

AN ABSTRACT OF THE THESIS OF

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ABSTRACT

Phytophthora cactorum lacks sterol synthesis capability. Nevertheless, it exhibits vegetative growth in the total absence of sterol. Supplementation of the growth medium with sterol, however, resulted in a three-fold increase in cell yield. This increase was shown by whole cell and cell free experiments to be the consequence of altered energy transformation. A specific change in efficiency of oxidative phosphorylation was suggested.

Uptake of sterols by Phytophthora cactorum is by simple adsorption. The kinetics of adsorption were determined, yielding an apparent K_m of $18.0 \mu M$ and V_{max} of 4.81×10^{-5} $\mu moles$ per minute per mg dry weight. The absence of specificity in initial uptake, esterification, and membrane deposition with respect to sterol structure was demonstrated.

The Development of Phytophthora cactorum
as a Model System for the Study of Sterol Metabolism

by

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Typed by Carlene Ballew for Robert Alfonso Gonzales

To my mother and father, who enabled me
to seek my own destiny.

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The Development of Phytophthora cactorum
as a Model System for the Study of Sterol Metabolism

I. Introduction

Qualitative and quantitative correlations of the presence of sterols with both physical and physiological functions in the cell are major objectives in the studies of sterol metabolism. While sterol composition of some eukaryotic organisms may be altered by genetic or chemical means, the condition of absolute sterol depletion is, as a rule, unobtainable. Therefore, many experiments are compromised by a background of endogenous sterol. An exception to the rule is found in the Pythiaceae fungi.

The Pythiaceae fungi, comprised of the genera Pythium and Phytophthora, are oomycetes belonging to the class Phycomycetales or lower fungi, characterized by nonseptate hyphae. These organisms do not possess the ability to synthesize sterol (24,25,31,68,85,86), however, they are capable of vegetative growth in the total absence of sterol (51). This phenomenon is unique among eukaryotic organisms. Moreover, when exogenous sterols are supplied in the growth medium, these fungi will readily incorporate them into their membranes (33). Incorporation of sterols results in two significant physiological modifications. In the absence of sterol the Pythiaceae fungi are incapable of both sexual and asexual sporulation (Figure 1). However, these modes of reproduction are fully functional in the presence of sterol. Enhancement of growth

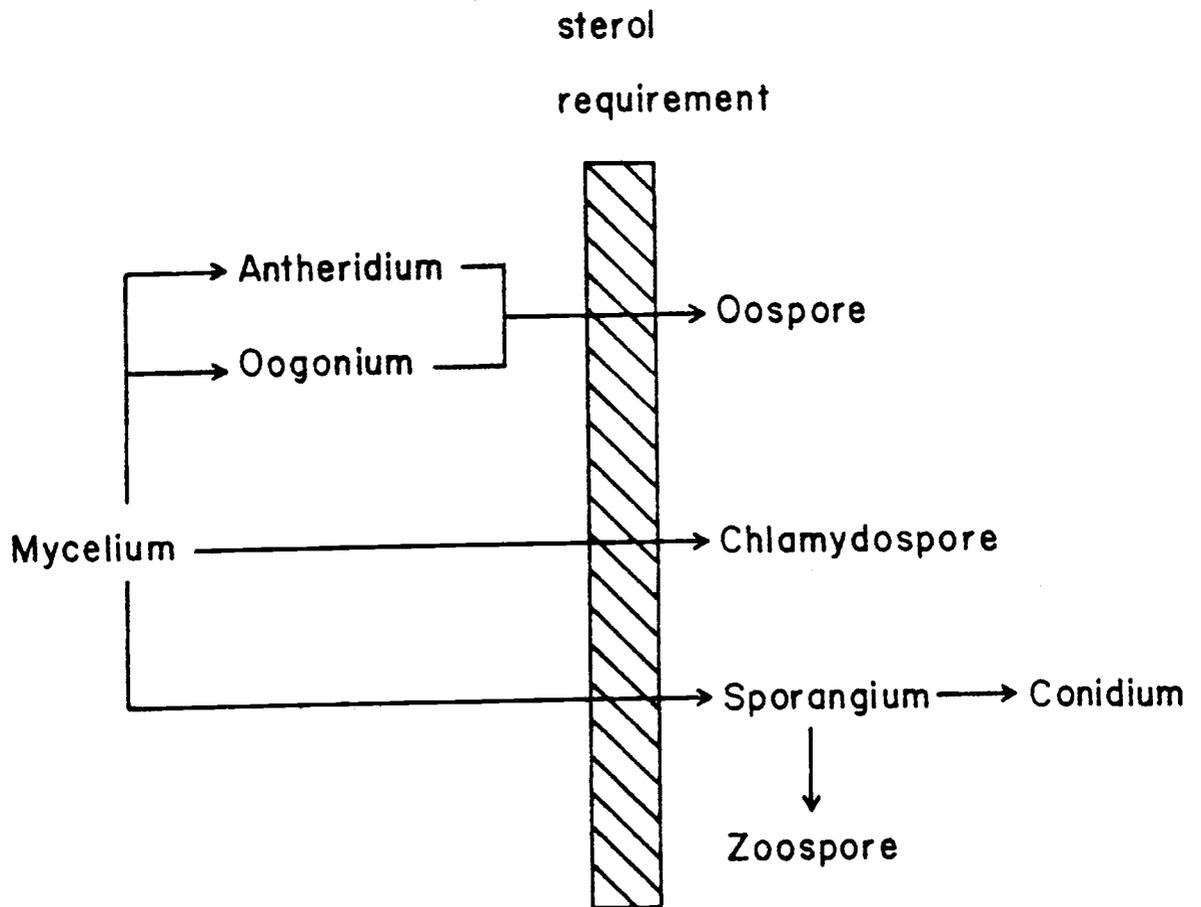


Figure I. Reproduction in *Phytophthora cactorum* (5).

and concomitant elevation of cellular metabolic rates of many of the Pythiaceae fungi are observed when the growth medium is supplemented with sterol. The mechanisms by which sterol effects these modifications in the Pythiaceae fungi have not been resolved.

It is the purpose of this study to develop criteria for the use of Phytophthora cactorum as a model system for the study of sterol metabolism. A correlation between the presence of sterol and a specific cellular function is made, and the conditions of sterol uptake determined.

The sterol numbering system and a listing of common sterols are presented in Figure II and Table I, respectively, for convenient reference.

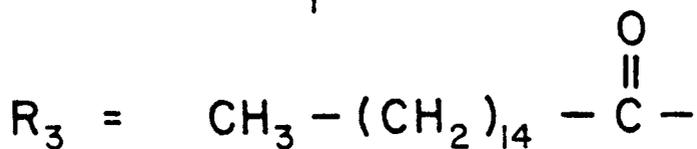
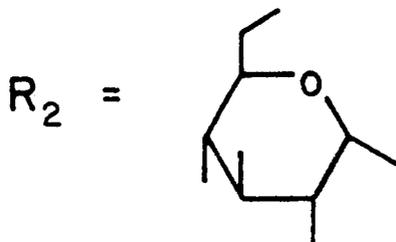
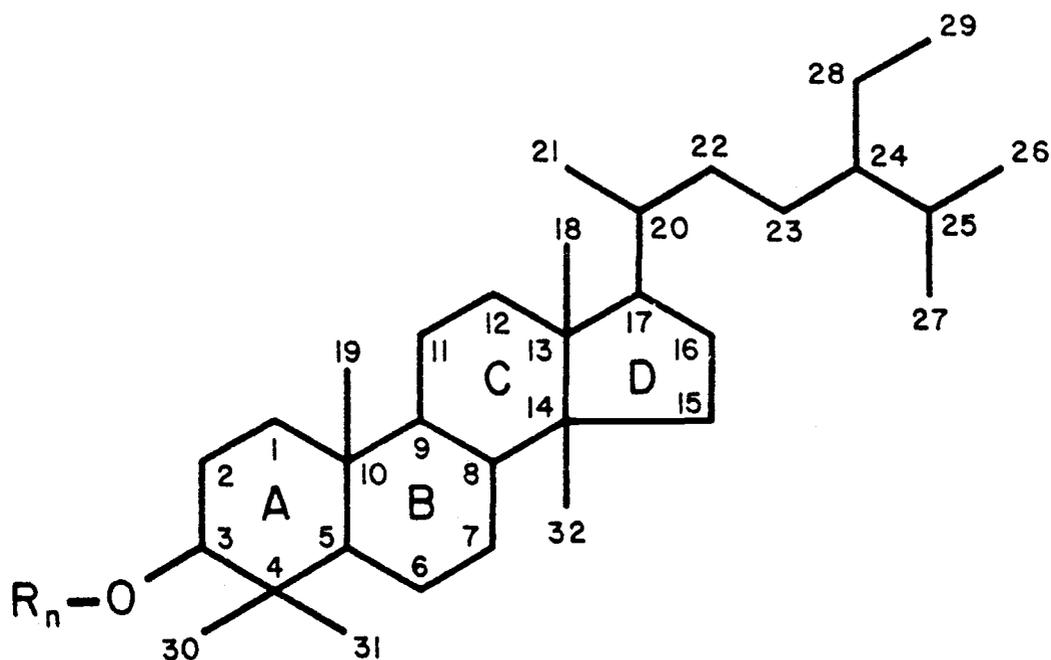


Figure II. The Sterol Numbering System. Each carbon is assigned a specific number. Unsaturation is designated by the first carbon of the bond. The second carbon is assumed to be the succeeding number of the sequence. Where this is not the case, the second carbon is enclosed in parentheses. Sterol derivatives of concern are modified at the C3 position: R₁ free sterols; R₂ steryl glycosides; R₃ steryl esters.

