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Partial characterization of Elytroderma deformans

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A PARTIAL CHARACTERIZATION OF ELYTRODERMA DEFORMANS

By

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INTRODUCTION

Relatively little is known about the physiology of Elytroderma deformans (Weir) Darker, the etiological agent of a needle cast foliage disease characterized by the production in some hosts of a deformity known as (witches') brooming. Studies dealing with the physiology, metabolism, and nutrition of this organism have been hindered by the inability to isolate and cultivate this organism. However, with adequately controlled isolation techniques the fungus may be obtained in pure culture. Studies on the enzymology of E. deformans will shed information on the metabolism of this organism. Knowledge of the enzymes and metabolic processes of this fungus should lead to a specific approach to the chemotherapy of the needle cast diseases and possibly to the elucidation of the mechanism of pathogenicity.

The physiology of E. deformans has remained largely unexplored, mainly because of the difficulties encountered in proving that the fungus obtained in pure culture is actually the causative agent of the infection. Lightle (47) was not able to isolate the fungus, but Weir (87) obtained growth from the germination of the ascospores. However, he was unable to maintain the organism, as after several transfers the mycelium died. Sikirowski (72) reported the successful culture of the organism on a medium prepared by the addition of juice from macerated pine needles to agar. Gordon (31) reported isolation of the organism on a medium containing macerated pine needles as well as some successful isolation on potato dextrose agar without containing supplements from

pine needles. After initial isolation, the fungus could be maintained on potato dextrose agar containing no supplements from pine needles.

The life cycle of the organism was found to have varied activity at different times of the year, and, therefore, isolation may be influenced by a variation in the season or the composition of the needle from which the isolation was attempted.

The growth factors for fungi are those organic substances which in minute amounts, are necessary or stimulatory for growth and do not serve simply as energy sources. The term "growth factor" indicates that growth is the mode of response ordinarily observed, and can be measured by the change in weight of the mycelium. Certain organic substances (e.g., amino acids, nucleotide constituents, and fatty acids) which serve as building blocks of intracellular structures are not included as growth factors because the concentrations required are considerably higher than the coenzyme vitamins (1.0-10.0 µg/ml instead of 0.001-0.00001 µg/ml) (Table 1).

TABLE 1. A comparison of the activity of four common growth factors.

Growth factor	Amount of growth factor necessary for production of 1.0 mg mycelium (dry weight) (µg)	Lowest concentration of growth factor giving maximum growth rate (µg/ml)
Biotin	0.00025	ca. 0.0001
Thiamine	0.0004 - 0.0025	ca. 0.001
Pyridoxine	0.0003 - 0.0007	ca. 0.001
Inositol	1.1 - 1.7	ca. 0.5

From Fries (29).

The capacity to synthesize a certain growth factor may change with time as a result of changes in the environment and genetic or metabolic mechanisms of the organism. Variations in biosynthetic ability may also occur from one stage to another during the life cycle of an organism (Pine, 65). The number of growth factors required by fungi varies from none to at least six, with the most exacting species being found among the yeasts (Table 2).

TABLE 2. Examples of fungi requiring different growth factors.

Species	Thiamine	Riboflavin	Biotin	Niacin
Lophodermium pinastri	+		+	
Phycomyces blakesleeanus	+			
Melanospora destruens			+	
Torula sphaerica			+	+
Nematospora gossypii			+	
Poria vaillantii	+	+	+	

From Fries (29).

A complete requirement for a growth factor is not usually influenced by cultural conditions. A partial requirement may well be more or less pronounced, depending on the external conditions (Lichstein and Begue, 45). At times the requirement for a certain factor depends on the composition of the medium at any temperature in the biokinetic life zone of that organism, as Pythium butleri becomes thiamine-dependent as soon as the mineral salt concentration exceeds a certain concentration (Robbins and Kavanagh, 67).

Most fungi grow within the pH range of 4.0 - 8.0. Many fungi will grow over a wider range, and a very few have been reported to have a narrower range (Lilly and Barnett, 46). This growth is observed to be maximal at a particular pH, and the growth of the organism diminishes or ceases at the high and low extremes. Many factors will change the shape of a pH-growth relationship curve, including temperature, growth factor supply, and nitrogen and carbon sources (Cochrane, 16). An organism may be able to grow well at a pH which does not permit the initiation of growth (Jensen, 40). However, most plant pathogens examined grow best in media with an initial pH of 5.0 - 6.5 (Hugo, 36). In physiological studies pH must necessarily be controlled by a buffering system. Phosphate usefulness is limited by the tolerance of the fungus to the ion, with this ranging from 0.003 M to 0.20 M (Clark, 17; Herrick, 35).

The growth of a fungus or the production of a fungal metabolite is the result of many enzymatically controlled reactions and processes, each of which may have different coefficients and optima. It is obvious, therefore, that a fungal activity will start at a minimum value, maximize at an optimum, and decline and cease at the maximum temperature. The difficulty of defining the optimum temperature was reviewed by Fawcett (24) who measured daily increments of growth on agar of four fungi. Cartwright and Findlay (15) surveyed many wood-destroying fungi and obtained most optima in the 20 - 30°C range. The observed cardinal growth temperatures of species within the same genus differ greatly and are thought to reflect the temperatures in the natural environments of the fungi (Weimer, 86). The relation between a plant disease of fungal

etiology and temperature is not a simple relationship as there are of necessity two or more organisms involved. The temperature optima for selected plant pathogens in a study by Togashi (83) were in the 20 - 30°C range with about 50 percent between 26 and 30°C. The vascular wilts of plants caused by Fusarium spp. have an incidence at temperatures close to the optimum range for growth of the pathogen (Jones, 41). For a few plant pathogens it has been shown that differences between the temperature optima for disease and for growth of the pathogen occur and reflect the effect of temperature on the host (Foster, 26). The latter effect has been observed in seedling blight of wheat (Dickinson, 19).

Infections with plant pathogens of either an obligate or a facultative nature are followed by marked increases in the rates of respiration of the host tissues. Photosynthesis in the host may or may not be stimulated at first. However, it always decreases within a few days after infection begins (Allen, 2). With the rusts and powdery mildews, increased respiration and decreased photosynthesis are accompanied by the accumulation of various substances marked by local increases in dry weight, and with the pycnial, aecial, and telial stages of many rusts, by localized hypertrophy or brooming (Shaw, 70). It has also been shown that there are large increases in the concentrations of free amino acids at the sites of rust infections (Yarwood, 88), and that indoleacetic acid or its metabolic products are present in similar high concentrations. The transport of indoleacetic acid and its metabolic products to the site of infection has been postulated by Pilet (64) in his observations of rust infections. The hypothesis of a diffusable substance (or substances) produced at the site of the infection either by the fungus or

the host cells under attack, or both, stimulating the metabolic activity of the host tissue in the vicinity of the infection, has been put forth by Shaw (70).

Although filamentous fungi are commonly thought of as being strictly aerobic, the relationship between growth and oxygen supply varies considerably in different species. Many fungi can utilize carbohydrates anaerobically, producing typical fermentation products, although most cannot grow without some oxygen (Cochrane, 16).

The metabolic activities of fungi, with respect to the ability of fungi to utilize certain sugars as sole sources of carbon and energy as well as the efficiency of conversion of these compounds into cell mass, provides valuable clues to the glycolytic pathways available to the cell. The presence of a glycolytic enzyme in a cell extract suggests that the cell has the potential to use it.

Fungi usually utilize simple inorganic nitrogen compounds. Ammonium salts or nitrates are suitable sources of nitrogen for most fungi, although with nitrate there may be a lag in growth before the necessary enzymes concerned in nitrate reduction are produced (Nicholas, 59). There is no optimum amount of nitrogen for a culture, the demand being dependent directly upon the carbon supply. This relationship may be altered by any change of concentration of any other factor, depending on the factors required for metabolism of nitrogen. The growth of Scopulariopsis brevicaulis on different nitrogen sources is shown in Table 3.

Nitrate reduction in the fungi is believed to be of strict assimilatory nature, incorporating all of the reduced nitrate into the cell

TABLE 3. Growth of Scopularopsis brevicaulis on different nitrogen sources (with glucose).

Parameter	Nitrogen source				
	KNO_3	NH_4NO_3	$(NH_4)_2SO_4$	$(NH_4)_2SO_4$ + tartrate	$(NH_4)_2SO_4$ + malate
Mean dry matter (mg/flask)	200	18	12	160	225
Nitrogen assimilated (% of initial)	100	15-20	15-20	100	100
Range of final pH	8.0-8.1	3.0-3.9	2.7-3.8	3.4-4.4	7.5-8.6

From Morton and MacMillan (57).

(Cochrane, 16). As in other micro-organisms, the specificity of response to organic nitrogen sources such as amino acids in fungi, shows that they are assimilated as such (Thorne, 81). Beckman et al. (7) found that glycine, asparagine, glutamic acid, and aspartic acid are capable of supporting good growth of Chalera quercine with growth of mycelium utilizing asparagine superior to that growth obtained with aspartic acid. It has been observed that other substances available for nitrogen sources to the fungi are the proteins, the nucleic acids, and urea. In pure culture, proteins such as gelatin, casein, and albumin can serve as sources of nitrogen for common saprophytic fungi and the actinomycetes (Jermyn, 39). Surveys among the culture filtrates indicate that the occurrence of an enzyme (or enzymes) able to hydrolyze gelatin is common among the fungi (Dion, 21). Waksman and Starkey (85) have shown enzyme complements capable of hydrolyzing casein, gelatin, fibrin, and albumin in Streptomyces violaceus-ruber, and Rhizopus spp.

Nucleic acids, nucleotides, and the purine bases can also be used as nitrogen sources by fungi. Early work was performed utilizing non-specific, crude tests such as liberation of phosphorus from nitrogen bases and the utilization of nucleic acid nitrogen for growth (Melin, 50). It has now been established that DNA is acted upon by DNase yielding primarily mononucleotides, and occurs with Neurospora crassa (Kaplin, 43) and Streptomyces sp. (Muggleton, 58). Urea is recognized as a utilizable nitrogen source among the fungi (Bach, 4; Crewther, 18). The urease of Aspergillus niger is most active at pH 7.6, although its production is favored to a marked degree by low pH (Bach, 4; Miwa, 55).

Hydrolytic activity is obviously not limited to the nitrogenous substances and amylase activity, defined as the ability of cell-free materials to hydrolyze starch or glycogen, is nearly universal among fungi and the actinomycetes (Cochrane, 16). Amylase with some exceptions is found in the culture medium as well as in the cells. The amount of enzyme is usually increased by the inclusion of starch in the growth medium and is affected by the nitrogen source and by small amounts of salts (Erb, 22; Shu, 71). Inasmuch as many fungi utilize cellulose as a carbon source, it is to be expected that an enzyme or enzymes are released from the cell and hydrolyze cellulose to soluble sugars. Microbial degradation of cellulose has been shown by Siu (73) to occur in ascomycetes and basidiomycetes, both groups being particularly rich in species with cellulase activity.

Because of the nature of the life cycle observed in E. deformans, it would be of interest to determine the length of time of survival of the organism in the soil under the infected tree to elucidate the

possible mechanism for additional dispersion of the fungal spores. Sporulation of E. deformans has not been observed in culture or in the soil; however, it is obvious that the great number of spores present in the spore discharge are not all involved in establishing an infection in the host and, therefore, must fall either on a tree branch or limb or onto the soil beneath the infected tree. It is also of interest to observe the effect of desiccation on viability in the establishment of host-parasite relationships enumerated above.

The effects of fungicides on plant pathogens are only recently receiving attention, and the two particular ones involved in this study were chosen on the basis of their value to the control of the parasite in the host in areas of endemicity. Cyclohexamide[1] is an inhibitor of protein synthesis and is thought to act upon the ribosome in some capacity. Recent investigations have not yet elucidated the mechanism of its inhibition, but the protein synthesis of the cell becomes the limiting factor after the application of the inhibitor (fungicide). The second inhibitor chosen for this study was phytoactin[1] and it also has a predilection for the ribosome as recent studies indicate (Dimond, 20). Very little seems to have been done with respect to the influence of temperature and/or light on the efficacy of the inhibitor. Richmond and Somers (66) examined the effect of uptake of captan on Neurospora crassa and observed a linear uptake with temperature. McIntosh (49) examined a number of other compounds and found an increase in temperature increased toxicity to a greater extent, which varied from compound

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[1]Actidione* and Pabst L-318, respectively.

to compound. The activity of fungicides is also modified by pH. The knowledge of the life history of E. deformans does enable the application of the fungicide at a time to give the chemical the optimal chance of inhibiting the fungus. Several studies have been carried out with the (so-called) systemic fungicides which act within plant tissues. It is hoped that by a determination of the minimum lethal dose (MLD) in vitro, much can be learned of the practicality of usage of these two compounds. If this knowledge is obtained and it is determined that the MLD is far too high for the host cell, then a look at the fungicides in the area of the residual or contact classification is worthwhile.

STATEMENT OF THE PROBLEM

It is apparent that very little is known about E. deformans with respect to its physiology and variations in metabolic activities. It is therefore the purpose of this investigation to recover the organism from infected needles and determine its growth requirements in pure culture, its metabolic activities, and its susceptibility to physical and chemical agents.

