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EFFECT OF GLUCOSE AND PHOSPHATE

ON GROWTH OF THE DERMATOPHYTE <u>TRICHOPHYTON</u> <u>MENTAGROPHYTES</u>

by

Miriam L. Greenberg

A THESIS SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL

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1975

DEDICATION

TO CARING, AND TO THOSE WHO CARE.

THAT IS THE AIM TO WHICH SCIENCE
SHOULD DEDICATE ITS VAST POTENTIAL.

ACKNOWLEDGEMENTS

Miriam L. Greenberg is the daughter of Betty Greenberg and the late Jack Greenberg. She was born January 5, 1951 in Miami Beach, Florida.

Her elementary education was obtained at the Hebrew Academy of Greater Miami, and secondary education at Miami Beach High School where she graduated valedictorian and National Merit Finalist in 1968.

In September of 1968, she entered Reed College in Portland, Oregon with an Elliot Scholarship. She spent her junior year at Hebrew University of Jerusalem in Israel, having received the Popick-Civic League of Miami Academic Scholarship. She graduated from Reed in May, 1972. In September of 1972 she began her studies for a Master's degree in the Department of Microbiology at Loyola University of Chicago, Stritch School of Medicine. From then until September of 1973, Ms. Greenberg received an assistantship from the Department of Pediatrics in order to carry out a screening program for Tay-Sachs disease, in conjunction with the Chicago chapter of the National Tay-Sachs Foundation. She helped form the Graduate Student Council at the Medical Center in 1974. Also in that year she was second place winner in the Graduate Student Research Forum for student papers sponsored by the Loyola University Chapter of Sigma Xi.

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ABBREVIATIONS

C ....centigrade

cm ....centimeters

conc ....concentration

g ....grams

GP ....glucose and phosphate

hr ....hours

lbs ....pounds

M ....molar

μg ....micrograms

μm ....micrometers

mg ....milligrams

ml ....milliliters

mm ....millimeters

mM ....millimolar

min ....minutes

nm ....nanometers

O.D. ....optical density

% ....percent

r.p.m. ....revolutions/minute

v ....volume

wt ....weight

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I. INTRODUCTION

The dermatophytes are a well-defined group of pathogenic fungi consisting of three genera: Epidermophyton, Microsporum, and the subject of this work, Trichophyton. In contrast to many other mycotic infections which are potentially systemic in nature, these fungi are capable of invading only the superficial tissue, i.e., the hair, nails, and superficial layers of skin, in humans and other animals.

"Dermatomycoses," as dermatophyte infections are called, are classified according to the site of infection rather than according to the etiologic agent of infection. This is true because even a single species of dermatophyte can present a variety of clinical symptoms, depending upon the part of the body in which it is found (Emmons et al., 1970). Trichophyton mentagrophytes infections, or "tinea" as they are called clinically, can occur in many different parts of the body. This organism is the leading cause of tinea pedis, or "athlete's foot." In a more generalized form known as "jungle rot," the organism contributed to the disability of many soldiers in Southeast Asia. In general, poor hygienic conditions are predisposing factors in the development of the disease.

Most strains of T. mentagrophytes are capable of producing both microconidia and macroconidia, the former occurring predominantly. When placed in favorable environmental and nutritional conditions, microconidia undergo a process of germination, which has been character-

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ized by Hashimoto et al. (1972). Following germination, a germ tube
develops and elongates gradually to form a hypha. In this stage of
growth, the vegetative stage, the hyphae branch and intertwine to form
mycelial masses. After two to three days of incubation in liquid media,
septation of the hyphae leads to the formation of arthrospores, which
appear under the phase contrast microscope as long "strings of pearls."
Arthrospores are evident in clinical specimens, although they do not
develop when cultured on agar, an environment which is more conducive
to microconidiation.

