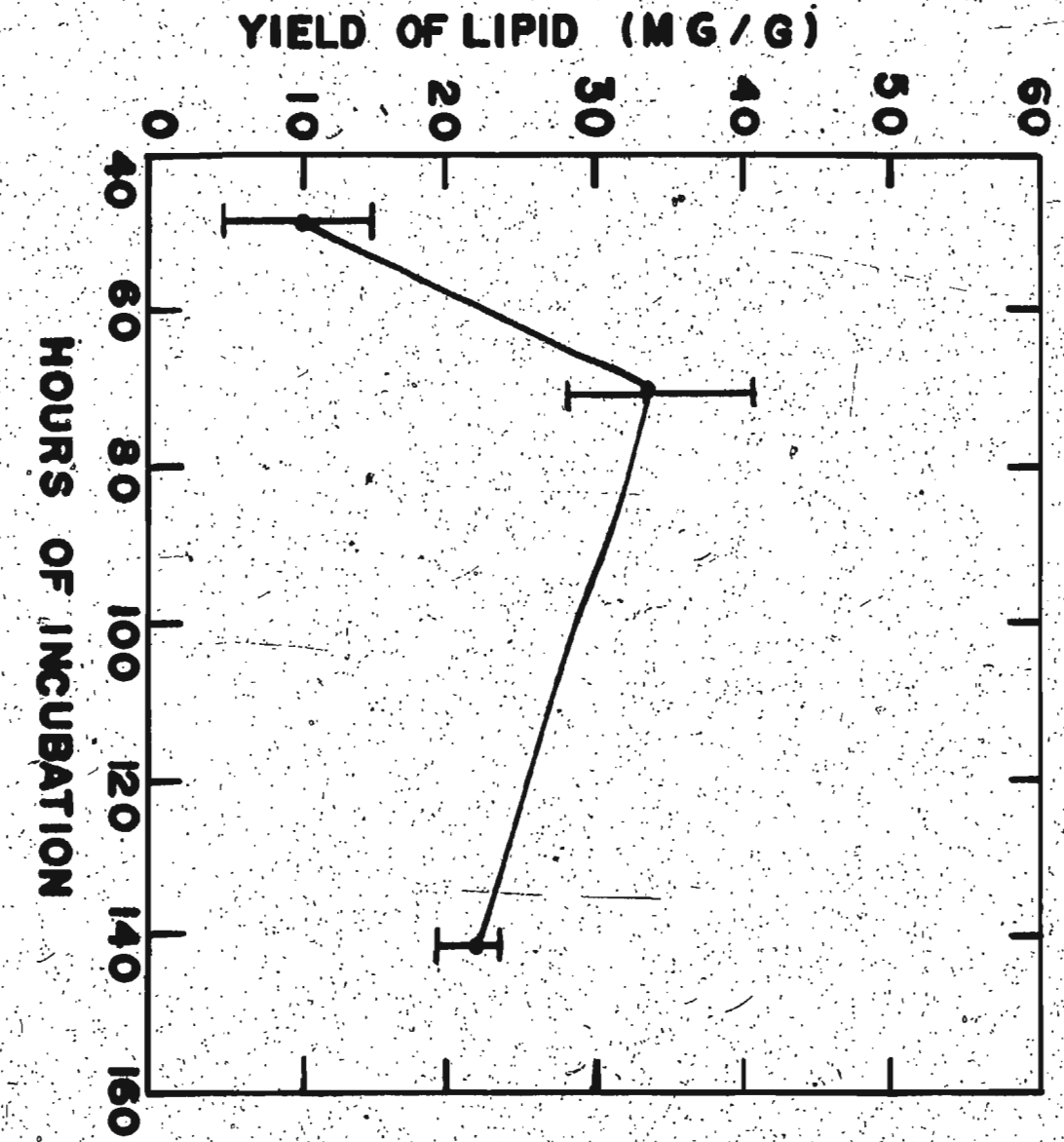
Table 34

Production of lipid by E. egressa during six days growth in vitamin free medium M3Aa[a]

Hours of incubation	Lipid yield (mgm/gm)
49.5	10.9 ± 1.3
72.5	35.3 ± 1.3
142.0	22.5 ± 0.6

[a] based on three replicates

Figure 13. Lipid production by E. egressa in medium M3Aa without vitamins.