<u>Common Name</u>	<u>Structural Nomenclature</u>
sitosterol	24 β -ethyl-cholesta-5-ene-3 β ol
campesterol	24 β -methyl-cholesta-5-ene-3 β ol
stigmasterol	24 β -ethyl-cholesta-5,22-diene-3 β ol
cholesterol	cholesta-5-ene-3 β ol
lanosterol	lanosta-5,24-diene-3 β ol

Table I. List of Common Sterols. See Figure I legend for numbering details.

II. Review of the Literature

A. Sterol Requirement

The requirement of an exogenous substance for the formation of oospores by Phytophthora cactorum was first described by Leonian (51). He observed that P. cactorum was incapable of oospore formation in defined media unless a pea infusion was added. In experiments where mycelia were exposed to the infusion and then returned to defined medium, a minimum exposure of one hour was required to allow for oogonia production. The maximum number of oogonia was formed after eight hours exposure to the infusion. A second transfer of this mycelium to fresh defined medium resulted in the subsequent loss of sporulation capability in newly generated hyphae. These observations were ascribed to the uptake and eventual exhaustion of a factor contained in the pea infusion. Stimulatory activity for sporulation was localized in the nonsaponifiable fraction of organic solvent extractable material (52). The substance was found to be a component of a wide variety of complex nutrient additions (eg., oatmeal, lima bean, and hemp seed, etc.) (26,29); its identification as sterol was made simultaneously and independently in four different laboratories (20,27,30,49).

Comparative studies of the ability of various steroids to promote sporulation were made. Haskins et al. (27) and Elliott et al. (19) observed that a hydroxyl group at position C-3 of ring A and a 5 α configuration of the sterol molecule were absolutely required. A hierarchy of sterols with respect to ability to induce sporulation was found based

on side chain modifications and ring structure unsaturations (17,19). However, the reported effect due to ring structure unsaturations is suspect as Knights and Elliott (43) have since shown that Δ^7 - and $\Delta^{5,7}$ -sterols are converted in vivo to their Δ^5 -sterol counterparts by Phytophthora cactorum.

To clarify the data concerning side chain modifications, Elliott (18) reexamined the sterol effect by comparing a series of Δ^5 -sterols. For completely saturated side chains the ability to induce sporulation was in the order $C_{24}:C_2H_5 > CH_3 > H$. It was observed that 24 α -methyl-sterol exhibited greater activity than 24 β -methyl-sterol. There was less difference between 24 α - and 24 β -ethyl-sterols. A trans-unsaturation at C-22 (Δ^{22}) had no effect while a cis-unsaturation reduced the ability to induce sporulation. The structural requirements for sterols to induce sporulation in the Pythiaceae fungi are very similar to those for sterols to support anaerobic growth of yeast (2,61,62) and for sterol penetration into phospholipid liposomes (15).

B. Sterol Effect on Growth and Catabolism

Hendrix (29) observed that ether extracts of oatmeal or the non-saponifiable fraction of vegetable oils stimulated the growth of Phytophthora parasitica var. nicotianae on defined media. Purified sterols were then supplemented to the growth medium and were found to stimulate growth (30). Sterols have since been found to enhance growth of a variety of Phytophthora and Pythium species (7,37,47,60,70).

Elliott et al. (19) observed that the ability of individual sterols to promote sporulation in Phytophthora cactorum seemed related to the ability to enhance growth. However, these investigators noted great variability from one experiment to another. Nes et al. (60) showed that log phase growth rates are unchanged by the addition of sterol to the medium. Differences are noted, however, in the length of the lag phase of the culture cycle. Cholesterol, campesterol, and sitosterol decrease the lag phase while cholestanol, 5 α -cholestan-3-one, and epicholestanol have no significant effect on growth. From the graphs of the growth curves presented by Nes et al. (60, Fig. 2) there appears to be an increase in cellular yield when the culture is supplemented with cholesterol. The growth curves were not of sufficient duration to confirm this observation and no mention is made by the authors.

The enhancement of growth of various Phythiaceous fungi by sterol presents an interesting situation. A direct correlation of the presence of sterol with specific physiological functions might, therefore, be possible. An effort was made to define the sterol effect on growth and to determine the direct mode of action.

Hendrix et al. (37) observed that shake cultures of Phytophthora parasitica var. nicotianae grew much better than still cultures. More importantly, however, the enhanced growth response due to sterol was much greater. This was viewed as a possible clue to the physiological shift taking place. That a shift was indeed occurring was suggested by Troast and Norman (81), who observed an increase in the ratio of

insoluble to soluble protein with the addition of sterol to Phytophthora cactorum cultures.

Sietsma and Haskins (75) observed no change in the rate of glucose uptake in a Phythium sp. PRL 2142 culture supplemented with sterol. However, the amount of lactic acid and acetoin produced was doubled while the amount of oxygen consumed was halved. The authors suggested that these changes were the result of the organism following a more anaerobic pattern of metabolism with the incorporation of sterol. On the other hand, Schlosser and Gottlieb (70) observed a 25% increase in glucose uptake and a two fold increase in CO₂ evolution with sterol supplementation for Pythium ultimum. This was calculated to represent a 65% increase in the amount of CO₂ produced per unit glucose consumed. These authors suggested that sterol may be incorporated into the mitochondrial membrane resulting in the higher energy level observed. Since the same experiments or experimental methods were not used in these two reports, it is difficult to compare the results.

Further evidence of increased metabolic activity with sterol supplementation was presented by Sietsma (73) and Calderone and Norman (10). Three key enzymes of fermentative glucose utilization, aldolase, D-lactate dehydrogenase, and glucose-6-phosphate dehydrogenase, were found to exhibit increased activity. Most enzymes of the tricarboxylic acid cycle and some amino transferases were also found to exhibit higher activity.

Unfortunately, measurements of electron transport activities and oxidative phosphorylation efficiencies with respect to sterol supplemen-

tation have not been made. Confirmation of the suggestion of Hendrix et al. (37) and Schosser and Gotlieb (70) that a shift in the energy state of the organism occurs with sterol supplementation would require such experiments.

Some effects of sterol on the permeability of the membranes of the Pythiaceae fungi have been observed. When mycelium of Pythium PRL 2142, grown in the absence of sterol, is placed in distilled water leakage of intracellular components is observed (11). The rate of leakage is reduced if the organism is grown in the presence of sterol. Addition of polyene antibiotics, whose mode of action involves complexing with sterol (63), to the distilled water has no effect on leakage from mycelium lacking sterol. However, leakage is increased if sterol is present. A similar effect on growth has been observed. Growth is inhibited by filipin only when sterol is present in the medium (69). Transfer of the sterol-containing mycelium to sterol-less medium results in the reversal of sensitivity to the antibiotic.

C. Sterol Uptake and Metabolism

One important parameter of the study of the sterol effect in the Pythiaceae fungi is the actual uptake of sterol by the organism. The experiments of Leonian (51) in which mycelia of Phytophthora cactorum were exposed to a pea infusion for varying lengths of time and then tested for sporulation capability, were, in effect, the first kinetic studies of sterol uptake by these organisms. Within one hour sufficient

sterol was accumulated to allow or induce the production of oogonia. The number of oogonia formed increased with extended exposure times of up to eight hours.

Calderone and Norman (10) found that the specific activities of aldolase, glucose-6-phosphate dehydrogenase, and glutamate transaminase were increased after the addition of sterol to the culture medium of Phytophthora cactorum. The shift to higher activities, however, was not apparent until 24 hours after the addition of the sterol. Defago and Kern (13) measured the rate of incorporation of adsorbed cholesterol into the membranes of Pythium paroecandrum by monitoring the sensitivity of the organism to digitonin, a plant saponin. Sensitivity is manifest with increased permeability of the protoplasmic membrane. They observed that P. paroecandrum exhibited sensitivity within four to five minutes after the addition of cholesterol to the culture medium. Fifty percent of maximal sensitivity was observed within two hours. Although these techniques involve an indirect assessment of uptake, the information is useful in the interpretation of direct measurements.

Direct measurements of the uptake of sterol by the Phythiaceous fungi have been reported by two groups with significantly different results. Defago and Kern (13) observed very rapid uptake of ^{14}C -cholesterol from the medium of a non-growing culture of Pythium paroecandrum, with 90% of the label being removed from the medium in 80 minutes. Development by the organism of sensitivity to digitonin within four to five minutes after exposure to sterol lends support to this observation.

Elliott and Knights (21) on the other hand, observed much slower accumulation of ^{14}C -cholesterol in the acetone extracts of the mycelium of growing cultures of Phytophthora cactorum. The data show that the amount of sterol per unit dry weight increased with time until 24 hours. Sterol levels then decreased by 30% over the next 36 hours. They reported 90% of the label removed from the medium by 48 hours. The decrease in sterol levels is most likely due to a dilution effect of growth. Although different culture conditions were used by the two groups, it is not likely that the difference in sterol uptake rates observed in these two organisms is due to methodology.

One possible mechanism of sterol specificity the fungus might exhibit would be a differential uptake with respect to sterol structure. Langcake (48) grew Phytophthora infestans in media supplemented with different sterols and then analyzed for content. Cholesterol was accumulated to a greater extent than stigmasterol, and stigmasterol greater than sitosterol. However, no correlation between amount of sterol accumulated and ability to enhance growth could be made. Similar data were reported by Knights and Elliott (43) for the uptake of sterols whose structural differences were limited to modifications of the ring structure. Levels of total sterols accumulated after 36 hours from the time of addition were in the order: Δ^5 - $>\Delta^7$ - $>\Delta^{5,7}$ -sterols. This was interpreted by the authors to imply differential uptake rates in the same order. One criticism of this interpretation may arise from the fact that the sterols were added as acetates. Elliott and Knights (21) have observed that cholesteryl oleate is taken up more slowly than

free cholesterol. However, Defago and Kern (13) have shown that Pythium paroecandrum produces an exoesterase to break down sterol esters in the medium prior to adsorption. If an exoesterase exists in Phytophthora cactorum, the specificity of this enzyme could possibly affect the uptake results for sterol acetates.