In the process of increasing the phosphate concentration in T.
mentagrophytes growth media in order to strengthen the buffering
capacity, Hashimoto (personal communication) noticed that the increased
phosphate concentration resulted in inhibition of growth. Further in-
vestigation revealed that inhibition due to higher concentrations of
phosphate occurred only when the medium also contained relatively high
concentrations of glucose. The concentrations that were found to
severely inhibit growth were 0.1 M phosphate and 0.2 M glucose, concen-
trations that are well within the range used in many microbiological
systems, as discussed below. However, as inhibition of this type has
not been described in other systems, the observation merited further
investigation, particularly with regard to a characterization of the
nature of inhibition.

In the course of further experimentation, it was noted that inhi-
bition due to high concentrations of glucose and phosphate was affected
by the method of sterilization of the medium. At some stage in the
growth cycle of the organism, inhibition was dramatically increased when
all the components of the media were autoclaved together (at 121 C and

15 lbs/inch^2) as compared to that which occurred when glucose was auto-claved separately and added aseptically to the rest of the medium.

Changes in autoclaved sugars have been noted since the 1930's. Smith reported (1932) that glucose and maltose undergo 29% and 62% destruction, respectively, when autoclaved with phosphate for 40 min. Davis and Rogers (1939) confirmed that autoclaving sugar-containing solutions for 30 min at 121 C was very much more destructive than 'momentary' autoclaving. The most unstable sugars tested were shown to be fructose, glucose, arabinose, and lactose.

Polarimetric data and chemical analyses indicated that autoclaving glucose with phosphate buffer at pH 6.4 to 6.6 resulted in conversion of the sugars to ketoses (Englis and Hanahan, 1945). The amount of con-version increased with an increase in the amount of phosphate present.

Most recently, Namiki et al. (1973) demonstrated that free-radical products were formed when certain amino acids and sugars were heated. Specifically, both browning and free-radical generation were seen in a mixture of D-arabinose, alanine, and distilled water. The free-radicals were detected spectroscopically by ESR (electron spin resonance), and the extent of browning was determined by measuring absorbance at 420 nm.

Most of the work done with autoclaved media dealt primarily with the biological effects upon various microorganisms, although a number of investigators did attempt partial chemical characterization, as will be seen in the discussion which follows.

Lewis (1930) noted that Phytomonas malvaceara was inhibited by the presence of sugars in the culture media, under certain conditions. Inhibition was found to be dependent upon the composition of the medium, the amount of sugar present, and the method used to sterilize the medium.

Temperature and the combination of components which were sterilized together were critical factors. In all cases in which glucose was sterilized in the presence of phosphate, growth was inhibited.

The pH at which sterilization occurred was also seen to be critical. If the pH of the medium was adjusted to 5.4 before being autoclaved, and to pH 7.2 following autoclaving, there was no color change, and the medium supported good growth. If the pH before sterilization was adjusted to 8.8, growth did not occur. Lewis concluded that inhibition was caused by the reaction product of a glucose-nitrogen compound with phosphate in alkaline conditions.

Caramelization alone did not inhibit growth. If the glucose were caramelized in the dry state and added to the medium, growth was not inhibited.

Other organisms that were inhibited included Staphylococcus epidermidis, Staphylococcus aureus, Sarcina lutea, and Bacillus anthracis, whereas Serratia marcescens, Salmonella enteritides, Escherichia coli, Enterobacter aerogenes, Pseudomonas fluorescens, and Pseudomonas aeruginosa were not inhibited by medium in which sugars were autoclaved.

Lewis concluded that in the presence of phosphate during heating, sugars reacted with nitrogen compounds to form altered nitrogen compounds which could be utilized by some but not all organisms studied.

Heat-sterilized sugars were reported to deleteriously affect both the lag time and the thermal resistance of Escherichia coli. Baumgartner (1938) reported that sterilizing media containing sugars by autoclaving resulted in the production of material which was directly toxic to E. coli when grown at 54 C, and which caused a delay in growth at 37 C. The toxic material was produced simultaneously with the caramelization

of media containing reducing sugars and phosphate or nutrient broth. When sugar-containing solutions were filter-sterilized, the toxic material was not formed, and when sugar was sterilized in distilled water, only heat treatment drastic enough to cause caramelization could produce the toxic product.