YIELD OF LIPID (MG/G)
0
10
20
30
40
50
60
HOURS OF INCUBATION
40
60
80
100
120
140
160

during cellular aggregation and extensive cell wall synthesis. Singh and Walker (see Weete et al., 1973) reported a decrease in lipid levels presumably for energy and to provide substrates for polysaccharide synthesis during stalk dormation of Dictyostelium discoideum Agg.

2. Fatty acid analysis of E. egressa

The results (Table 35) indicated that the major fatty acids were C14:0 and γ-C18:3 at 30.7% and 24.1% respectively. Trace amounts of C8:0, C10:0, C15:1, C20:3, C22:1 and C24:0 were also detected. The percentage of short chain fatty acids (less than C16:0) was 35.8%.

Tyrrell (1967) reported the presence of C20:4 and high levels of C14:0 for 17 isolates of Entomophthora to be a characteristic feature. All Entomophthora isolates tested had low levels of C18:3. The present result of a high level of C18:3 appears contradictory to Tyrrell's results. The present results did not include C13:0, C14:1, C16:2 reported by Tyrrell (1967). However, the author obtained fatty acids of the C22:0, C22:1 and C24:0 levels. Tyrrell (1967) reported fatty acids in excess of the C20 series ranging from 0% to 24.4%.

Because of the differences in growth media, harvest time and the non-walled stage of E. egressa in terms of Tyrrell's work (1967) the quantitative values of the fatty acids were not rigidly compared and the taxonomic grouping proposed by Tyrrell (1967) was not applicable.

Table 35

Total fatty acid analysis of *E. egressa* after three days growth in vitamin free medium M3Aa

Fatty acid[a]	Per cent total	Fatty acid	Per cent total
8:0	trace[b]	18:1	9.0 ± 0.5
10:0	trace	18:2	2.7 ± 0.2
12:0	4.0 ± 0.3	18:3	24.1 ± 3.0
14:0	30.7 ± 2.1	20:1	3.6 ± 0.1
15:0	1.1 ± 0.2	20:2	3.4 ± 0.5
15:1	trace	20:3	trace
16:0	6.4 ± 1.2	20:4	4.0 ± 0.7
16:1	1.2 ± 0.1	22:0	1.1 ± 0.1
17:0	2.2 ± 0.0	22:1	trace
18:0	4.4 ± 0.3	24:0	trace

[a] First number is the number of carbon atoms, the second is the number of double bonds.

[b] All values less than 1%

Summary

1. The optimum physical growth parameters of fetal calf serum concentration, type of osmotic stabilizing agent, osmotic pressure, temperature, type and concentration of buffer and pH were determined.

2. Total lipid and fatty acid analysis of stages of L. fiscellaria fiscellaria were studied for possible insight into the nutritional requirements of the protoplasts.

3. Ninhydrin-positive compound analysis of the larval haemolymph resulted in the development of a medium superior to that of the standard medium in terms of generation time, maximum cell yield and protoplast morphology.

4. Protoplasts utilized glucose when grown in the standard medium but only late in the life cycle.

5. Ninhydrin-positive compound analysis of amino acid uptake by the protoplasts from the standard medium allowed reduction of the number of types of compounds from 21 to 3 amino acids.

6. The protoplasts were found to be vitamin autotrophic when grown in two types of media. The cells also require protein for membrane integrity in shaken culture but not in stationary culture.

7. The protoplasts readily utilized glutamine. The cellular yield increasing in number with increasing glutamine concentration.

8. The cells were found to increase in lipid content during exponential growth followed by a reduction associated with a change in development. The major fatty acids were C14:0 and C18:3.

9. Analysis of the uptake of ninhydrin-positive compounds from the medium based on haemolymph analysis revealed differences in amino acid utilization compared with the uptake from the standard medium.

10. Morphological development was observed throughout the experiments, and the eleven different stages detected were compared.

Appendix I

Nuclear staining procedure

A sample of 24 hour old protoplast cells grown in Grace's modified insect tissue culture medium was centrifuged at 155 xg until a pellet was detected. The cells were transferred to microscope slides and exposed to osmium tetraoxide (2%) fumes for five minutes. To this two drops of 2% OsO_4 was added followed 30 seconds later by Mayer's albumen. The slides were heated at 70°C for 1 to 1.5 hours.