METHODS AND MATERIALS

(1) Isolation of Organisms

Diseased needles between 12 and 18 months of age were used for obtaining isolates of Elytroderma deformans. Needles were placed in a sterile Petri plate and externally sterilized for 5 minutes in a 50 percent v/v Purex[2] solution with 5.0 percent wetting agent Tween 80. The needles were washed in two changes of sterile distilled water. Following the removal from the second rinse with forceps, the needles were sectioned into 100 mm lengths and placed into Petri plates containing modified variations of potato dextrose agar (PDA). Incubation was at room temperature (approximately 23-28°C). Transfers of hyphal tips after the establishment of a culture were made to slants of PDA.

(2) Culture Media and Methods Employed

The basal medium used for isolation was potato dextrose agar prepared by boiling and filtering 200 g of potatoes, and adding 20 g dextrose and 20 g agar.

Enriched media for isolation were prepared as follows:

Ponderosa pine needles 12 to 16 months in age were macerated in a Waring blender (350 g needles in 1000 ml distilled water) for 3 minutes and the resulting decoction was divided into 5 aliquots; each aliquot of the decoction was extracted with 100 ml of one of the following

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[2]Sodium hypochlorite - Commercial bleach manufactured by Purex Corporation Ltd., Lakewood, California.

solvents: benzene, chloroform, ether, or acetone; the other extract was used both as such and after further extraction with water; 25 ml of each extract were concentrated by evaporating almost to dryness. The resulting concentrates were weighed and added separately to aliquots of the basal isolation medium.

(3) Determination of the pH of Needle Homogenate

The pH of needle homogenates was determined on biweekly samplings of infected and uninfected needles from collection sites at the same height above ground and same side of the tree to minimize variation. Ten g of collected needles were suspended in 100 ml of distilled water and the mixture was macerated in a Waring blender for 3 minutes. The needles were allowed to settle and the pH was measured using a Beckman model 76 Expanded Scale pH Meter.

(4) Variation of Isolation with Needle Composition

Infected and uninfected Ponderosa pine needles were placed in a Waring blender (10 g in 100 ml distilled water) and macerated for 3 minutes. The steam distillate was extracted according to the procedure of Mirov (53) in an attempt to isolate the fraction which would be the most probable volatile factor stimulating the growth of the organism. The homogenate was distilled and the distillate collected in 10 ml quantities. The analysis was performed by vapor-phase chromatography. A Wilkens Aerograph Hi-Fi 600-C Instrument in combination with a Minneapolis Honeywell Brown Recorder equipped with automatic integrator was employed. The amount injected was 1.0 µl. A 1/4 inch x 5 foot column, Silicone 550-20 percent on acid washed 60/80 chromsorb with nitrogen

flow rate adjusted to 12-15 ml/minute, 60-62°C column, 145 to 150°C detector and 120 to 130°C injector temperatures, was used for optimum separation of components. The percent composition of the individual terpene components was computed directly from the integration results with no response correction, as the variation of the terpene composition between individual trees exceeds by far the error committed.

(5) Vitamin Requirements in Culture

Following initial isolations of the fungus from infected needles, cultures were transferred to malt extract broth (MEB), which was adjusted to pH 4.25, and incubated at ambient temperature for 9 days. A basal medium was then prepared utilizing a 0.01M phosphate buffer and enrichments according to Table 4.

Inocula were prepared by centrifuging the growth from the MEB in 250 ml Nalgene centrifuge bottles at 2,000 rpm in an International Clinical Centrifuge for 10 minutes. The mycelium obtained was then washed four times with 0.85 percent saline, transferred to a sterile Waring blender cup, resuspended in 9 volumes of 0.85 percent saline, and macerated for 30 seconds. The above media were inoculated with 0.5 ml of the resulting homogenate. Dry weight determinations of the inoculum were made and subtracted from the weight of the mycelium obtained after incubation for 14 days to measure growth in the medium. A chemically defined medium was also prepared for use as Basal Medium II:

1.) KH_2PO_4	1.00 g
2.) $MgSO_4$	0.50 g
3.) NaCl	0.10 g
4.) $CaCl_2$	0.10 g
5.) $ZnSO_4$	0.05 g
6.) glucose	10.00 g
7.) distilled water to make	1000 ml

TABLE 4. Basal Medium I composition.

Medium	Glucose 0.1M	Na Glutamate 0.1M	Yeast Extract 0.05%	KNO_3 0.1M	$(NH_4)_2SO_4$ 0.1M	Pine Needle Extract (aqueous) 1.0%
1	X					
2		X				
3	X		X			
4		X	X			
5	X			X		
6	X				X	
7		X		X		
8	X					X
9						X

The following vitamins were used as enrichments:

1.) Biotin	002 µg per liter of medium.
2.) Pyridoxine HCl	400 µg
3.) Thiamine HCl	400 µg
4.) Riboflavin	200 µg
5.) Niacin	400 µg
6.) Folic acid	002 µg
7.) Inositol	002 µg
8.) Calcium pantothenate	400 µg
9.) PABA	200 µg

The following inorganic and organic nitrogen sources were used:

1.) $(NH_4)_2SO_4$	0.25 g per 50 ml of medium.
2.) KNO_3	0.25 g
3.) alanine	0.15 g
4.) glycine	0.12 g
5.) serine	0.17 g
6.) asparagine	0.11 g

Determination of the vitamin requirements was performed by utilizing glucose, a nitrogen source, and all vitamins. Subsequent experiments were performed deleting vitamins until growth was obtained with a minimum of vitamins.

(6) The pH Requirements

The pH determinations for optimum growth were performed for the following media:

1.) Sabouraud dextrose broth (Difco)
2.) Potato dextrose broth (Difco)
3.) Malt extract broth (Difco)
4.) Chemically defined medium

The media were rehydrated in 1000 ml 0.01M phosphate buffer and a range of pH values was then established from 3.0 to 9.0 with 0.5 pH unit increments. The resulting culture media were inoculated with equivalent blended inocula of E. deformans. At the end of 5 days of incubation, each mycelium was centrifuged at 2200 rpm in an International Clinical Centrifuge and collected in a Gooch filter, dried, and weighed for

determination of growth. The results were then graphed (dry wt. vs pH of the culture medium).

(7) Cardinal Temperatures for Growth

Malt extract broth was adjusted to pH 4.5 and was divided into aliquots of 50 ml each. A blended inoculum was prepared as above and used to inoculate each flask. The flasks were then placed at the following temperatures in order to determine the gross points of the temperature curve: 37, 30, 25, 20, 15, 12, and 8 degrees C. Dry weight determinations were used for the measurement of growth after a 27-day incubation period. The temperature range limits were then varied to determine plus or minus 2 degrees where there was no indication of growth as measured by dry weight increase.

(8) Indoleacetic Acid Content of Normal and Infected Needles

Infected needles (350 g in 50 ml distilled water) were macerated in the cold (15°C) and in the absence of light. The resulting homogenate was then extracted with ether, concentrated by evaporation and chromatographed in isopropanol : acetic acid : water (10 : 1 : 1). The same procedure was utilized for normal needles. The chromatograms were developed in Salkowski reagent modified according to Gordon and Weber (32). The spots giving the usual color reactions were then measured and the Rf values determined. Known concentrations of indoleacetic acid, indolebutyric acid, and indole were run to determine reference Rf values in this solvent system. The mycelium after 14 days of incubation was macerated in the absence of light and in the cold (12°C) and extracted with ether. The extract was concentrated and chromatographed in the same procedure as above.

(9) Oxygen Requirements

In an analysis of the oxygen requirements an all-or-none requirement was performed instead of a detailed analysis of strict quantitative amounts. An inoculum of E. deformans was transferred to plates of MEB with agar added to 1.5 percent concentration and the inoculated plates transferred to a Case anaerobic jar. The latter, containing the inoculated plates, was then flushed two times with nitrogen and filled a third time before incubation at ambient temperature. A positive test for the requirement of oxygen was then obtained if no growth occurred as measured above that of the inoculum.

(10) Metabolic Studies on Carbohydrate Metabolism

Inocula were prepared by growing the fungus in malt extract broth for 14 days and harvesting by centrifugation in Nalgene 250 ml centrifuge bottles at 2200 rpm for 15 minutes. The mycelium so recovered was then washed three times with sterile distilled water. The washed mycelium was then transferred to a graduated centrifuge tube and resuspended in 9 volumes of water. This suspension was then macerated for 30 seconds in a Waring blender cup and used for inoculation. The dry weight of the inoculum was determined.

The Warburg apparatus was utilized for manometric measurement of oxygen uptake. Glucose was used as a substrate and runs were made at different temperatures according to the procedure of Warburg's direct technique (Umbreit, Burris and Stauffer, 84). Flasks contained 1.0 ml cell suspension of E. deformans (24 mg dry weight), 1.0 ml M/100 phosphate buffer at a pH within the range 3.5 through 7.0 (0.5 pH unit increments), and 0.5 ml M/10 glucose in side arm. Temperature was adjusted to 24°C or 34°C to attempt demonstration of the Q_{10} effect.

(11) Metabolic Studies on Nitrogen Utilization

Inoculum was prepared as in the above manometric determinations and was standardized by dry weight.

Flasks contained 1.0 ml cell suspension of E. deformans (26 mg dry weight), 1.0 ml M/100 phosphate buffer at pH 3.5 through 7.0 with 0.5 pH unit increments, and 0.5 ml M/10 asparagine source in side arm. Temperature was 24 and 34°C to attempt demonstration of Q_{10} effect.

(12) Hydrolytic Enzyme Complement of E. deformans

(a) Gelatinase

Three isolates of E. deformans were assayed for gelatin-hydrolyzing ability. All strains were grown on potato dextrose agar prior to transfer of hyphal tip for inoculation of assay medium. The method utilized was that of Smith (75) known as the agar-gelatin plate test. This consisted of preparing 1.0 percent agar plates containing 0.4 percent gelatin. The plates were set in series at pH 7.0 and 5.8, the latter being the lowest pH obtainable without precipitation. The plates were inoculated and incubated with a rubber dish seal at ambient temperature for 17 days. The plates were then flooded with approximately 10 ml of mercuric chloride in hydrochloric acid (15 g HgCl/120 ml 0.37 N HCl), a protien precipitant. A cloudy, white precipitate in the agar indicated the presence of gelatin; clear areas indicated zones of hydrolysis.

(b) Deoxyribonuclease

A modification of the method of Jeffries et al. (38) was employed to study the nuclease activity of E. deformans. The deoxyribonuclease test agar by Difco was rehydrated, sterilized, adjusted to pH 7.0 or 4.9, and plated. The fungi were grown on potato dextrose agar prior

to transfer of hyphal tips for inoculation of the assay medium. The plates were inoculated and incubated with rubber dish seals at ambient temperature for 21 days. After incubation, the plates were flooded with approximately 15 ml of 0.1 N HCl. The positive reaction for deoxyribonuclease activity was indicated by a clear zone in the agar. The polymerized DNA was precipitated by the acid.

(c) Amylase

A modification of the method described by Robinson et al. (69) was utilized for the determination of amylase activity. The organisms were again three isolates and the media were adjusted to pH 7.0 or 5.3. The starch agar (Difco) was rehydrated and sterilized. Hyphal tips from E. deformans grown on PDA were used for inocula. The inoculated plates were incubated at ambient temperature for 21 days. After incubation, the plates were flooded with Gram's iodine. The positive test or hydrolysis of starch was indicated by a clear zone around the organism, while the unhydrolyzed starch reacted with the iodine to give a purple color.

(d) Cellulase

The test for cellulase was modified from many reports in literature. The substrate was a strip of Whatman No. 1 filter paper suspended from the neck of a flask with the bottom of the strip immersed in malt extract broth. The flask was sterilized and inoculated with 1.0 ml of a culture of E. deformans. Three strains were utilized and dry weight determination of the inoculum size was performed. The flask, after inoculation, was incubated at ambient temperature with a tape seal around the cap for 47 days. After incubation a thorough examination of the filter paper was performed. A positive test indicating the utilization of cellulose was the disintegration of the filter paper. The negative

test was intact filter paper with or without growth of the organism on it.

(e) Nitrate reduction

The test for the reduction of nitrate to nitrite or further to ammonia or nitrogen was that reported by Tittsler (82). A trypticase-nitrate-broth solution was made by rehydration and sterilization of the Difco medium. The pH was adjusted to 4.5. The sterilized medium was inoculated and incubated at ambient temperature for 14 days with a tape seal around the cap of the tubes. Three isolates were used. After incubation 1.0 ml of sulfanilic acid solution and 1.0 ml of dimethyl-alpha-naphthylamine were added to the tubes. A positive test indicating that the NO_3 had been reduced to NO_2, was the development of a red, purple, or maroon color. The negative test (no color development) was examined further by the addition of a trace amount of powdered zinc as catalyst.

(f) Urease

The method of Stuart, Van Stratum, and Rustigan (79) was followed utilizing urea broth by Difco. The product was rehydrated, sterilized, and pH adjusted to 4.5. The sterilized medium was inoculated with 0.5 ml of a blended mycelium of E. deformans previously grown in malt extract broth. Three isolates were again used. A positive test was the observation of color change of the indicator from yellow to red in the alkaline range indicating the production of ammonia.