Defago and Kern (14) described differential uptake rates of campesterol greater than stigmasterol and cholesterol greater than sitosterol in competition experiments. These sterols were added to non-growing cultures of Pythium paroecandrum at known compositions. Changes in the ratios of the sterols remaining in the medium were then monitored. One is reminded that, in this system, 90% of the added label is removed from the medium in 80 minutes (13). Sitosterol was found to represent a greater percentage of the sterol remaining after 50 minutes. This was interpreted as a slower rate of uptake. The changes observed, however, were small. In the first 20 minutes after addition of the sterols the standard deviations of the sterol levels were greater than the actual differences reported.

Uptake of sterol by Pythium paroecandrum was observed to occur in killed or latent cultures (13). Elliott and Knights (21) reported that uptake in Phytophthora cactorum followed simple adsorption kinetics. These results suggest the uptake of sterols by the Phythiaceous fungi is not an energy requiring transport phenomenon.

The fate of the sterol accumulated from the medium by the Phythiaceous fungi is important in the determination of its cellular function. In experiments where ^{14}C -cholesterol is supplemented to the growth medium,

most of the label recovered from the mycelium is associated with the protoplasmic membrane fraction of cell homogenates (48,72,74,75). However, a significant amount of label is found associated with the soluble cytoplasmic fraction.

The differential distribution of sterol within the hyphae suggests some modification of the molecular structure. Free sterols are quite insoluble in water and are thought to be directly associated with the particulate membrane fraction. Modifications to the sterol structure can be described at two possible levels. The first level would consist of chemical alterations within the molecule itself (ie. shifts in unsaturations, addition or removal of carbon atoms, or the addition of other atoms). These alterations would be in accordance with a functioning sterol biosynthetic pathway. Although the Pythiaceae fungi are incapable of de novo sterol biosynthesis (31), there appears to be some capability to alter certain sterols. Knights and Elliott (43) have shown that Δ^7 - and $\Delta^{5,7}$ -sterols are converted in vivo by Phytophthora cactorum to the Δ^5 -sterol counterparts. Modifications to the side chains, however, are not evident in their data. The inability to modify the side chains is consistent with the observation that Pythium and Phytophthora species are unable to convert lanosterol to products further along the pathway of sterol biosynthesis (71,85). In any event, chemical modification of this type would not adequately explain the differential distribution of sterols within the hyphae.

The second level of modification would comprise the covalent attachment of independent chemical moieties to the sterol molecule.

Two major classes at this level are steryl esters and steryl glycosides (Figure II). The metabolism of ^{14}C -cholesterol by Pythium periplocum to at least two different compounds, one more polar and one less polar than free cholesterol, was first reported by Hendrix et al. (36) and later shown to be a general phenomenon in the Pythiaceae fungi (32,34,35). The less polar metabolite has an identical R_f value on thin layer chromatography (TLC) as steryl ester standards (22) and upon alkaline hydrolysis, the ^{14}C -label co-migrates with free cholesterol standards (36). The more polar metabolite was identified as esterified steryl glucoside (57). The occurrence of UDPG:sterol glucosyltransferase activity in Phytophthora infestans has been reported (84). The presence of steryl esters is consistent with the differential distribution of sterol within the hyphae. It has been shown that steryl esters are incompatible with phospholipid membranes (46) and are major components of the lipid droplets that are known to accumulate in the cytosol of yeast (12,55). On the other hand, both steryl glycosides and acylated steryl glycosides have been shown to be fully compatible with phospholipid membranes (59). Therefore, their cellular location would most likely be in the membrane fraction.

An important aspect of the modification of sterol is the possible involvement in the reproductive process of the Pythiaceae fungi. It has been postulated that the mode of action of the sterol effect on reproduction is induction by hormones that are direct products of the sterols accumulated from the medium (18). Such a system is found in Achlya bisexualis where a steroid hormone, antheridiol, is directly

involved with the initiation of the sexual response (3,58). Evidence for the existence of a sex hormone in Phytophthora has been reported (44). However, the compound has not yet been isolated for identification. Conversely, Pratt and Mitchell (65) have shown that, in heterothallic strains, sterol is not required for the initiation of the sexual interaction but is necessary for the production of mature oospores.

III. Materials and Methods

A. Organism and Culture Conditions

Phytophthora cactorum, obtained from E. Hansen, Department of Botany and Plant Pathology, Oregon State University, was used throughout this study. The organism was subcultured routinely on medium containing (per liter) sucrose 10g, tryptone 10g, yeast extract 5g, and Difco agar 15g.

For most experiments, large batch cultures were grown in a New Brunswick Scientific Co. model MF-114 Microfermentor (14 liter capacity) containing eight liters of a basal medium. The basal medium was composed of (per liter) sucrose 10g, phthalic acid 2g, KH_2PO_4 1g, NH_4NO_3 1g, MgSO_4 0.5g, NaCl 0.1g, CaCl_2 0.1g, thiamine·HCl 0.1g, H_3BO_3 0.00001g, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.00001g, KI 0.0001g, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 0.00005g, and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.00007g (pH 6.0). Sterol was added to the indicated level as a solution in ethanol containing 1% TWEEN-40. When cultures were to be grown in the absence of sterol, ethanol containing 1% TWEEN-40 was added as a control. The maximum level of ethanol added to the cultures, however, never exceeded 0.5% v/v. Growth was initiated by inoculating with approximately 10mg wet weight of mycelial mat from a 20ml still culture and monitored by removing 100ml aliquots at various times for dry weight and pH determinations. Dry weights were taken from lyophilized samples.

For experiments describing growth in the presence of low substrate concentrations, cultures were grown in 250ml flasks containing 20ml of

the basal medium. The cultures were incubated at 25°C in a New Brunswick model G24 Environmental Shaking Incubator (200 rpm).

B. Whole Cell Experiments

Multiple aliquots, yielding 20-30mg dry weight each were removed from the eight liter batch culture at the times indicated. The mycelium was harvested by filtration, washed once with distilled water, and re-suspended in 10mM phthalic acid (pH 5.5).

Respiration was measured manometrically (83) at 25°C in a Gilson model G8 Differential Respirometer after the addition of glucose (5mM final concentration). Oxygen uptake was reported as $\mu\text{l O}_2$ per min per mg dry weight.

Glucose uptake and CO_2 evolution rates were measured by the method of Schlosser and Gottlieb (70). ^{14}C -Glucose (5mM final concentration; 4.4×10^4 dpm/ml) was added to 10ml mycelial suspensions contained in 50ml serum bottles. Aliquots of 0.1ml were removed at regular intervals and placed into counting vials containing five milliliters of a scintillation cocktail composed of 4.0g 2,5-diphenyloxazole (PPO), 0.2g 1,4-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP), and 60.0g naphthalene per liter 88% p-dioxane, 10% methanol, and 2% ethylene glycol. Glucose uptake rates were determined by monitoring the disappearance of ^{14}C -label from the medium and were reported as pmoles per minute per mg dry weight. For CO_2 evolution, the serum bottles were fitted with rubber stoppers to which long-stem collecting ampules were attached. The ampules contained 0.5ml of 1M Hyamine hydroxide (in methanol) and were

suspended in the serum bottle to trap the evolved CO_2 . The collecting ampules were exchanged with fresh ampules at regular intervals during the course of the experiment. The contents of each ampule were transferred to counting vials containing 5ml of a toluene scintillation cocktail composed of (per liter) PPO 3.0g and POPOP 0.1g. CO_2 evolution rates were determined by monitoring the total accumulated ^{14}C -label in the Hyamine hydroxide. Rates of evolution were reported as Δcpm per min per mg dry weight.

Sterol uptake experiments were performed at 25°C with 20ml mycelial suspensions contained in 250ml flasks. The uptake buffer contained, in addition to 10mM phthalic acid, 1% sucrose w/v. Samples were equilibrated approximately 30 minutes prior to the addition of sterol. Due to the propensity for the mycelium to adhere to the sides of the flask, all uptake incubations were at slow shaking (75 rpm) in the New Brunswick G24 Incubator. After the desired time interval, the mycelium was harvested by filtration and washed three times with 10ml volumes of a 0.5% Tergitol NP-40 solution to remove extracellular sterol. The mycelium was then washed three times with distilled water. The sample was lyophilized for dry weight determination and extracted for sterol.

C. Preparation of Cell Free Extracts

For the preparation of cell free extracts and mitochondrial fractions, the harvested mycelia were resuspended in 0.6M sorbitol, 10mM Tris-HCl, and 0.5mM ethylenediamine tetraacetic acid (pH 6.6) at approximately 0.8g/ml. Hyphae were disrupted in a French pressure cell

at 1,500 psi. Intact hyphae and cellular debris were removed from the homogenate by centrifuging three times at 1,000 x g for two minutes. The supernatant was used as a crude protein preparation or was centrifuged at 27,000 x g for 30 minutes to yield a crude mitochondrial preparation. Gradient purified mitochondria were prepared according to the method of Bottema and Parks (6).

D. Enzyme Assays

Phosphofructokinase and aldolase were assayed using coupled enzyme reactions based upon the oxidation of NADH (77). D(-)-Lactate dehydrogenase was assayed in the reverse direction by following the conversion of pyruvate plus NADH to lactate and NAD^+ (50). Endogenous NADH oxidase activity was measured in the absence of substrate and subtracted as background. Enzyme activity was reported as $\mu\text{mole NADH}$ oxidized per minute per mg protein.