Hydrolysis of the nucleic acid occurred by immersion of the material into 1N HCl for 1.25 to 1.50 hours followed by rinsing with 1N HCl and distilled water. The cells were stained by soaking in Schiff's reagent for 1.5 hours. The slides were rinsed in three separate one-minute washings in sulfurous acid (1 gm $NaHSO_3$, 10 ml 1N HCl, 200 ml distilled water) followed by rinsing with distilled water. The slides were held in a 60% ethanol bath until a pink colouration occurred. This was followed by three ten-minute immersions in 80% and 100% ethanol. The nuclei stained pink against a brown background.

Literature Cited

Akimenko, V.K., A.G. Medentsev, and V.V. Dmitriev, 1975. Isolation of protoplasts from the yeast Candida lipolytica. Miblao 44: 101-152.

Alexopolous, C.J., 1962. Introductory Mycology. 2nd edition. John Wiley and Sons, Inc., New York, N.Y. pp. 202-207.

Ambrose, E.J. and D.M. Easty, 1970. Cell Biology. Thomas Nelson and Sons Ltd., London, England. p. 280-281, pp. 438-439.

Aoki, J., 1966. Studies on the hyphal body formation of the muscardine fungi. III. Effects of amino acids and other nutrients. J. Sericult. Sci. Japan, 35: 429.

Bachmann, B.J. and D.M. Bonner, 1959. Protoplasts from Neurospora crassa. J. Bact. 78: 550-556.

Bade, M.L. and G.R. Wyatt, 1962. Metabolic conversions during pupation of the cecropia silkworm. I. Deposition and utilization of nutrient reserves. Biochem. J. 83: 470-478.

Barnett, H.L. and V.G. Lilly, 1955. The effects of humidity, temperature, and CO_2 on the sporulation of Choanephora cucurbitarum in culture. Mycologia 47: 26-29.

Berliner, M.D. and M.E. Reca, 1970. Release of protoplasts in the yeast phase of Histoplasma capsulatum without added enzymes. Science 67: 1255-1256.

Bheemeswar, B., 1958. Some aspects of amino acid metabolism in insects in Proceedings of the Fourth International Congress of Biochemistry, Vienna. L. Levenbook, Editor. Pergamon Press, New York, N.Y., pp. 78-87.

Bligh, E.G. and W.J. Dyer, 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911-917.

Boctor, I.Z. and S.I. Salem, 1973. Free amino acids of the haemolymph of the cotton leaf-worm, Spodoptera littoralis Boisduval larvae (Lepidoptera: Noctuidae). Comp. Biochem. Physiol. 45B: 785-790.

Cantino, E. and G.F. Turion, 1959. Physiology and development of lower fungi (Phycomycetes). Ann. Rev. Microbiol. 13: 97-124.

Chen, P.S., 1966. Amino acid and protein metabolism in insect development in Advances in Insect Physiology. Academic Press, New York, N.Y., pp. 53-114.

Child, J.J., C. Knapp, and D.E. Eveleigh, 1973. Improved pH control of fungal culture media. Mycologia 65: 1078-1086.

Cochrane, V.W., 1958. Physiology of Fungi. John Wiley and Sons, Inc. New York, N.Y.

Emerson, S. and M.R. Emerson, 1958. Production, reproduction and reversion of protoplast-like structures in the osmotic strain of Neurospora crassa. Nat. Acad. Sci., Proc. 44: 668-671.

Emerson, S., 1963. A plasmoid variant of Neurospora crassa. Genetica 34: 162-182.

Fast, P.G., 1966. A comparative study of the phospholipids and fatty acids of some insects. Lipids 1: 209-215.

Fast, P.G., 1970. Insect lipids. Prog. Chem. Fats, 11: 181-242.

Fawcett, D.W., 1966. An atalas of fine structure, the cell, its organelles and inclusions. W.B. Saunders Co., N.Y., pp. 415-422.

Florkin, M. and C. Jeuniaux, 1974. Hemolymph composition in the Physiology of Insecta. Vol. 5. M. Rockstein, editor. Academic Press, pp. 255-307.