The cytophagae, or cellulose-decomposing soil bacteria, were also studied in relationship to growth in sugar-containing media. Stanier (1942) reported that autoclaving glucose together with the culture medium inhibited growth of the cytophagae, whereas no toxic effect was seen if the glucose were autoclaved by filtration. The toxic material, Stanier noted, must have been active in exceedingly low concentrations, since less than 5% of the glucose was degraded during autoclaving. Thus, in an 0.3% glucose solution, the concentration of degradation product was only 0.015%. Sijpesteijn's results (1949) on the effect of sugars on the growth of Sporocytophaga myxococcoides differed from those of Stanier with regard to toxicity of high concentrations of sugar. Stanier reported glucose utilization and cellulose breakdown even in media containing greater than 0.5% glucose (up to 2%) if the glucose was sterilized by filtration. Sijpesteijn noted that increasing the glucose concentration from 0 to 0.5% increased the time necessary for obtaining visible growth. (Since the cytophagae are cellulose-decomposing organisms, it can be assumed that the effects of increased glucose concentration reflect a type of end-product inhibition of cellulose breakdown). The lag in growth due to increasing glucose was reduced when incubation temperature was reduced from 30 C to 25 C, and when the cell suspension in the inoculum was increased. Both were in agreement, however, that if the glucose were autoclaved in the growth medium at alkaline pH, growth inhibition

occurred.

In a study of the growth requirements of Neisseria gonorrhoeae (Gould et al., 1944), mild autoclaving of the whole medium was not inhibitory, nor was medium in which glucose was sterilized separately in distilled water. However, several strains of N. gonorrhoeae were inhibited if glucose and phosphate were autoclaved together at pH 7.4. This inhibitory effect could be alleviated if meat infusion were added to the medium. In 1950, Lankford reported that glucose degradation products in autoclaved media inactivated the cysteine in the peptone, and that a supplement of cysteine was necessary when glucose was sterilized with the medium.

Growth of mammalian tubercle bacilli was also shown to be retarded, as reflected by a loss of 60% in dry weight yield of the bacilli, when grown in caramelized medium (Corper and Clark, 1946). In this report, however, it must be noted that no distinction was attempted between caramelization-caused inhibition, and inhibition due to products formed simultaneously with the process of caramelization of sugars.

The lactic acid bacteria Lactobacillus arabinosus, Lactobacillus casei, and Leuconostoc mesenteroides were studied by Lankford et al. in 1947. Growth of these organisms was normal when glucose was autoclaved separately from the other components of the media. But autoclaving glucose in the medium resulted in lower growth maxima, and partial "inactivation" of essential nutrients. It was suggested that inactivation occurred as a result of combining the reactive functional groups with the aldehyde degradation products of glucose. In particular, increasing the pH of the medium above 6.8 before autoclaving or increasing the autoclaving time resulted in increased inactivation of cysteine.

Hill and Patton (1947) noted that if the Maillard reaction (the browning reaction which occurs when amino acids and reducing sugars are heated) could be avoided by separate autoclaving of glucose, the organism Streptococcus faecalis grew better. Decreased growth of this organism in a medium which had undergone the Maillard reaction was attributed to the destruction of nutrients, in particular amino acids and vitamins (Patton and Hill, 1948). Inactivation of these nutrients was minimized when glucose was replaced by sucrose.

In disagreement with the hypothesis that inhibition was due to inactivation of nutrients by glucose degradation products, Finkelstein and Lankford (1957) suggested that specific substances toxic to Vibrio cholerae were formed when glucose and phosphate were autoclaved together. When glucose was autoclaved in medium containing 0.2% phosphate, they noted a delay in the appearance of visible growth, the duration of which was inversely related to the inoculum size. At phosphate concentrations of 0.5 to 1% (about 0.07 M), no growth at all occurred with small inocula. Additional factors affecting inhibition included the initial pH of the glucose-phosphate solution before sterilization, and the temperature and duration of autoclaving. Maximum inhibition occurred at pH 7 to 10, and with 20 min of autoclaving. Finkelstein and Lankford ruled out the possibility that the cause of inhibition was the binding of essential nitrogenous components by glucose degradation products, as stoichiometric evidence did not confirm this hypothesis. Consistent with the findings of Gould et al. (1944) mentioned above, inhibition could be reversed with the addition of reducing agents to the medium. Cysteine and glutathione in concentrations of 10 to 100 μg/ml could counteract toxicity, as could small amounts of complex culture media, such as yeast extract.