(13) Survival in Soils

The method utilized in this study was a modification of that by Atkinson (3). A mycelial inoculum was transferred to moist soil that

had previously been sterilized, and the pH of the soil was adjusted from 4.5 through 7.0 utilizing 0.5 pH unit increments. Soil from beneath several infected trees was also utilized in an attempt to reproduce that natural environment encountered by the organism in the Bass Creek Drainage. Soils were then sampled for viable E. deformans on the 1st, 3rd, 7th and 14th days by transferring rehydrated mycelial inoculum and some surrounding soil particles to tubes of potato dextrose agar. If growth was not evident after the 1st, 3rd, 7th, and 14th days of incubation, viability was reported as negative.

(14) Effects of Desiccation

The procedure utilized the transfer of mycelium to sterile vessels and subjecting it to a variety of desiccation methods: (1) vacuum for 12 hours; (2) desiccant ($CaCl_2$) in desiccator with vacuum drawn for 12 hours; and (3) rapid filtration on a Büchner funnel. All desiccation experiments were performed at ambient temperatures. Assay of the dried products was along the same time schedule as above: 1, 3, 7, and 14 days.

(15) Effects of Inhibitors

Phytoactin and cyclohexamide were added to potato dextrose broth cultures in sufficient concentrations for lethal effect. Then the media plus inhibitor were serially diluted to the point where growth would be observed. Following the determination of the ranges of the inhibitory concentrations as stated above, manometric studies were undertaken to show the effects of the addition of the inhibitor upon resting cells. The procedure was standard Umbreit, Burris, and Stauffer (84) and flasks contained: 1.0 ml M/100 buffer, 1.0 ml blended cell

inoculum prepared as in (10) above, 0.5 ml M/10 glucose in one side arm, and maximal inhibitory concentration of inhibitor as determined above in the other side arm. The temperature was ambient (25°C).

RESULTS

Isolation of Organisms

As it will be seen from Table 5, the isolation on the pine needle-extract media was almost as rapid as the actual macerated pine needle agar previously utilized. Ether and chloroform extracts gave the results most nearly comparable to the pine needle maceration agar. Each dish had four 10-mm sections of infected needles from the same aged bundle allowing a possibility of eight rapid isolations assuming that the fungus would grow out of the open ends of the needle more readily than it would through the altered cuticles and stomata. In any case, it will be seen from the number if isolates obtained and the length of time taken before hyphal tips could be transferred, that either and chloroform were extracting some factor which facilitated isolation of the fungus. The non-volatile factor extracted by the solution was not essential for continued growth, however. The water extract of pine needles also provided adequate nutrients for primary isolation. The water-extracted ether and the benzene gave longer primary isolation times, indicating that neither solvent system was extracting the same chemicals or quantities of the same chemicals, as the ether or chloroform system. The acetone extract provided even less a chemical environment for primary isolation. Microscopic examination showed sufficiently greater penetration of the agar by the fungus on the ether, chloroform, and pine needle extract agars after growth outward from the needle sections. After 20 days growth appeared uniform on all extract agars except the acetone and benzene which showed only small amounts of growth.

TABLE 5. Primary isolation of E. deformans on enriched media.

Culture medium	Number of isolates	Time required $\bar{X}$ hours
(1) Pine needle	6/8	48
(2) Benzene extract	2/8	96
(3) Chloroform extract	5/8	48
(4) Ether extract	5/8	48
(5) Water-extracted ether	2/8	96
(6) Acetone extract	1/8	192
(7) Water extract	4/8	72

Determination of the pH of Needle Homogenate

The pH of the needle homogenates on a biweekly basis will be seen in Figure 1. The infected needle homogenates showed a higher pH continually throughout the study. Until the 14th sampling (27 February 1966) the values of the infected and uninfected needle pH roughly parallelled each other with the infected maintaining a pH about 0.40 unit higher than the uninfected needles. It will be seen also that the infected needles maintained a more gradual change in the early portion of the samplings, in comparison to those of the uninfected needle homogenates noted on the 2nd, 5th, and 8th samplings. From the 14th sampling through the 17th sampling, the pH of the infected needle homogenates rose at a greater rate than that of the corresponding uninfected needle homogenates. Both pH curves showed an initial drop followed by a gradual decline to the lowest pH values reached by both homogenates on the 11th sampling (16 January 1966). From this point on, both curves showed a rise in the pH values, with the infected homogenates increasing at a greater rate than that of the uninfected, and the infected continuing to increase after the uninfected had leveled off at a constant value.

In conjunction with the above sampling of the needle pH values, primary isolations were attempted with the results observed shown in Table 6. The length of time required for four isolates to grow out sufficiently to be recognized and transferred to other media was a variable with extremes ranging from 48 hours to 58 hours. The number of isolates was also a variable ranging from one of the four possible isolates to all four yielding a primary isolate. A distinct variation

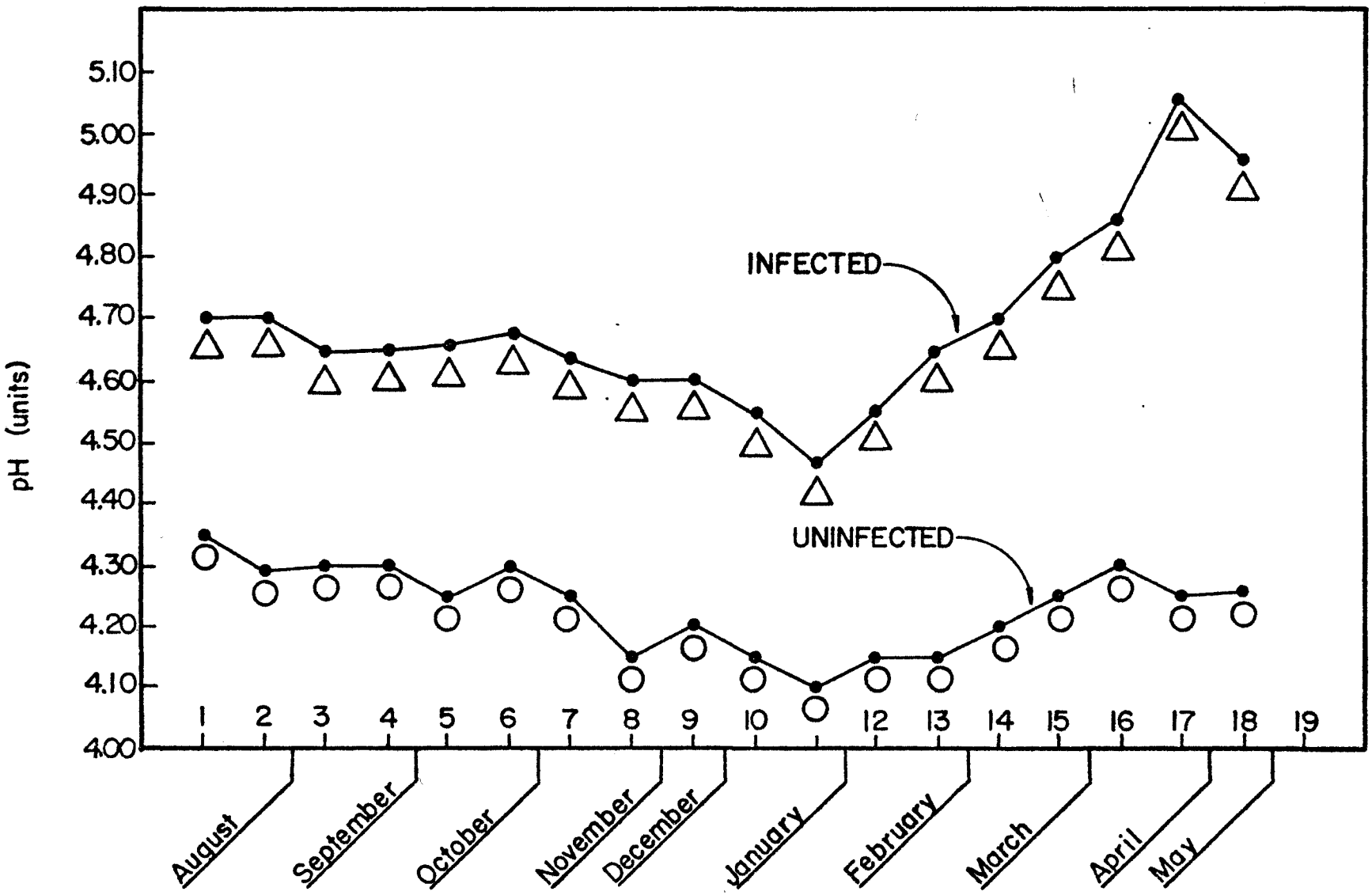


Figure 1. A comparison of the pH of infected and uninfected needle

TABLE 6. Schedule of collections correlated with isolations.

Sample No.	Date of Collection	Number of Isolates	Average Time Required
1	8/ 1/65	3/4	48
2	8/15/65	3/4	48
3	9/12/65	3/4	48
4	9/26/65	2/4	48
5	10/10/65	3/4	48
6	10/24/65	2/4	48
7	11/ 7/65	3/4	48
8	11/21/65	2/4	55
9	12/ 5/65	2/4	58
10	1/16/66	2/4	55
11	1/30/66	1/4	58
12	2/13/66	1/4	56
13	2/27/66	2/4	55
14	3/13/66	2/4	50
15	3/27/66	3/4	50
16	4/10/66	4/4	48
17	4/24/66	3/4	44
18	5/ 8/66	4/4	45
19	5/22/66	3/4	45
20	6/ 5/66	3/4	44

was observed indicating that there was indeed a correlation with the time of the season or year and the success of recovery.

Variation of Needle Composition

There was a variation in the infected and uninfected Ponderosa pine needles with respect to the needle terpene composition as measured by gas chromatography. In both cases there were six peaks obtained as correlated with about nine that are found with regularity in literature (Mirov, 54). The results can be seen from the table below.

TABLE 7. Amount of terpenes in steam distillate of Ponderosa pine needles.

Peaks	Uninfected		Infected		Percent change from normal	Probable identity
	Units^2	Percent*	Units^2	Percent*		
1	11.6	18.8	02.0	13.6	-27.6	limonene
2	29.0	47.0	07.7	52.4	-14.9	d-Δ_3-carene
3,4	16.0	25.9	04.5	30.6	-18.2	β-pinene, ?
5,6	05.0	08.1	00.5	03.4	-58.0	? , ?

* Percentage of total square units under all peaks.

It can be seen from the above table that the amounts of the terpenes obtained showed a considerable difference between the infected and the uninfected needles. Peak 2 was the largest in both cases, and was a distinct, readable peak. Peaks 3 and 4 were joined or overlapping in both infected and uninfected needle samples. Peaks 5 and 6 could be measured in the uninfected needles, but were not determinable in the infected needle sample because too little was there. It can be seen, therefore, that the total amount of terpene was reduced in every case

in the infected sample by about 75 percent. The relative proportions were also changed, but not quite as drastically as the amounts.

Vitamin Requirements in Culture

As can be seen from the schedule of media, Figure 2, good growth was obtained from those containing a carbon source and yeast extract. The mycelial dry weight was a factor of three greater when grown in the presence of yeast extract. KNO_3 in combination with glucose gave fair growth in a strictly synthetic medium even when transferred in a serial dilution series to the point of extinction by dilution of the accessory growth factors that might have been transferred in the glucose sample or the KNO_3. Glucose and pine needle extract (aqueous) gave good results indicating that growth factors in the pine needles were well utilized by the fungus.

The vitamin requirements were found to be of a nature that stimulated growth, but were not necessary for growth in culture. When vitamins were added with glucose the resulting growth was the greatest amount obtained in a defined medium. When the vitamins were then divided into two groups, the one series consisting of biotin, pyridoxine HCl, thiamine HCl, riboflavin, and niacin; and the other series, folic acid, inositol, calcium pantothenate, para-aminobenzoic acid; the results showed that the complement of all was more stimulatory for growth than either of the two groups alone.