Floch, J., M. Lees, and G.H.S. Stanley, 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226: 497-509.

Fries, N., 1965. The chemical environment for fungal growth. 3. Vitamins and other organic growth factors in The Fungi on Advanced Treatise, Vol. I. G.C. Ainsworth and A.S. Sussman, editors. Academic Press, New York, N.Y., pp. 491-524.

Gagnon, J.D., 1964. A note on the free amino acid content in needles of Abies balsamea and Picea mariana growing on two site qualities. Oxf: 160.2.182-41 114.27 64-0-23.

Gabriel, M., 1968. Formation and regeneration of protoplasts in the mold Rhizopus nigricans. Folia Microbiol. 13: 231-234.

Garcia-Acha, I., F. Lopez-Belmonte, and J.R. Villanueva, 1966. Regeneration of mycelial protoplasts of Fusarium culmorum. J. Gen. Microbiol. 45: 515-523.

Giese, A.C., 1968. Cell physiology, 3rd edition. W.B. Saunders Co., Toronto, Ontario, pp. 260-261.

Gilbert, L.I., 1967. Lipid metabolism and function in insects. Adv. Insect Physiology 4: 69-208.

Gleason, F.H., 1968. Nutritional comparisons in the Leptomitales. Am. J. Botany 55: 1003-1010.

Gleason, F., 1976. The physiology of the lower freshwater fungi in Recent Advances in Aquatic Mycology. E.B. Gareth Jones, editor. Elek Science, London, pp. 543-572.

Gleason, H., C.R. Rudolph, and J.S. Price, 1970. Growth of certain aquatic Oomycetes on amino acids I. Saprolegnia, Leptolegnia and Dictyuchus. Physiologia Plantarum 23: 513-516.

Good, N.E. and S. Izawa, 1966. Hydrogen ion buffers in Methods in Enzymology, Vol. 24, pp. 53-68.

Gordon, R. and C.H. Bailey, 1976. Free amino acids, ions and osmotic pressure of the hemolymph of three species of blackflies. Can. J. Zool. 54: 399-404.

Grace, T.D.C., 1962. Establishment of four strains of cells from insect tissue grown in vitro. Nature (London) 195: 788-789.

Grace, T.D.C., 1966. Establishment of a line of mosquito (Aedes aegypti L.) cells grown in vitro. Nature

(London) 211: 366-367.

Gustafsson, M., 1965a. On species of the genus Entomophthora Fres. in Sweden II. Cultivation and physiology. Lantbrukshogskolans Annaler 31: 405-457.

Gustafsson, M., 1965b. On species of the genus Entomophthora Fres. in Sweden. Lantbrukshogskolans Annaler 31: 103-212.

Hall, I.M. and J.V. Bell, 1961. Further studies on the effect of temperature on the growth of some entomophthoraceous fungi. J. Invert. Pathol. 3: 289-296.

Hamilton, J.C. and J. Calvet, 1964. Production of protoplasts in an osmotic mutant of Neurospora crassa without added enzyme. J. Bacteriol. 88: 1084-1086.

Hawker, L.E., 1966. Environmental influences on reproduction in the Fungi on Advanced Treatise. G.C. Ainsworth and A.S. Sussman, editors. Academic Press, New York, N.Y.

Hawker, L.E. and A.H. Linton, 1972. Colonial growth in Micro-organisms Function Form and Environment. Edward Arnold (Publishers) Ltd., London, England, p. 185.

Heimpel, A.M., 1950. Further observations on the pH in the gut and blood of Canadian forest insects. Can. J. Zool. 34: 210-212.

Hoddinott, J. and O.A. Olsen, 1972. A study of the carbohydrates in the cell walls of some species of the Entomophthorales. Can. J. Bot. 50: 1675-1679.

Hutchison, J.A., 1962. Studies on a new Entomophthora attacking calyptrate flies. Mycologia 54: 258-271.

Izawa, S. and R.M. Singh, 1966. Hydrogen ion buffers for biological research. Biochem. 5: 467-477.

Keough, K.M.W., G. MacDonald, and W. Thompson, 1972. A possible relation between phosphoinositides and the diglyceride pool in rat brain. Biochem. biophys. Acta 270: 337-347.