NADH-cytochrome c reductase and succinate-cytochrome c reductase of the electron transport chain were assayed by the method of Dowler et al. (16). Endogenous cytochrome c reductase was measured in the absence of NADH and succinate and subtracted as background. Cytochrome c oxidase was assayed by the method of Thompson and Parks (80). Enzyme activity was reported as $\mu\text{mole cytochrome c}$ reduced or oxidized per min per mg protein.

All enzyme activities were measured spectrophotometrically at 25°C in a Beckman model 25 Double Beam Spectrophotometer set at 340nm (oxidation of NADH) or 553nm (oxidation/reduction of cytochrome c).

Reaction mixtures in one centimeter quartz cuvettes contained one milliliter total volume. Reactions were initiated by the addition of an aliquot of the indicated enzyme preparation. To calculate enzyme activities, millimolar extinction coefficients of 19.2 for cytochrome c (40) and 6.22 for NADH (39) were used. Protein was determined by the method of Lowry using bovine serum albumin as standard (53).

E. Cytochrome Spectra

Mitochondrial cytochromes were extracted by the method of Tzagoloff et al. (82). A solution of 1% deoxycholate, 1M KCl, and 50mM Tris (pH 8.0) was added to the mitochondrial preparation to yield a final concentration of seven to eight milligrams protein per milliliter. The mixture was vortexed and then centrifuged at 27,000 x g for 30 minutes. The supernatant was divided into two portions. One portion was reduced with sodium dithionite and the other oxidized with potassium ferricyanide. The difference spectrum was then scanned between 500nm and 650nm at 25°C using a Beckman model 25 Double Beam Spectrophotometer with scanning attachments.

F. Sterol Quantitation

Sterols were extracted from lyophilized mycelial samples by either alkaline pyrogallol saponification (1) or a DMSO extraction regimen (79). Extracts were chromatographed on silica gel 60 F-254 thin layer chromatography plates using the system of Skipski et al. (76). The free sterol band and the steryl ester band, when present, were scraped

and eluted with a mixture of chloroform/methanol (4:1). Steryl esters were saponified to free sterols prior to quantitation. Quantitation of free sterol was by integration of detector analog output from a Varian series 2700 Gas Chromatograph linked to a Varian CDS 111 integrator using cholestane as an internal standard.

IV. Results and Discussion

A. Growth Conditions

In many of the studies described in the literature, involving the Pythiaceae fungi, growth of the organisms in liquid medium was performed in still culture. The culture flasks were simply incubated without shaking on a laboratory bench top. Oxygen tension is, therefore, dependent not only on the composition of the medium but also the prevailing ambient conditions. As this group of fungi is obligately aerobic (78), it is not surprising then that growth is quite slow and fairly erratic (data not shown). The observation that shake cultures exhibit enhanced growth as compared to still cultures substantiates the point (8,37). A very distinct disadvantage of the still culture technique lies in the heterogeneity of the mycelial mat that is formed at the surface. Hyphae on the periphery of the mat are highly diffuse while those proximal to the center of growth are much more densely packed. Yanagita and Kogane (88) have observed a differential distribution of nucleic acids, cell wall carbohydrates, and reproductive activity along the radius of surface colonies of Aspergillus niger and Penicillium urticae. Undoubtedly a similar situation exists in Phytophthora cactorum.

On the other hand, shake cultures, while avoiding the problem of oxygen limitation, do not resolve the enigma of culture heterogeneity. In fact, heterogeneity is actually more extreme. Growth of mycelium with rigorous shaking resulted in what is described as a pellet culture (9,87). The mycelium formed a number of hollow spheres ranging in size

from two to ten millimeters in diameter depending on the size of the culture flask utilized. The walls of the spheres are very densely packed and composed of distinct layers. Fungal pellets have been shown to exhibit differential distribution of cellular components along the pellet radius in a pattern similar to that found in the surface colonies (87).

One objective of this study was to correlate specific intracellular functions with the presence of sterol. Therefore, it was believed that the anticipated in vitro experiments would necessitate a more homogeneous culture. Attempts to grow the organism in a large culture vessel (32 liters) fitted with a gas dispersion tube proved unsatisfactory. The mycelium grew in a single mass around the fritted glass cylinder of the dispersion tube. The problem of heterogeneity was finally resolved satisfactorily, when the organism was grown in a stirred tank reactor (STR) (i.e. the New Brunswick Microfermentor) as described by Bull and Bushell (8). While mycelial pellets were still produced, they rarely exceeded two millimeters in diameter. Moreover, they were quite diffuse throughout the entire pellet. Uptake of nutrients may occur at the growing tip of the hyphal filament, however, it was considered important that there be access to the majority of hyphae for the sterol uptake experiments. An additional advantage of the STR culture method is the facility with which growth can be monitored. Instead of harvesting a number of small replicate flasks, as in still culture, aliquots can be aseptically removed from a single batch culture at various intervals through the entire growth cycle.

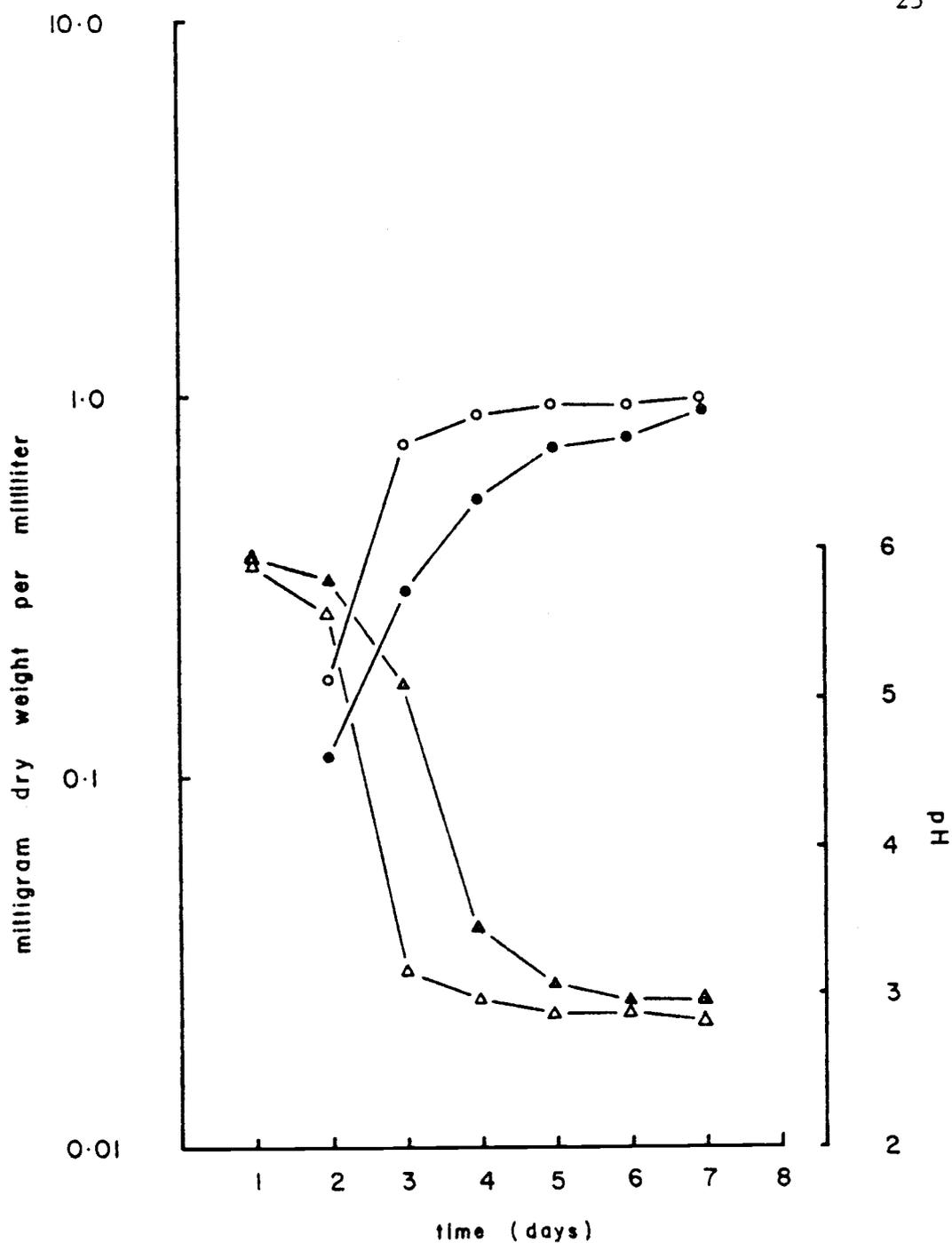


Figure III. Growth of *Phytophthora cactorum* in an Unbuffered Medium. The composition of the medium is as described in Materials and Methods. ○-○, dry weight in the presence of 10mg/l sterol (mixture of sitosterol, campesterol, and stigmasterol); ●-●, dry weight in the absence of sterol; Δ-Δ, pH in the presence of sterol; ▲-▲, pH in the absence of sterol.

Fluctuations in cell production in still culture samples due to differences such as inoculum size, volume of medium, and nutrient concentrations are thus avoided.