Nitrogen sources were varied in an effort to determine which was better utilized by the fungus. As it can be seen from Figure 3, glycine, serine, and asparagine were better utilized as organic nitrogen sources than was alanine. The amount of growth as measured by dry weight was

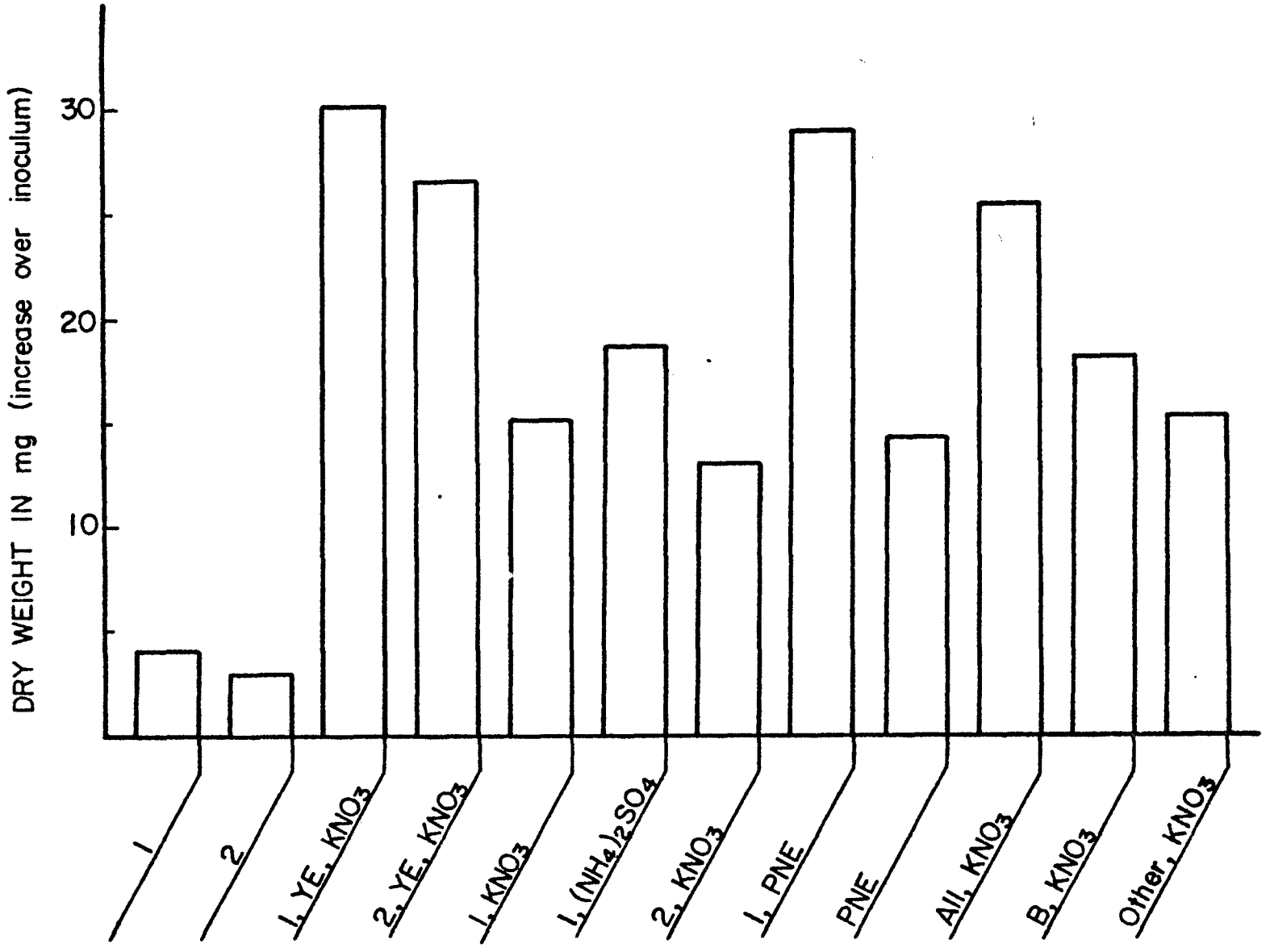


Figure 2. A comparison of growth in enriched media. (1) Glucose substrate and (2) sodium glutamate substrate.

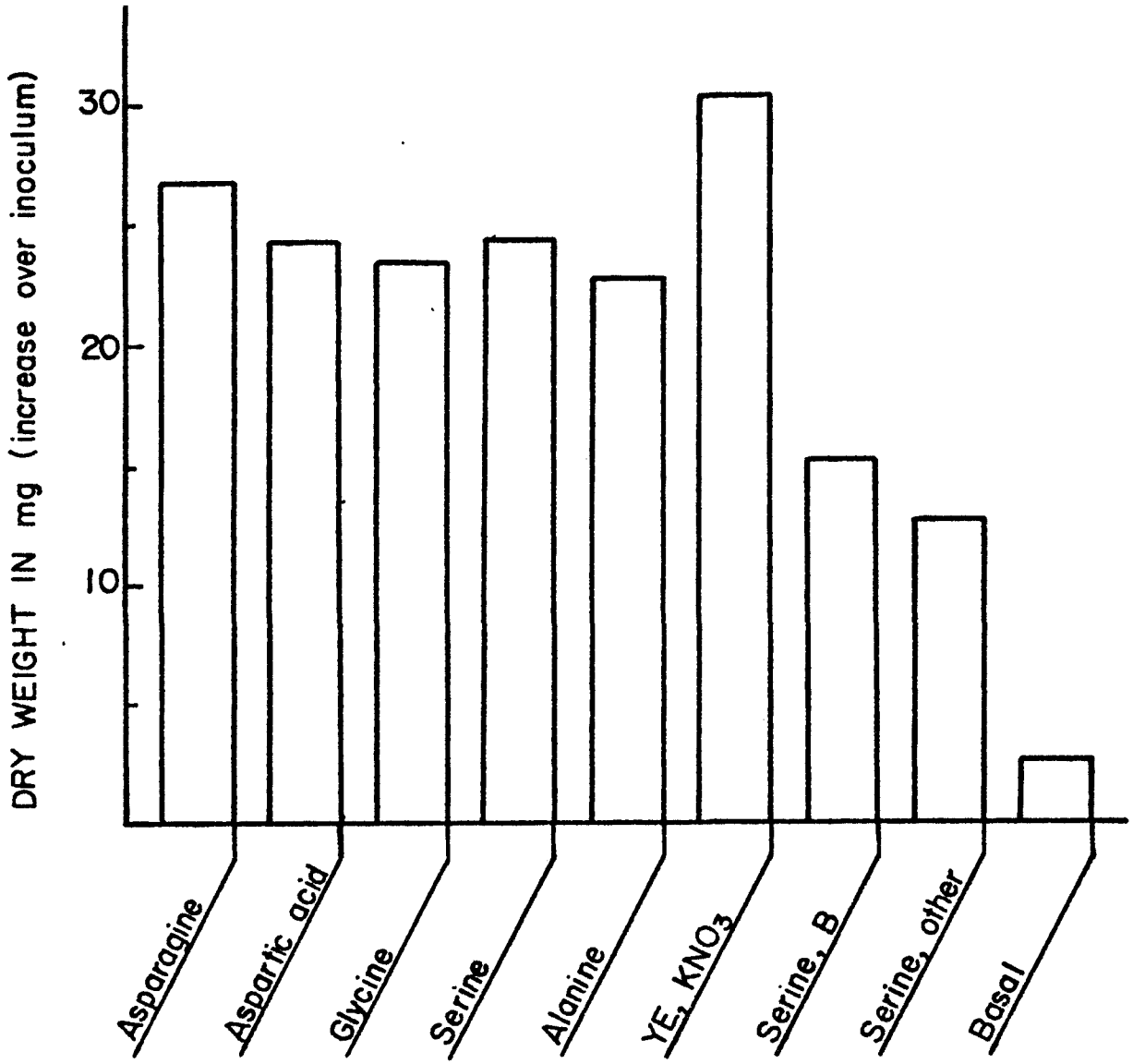


Figure 3.. A comparison of growth in chemically defined media (Glucose substrate).

reduced in the chemically defined medium with only 15.5 mg as the maximum obtained as compared to 38.5 mg obtained with potato dextrose broth (Figure 4).

pH Requirements for Culture

Determinations of the optimum pH (Figure 4) for growth of E. deformans isolates were performed in the three commercially available broths and the chemically defined medium and all showed a distinct optimum growth at pH 4.0 - 4.5. The dry weight values varied, but all dropped rather sharply after maximizing at the 4.0 - 4.5 value.

Temperature Relations in Culture

The temperature tolerances for E. deformans were shown by the 27-day incubation of flasks at six different temperatures. The highest temperature at which growth was obtained was 30°C and the lowest was 8°C. The optimum growth was obtained at 15°C. Upon alteration of the ranges between the observation of growth and the absence of observation of growth, a high temperature tolerance of 34°C was obtained, as at 35° the fungus did not survive. The lower limit for growth was 10°C where growth was observed with the fungus tolerating 8°C and surviving. The rate of growth at either of the extremes was exceptionally slow.

Determination of Indoleacetic Acid

The indoleacetic acid (IAA) content of normal and infected needles was determined as well as any IAA produced in culture by the fungus E. deformans. The reference values in Table 8 were determined and the solvent systems were run yielding good results. There was no IAA detected by these methods in the cultures or culture filtrates; however,

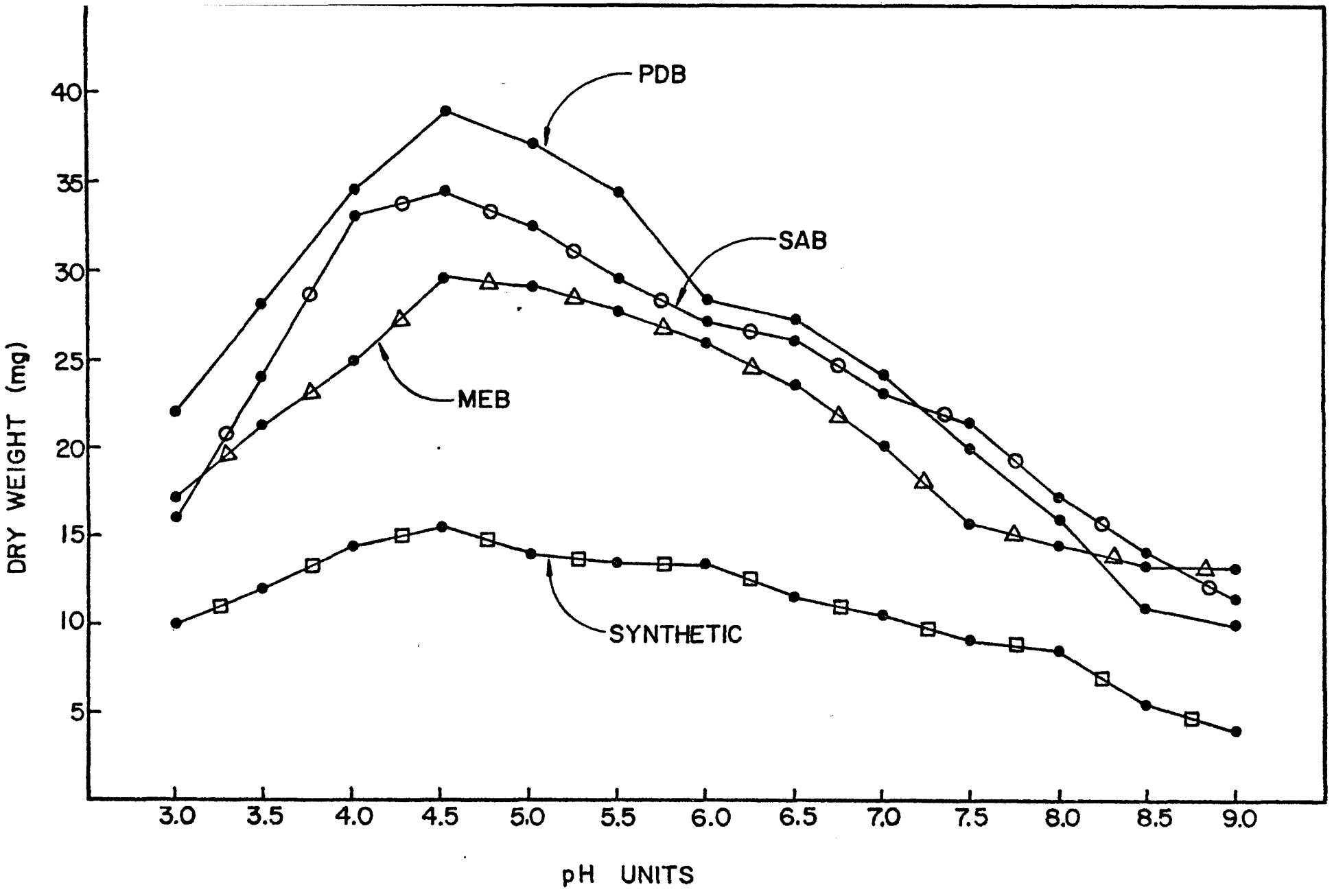


Figure 4. Dry weight of mycelium obtained from broth cultures maintained

TABLE 8. Rf values of indole compounds.

Compound	Reference Rf	Experimentally Determined Rf	Color Salkowski Reactions
Indole	0.79	0.78	Red
Indole-3-acetic acid	0.25	0.23	Red
Indole-3-propionic acid	0.35	0.38	Yellow
Indole-3-butyric acid	0.44	0.41	Yellow

(Block, Durrum, and Zweig, 9)

the infected needles showed a heavy spot of IAA as compared to the absence of any such spot in the uninfected needle extracts. The color reactions were deep red and yellow as those described in the literature. There were no indications of indole, indole propionic, or indole butyric acids by the above method. All extractions were run in the absence of light and in the cold to minimize oxidation.

The production of indoleacetic acid in culture was not detected by the extraction and chromatographic methods employed in this study. There were no derivatives in the indole series detected, such as indolebutyric, indoleproprionic acid, or indole itself as measured against known standards employed.