Koidsumi, K., 1957. Antifungal action of cuticular lipids in insecta. J. Insect. Physiol. 1: 40-51.

Kopecka, M., 1974. Sporulation in protoplasts of the yeast Saccharomyces cerevisae. J. Gen. Microbiol. 83: 171-178.

Krejzova, R., 1970. Submerged cultivation of Entomophthora virulenta Hall and Dunn, 1957. Ceska Mykologie 24: 87-94.

Latgé, J.P., 1975a. Croissance et sporulation de 6 especes d"Entomoththorales I. Influence de la nutrition carbonee. Entomophaga 20: 201-207.

Latgé, J.P., 1975b. Croissance et sporulation de 6 especes d'Entomophthorales II. Influence de diverses sources d'azote. Mycopathol. 57: 53-57.

Lehninger, A.L., 1975. Biochemistry 2nd. Edition. John Hopkins University, School of Medicine. Worth

Publishers, Inc. New York, N.Y.

Levenbook, L., 1950. The composition of horse bot fly (Gasterophilus intestinalis) larva blood. Biochem. J. 47: 336-346.

Lindsay, O.B. and J.S. Barlow, 1970. Distribution of long-chain fatty acids among the lipid classes of Lucila sericata (Meigen) and Galleria mellonella (Linnaeus). Can. J. Zool. 48: 775-778.

MacLeod, D.M., 1976. Personal communication. Insect Pathology Research Institute, Canadian Forestry Service, Environment Canada, Sault Ste. Marie, Ontario.

Maddy, A.H., 1969. Some problems relating to the chemical composition of membranes in The Molecular Basis of Membrane Funotion. D.C. Tosteson, editor. Prentice-Hall Inc., New Jersey, pp. 95-108.

Matanmi, A. and J.L. Libby, 1976. The production and germination of resting spores of Entomophthora virulenta (Entomophthorales: Entomophthoraceae). J. Invert. Pathol. 27: 279-285.

McMorran, A., 1965. A synthetic diet for the spruce budworm Choristoneura fumiferana (Clems.) (Lepidoptera: Tortricidae). Can. Ent. 97: 58-62.

Meister, A., 1965. Biochemistry of the amino acids. Vol. 1, 2nd edition. Academic Press, New York, N.Y. pp. 269-369.

Michel, J., 1961. Metabolism of cells in tissue culture in vitro. The influence of serum protein fractions on

growth of normal and neoplastic cells. Exp. Cell. Res. 23: 324-334.

Moore-Landecker, E., 1972. Fundamentals of the fungi. Prentice-Hall, Inc., Englewood Cliffs, New Jersey, pp. 147-183.

Morgan, T.E., D.J. Hanahan, and J. Ekholm, 1963. A rapdd method of deacylation of phospholipids and neutral lipids. (Abstr.) Federation Proc. 24 pt 1, 414. p. 1514.

Muller-Kogler, E., 1959. Zur isolierung und kultur insektenpathogener Entomophthoraceae. Entomophaga 4: 261-274.

Musilkova, M. and Z. Fencl, 1968. Some factors affecting the formation of protoplasts in Aspergillus niger. Folia Microbiol. 13: 235-239.

Natrella, G., 1972. The realtionship between confidence intervals and tests of significance in Statistical Issues. Rodger E. Kirk, editor. Brooks/Cole Publishing Company, Monterey, California, p. 113-117.

Nečas, O., 1965. Mechanism of regeneration of yeast protoplasts II. Formation of the cell wall de nova. Folia Biol. (Prague) 11: 97-99.

Nečas, O., 1971. Cell wall synthesis in yeast protoplasts. Bacteriol. Rev. 35: 149-170.

Nicholas, D.J.D., 1965. Utilization of inorganic nitrogen compounds and amino acids by fungi in the Fungi on Advanced Treatise, Vol. 1, G.C. Ainsworth and A.S.

Sussman, editors. Academic Press, New York, N.Y.

Niederprueum, D.J., 1965. Carbohydrate metabolism in The Fungi on Advanced Treatise, Vol. 1, G.C. Ainsworth and A.S. Sussman, editors. Academic Press. New York, N.Y.

Nolan, R.A., 1970. The phycomycete Catenaria anguillulae: Growth requirements. J. Gen. Microbiol. 60: 167-180.