With the culture techniques firmly established, another problem concerning the growth of the organism was broached. One specific effect induced by sterol supplementation of the Pythiaceae fungi is the elevated in vitro activities of three key enzymes of glucose utilization: aldolase, glucose-6-phosphate dehydrogenase, and lactate dehydrogenase. Attempts were made to repeat these results as part of this study. The results did not confirm the presence of elevated activities in sterol supplemented cultures (data not shown), however, there was great variation in the data from one set of experiments to another. Cultures utilized in these experiments were grown in the basal salts medium described in the Materials and Methods section excluding the phthalic acid. Growth curves for Phytophthora cactorum in this medium are shown in Figure III. The pH curve indicates that the culture is exposed to drastic variations in culture conditions. A staling effect is observed when the pH of the medium approaches 3.0. Microscopic examination revealed the presence of perennating mycelium throughout the culture. Since there was some concern that the pH of the medium was responsible for the inconsistent and conflicting results, the medium was examined for buffering capacity. Titration revealed a very poor buffering capacity below pH 6.0; a characteristic shared by many of the defined media commonly used for growth and sporulation of the Pythiaceae fungi (Figure IV) (19,38,73), see ref. # 67 for a compilation of defined

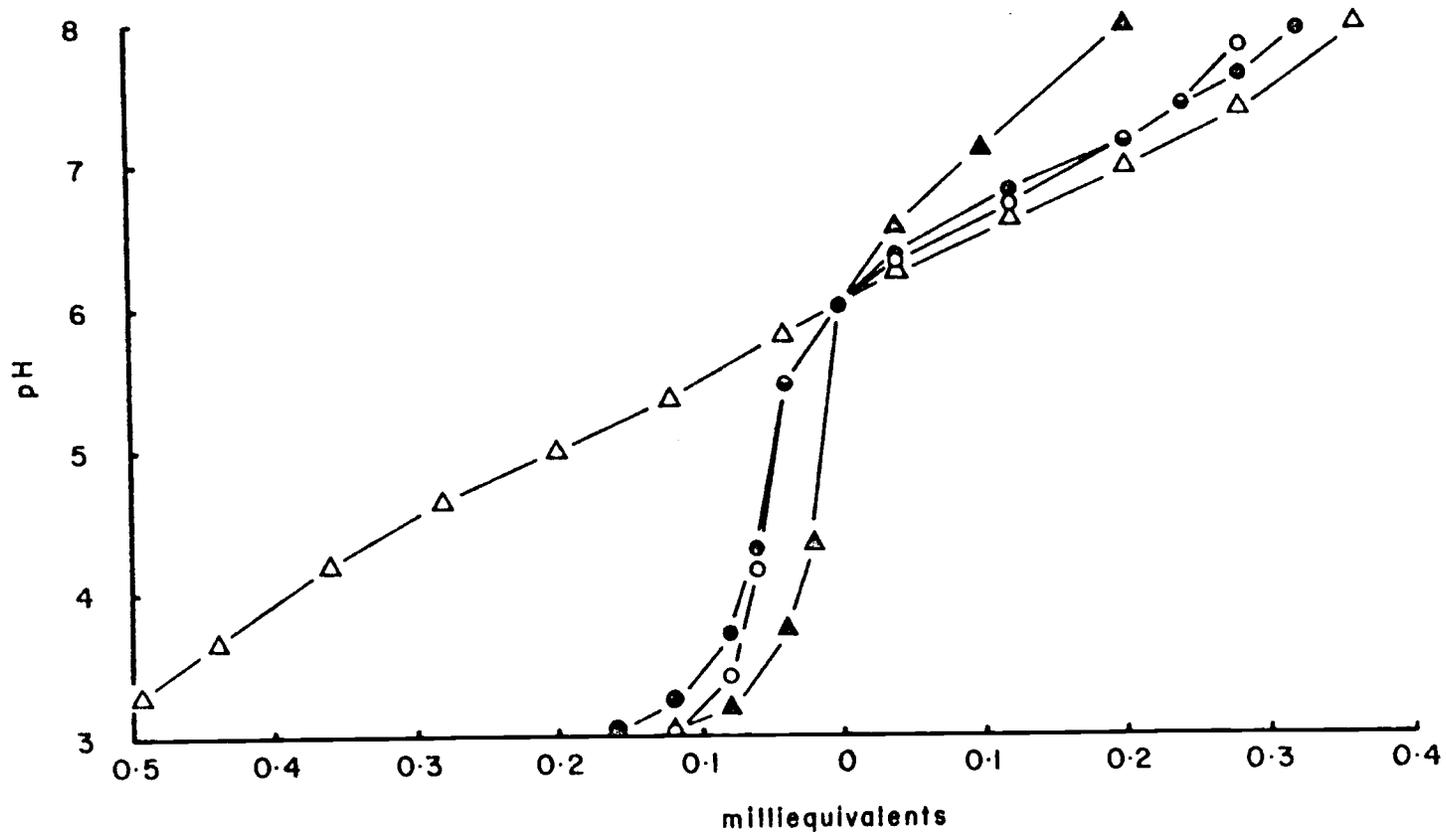


Figure IV. Titration of Basal Media Commonly Used for Growth and Sporulation of *Phytophthora cactorum*. Initial pH was adjusted to 6.0 with KOH. Each sample was divided into two aliquots; one was titrated with 0.1N KOH and the other with 0.1N HCl. ▲-▲, Elliott, et al., Journ. Gen. Microbiol. 42(1966)425; Δ-Δ, Phthalate Buffered medium as described in Materials and methods; O-O, Hohl, Phytopath. Z. 84(1975)18; ●-●, Sietsma, Biochim. Biophys. Acta 244(1971)178.

media). To circumvent this problem, phthalic acid ($pK=2.95$ and 5.41) was added to ten millimolar. Other common organic buffers with pK between 4.5 and 5.5 , such as succinate, citrate, and acetate were observed to inhibit the growth of Phytophthora cactorum (L. W. Parks, unpublished results).

The results of growth experiments performed with the phthalate buffered medium are shown in Figure V. The additional buffering capacity of the medium allowed a ten fold increase in the cell yield for the sterol supplemented culture. This suggests that the reduced growth in the unbuffered medium is due to the hydrogen ion concentration or its indirect effects and not to the accumulation of a growth inhibitory compound.

The culture age at the time of the sampling was probably responsible for the inconsistent results obtained for the enzymes. While this factor remains an important consideration in physiological studies, the increased cell yield and more restricted pH change offer more flexibility in this system. Data from physiological studies, to be presented in the next section, were consistent and repeatable from one batch culture to another.

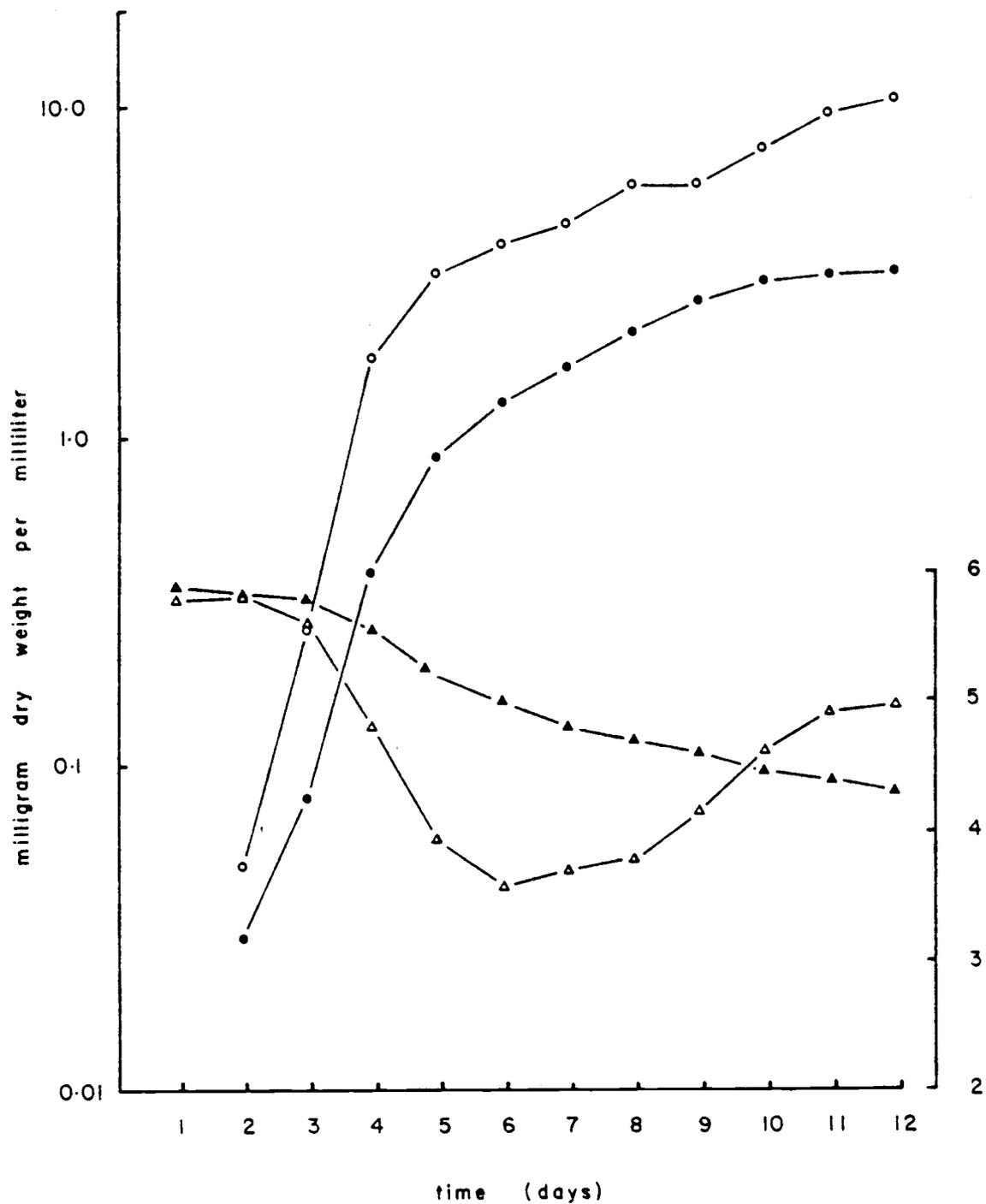


Figure V. Growth of *Phytophthora cactorum* in Phthalate Buffered Medium, symbols are as described in Figure III.