Oxygen Requirements

In the analysis of oxygen requirements, it was observed that the fungus would neither tolerate nor grow in the absence of oxygen. The controls were characterized by a very heavy overgrowth of the plates of medium in the same length of time. The plates, when removed from the

environment lacking oxygen, showed no evidence of growth over the original inoculum.

Warburg Studies

The metabolic studies on carbohydrate metabolism can be seen from the following figures and tables. The optimum utilization of glucose by the fungus was observed at pH 4.5, with the pH values closest to this being next most active. The endogenous respiration in all cases was observed to be fairly high. When the temperature was increased from 24 to 34°C, there was a noticeable change in the rate of utilization of glucose. The rate was almost doubled giving rise to a demonstration of the Q_{10} effect.

The metabolic studies on nitrogen utilization showed that asparagine was utilized to a great degree. The results obtained upon the increase of the temperature 10 degrees did not show a doubling of the µl of oxygen uptaken. The pH of 4.5 was the optimum pH value for the utilization of asparagine. The endogenous was not as active in some of the runs in the nitrogen utilization series as it had been in the carbohydrate metabolism series.

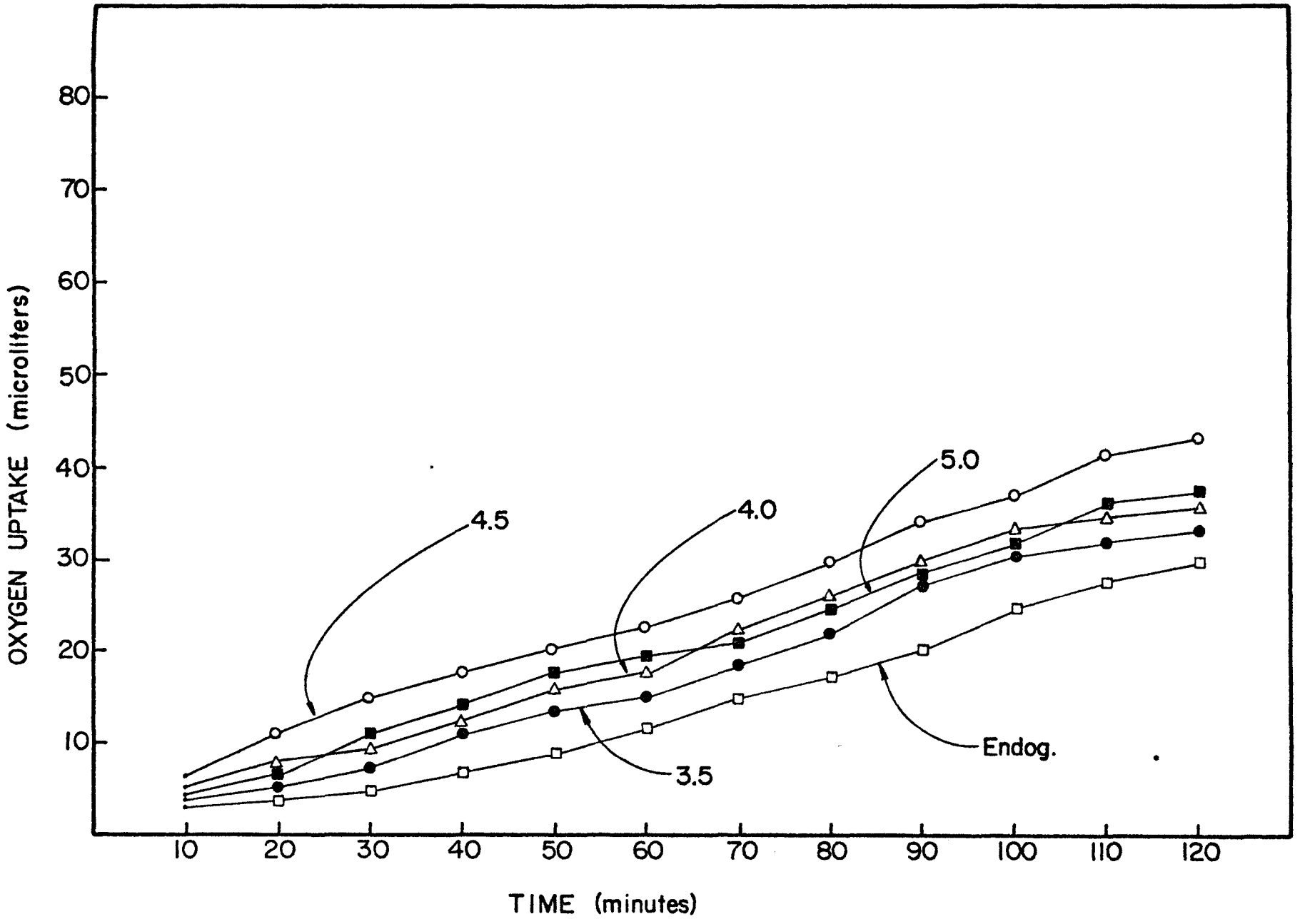
Hydrolytic Enzymes

Gelatinase was produced by the fungus, as evidenced by the wide zones of clear areas after the addition of mercuric chloride solution. The fungus penetrated the agar to a greater degree, and there was a greater area cleared in the lower pH value of 5.8 as compared to that at pH 7.0.

Deoxyribonuclease was also found to be produced by E. deformans. In the series of DNase test media, growth at the lower pH exhibited a

TABLE 9. Oxygen uptake with glucose substrate at 24°C and 34°C.

Time in Minutes	pH Values									
	3.5		4.0		4.5		5.0		Endogenous	
	24°	34°	24°	34°	24°	34°	24°	34°	24°	34°
10	3.3	6.7	4.0	5.9	5.1	9.0	3.4	5.5	2.7	4.8
20	5.0	8.2	7.1	9.4	10.4	14.8	6.6	7.4	4.0	6.1
30	7.6	10.1	9.9	15.5	14.8	16.3	10.7	11.2	5.1	7.2
40	11.1	12.6	12.6	18.2	18.0	24.4	14.1	14.4	7.4	9.3
50	13.8	15.2	15.9	27.3	21.7	33.0	17.7	20.1	9.6	12.5
60	15.3	19.0	18.5	31.9	23.2	38.7	19.5	24.4	12.7	14.0
70	18.9	22.6	22.1	35.4	26.8	44.3	21.3	29.7	15.1	17.3
80	22.8	27.9	26.2	39.8	30.0	48.9	25.2	33.8	17.9	21.4
90	27.5	31.3	29.8	44.2	34.7	53.0	28.7	39.4	21.8	24.0
100	30.8	38.4	33.2	48.9	37.3	62.1	32.2	45.2	25.5	27.8
110	32.5	44.8	34.9	53.5	41.2	70.6	36.0	51.1	28.9	30.6
120	33.4	52.9	36.5	61.7	43.0	74.7	37.4	55.7	30.4	36.3


OXYGEN UPTAKE (microliters)
80
70
60
50
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TIME (minutes)
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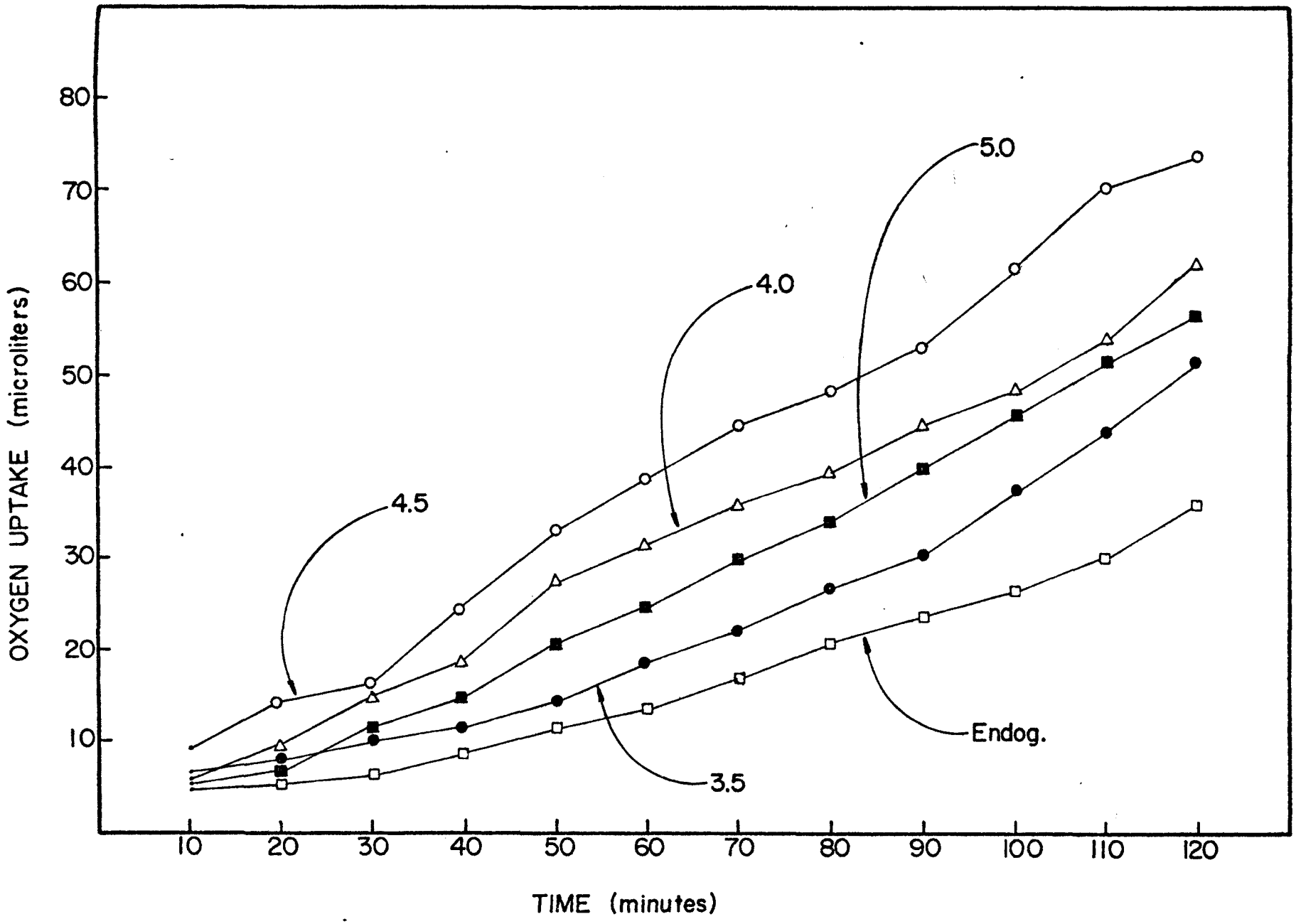

Figure 6. The oxidation of glucose by Elytroderma deformans at 34°C.

TABLE 10. Oxygen uptake with asparagine substrate at 24°C and 34°C.

Time in Minutes	pH Values									
	3.5		4.0		4.5		5.0		Endogenous	
	24°	34°	24°	34°	24°	34°	24°	34°	24°	34°
10	4.4	4.9	4.8	5.1	5.7	5.9	4.9	5.2	3.8	3.9
20	5.0	5.9	6.0	6.8	7.5	8.8	6.3	7.5	3.8	4.2
30	5.3	8.5	6.9	9.0	9.1	12.3	7.6	10.1	3.9	4.9
40	6.1	10.4	7.9	11.9	10.6	18.2	9.4	13.4	4.5	6.0
50	7.9	13.8	9.4	15.3	13.3	23.1	11.7	17.2	4.9	7.2
60	9.2	15.0	11.6	18.2	18.4	27.9	13.5	20.5	5.4	8.5
70	11.0	16.9	14.0	21.1	22.1	33.0	17.0	24.9	6.0	10.0
80	12.1	19.3	16.1	23.3	25.7	38.1	18.9	28.8	6.9	11.9
90	13.8	21.6	19.2	27.2	28.2	43.2	22.4	32.9	7.3	13.2
100	16.9	23.9	21.6	31.7	30.3	47.4	24.9	37.5	14.1	14.1
110	18.8	27.1	23.7	35.9	32.9	50.5	26.8	43.7	15.3	16.2
120	20.5	30.4	26.2	39.4	34.8	53.2	29.6	45.9	16.8	17.7