Nolan, R.A. and D.J. Lewis, 1974. Studies on Pythiopsis cymosa from Newfoundland. Trans. Br. Mycol. Soc. 62: 163-179.

Nolan, R.A., 1975. Physiological studies with the fungus Saprolegnia megasperma isolated from the freshwater nematode Neomesomermis flumenalis. Can. J. Bot. 53: 3032-3040.

Nolan, R.A., 1976. Physiological studies on an isolate of Saprolegnia ferax from the larval gur of the blackfly Simulium vittatum. Mycologia 68: 523-540.

Nolan, R.A., G.B. Dunphy, and D.M. MacLeod, 1976. In vitro germination of Entomophthora egressa resting spores. Can. J. Bot. 54: 1131-1134.

Oberling, C. and W. Bernhard, 1961. The morphology of cancer cells in The Cell, Biochemistry, Physiology, and Morphology, Vol. V. Jean Brachet and Alfred E. Mirsky, editors. Academic Press, New York, N.Y.

Otvos, I.S., C. Clark, and L.J. Clarke, 1971. The hemlock lobper in Newfoundland: The outbreak, 1966 to 1971, and aerial spraying, 1968 and 1969. Newfoundland

Forest Research Centre, St. John's, Newfoundland. Information Report N-X-68.

Otvos, I.S., 1973. Biological control agents and their role in the population fluctuations of the eastern hemlock looper in Newfoundland. Newfoundland Forest Research Centre, St. John's, Newfoundland. Information Report N-X-102.

Otvos, I.S., D.M. MacLeod, and D. Tyrrell, 1973. Two species of Entomophthora pathogenic to the eastern hemlock looper (Lepidoptera: Geometridae) in Newfoundland. Can. Ent. 105: 1435-1441.

Patterson, Jr., N.K., 1965. Uptake and utilization of amino acids by cell cultures in Growth, Nutrition and Metabolism of Cells in Culture. G.H. Rothblast and V.J. Cristofalo, editors, Vol. 1. Academic Press, New York, N.Y., pp. 172-209.

Peberdy, J.F. and R.K. Gibson, 1971. Regeneration of Aspergillus nidulans protoplasts. J. Gen. Microbiol. 69: 325-330.

Pelletier, R.L. and G.W. Keitt, 1954. Venturia inaequalis (CKe) Wint. VI. Amino acids as a source of nitrogen. Am. J. Bot. 41: 362-371.

Peters, V.J., J.M. Prescott, and E.E. Snell, 1953. Peptides and bacterial growth, IV. Histidine peptides as growth factors.

Prasertphon, S. and Y. Tanada, 1968. The formation and circulation in Galleria mellonella of hyphal bodies

of Entomophthoraceous fungi. J. Invert. Pathol. 11: 260-280.

Reischer, H.S., 1951. Growth of Saprolegniaceae in synthetic media II. Nitrogen requirements and the role of Kreb's cycle acids. Mycologia 43: 319-328.

Rhodes, A. and D.L. Fletcher, 1966. Principles of Industrial Microbiology. Pergamon Press Ltd., London, England.

Sawyer, W.H., 1929. Observations on some entomogenous members of the Entomophthoraceae in artificial cultures. Am. J. Bot. 16: 87-121.

Shaw, R., 1965. The occurrence of γ-linolenic acid in fungi. Biochem. biophys. Acta 98: 230-237.

Shaw, R., 1966. The fatty acids of phycomycete fungi and the significance of the γ-linolenic acid component. Comp. Biochem. Physiol. 18: 325-331.

Sietsma, J.H. and W.E. DeBoer, 1973. Formation and regeneration of protoplasts of Pythium PRL 2142 in Yeast, Mould and Plant Protoplasts. J.R. Villanueva, I. Garcia-Acha, S. Gascon and F. Uruburu, editors. Academic Press, London, England, pp. 275-283.

Smith, M.C.W., 1953. The nutrition and physiology of Entomophthora coronata (Cost.) Kevorkian. Ph.D. Thesis, University of Michigan, 91 pp.

Sokal, R.R. and F,J. Rohlf, 1969. Biometry the Principles and Practise of Statistics in Biological Research. W.H. Freeman and Company, San Francisco.