These workers characterized the toxic components as nonvolatile, and capable of being adsorbed with acid-washed activated charcoal. It was suggested that at least two related acidic carbonyl compounds, probably aldehydes, were the components toxic to Vibrio cholerae and to certain other gram negative bacteria tested.

The effect of sugar sterilization has been observed also in relation to fungal growth. The fungus Thielaviopsis basicola was inhibited by medium in which fructose and, to a lesser extent, glucose was autoclaved (Barnett et al., 1953). The degree of inhibition increased with increased incubation temperature, up to approximately 31.5 C, and was not evident at 20 to 25 C.

The interaction of amino acids and a number of reducing sugars during autoclaving caused inhibition of growth of Phytophthora fragariae, a fungus infecting strawberries (McKeen, 1956). Autoclaved non-reducing sugars were not inhibitory, nor was medium to which were added filter sterilized sugars.

In plants, growth of fruit bodies of Flammulina velutipes was greatly inhibited by the products of autoclaving glucose with amino acids, in particular glucose and glutamine (Gruen and Wu, 1972).

Autoclaved media were also seen to affect the susceptibility of Escherichia coli to copper toxicity (Burke and McVeigh, 1967). It was noted that copper ions could be toxic to E. coli, and that toxicity increased with increased incubation temperature and with increased exposure of the medium to heat during sterilization.

Shortly after Lewis' work (1930) on inhibition of Phytomonas in autoclaved media, Fulmer et al. (1931) reported that autoclaving glucose with phosphate-containing media stimulated the growth of

Enterobacter pectinovorum, as reflected in the time necessary for first appearance of growth. Stimulation in autoclaved medium containing NH_4Cl, K_2HPO_4, and 5% glucose was found to be proportional to the extent of caramelization of the medium. However, extraction of the caramel products with activated charcoal revealed that stimulation was not due to the caramelization products but to some other product(s) produced at the same time. Other organisms that were stimulated included Enterobacter aerogenes, Serratia marcescens, Escherichia coli, Bacillus subtilis, Escherichia freundii, Enterobacter faeni, and a species of Actinomyces.

The group of organisms most studied with reference to stimulation in autoclaved media were the lactic acid bacteria. In discussing production of different cheeses by slight variation of production techniques, Orla-Jensen (1933) reported that Streptococcus lactis was the only species of the lactic acid bacteria that could ferment glucose which had been sterilized alone in distilled water. Heat sterilization of sugar-containing media resulted in conversion of sugars into other forms; 9% of the glucose was converted, mainly into fructose, 20% of the fructose was converted, mainly into glucose, and other volatile substances were formed that included furfuraldehyde, methylglyoxal, and a diacetyl compound. Snell et al. (1948) further noted that even greater stimulation of growth of Lactobacillus bulgaricus occurred when glucose was separately heated with phosphate at pH 7 and added aseptically to the medium. Optimum stimulation occurred with a large inoculum consisting of young cultures in medium which had been autoclaved for a prolonged time period. Stimulation also occurred when glucose was autoclaved with amino acids and yeast extract. Since pyruvate cause stimulation, Snell et al. (1948) suggested that it was one of the main products formed. Stimulatory