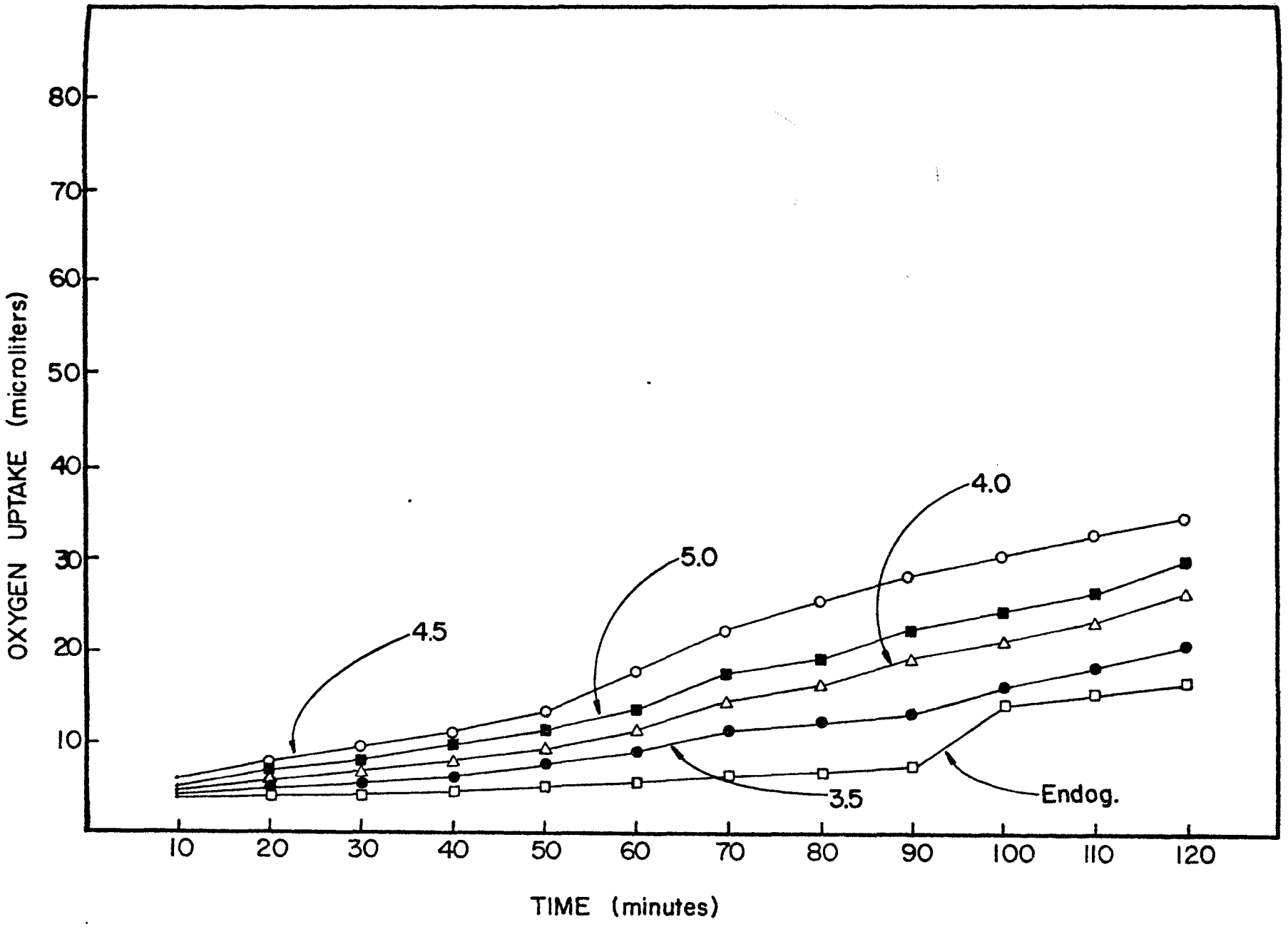

Figure 7. Oxygen uptake by Elytroderma deformans on asparagine at 24°C.

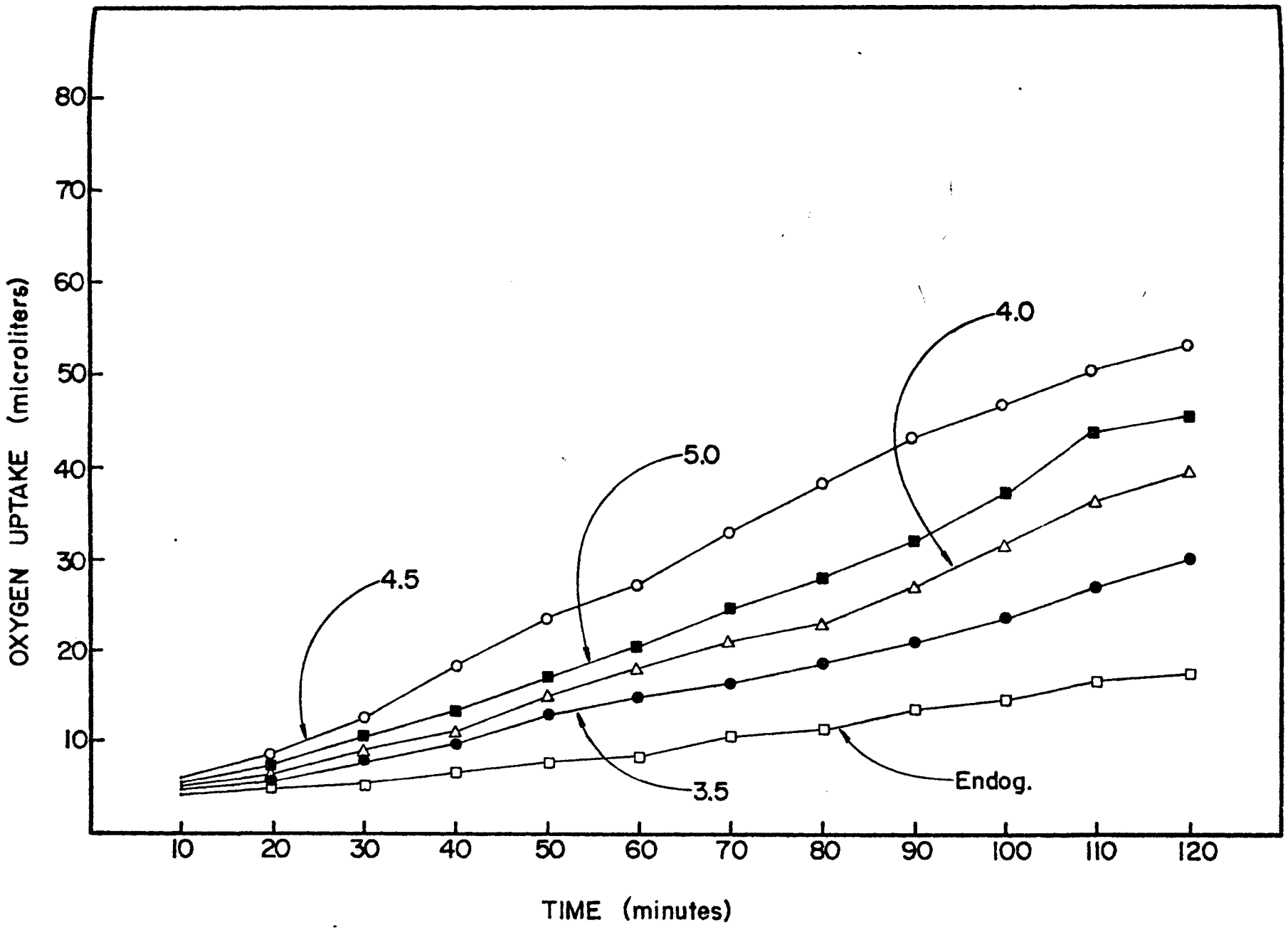

Figure 8. Oxygen uptake by Elytroderma deformans on asparagine at 34°C.

TABLE 11. Effect of inhibitors on oxygen uptake at 24°C.

Time in Minutes	Cyclohexamide Inhibition Glucose Substrate; 24°C; 100 ppm					Phytoactin Inhibition Glucose Substrate; 24°C; 1 ppb				
	pH Values					pH Values				
	3.5	4.0	4.5	5.0	Endogenous	3.5	4.0	4.5	5.0	Endogenous
10	4.0	4.9	5.2	4.2	3.9	5.1	5.5	5.7	5.0	4.9
20	5.4	7.2	8.4	6.1	4.9	5.9	7.8	9.9	7.1	5.3
30	7.0	8.9	10.6	7.9	5.2	7.2	10.1	14.0	9.2	6.2
40	8.1	10.1	12.7	9.2	5.9	9.2	12.1	18.4	10.8	7.9
50	8.7	10.7	13.6	9.8	6.3	10.8	15.2	21.3	13.1	8.9
60	9.1	11.0	14.0	10.2	6.5	13.1	18.0	24.2	15.9	10.3
70	---	---	---	---	---	13.9	19.2	26.1	17.1	11.2
80	---	---	---	---	---	14.2	20.5	27.0	17.8	11.7
90	---	---	---	---	---	---	---	---	---	---
100	---	---	---	---	---	---	---	---	---	---
110	---	---	---	---	---	---	---	---	---	---
120	10.6	12.2	15.4	11.4	8.2	15.9	22.3	29.1	19.5	13.0

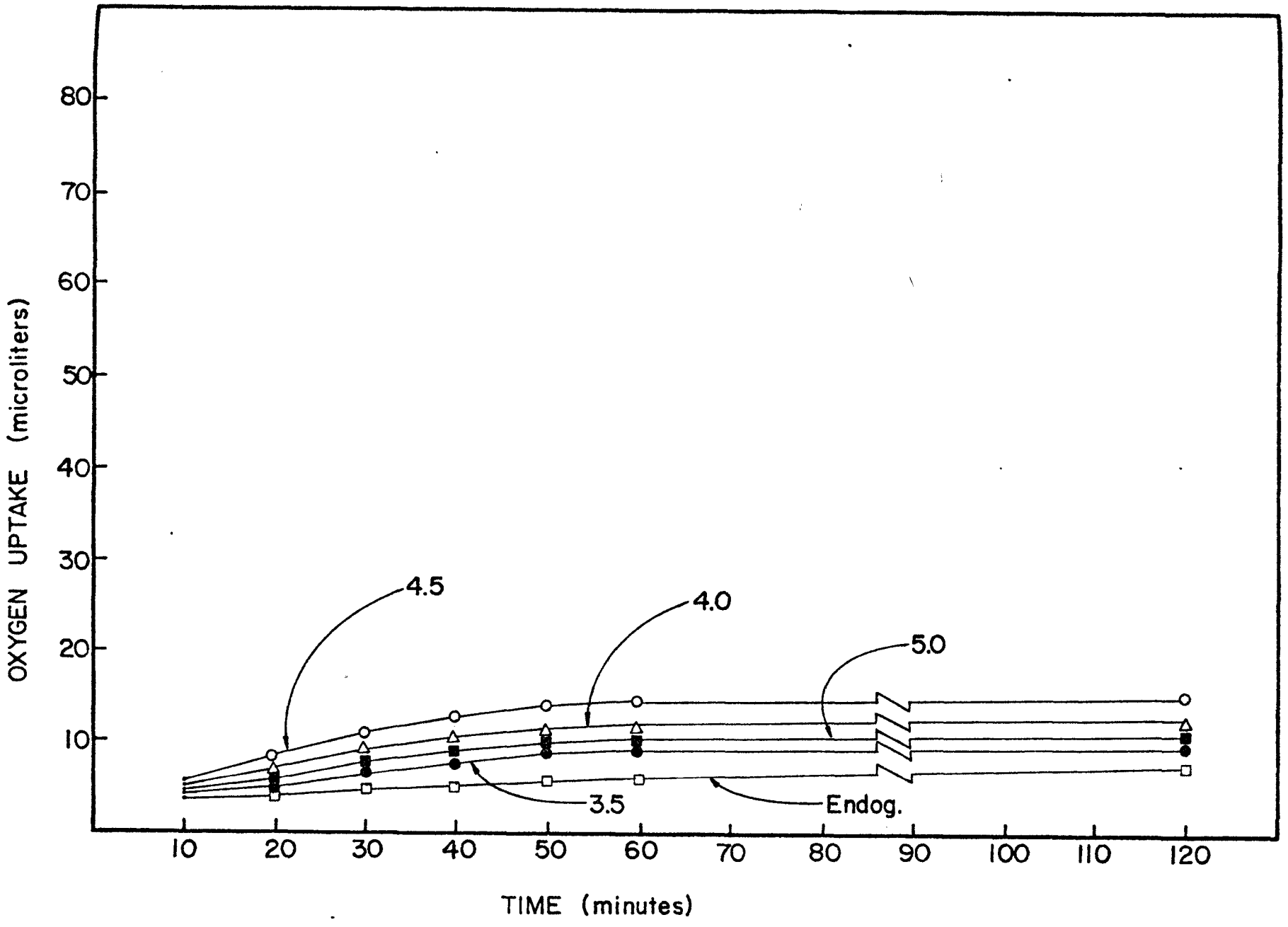

Figure 9. Cyclohexamide inhibition; glucose substrate; at 24°C.