B. Effect of Sterol on Growth and Metabolism

The enhanced growth of the Pythiaceae fungi, when supplemented with sterol, was shown to be accompanied by a general elevation in the levels of cellular metabolism (10,70,73). However, the mechanism of the sterol effect remained unresolved. To account for this sterol induced stimulation, two mechanisms have been postulated. The response may simply be the result of increased permeability to nutrient compounds by the protoplasmic membrane. Elevated glycolytic (10) and tricarboxylic enzyme activities (73) are, therefore, the consequence of increased substrate levels within the cell. An alternative postulation was proposed by Hendrix (33) and Schlosser and Gottlieb (70) involving an interaction between sterol and the mitochondrial membrane system resulting in a shift to a higher energy state.

The growth curves presented in Figure V show the extent of growth enhancement due to sterol supplementation for the strain of Phytophthora cactorum used in this study. These data show that the rate of growth during the log phase of the culture cycle was unchanged, while the length of the lag phase was decreased by approximately 18 hours. These specific results concur with the observations of Nes et al. (60). However, when the cultures were allowed to continue through stationary phase, the sterol supplemented culture exhibited a three fold higher final cell yield than the unsupplemented culture. When Phytophthora cactorum was grown in media containing a limiting concentration of substrate (10mM sucrose), the final cell yields were 0.74mg dry weight per

milliliter for the sterol supplemented culture and 0.27mg dry weight per milliliter for the unsupplemented culture. The maintenance of the approximately three-fold enhancement of final cell yield due to sterol at low substrate levels indicates that the effect is a result of altered metabolism rather than to differential sensitivity to a secondary metabolite.

Kormancikova et al. (45) have shown that levels of oxidative phosphorylation efficiency in Saccharomyces cerevisiae can be evaluated based on molar growth yields (grams dry weight cells formed per mole substrate catabolized). Cell yield is, therefore, directly related to the stoichiometry of ATP formation. Altered permeability of the protoplasmic membrane may allow more rapid access to catabolic substrates. This might be reflected as an increased rate of log phase growth, however, changes of this nature would not be expected to alter the stoichiometry of ATP formation. It is not likely then that energy reserves could be augmented to a significant degree by more rapid substrate processing to account for the increased production of cell mass in Phytophthora cactorum. Conversely, an improved efficiency of oxidative phosphorylation, anticipated by a shift to a higher energy state, would result in an increased cell yield.

Whole cell measurements of glucose metabolism by log phase cultures of Phytophthora cactorum are shown in Table II. Sterol supplementation resulted in a decreased rate of glucose uptake and a three fold higher rate of carbon dioxide production. The rate of oxygen utilization, however, was unchanged. ^{14}C -Glucose uptake was determined by measuring

	Glucose Uptake*	CO ₂ Evolution*	O ₂ Consumption**
Unsupplemented	40.6	0.59	0.33
Supplemented	30.7	1.74	0.31

* change in cpm per minute per mg dry weight

** μ l O₂ per minute per mg dry weight

Table II. Glucose Catabolism by Whole Cells. Uniformly labeled ¹⁴C-glucose was added to aliquots of log phase mycelium to a final concentration of 5 mM.

the rate of disappearance of ^{14}C -label from the medium. One criticism of this method is that it does not account for accumulation of lactic acid, a product of glucose metabolism in the Pythiaceae fungi (80). However, if the ^{14}C -label removed from the medium is simply considered as substrate available for energy production and structural components, the rates observed may be regarded as significant. In this respect the data in Table II suggest an alteration in the metabolic fate of glucose in the presence of sterol. It is of interest to note that Schlosser and Gottlieb (70) observed an increase in the rate of substrate uptake in sterol supplemented cultures of Pythium ultimum using the same technique.

The enhanced growth and altered fate of absorbed glucose in the presence of sterol suggest that the interaction of sterol in the cell leads directly to an increased level of oxidative phosphorylation. This supposition was corroborated when enzymes of fermentative glucose utilization were assayed. The results are shown in Table III and were somewhat unexpected. The presence of sterol resulted in little or no change in glycolytic activity. A substantial increase would have been expected if the sterol effect was simply altered permeability of the protoplasmic membrane. These data were in direct disagreement with the results of Sietsma (73) and Calderone and Norman (10) who observed increased levels of glycolytic activity with sterol supplementation. However, the organisms used by these authors were species of the genus Pythium. Conflicting results concerning glucose uptake by Phytophthora cactorum and Pythium ultimum have been mentioned previously. The

	Phosphofructokinase	Aldolase	Lactate Dehydrogenase	NADH-cyt c Reductase	Succ-cyt c Reductase	Cytochrome Oxidase
Unsupplemented	0.013*	0.003	1.928	0.100	0.040	0.128
Supplemented	0.014	0.001	1.568	0.130	0.104	0.133

*activity expressed as umole converted per minute per mg dry weight

Table III. Glycolytic and Electron Transport Enzyme Activities in Cell Free Extracts.

enhancement of growth due to sterol, although wide spread, is not an absolute character of the Pythiaceus fungi. In particular, the species of Pythium used by Sietsma (73) did not exhibit an alteration in growth with sterol supplementation. The specific function of sterol in the cell may be constant in the Pythiaceus fungi, however, the consequences of the presence of sterol may not be as dramatic in some strains as in Phytophthora cactorum. Although the contradicting results may be a reflection of a real generic difference, the culture conditions utilized must also be considered. The results reported by Schlosser and Gottlieb (70), Calderone and Norman (10), and Sietsma (73) were obtained from mycelia grown in either still culture or shake culture in unbuffered media. The problems inherent with these culture techniques have been discussed.

The suggested alteration in energy transformation implies that sterols accumulated from the medium were incorporated into the membranes of the mitochondria, the site of oxidative phosphorylation. Mitochondria, isolated from sterol supplemented cultures of Phytophthora cactorum, were, in fact, found to contain sterol (see Table V).

A potential mechanism, by which the sterol interaction with the mitochondria gives rise to altered energy transformation, is the induction of the synthesis of mitochondrial components directly involved in respiratory metabolism (eg., electron transport chain). In the absence of sterol, these components may be lacking entirely or present in reduced levels. A relationship of this type is observed in aerobic adaptation of yeast. Aeration of anaerobic yeast cultures

results in respiratory adaptation (64) and sterol synthesis (41,42). Inhibitors of respiration were found concomitantly to inhibit sterol synthesis (64).

Cytochromes were extracted from crude mitochondrial preparations from sterol supplemented and unsupplemented cultures of Phytophthora cactorum. The difference spectra were recorded and are shown in Figure VI. A spectrum for a typical strain of Saccharomyces cerevisiae is included for comparison. These data show that the mitochondria from unsupplemented cultures contained a full complement of cytochromes. However, they were present in reduced amounts. To confirm the presence of a functional electron transport chain in these cultures, the activities of NADH-cytochrome c reductase, succinate-cytochrome c reductase, and cytochrome oxidase were assayed. These data are shown in Table III. The reductases both exhibited elevated activity in the sterol supplemented culture. Cytochrome oxidase, however, was essentially unchanged. It is clear that the presence of sterol does not result in the de novo synthesis of the electron transport chain.

The question remains as to whether the increased activity of the early components of the electron transport chain alone can account for the increase in cell production due to sterol. If this is the case, then the specific mode of action of the sterol remains obscure. It is possible, however, that sterol interaction in the membrane allows increased access of respiratory substrates. In this manner, a more efficient utilization of substrate is realized.