substances produced upon heating enhanced the growth of Lactobacillus fermenti too (Snell and Lewis, 1953). Glucose was always involved in the production of stimulants, especially when autoclaved with potassium phosphate or with sodium acetate, and to a lesser extent with casein hydrolyzate, sodium hydroxide, or tap water; but glucose autoclaved in distilled water was not stimulatory. Snell and Lewis indicated that reducing agents such as cysteine could substitute as the stimulatory agent(s) in some media. N-D-glucosylglycine could replace the stimulatory effect of autoclaved media in Lactobacillus gayoni (Rogers et al., 1953). Further work with Lactobacillus fermenti (Ramsey and Lankford, 1956) revealed that the nature of stimulation of growth was that of reduction of the lag phase, whereas the rate of growth in the exponential phase was not affected. Their attempts to characterize the stimulatory components seemed to rule out the possibility of an aldehyde, a ketone, a phosphorylated glucose derivative, a volatile compound, a reducing agent, or an acidic substance.

In a study of the oxygen requirements of lactobacilli, streptococci, leuconostocs, pediococci, and aerococci, Whittenbury (1963) showed that the method of sterilizing the fermentable substrates greatly affected growth. Growth of Lactobacillus brevis, L. buchneri, and L. viridescens differed according to the method of sterilization. Although aerobic growth was similar in both kinds of media, anaerobic growth developed rapidly in a medium in which the substrate was sterilized by autoclaving directly in the medium, but slowly or not at all in medium containing separately-sterilized substrate. Growth of lactobacilli also differed at different incubation temperatures when soft agar medium in which glucose was autoclaved was used for growth. Some strains of Lactobacillus brevis

and _Lactobacillus bucheri_ grew aerobically and anaerobically at 30 C, but only anaerobically at 37 C.

Further studies with _Lactobacillus brevis_ (Stamer and Stoyla, 1967) showed that the presence of glucose at the time of autoclaving stimulated growth of the organism under stationary and anaerobic conditions, although there was no difference in aerobic growth between autoclaved and filter-sterilized media. Stimulation was shown to be dependent upon the concentration of glucose present at the time of autoclaving. Comparison of the carbohydrate composition of autoclaved and filter-sterilized media suggested that fructose was produced upon autoclaving, and when fructose was added to filter-sterilized medium, stimulatory effects were evident. Further evidence that fructose may be capable of stimulatory activity was suggested by Stamer and Stoyla (1970) with regard to the organism _Leuconostoc citrovorum_. Filter-sterilized plant extracts (tomato) were shown to contain stimulatory material which, upon fractionation, revealed only two components after acid hydrolysis: glucose and fructose. Both the stimulatory factors and increased CO_2 tension were necessary to reduce the lag in growth. It was suggested that fructose stimulated growth by acting as a hydrogen acceptor. The specificity of stimulation was reflected in the fact that L-sorbose (an epimer of fructose) was inactive insofar as stimulation was concerned. Pyruvate was also inactive, contrary to the findings of Snell et al. (1948) with _Lactobacillus bulgaricus_.

The inability of pyruvate to cause stimulation in _Leuconostoc citrovorum_ was also contrary to earlier data with _Streptococcus salivarius_ (Smiley et al., 1943), in which increased pyruvate caused increased stimulation of growth of the organism. Stimulation by autoclaved media

here too depended on prolonged heating of the media and was not associated with caramelization.

In <u>Streptococcus</u> <u>faecalis</u>, Rabinowitz and Snell (1947) showed that although autoclaving the medium was necessary to support growth, prolonged autoclaving rendered the medium incapable of supporting maximum growth. In addition, medium in which the glucose was separately autoclaved could be used if the glucose were alkali-treated, or if ascorbic acid were added to the medium. Sodium thioglycollate and cysteine were also effective to a lesser degree, although cystine was not. They concluded that <u>S</u>. <u>faecalis</u> required a non-specific reducing agent for growth.