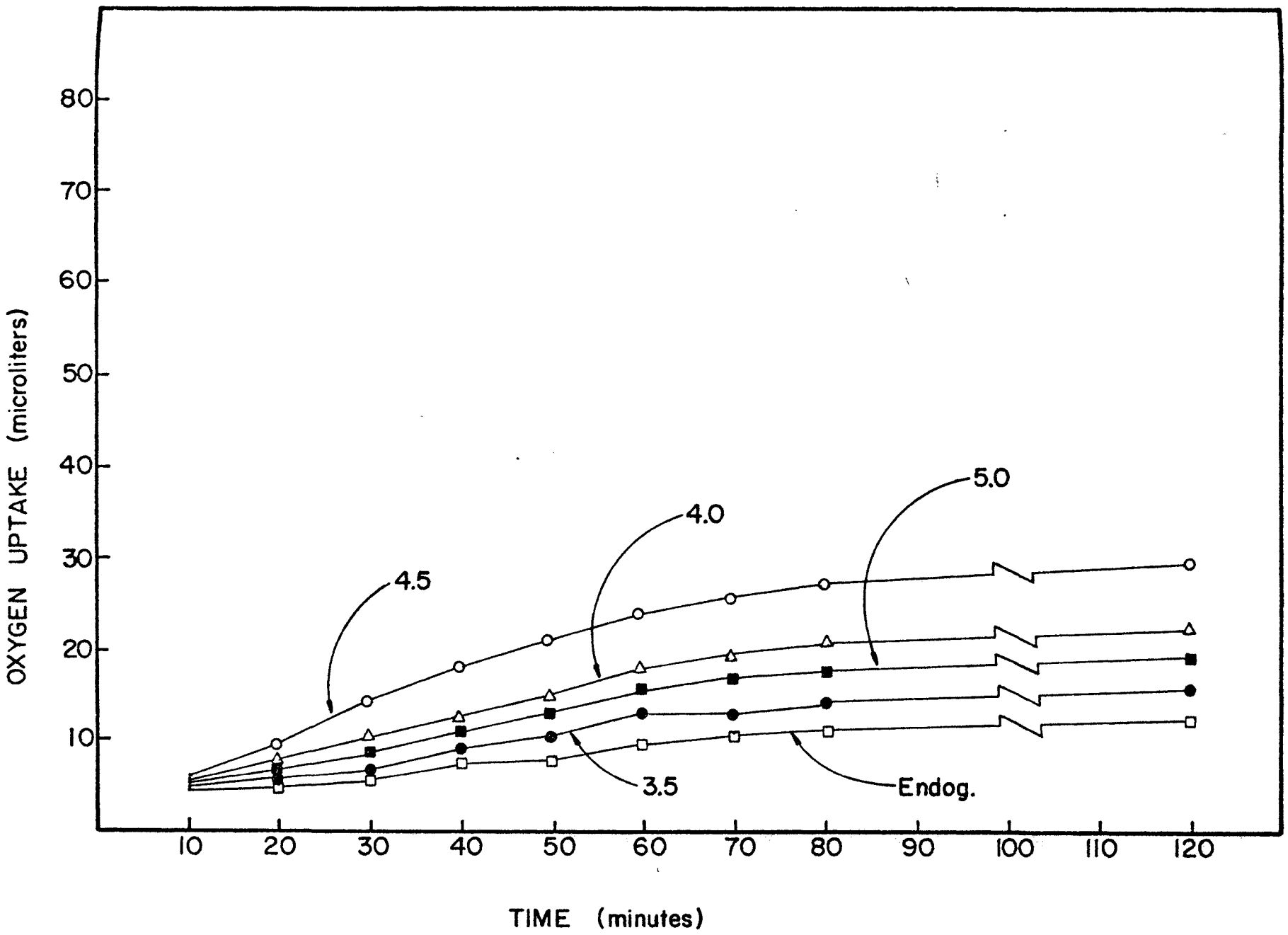

Figure 10. Phytoactin inhibition; glucose substrate; at 24°C.

greater degree of clearing of the agar medium around the growth than was observed at the higher pH value of 7.0. More clearing was observed around the growth of the mycelium in subsurface growth than near-surface growth.

Amylase was also found to be produced by E. deformans. The areas of hydrolyzed starch were also observed to be greater at a greater depth from the surface of the medium. The lower pH of the two employed, 5.3, was found to show the greatest degree of starch hydrolysis as measured by hydrolytic areas after the addition of the iodine.

Nitrate was reduced to nitrite by the fungus. There was no second series with which to compare the efficacy of nitrate reduction, as only one was utilized and that was adjusted to pH 4.5. The characteristic positive test was obtained showing the reduction to nitrite.

Urease was also found to be produced by the fungus. The pH of the medium was adjusted to pH 4.5 and no second series was prepared for comparison of urease production at a higher pH value. The characteristic positive test (color change from yellow to red) was observed.

Soil Survival

The survival of E. deformans in soil was not found to be great. Soil pH was adjusted over a range and at none of these pH values did the fungus survive for a very long period of time. There was no growth obtained from the rehydrated soil samples after the third day of sampling. There was no variation in the survival time at any of the pH values as measured by these methods.

Effects of Desiccation

The effects of desiccation were found to be severe upon E. deformans. Of the three methods utilized, desiccation by vacuum, desiccation by desiccant, and desiccation by extended filtration, the last method proved the most favorable to the fungus. The fungus was not found to be viable after exposure to vacuum for 12 hours nor was it found to be viable after exposure to a desiccant and vacuum for 12 hours. After 3 hours of drying by Büchner funnel, the fungus survived for at least 3 days.

TABLE 12. Effects of desiccation on E. deformans.

Method	Time in days			
	1	3	7	14
Vacuum	-	-	-	-
Desiccant	-	-	-	-
Air dry (funnel)	+	+	-	-

+ = growth after desiccation treatment
- = no such growth

Inhibitor Effects

The effects of the inhibitors phytoactin and cyclohexamide on E. deformans were found to be most pronounced after 60 minutes of exposure. The efficacy of the inhibitors did not appear to be affected by pH. Very little oxygen was utilized after 60 minutes of exposure, as can be seen from Figures 9 and 10.

DISCUSSION

It can be seen from the results in Table 5 that chloroform and ether extracted some factors which facilitated isolation and subsequent growth of E. deformans from the needle sections. The water extract provided almost as good an isolation medium as did the above two extracts; however, the ether extract proved considerably better in isolation than did the water-extracted ether. The acetone and benzene extracts also proved poor additives for isolation and recovery of the fungus from the needle sections.

Although chloroform and benzene both have a very low water solubility, results observed with these differed widely inasmuch as the chloroform extract supported a higher number if isolations than did the benzene extract. Ether and acetone are similar in that they are both more soluble in water (7.5 g/100 ml and ∞g/100 ml at 25°C, respectively), however differ in the resulting isolation and recovery capacity. It is possible that the amount of residual solvent remaining could be toxic to the fungus or that the extracts were not providing the same chemical extractives. In the latter case it is probable that the solvents employed extracted different materials selectively, and that a growth-enhancing factor appears to be provided by the chloroform, ether, and water extracts which was not present in the other systems. Alcohols, benzenes, and acetone give a more complete extraction of the phenolic compounds (Buchanan, 13). The results in Table 5, where benzene and acetone extracts failed to facilitate isolation and subsequent growth

of E. deformans to that extent observed in the ether and chloroform extract additives, suggest that the mechanism for inhibition may have been the presence of phenolic compounds in such concentrations as to be inhibitory, as these are reported to be strongly fungistatic and fungicidal (Dimond, 20).

In the extraction procedures employed, there are many chemicals which would be extracted from needles and which might influence the growth of the fungus. Among these factors would be the volatile oils, resin acids, fatty acids, flavonoids, lignins, nitrogenous compounds, aromatic acids, aldehydes, and alcohols.

Among those factors extracted which would be known to influence the growth of E. deformans would be the carbohydrates. Despite the fact that the major carbohydrate components are non-extractable cell wall components, certain soluble substances and some materials which appear to be extraneous components are present in many species of pine. The sugars and glycosides transported by the sap are present in sufficient quantities as to be extractable. Sucrose, glucose, and fructose are common in the sapwood, heartwood, and the needles of many pines (Smith and Zavarin, 74). The function of these carbohydrates would be primary energy sources for the fungus and have been shown to be utilized in laboratory culture media.

Among other factors known to influence the growth of E. deformans was the steam distillate. Results of the addition of the steam distillate to the PDA medium suggested a stimulatory effect by that addition, as the growth was heavier and more rapid than that observed on the control PDA plates.

Among those substances known to be extractable, but whose influence on the growth of the fungus is unknown, are the polyhedric cyclohexanes. These have been reported in literature in the usual alcohol or acetone extractions because of the presence of some water, as they are insoluble in anhydrous alcohol or acetone (Browning, 12). Certain phenolic substances are transported by the sap, and small amounts are present in the sapwood. Some phenols are extracted with benzene and thus accompany the fats and resin acids.

Other substances whose influence on the fungus is unknown are the fats. Although in plants the fats are normally concentrated in seed tissues, appreciable amounts of fatty acids are obtained upon saponification of ether or benzene extracts of many species of pine. The predominant acids in pine and at least one species of spruce are oleic and linoleic, with small amounts of linolanic acid reported (Buchanan, 13; Inglett, 37). The resin acids normally occur as free acids, and they do not esterify with alcohols. Levopimaric acid is the chief acid in pinewood oleoresin and abietic acid is the predominant resin acid in the rosin of pine (Buchanan, 13). Data published on the composition of oleoresins and rosins are somewhat variable owing to the appreciable variation between samples and different methods of analysis. The acids in Table 13 show graphically the contrast of the literature reports.

In the volatile oils of the needles, the constituents consist of terpenes and related compounds (which will be discussed in greater detail below), paraffin compounds, and aromatic compounds. Mirov (51) has compiled data on the composition of gum turpentines from the oleoresin of pines. Oleoresin from Jeffrey pine and digger pine are unusual

TABLE 13. Composition of rosin samples[a]

Acid	Gum Rosin Lawrence[b]	Gum Rosin Genge[c]
Abietic	20	33
Palustric	17	05
Neoabietic	17	14
Dehydroabietic	05	06
Isopimaric	--	18
Pimaric	--	05
Dihydropimaric	--	<01
Dihydropalustric	--	<01

[a] All values in percent

[b] Lawrence (44)

[c] Genge (30)

in that they contain n-heptane as the major volatile constituent (Mirov, 52). Small amounts of n-heptane and n-decane have been found in the turpentines of other pines (Mirov, 52), and small amounts of octyl, nonyl, and decyl aldehydes are present in some turpentines. The influence upon fungal growth of these compounds is not known.

It can be seen from the foregoing discussion of extractable components that there is (1) a marked variation in the types of compounds extracted, (2) a variability in the isolates with respect to the capacity to exist and to grow well on artificial media, and (3) a need for further exploration before compounds are actually identified on the basis of their chemical properties and their efficacy in facilitating isolation of E. deformans.

The determination of pH of the needle homogenate was undertaken to detect whether variations in pH throughout the year may influence, in some manner, the growth of E. deformans in vivo. The pH was observed to vary throughout the study. The observed drop in pH would be expected as the seasons progress to colder temperatures, with the concommitant decrease in water content. It was found by Thimann (80) that more acidic components increased from October to December. The chemicals showing the greatest increase in concentration were sucrose, aspartic acid, glutamic acid, and other organic acids. The variation, then, appears to be in direct relation to the variation of the chemical constituents as found in nature (i.e., a decrease in the water content of sap leading to an increase in acidity and in concentration of the chemicals present). The change in pH may also be correlated with the chemicals present as synthesized by the tree either normally or in

response to the infection, and/or as synthesized by the tree and altered by the pathogen. The latter situation obviously may give rise to metabolic by-products which may result in changes in pH. It will be observed that the values of the infected needle homogenates were considerably higher than those of the uninfected needle homogenates throughout the sampling study. It must be observed that the method of pH determination is an acceptable one previously utilized (Gordon, 31; Stamm, 78); however, the pH is measured on a homogenate, not with a pair of electrodes positioned intercellularly or intracellularly. The resultant value is only a reflection of the ionizable compounds present in the needle.

An important consideration is the increase in acidity during homogenizing caused by the hydrolysis of acetyl groups found in the glucomannans and the xylans of the plant cell walls. These groups would be hydrolyzed to acetic acid in the presence of water. The pH of the extract may be as low as 3.5 in water extracts and could be similar to values obtained after extracting with a weakly acidic solution. Small amounts of hydrolysis products of polysaccharides and lignan appear in solution also (Browning, 12). Because the techniques were standardized these effects, if present, would probably have been expressed in both uninfected and infected needle mixtures, however. Therefore, the difference in pH between the two types of homogenates could be assumed to be real. The correlation between pH values of the homogenates and the pH values demonstrating optimum growth in laboratory culture media by E. deformans was highly positive.

The growth of E. deformans observed upon initial culturing suggested that a requirement was present, and that vitamins were possibly being

transferred in the inoculum. The latter possibility was considered to be less significant than the inoculum factor. The mycelium was therefore repeatedly transferred in vitamin-free chemically defined medium in order to eliminate, by dilution to extinction, any growth factors transferred by the inoculum. The results were such that the nature of a vitamin requirement, if any, could be explained in two ways. It is assumed that a fungus which is independent of an externally supplied vitamin is so due to its ability to synthesize that vitamin. In contrast to this explanation is the fact that a fungus may indeed be able to synthesize a vitamin, but at such a slow rate, that under the usual conditions of culture, the rate of all other processes is potentially faster than vitamin synthesis. In this case, the fungus obviously grows in the absence of an external vitamin supply, but responds to an external supply by a more rapid rate of growth. The latter illustration appears to be the effect observed with E. deformans. There are many other examples in literature where growth is stimulated upon the addition of such vitamins as thiamine (Blumer, 11; Fries, 28), biotin (Lindeberg, 48; Robbins and Ma, 68), and nicotinic acid (Fries, 28; Robbins and Ma, 68).