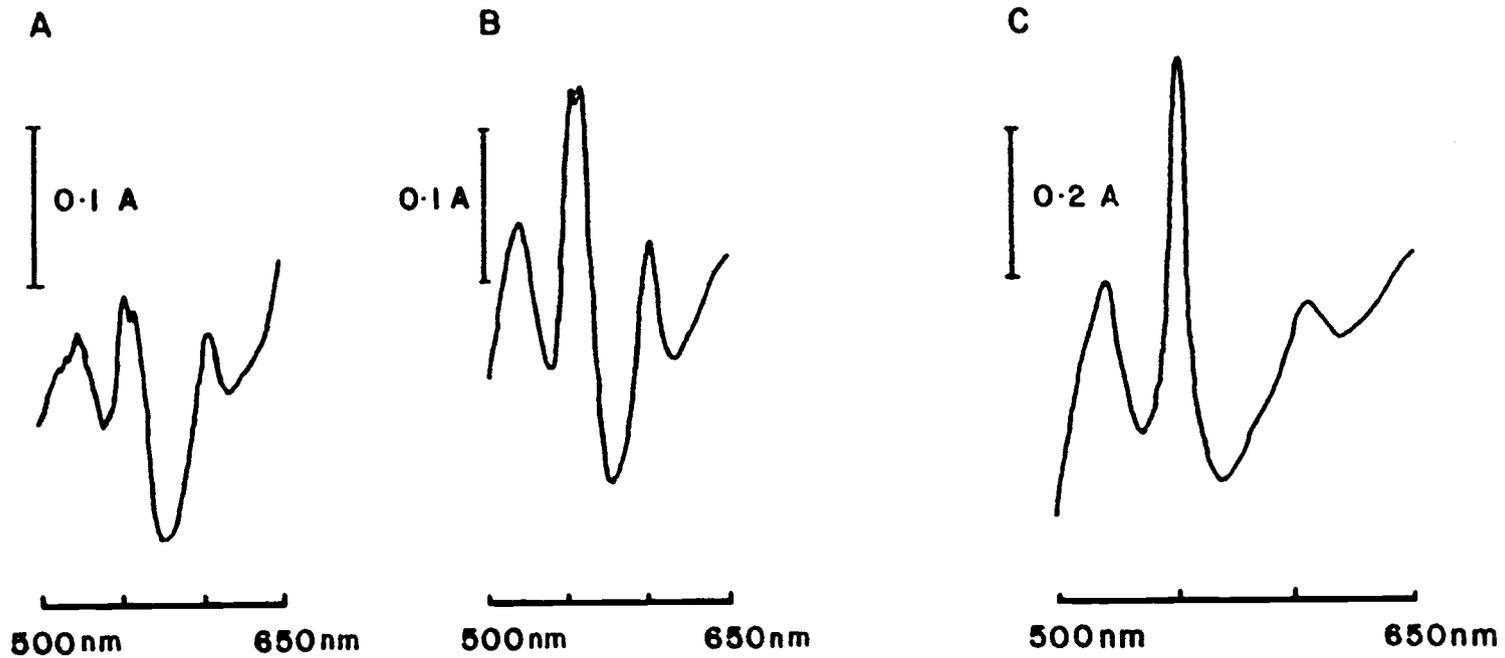


Figure VI. Cytochrome Spectra. Cytochromes were extracted and prepared as described in Materials and Methods. A. Difference spectrum of cytochromes from Phytophthora cactorum grown in the absence of sterol. B. Grown in the presence of 10mg/liter sterol. C. Difference spectrum of cytochromes from Saccharomyces cerevisiae, strain MCC.

An alternative mechanism for the modification of energy transformation assumes the presence of a functional electron transport chain. The sterol effect may involve the coupling of oxidative phosphorylation with electron transport. A close association between coupling and the lipid content of yeast mitochondria has been observed. Loss of oxidative phosphorylation with unsaturated fatty acid or sterol depletion (4,54,66) is reflected by a drop in P:O and respiratory control ratios (28). The loss of coupling with depletion is due specifically to the loss of the mitochondrial proton gradient (4).

The final determination of the specific mechanism of the sterol effect in Phytophthora cactorum awaits the isolation of intact mitochondria whose respiration is coupled to oxidative phosphorylation.

C. Sterol Uptake

The interest in the Pythiaceae fungi for the study of sterol function is focused on two aspects. These fungi are capable of vegetative growth in spite of the total absence of sterols for their membranes. The potential in this phenomenon is the possibility of isolating membrane systems (eg., mitochondrial) that, heretofore, have been unobtainable absolutely devoid of sterols. The other aspect is the readiness with which these organisms will incorporate exogenous sterol into their membranes. It should be possible to examine a wider array of sterol effects on membrane properties than previously have been possible in any one system.

A major consideration that must be made in studies concerning the effects of sterols in Pythiaceae fungi is the possible existence of an inherent mechanism of sterol specificity with regard to structure. Knights and Elliott (43) have shown that Phytophthora cactorum is capable of converting Δ^7 - and $\Delta^{5,7}$ -sterols to Δ^5 -sterols. The side chains of these sterols were not modified. It is not known if this is a reflection of a specificity for Δ^5 -sterols or simply a fortuitous conversion by remnants of a now lost sterol biosynthetic pathway.

There are three levels at which the organism may distinguish a more preferable sterol: 1) initial uptake of sterol from the medium, 2) differential esterification, and 3) differential deposition in specific membranes.

Uptake of sterol by both killed and latent cultures of Pythium paroeandrum (13) suggests that sterol accumulation is not an energy dependent transport phenomenon. The alternative is simple adsorption as suggested by Elliott and Knights (21). Accumulation of sterol was observed by these authors to follow first order kinetics.

The time course of sterol uptake for Phytophthora cactorum is shown in Figure VII. The samples harvested at the zero time point were exposed to sterol for no more than 15 seconds. Less than 0.2% of the total sterol accumulated after three hours exposure was found in the non-saponifiable fraction of these samples. Therefore, it was concluded that the Tergitol NP-40 wash at the time of harvest removed essentially all of the non-adsorbed sterol. Accumulation of sterol is linear with respect to time for at least five hours. The remaining experiments involving sterol uptake were not exposed to the exogenous sterol in excess of 4.5 hours.

The effect of concentration on the accumulation of cholesterol and sitosterol by Phytophthora cactorum is shown in the double reciprocal plot of Figure VIII. This experiment was performed with samples taken from the same batch culture to avoid any possible effect of culture conditions on sterol accumulation. The slopes of the curves for cholesterol and sitosterol were essentially superimposable with an average apparent K_m of 18.0 μM and V_{max} of 4.81×10^{-5} $\mu mole$ per minute per mg dry weight. Since cholesterol differs from sitosterol only by the absence of an ethyl group at position 24, these data argue that

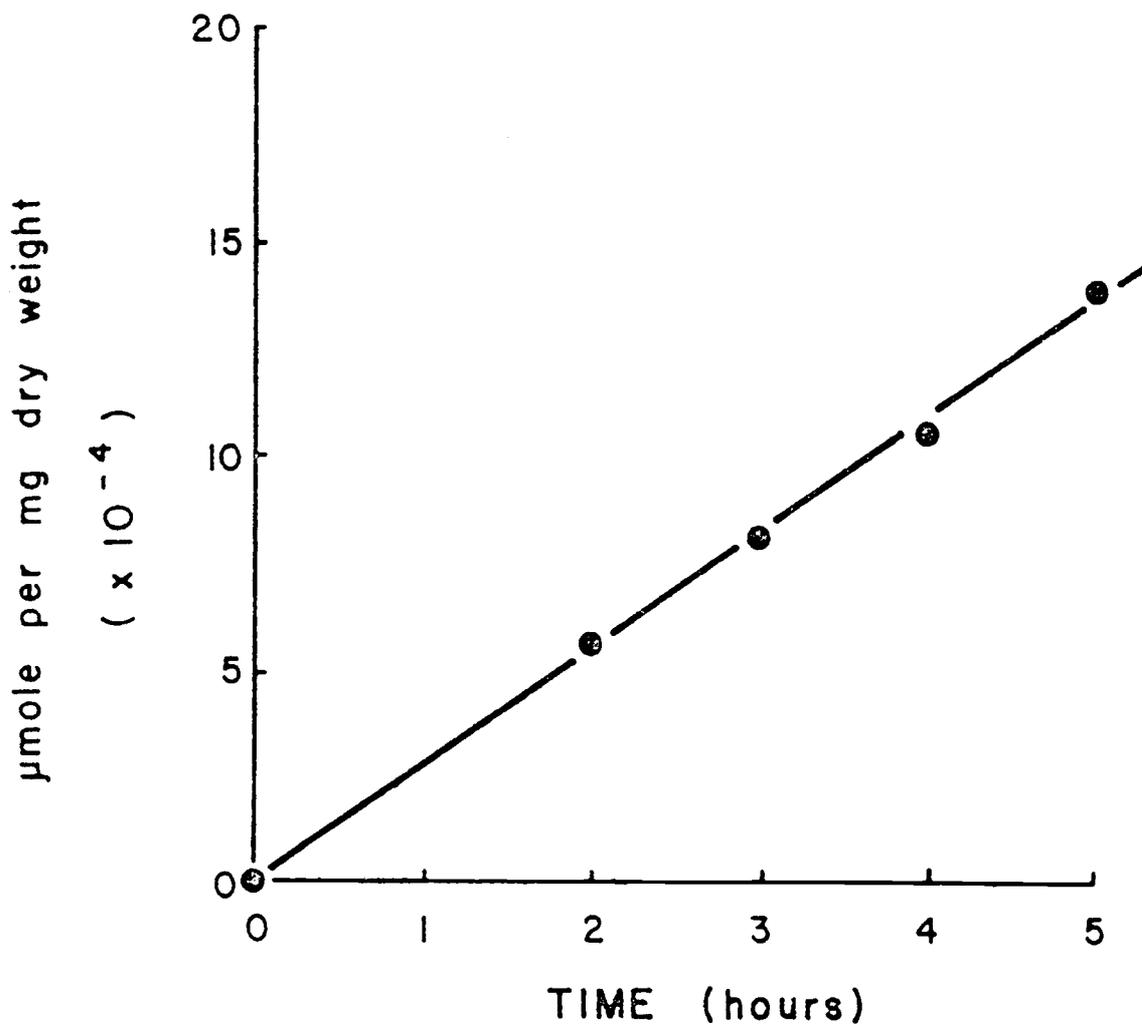


Figure VII. Time Course of Sterol Uptake by Phytophthora cactorum. A sterol mixture (sitosterol, campesterol, and stigmasterol) was added to aliquots of mycelia to a final concentration of 10mg/l. The aliquots were harvested at intervals and sterol quantitated as described in Materials and Methods. The data points represent total sterol accumulated in the mycelia and has been corrected for the individual molecular weights of each sterol in the mixture.