Temin, H.M., R.W. Pierson, Jr., and N.C. Dulak, 1972. The role of serum in the control of multiplication of avian and mammalian cells in culture in Growth, Nutrition and Metabolism of Cells in Culture, Vol. I. George H. Rothblat and Vincent J. Cristofalo, editors. Academic Press, New York, N.Y. pp. 50-75.

Terriere, L.C. and P.A. Grau, 1972. Dietary requirements and tissue levels of fatty acids in three noctuidae. J. Insect. Physiol. 18: 633-647.

Thompson, S.N. and J.S. Barlow, 1971. Aspects of fatty acid metabolism in Galleria mellonella (L.) (Lepidoptera: Pyralidoe): Isolation of the elongation system. Comp. Biochem. Physiol. 38B: 333-346.

Turunen, S., 1973. Role of labelled dietary fatty acids and acetate in phospholipids during metamorphosis of Pieris brassicae. J. Insect Physiol. 19: 2327-2340.

Turunen, S., 1974a. Metabolism and function of fatty acids in phytophagous leoidoptera. Ann. Zool., Fennici 11: 170-184.

Turunen, S., 1974b. Lipid utilization in adult Pieris brassicae with special reference to the role of linolenic acid. J. Insect. Physiol. 20: 1257-1269.

Tyrrell, D., 1967. The fatty acids of 17 Entomophthora isolates. Can. J. Microbiol. 13: 755-760.

Tyrrell, D., 1970. Production of resting spores of some Entomophthora species on artificial media. Can. For. Ser. Bi. Res. Notes 26: 12-13.

Tyrrell, D. and D.M. MacLeod, 1972. A taxonomic proposal regarding Delacroxia coronata (Entomophthoraceae). J. Invert. Pathol. 20: 11-13.

Tyrrell, D. and D.M. MacLeod, 1972. Spontaneous formation of protoplasts by a species of Entomophthora. J. Invert. Pathol. 19: 354-360.

Vandenberg, J.D. and R.S. Soper, 1975. Isolation and identification of Entomophthora species Fres. (Phycomycetes: Entomophthorales) from the spruce budworm Choristoneura fumiferana Clem. (Lepidoptera: Tortricidae). J.N.Y. Entomol. Soc. 83: 254-255.

Villaneuva, J.R., 1966. Protoplasts of fungi in The Fungi on Advanced Treatise, Vol. 2. G.C. Ainsworth and A.S. Sussman, editors. Academic Press, New York, N.Y., pp. 3-62.

Villeneuve, J.L., 1962. Influence due regime alimentaire sur la concentration en acids amines non proteiques dans l'hemolymph des larves agees d'Argia affinis. J. Insect Physiol, 8: 585-588.

Wallace, D.R., D.M. MacLeod, C.R. Sullivan, D. Tyrrell, and A.J. Lyser, 1976. Induction of resting spore germination in Entomophthora aphidis by lond-day light conditions. Can. J. Bot. 54: 1410-1418.

Weete, J.D., G.C. Lawler, and J.L. Laseter, 1973. Total lipid and sterol components of Rhizopus arrhizus identification and metabolism, Arch. Biochem. Biophys. 155: 411-419.

Weete, J.D., 1974. Fungal lipid biochemistry, distribution and metabolism, Vol. I. Plenum Press, New York, N.Y., pp. 3-32, 67-105.

Wolf, F.T., 1951. The cultivation of two species of Entomophthora on synthetic media. Bull Torrey Bot. Club. 78: 211-220.

Worthington, R.E. and J.A. Payne, 1974. Lipid characterization of four stored-product insects. Ann. Ent. Soc. Am. 67: 535-538.

Wyatt, G.T., T.C. Loughheed, and S.S. Wyatt, 1956. The chemistry of insect haemolymph, organic components of the haemolymph of the silkworm, Bombyx mori, and two other species. J. Gen. Physiol. 39: 853-868.

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* --- Carner, G.R., 1976. A description of the life cycle of Entomophthora sp. in the two-spotted spider mite. J. Invert. Pathol. 28: 245-254. (see p. 133)

** --- Kawase, S., 1966. Tyrosinase in the silkworm during the pupation period. J. Insect Physiol. 5: 335-340. (see p. 137)