The addition of the B vitamin series (biotin, pyridoxine, thiamine, riboflavin, and niacin) was considerably more stimulatory to growth as measured by dry weight increase over inoculum than was the addition of the remaining vitamins (folic acid, inositol, calcium pantothenate, and para-aminobenzoic acid). When vitamins were added individually a stimulation of growth was also observed; however, it was not near the magnitude observed upon the addition of the complement of the vitamins (the B series). It was concluded, then, that the vitamin requirement was of

a partial nature, i.e., E. deformans has the ability to synthesize all required vitamins in the absence of any external source, but the synthesis is slow enough that the addition of vitamins resulted in a stimulation of growth.

E. deformans demonstrated no amino acid requirement, despite the fact that such requirements are common in other micro-organisms reported in the literature, and appear with reasonable frequency in mutants. Cochrane (16) stated that such requirements are rare in nature and more common in laboratory isolates, and are partial requirements rather than absolute requirements. It is thought that, in general, the catabolism of amino acids is directly related to the amino acid oxidases and amino acid dehydrogenases which have been demonstrated to occur with frequency in fungi (Bentley, 8; Fincham, 25).

It was found that amino acids, especially asparagine, provided better growth than did inorganic nitrogen sources. The nitrate produced somewhat slower growth than did the ammonium nitrogen; however, this could be accounted for by a preferential utilization of the ammonium ion. Cochrane (16) found the same result with Neurospora crassa, and stated that when there was a utilization of ammonium nitrate in culture media, there was a corresponding drop in pH indicating an initial usage of the ammonium ion only. Morton and MacMillan (57), from their work on Scopulariopsis brevicaulis, stated that the nitrate utilization did not begin until the ammonium ion had been virtually removed from the medium. Further studies by Morton (56) indicated that when an available carbon source was present, nitrate utilization was stopped completely by the addition of ammonium salts. Attempts have been made to explain

the preferential utilization of ammonium ion as a phenomenon of permeability or as based upon the absorption capacity of cell colloids (Cochrane, 16). It would appear that the effects of external pH values as well as the pH values required for optimal activity of nitrate reductase or the toxicity of nitrate ion must also be considered.

The optimal pH range for growth of E. deformans in laboratory media was 4.0-4.5. Values within 0.5 units on either side yielded nearly equal amounts of growth. In direct contrast to bacteria and actinomycetes, the fungi are relatively more able to invade an acid environment. In culture, the larger basiodiomycetes are often unable to grow at pH values higher than 7.0 (Norkrans, 61). Most plant pathogens grow best in media with an initial pH of 5.0-6.5 (Cochrane, 16), although species of Streptomyces are marked by a relative intolerance to acidity. Other pH ranges optimal for growth may extend well below the average, e.g., Fomes annosus 4.6-4.9 (Jensen, 39), Phycomyces blakesleeanus 3.5-4.6 (Farr), and Geotrichum sp. 3.0 (Painter, 62). The principal effects of external pH on growth of fungi are thought to be related to permeability and other surface phenomena (Cochrane, 16).

The seasonal variation in the number of successful isolations was found to be in association with several factors. The results in Figure 1 suggest that the increased proliferative activity of the fungus is partially responsible for the high number of successful isolations from the spring needle sections. The maximum number of isolates was made at the time of the appearance of new needles and for a period of 4 weeks following the breaking of the young needles from the fascicle. The time required for growth from the needle in culture was also observed to be

the shortest at this time, also suggesting that the more active the fungus in vivo, the shorter the time required for the fungus to appear on laboratory media. Also, in the late spring, the mycelium was more extensive and had destroyed more needle components which might hinder its growth from the younger needle sections. The difference in pH was also observed to be a factor, as the younger needles had a lower pH value than did the older ones. This might correlate well with the pH found optimal for growth of the fungus in cultures on laboratory media. Another factor possibly related is the variety of chemicals translocated as metabolites for the plant, but which may also enhance growth of the pathogen at the site of the infection. Other factors influencing the activity of the host also could be stimulatory to the pathogen, among these being the translocation rates and rates of photosynthesis providing pentose sugars and other plant nutrients.

The steam distillate was extracted according to the procedure of Mirov (53) in an attempt to isolate the fraction which would be most responsible for any influence in the growth of the organism. The results of gas chromatographic analysis of the extracted steam distillate showed a difference in the amounts of the compounds present in the infected needles and in the normal, uninfected needles. This result might suggest that there was an alteration of these components by the fungus or that the synthesis was affected to a demonstrable degree by the infection. Six peaks were obtained in each case and compared with the results of terpene analysis by Mirov (53) and Smith (76). (See Table 14).

Both authors then agree that d-Δ_3-carene, β-pinene, limonene, and myrcene are present in the greatest amounts; however, the remaining

TABLE 14. Amount of monoterpenes[a] in oleoresin of Ponderosa pine.

Terpene	Smith (53)	Mirov (76)
d-Δ_3-carene	36.2	47.0
β-pinene	26.4	25.0
limonene	14.5	6.0
myrcene	13.3	5.0
α-pinene	6.3	3.0
β-phellandrene	1.8	-
terpinolene	-	4.0
longifolene	-	2.0
cadinene	-	5.0
unknown	1.5	-

[a] In percentages

terpenes may also be formed. The differences in Table 7 are suggestive of terpene concentration being altered in some way by the fungus. Most oleoresin is produced in the thin-walled epithelial cells surrounding the resin canals in the sapwood, and it is thought to be the same in the needles. Droplets may be found in any living parenchyma cells of the pine (Mirov, 54). E. deformans infection appears to alter the amount of terpenes present by reducing the total quantity as compared with that of the uninfected needles. There are also quantitative changes, both increases and decreases, among the individual terpenes. This alteration may be the result of degradation of the terpenes by the fungus for utilization or as a result of the action of its metabolic by-products upon the terpenes. Because E. deformans does considerable damage mechanically in the needle, it therefore may interfere with the synthesis of terpenoids either by physical damage to the synthesizing sites, or by altering those chemicals and enzymes necessary for the synthesis. A time-course sequence was not determined for the quantitative amounts of terpenes from the time of infection in young needles (immediately after bursting from a fascicle) to show whether the infection interferes with the terpene content initially or only after a period of time, which would therefore suggest mechanical damage as opposed to chemical damage.

Growth of the pathogen at cardinal temperature points showed that the highest temperature at which the fungus would grow was 30°C, while it would tolerate 34°C without showing any lethal effects as detected by viability in malt extract broth after exposure to this temperature for 28 days. The lower limit for growth was 10°C. Tolerance of 8°C

and possibly lower indicates that the fungus is well adapted to survive a cold environment. Freeland (27) found that Pinus sylvestris and P. nigra still photosynthesize at -6°C, and respiration was detectable down to -19°C. Hepting (34) found that there was a considerable accumulation of carbohydrates in P. schinata over the winter. Inasmuch as the tree has lower water contents and increased chemical concentrations resulting from this, the freezing point may be lowered, thus preventing any ice crystal formation, and the fungus may be protected. Overwintering in this way has been observed in basiodiomycetes and actinomycetes (Parkinson and Waid, 63).

No IAA was detected in cultures of E. deformans in malt extract broth at pH 4.5. IAA was detected in the extracts from infected, but not from uninfected needles. One explanation for the failure to detect any IAA in the uninfected needle extracts could be in the action of IAA oxidase. However, precautions were taken to protect the extracts from light which has been shown to activate IAA oxidase (Hare, 33). In addition, the Rf values observed and the positivity of the Salkowski reaction make the result with infected needles certain enough to eliminate any other compounds. The IAA detected in the infected needle extracts could have come from normal synthesis in the plant, although lack of detectable IAA in the uninfected needle extracts would appear to contradict this. Abnormal synthesis may be a factor, such as that suggested by Shaw (70) in which IAA may be produced at the site of the infection either by the infecting pathogen or by the plant in response to the pathogen. Yarwood (88) suggested that the IAA detected at the site of the infection in rust might be the cause of the brooming. Further study

utilizing labelled precursers for IAA might well be employed to determine the origin of the IAA detected in the infected needles.

The all-or-none oxygen requirements were determined to learn if the organism would function in an anaerobic environment. The growth obtained in control plates indicated that the fungal inoculum was viable, but that it was unable to grow in the complete absence of oxygen. The quantitative oxygen requirements were not determined and should be investigated in further studies.

The metabolic studies with the Warburg apparatus demonstrated greatest respiratory activity of E. deformans at pH 4.5. This would correlate well with the value previously shown to exist in young needles, and to be the optimum for growth of the organism in laboratory media. A Q_{10} effect was demonstrable as it has been among other fungi (Nickerson and Edwards, 60).

In the respirometric studies of nitrogenous compounds asparagine was utilized as the substrate and the resulting uptake of oxygen was somewhat the same as that obtained with the hexose substrate. A test of the reactants after the Warburg run was performed in an effort to detect the presence of ammonia; however, there was none, indicating that most of the amino groups had been incorporated into the organism or oxidized.

Gelatinase, DNase, and amylase activities were all shown to be present in E. deformans cultures. At least the latter two of these enzymes could be of excellent value to the fungus in tissue destruction in the needle creating a supply of nutrients therefrom. In every case the zones of clearance in the positive tests were larger at the lower pH. This fact would suggest that greater metabolic activity can occur

at lower pH values although no optimum value was determined. The absence of a positive test for cellulase was considered to be a result needing further exploration, as the cell walls of the transport system are destroyed by the fungus and the primary component of the walls is cellulose. Acidic metabolic by-products may alter the cellulose structure by hydrolysis to allow more degradation. Destruction through pressure may also be a factor as the fungus grows extensively during the second year of infection within the needle.

Survival of E. deformans in soil was found to be relatively poor. The fungus most probably utilized intracellular metabolites until their depletion, when the cells then lost viability. The pH of the soil was adjusted to values found more favorable for growth of the organism; however, it was felt that if the mycelium had sporulated, the spores were no more capable of surviving in the soils than the hyphal cells.

The results of the desiccation studies (Table 12) showed a correlation between the efficacy of water removal from the mycelium and the survival of the fungus. In nature, the fungus would be surrounded by the needle with the cellular structures mechanically protecting the fungus. The water and solutes within the needle may provide additional protection even after casting from the tree. The fungus has been observed to withstand the effects of lyophilization; however, this method is judged to be less severe than the desiccation treatments employed in this study.

Inhibitors employed in this study were effective in limiting the amount of oxygen utilized after 60 minutes of exposure. Cyclohexamide was found to be inhibitory at a concentration of 0.4 ppm and phytoactin

at 0.8 ppm. These concentrations are well below those used for the Forest Service foliar sprays between 1961 and 1965 (Dimond, 20). Assays indicated that the amount of phytoactin sprayed dropped from 4 ppm to 2 ppm after one year and to 1 ppm after 2 years. With radio-active labelling of the phytoactin, it was possible to demonstrate the fungicide in mature needles less than 2 years of age. The yearling needles and the mature needles 3 years of age were not found to accumulate phytoactin (Dimond, 20).

The foliar sprays were applied to determine the efficacy of the inhibitor upon blister rust, and not on a systemic pathogen of the E. deformans nature. The only comparison that can be drawn between the two infections is that the inhibitors acted upon the mycelial stage rather than a spore form of the pathogen. The spore stage of the rust fungus was found to be resistant to 10 ppm of phytoactin in the forest applications (Dimond, 20), while the mycelial stage was resistant to only 0.1 ppm. In the present study, the mycelial stage of E. deformans was found to be resistant to concentrations below 0.8 ppm. The tissue in the canker of blister rust, if killed by an inhibitor such as the two employed in this study, limits the spread of the disease. The nature of E. deformans is such that the mycelial stage is the most important to inhibit from the epidemiological standpoint, as the spores are not disseminated and followed with infection every year (Gordon, 31). If, therefore, an inhibitor were introduced or applied to an infected tree, the fungus could perhaps be controlled with concentrations near 1 ppm.

SUMMARY

1.) E. deformans was isolated from Ponderosa pine needles and partially characterized. The fungal pathogen was found to have no vitamin requirements, was found to grow only in the presence of molecular oxygen, was found to survive exposure to temperatures from 8°C to 34°C, and was found to grow in laboratory media at temperatures from 10°C to 30°C, but not at 34°C or 37°C.

2.) Isolation was more readily effected from infected young needles (5 weeks and older) than from infected mature needles.

3.) Pine needle extracts (ether, chloroform, and water extracts, and steam distillates containing a variety of compounds) provided excellent isolation upon their addition to the basal medium.

4.) Growth in culture media was optimal at pH 4.5.

5.) Phytoactin and cyclohexamide at concentrations of 0.8 and 0.4 ppm, respectively, were effective in inhibiting endogenous and exogenous oxygen uptake by the fungus with glucose as substrate.

6.) Infected needle distillates contained lower total concentrations of terpene constituents than uninfected needle distillates of the same age. The concentrations of individual terpenes in infected needles did not change uniformly from the normal uninfected needles, however.

7.) The fungus possesses the following enzymes: gelatinase, DNase, urease, and amylase. E. deformans was able to reduce NO_3 to NO_2.

8.) Survival of the organism in soil was very poor. pH of the soil was found to have no effect on survival.

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