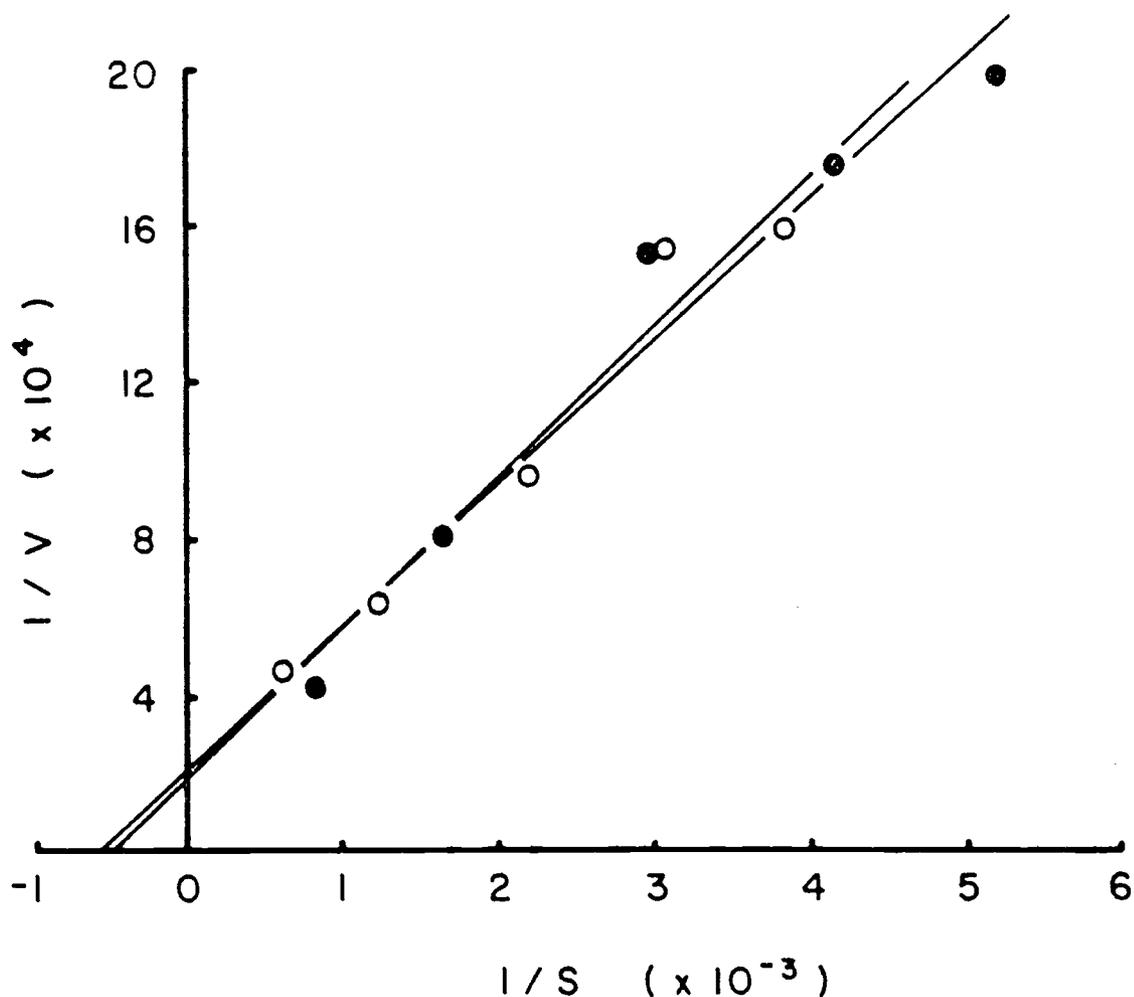


Figure VIII. Double Reciprocal Plots of Rate of Sterol Uptake Versus Concentration of Sterol. Purified cholesterol or sitosterol was added to aliquots of mycelium to the indicated concentration. The aliquots were harvested and sterol quantitated as described in Materials and Methods. The rate of uptake was reported as μ mole per minute per mg dry weight; sterol concentration as μ mole per liter. The data points represent the mean of three replicate samples.

there is no uptake specificity for the sterol side chain exhibited by Phytophthora cactorum.

The results of competition experiments are shown in Table IV. Samples of Phytophthora cactorum were exposed for three hours to mixtures of sterols in known compositions. There was no consistent pattern of change in the composition of total sterols accumulated from that originally present in the medium. The presence of different sterols had no countereffect on uptake. The composition of the free sterol pool and the steryl ester pool showed no change as well. This result shows that there was no preferential esterification of sterols based on side chain differences.

The differential deposition of sterol into specific membranes may be a complex protein-mediated phenomenon or simply a result of different solubilities of sterols in that particular membrane. In any event, it should be considered as a potential mechanism of specificity. In at least two instances evidence for the occurrence of preferential deposition has been observed in yeast. Anaerobically grown yeast, supplemented with cholesterol, will replace the cholesterol in the mitochondrial membranes with ergosterol upon aeration (23). A wild type yeast, in the presence of low levels of an azasterol inhibitor of sterol synthesis, preferentially incorporates ergosterol into the mitochondrial membranes although this sterol represents less than 60% of the total sterol under these conditions (56).

Experiment		Sitosterol	Campesterol	Cholesterol
1	added	<u>29.60</u>	<u>39.09</u>	<u>31.31</u>
	total sterol	29.54	33.48	36.98
	free sterol	22.22	37.29	40.48
	steryl ester	30.83	35.59	33.57
2	added	<u>45.68</u>	<u>30.17</u>	<u>24.16</u>
	total sterol	46.66	30.31	23.03
	free sterol	35.57	38.81	25.62
	steryl ester	47.56	31.31	21.13
3	added	<u>21.28</u>	<u>56.21</u>	<u>22.51</u>
	total sterol	19.23	57.91	22.86
	free sterol	18.17	57.32	24.51
	steryl ester	22.95	54.85	22.20
4	added	<u>22.54</u>	<u>29.77</u>	<u>47.69</u>
	total sterol	22.95	28.43	48.62
	free sterol	30.58	30.93	38.49
	steryl ester	25.32	27.56	47.12

Table IV. Sterol competition Experiments. Four different mixtures of sitosterol, campesterol, and cholesterol were added to aliquots of mycelium. Sterols accumulated in the mycelium after three hours were determined as described in Materials and Methods. Data is reported as percent composition.

The mitochondrion is an organelle that is highly dependent on the properties of its membranes. The interaction of sterol with the mitochondria was implicated in the sterol-induced physiological shift observed in Phytophthora cactorum. Therefore, it was thought that the mitochondrial membrane system would be the best candidate for preferential sterol deposition. A batch culture, grown in the presence of a mixture of sterols, was harvested in log phase. Gradient-purified mitochondria were isolated and the sterol composition determined. The results are shown in Table V. The composition of sterols in the mitochondria was the same as that for the whole cell sample. Preferential deposition is, therefore, not a factor of sterol specificity in Phytophthora cactorum. It should be noted that for this experiment lanosterol was included in the sterol mixture. The lanosterol stock contained two major peaks as determined by GLC. Both peaks were present in the whole cell sterols and mitochondrial sterols. However, one of the peaks was altered only in the mitochondrial sample. Its retention time on GLC was increased by 2.5 minutes. It is not certain if the alteration of this compound was a reflection of specificity or a fortuitous result. Modification of the structure may have circumvented the criteria for exclusion from the membrane. If this is indeed the case, then it can be concluded that the presence of a preferred sterol is more important in the mitochondrial membranes than in the protoplasmic membrane. On the other hand, the enzymes in Phytophthora cactorum that modify the sterol ring structure may have simply modified the sterol prior to its availability for insertion into the mitochondria.

	Relative Retention Time				
	0.82	0.89	1.00	1.07	1.19
Whole Cell	15.98	31.05	47.18	5.79	----
Mitochondria	19.98	31.11	43.16	----	5.75

Table V. Sterol Deposition in Mitochondria. A batch culture was supplemented with a mixture of sitosterol, campesterol, stigmasterol, and lanosterol. After four days growth, an aliquot was removed for determination of whole cell sterol composition. The remaining mycelia was used for the preparation of gradient purified mitochondria. In both cases, sterol composition was determined from the non-saponifiable fraction of lipid extracts. Retention times are relative to sitosterol. Data is reported as percentage of sterol mixture.

V. Summary

The supplementation of sterol to the culture medium of Phytophthora cactorum resulted in a three-fold increase in the final cell yield. Concomitant elevated production of carbon dioxide and reduced rate of substrate utilization, as evidenced by decreased uptake and reduced glycolytic activity, indicated a shift, in the manner of the Pasteur effect, to a more respiratory type of metabolism. The sterol induced oxidative phosphorylation was shown, however, not to be the result of de novo synthesis of the electron transport components.

Uptake of sterol by Phytophthora cactorum was shown to follow first order kinetics. Double reciprocal plots of the rate of sterol uptake versus concentration in the medium resulted in a straight line. The lines generated for cholesterol and sitosterol were essentially superimposable. These two sterols differ in structure only in the presence of absence of an ethyl group at position C24 of the sterol side chain. It was concluded, therefore, that Phytophthora cactorum did not exhibit specificity for sterol side chain structure in initial uptake. From the double reciprocal plot, an apparent K_m of $18.0\mu M$ and V_{max} of 4.81×10^{-5} $\mu moles$ per minute per mg dry weight were determined.

The absence of specificity in initial uptake was confirmed in competition experiments. The composition of sterol mixtures added to the medium was maintained in the total sterol extracted from the mycelium. In addition, these compositions were maintained in the free sterol pool and the steryl ester pool. Therefore, it was concluded

that sterol specificity at the level of esterification was also lacking in Phytophthora cactorum.

The sterol composition of purified mitochondria was essentially the same as that for the whole cell. Although at least one sterol or sterol derivative found in the mitochondria was structurally modified, no exclusion of individual sterols was observed. A lack of specificity in sterol deposition in specific membranes was suggested.

The accumulation of sterol by Phytophthora cactorum was shown to be relatively free from regulatory control. This is a distinct advantage for studies of the influence of sterol structure on biological membranes. Contrasting sterols may be incorporated into target membranes with much less difficulty than observed in many of the other systems used in similar studies.

The direct involvement of the mitochondria in the sterol effect on Phytophthora cactorum suggests a foundation for the use of the organism in the study of sterol function in the cell. The experimental methods and physiology of the mitochondrion have been well established in the literature, making this a very attractive system.

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