The nature of stimulation of <u>Bacillus</u> <u>globigii</u>, now called <u>B</u>. <u>subtilis</u> var. niger, was similar to that of <u>Lactobacillus</u> (Sergeant et al., 1957). The product of autoclaved glucose and phosphate stimulated <u>B</u>. <u>globigii</u> by reducing the lag time, proportionally, with increased glucose and phosphate concentrations. The size of inoculum was significant in that only large inocula could grow in separately autoclaved media. However, the stimulatory factors themselves varied. In contrast to Ramsey and Lankford's findings (1956) that the compound was probably not an acidic one, Sergeant et al. (1957) claimed that the nonvolatile compound was in fact a carboxylic acid with at least one hydroxyl group and some unsaturated bonds. In addition, whereas the stimulatory factor for some lactic acid bacteria and certain gram negative bacteria (Smiley et al., 1943; Ramsey and Lankford, 1956; and Fulmer et al., 1931) could not be adsorbed by activated charcoal, substance(s) which stimulate(s) growth of <u>B</u>. <u>globigii</u> was (were) charcoal adsorbable and partially ether-soluble.

Growth of four strains of propionibacteria was enhanced when glucose was autoclaved in the medium (Field and Lichstein, 1957). Here too, concentration of cells was a factor, as separate autoclaving resulted in growth only when a large inoculum was used. Evidence that further suggested the variable nature of stimulation was seen in that some substances which were capable of replacing the enhancement factor in other organisms, such as glyoxal, glyoxylic acid, pyruvate, and cysteine, were inactive in the propionic acid bacteria, while N-D-glucosylglycine, which was stimulatory to the growth of Lactobacillus gayoni (Rogers et al., 1956), was active to some extent in propionibacteria under certain conditions, at concentrations of 0.1 to 0.8 mg/ml. In these organisms, the amount of stimulation was not dependant upon the amount of browning of the medium (Field and Lichstein, 1958). However, the stimulatory material was in fact removed from the medium by treatment with activated charcoal, and could be eluted from the charcoal with 50% ethanol. CO_2 and some dicarboxylic acids could also replace stimulation caused by autoclaving. Field and Lichstein suggested that reducing carbohydrates such as glucose reacted with phosphates and amino acids during heating, resulting in the production of stimulatory substances which replaced the CO_2 requirement for initiation of growth in small inocula.

Growth stimulation due to autoclaved media was manifested in Mycobacterium paratuberculosis by "circumvention of the mycobactin requirement" (Morrison, 1965). That is, if autoclaved media were used (rather than filter-sterilized media), mycobactin was not required for growth. Morrison suggested that this organism was capable of only very slow growth on filtered media because the stimulating factors were formed very slowly at 37 C. Autoclaving the medium resulted in the formation of a compound

with an absorption maximum of 290 nm and minimum of 250 nm, possibly a glucose-phosphate compound.

In a description of the growth requirements of the Acidaminococci (Rogosa, 1969), it was reported that amino acids could serve as the sole energy source for growth. However, derivative products from glucose autoclaved in the culture media stimulated growth of these bacteria and in some cases were required for growth. It is interesting to note that the stimulatory effect described here was equally strong in strains utilizing glucose and with those not utilizing glucose.

The effect of sterilization of media upon ability to sustain fungal growth was studied as early as 1927 by Fulmer and Huesselmann. They reported that (a) growth stimulant(s) for the yeast Saccharomyces cerevisiae was (were) produced when the growth medium was autoclaved. Three types of caramel substances could potentially be produced: those containing sugar and ammonium chloride, sugar and potassium phosphate, and ammonium chloride and potassium phosphate. Fulmer and Huesselman concluded that the stimulant may be a caramel or (a) product(s) formed simultaneously with the caramelization process.

The aquatic fungus Pythiogeton was also noted to be stimulated by an autoclave-produced substance in the medium (Cantino, 1951). The fungus could grow on fructose as a carbon source only in a medium in which yeast extract had been autoclaved. Cantino characterized the stimulant by ruling out possibilities of it being a vitamin, amino acid, nucleic acid, or phosphorylated sugar. The factor exerts an all-or-none effect, Cantino reported, with a minimum threshold concentration. Although there was an interrelationship between the size of inoculum and the concentration of yeast extract required (with increased inoculum size the amount of required

extract decreased), the requirement for the stimulating factor could not be replaced with large inocula, which suggested that the fungus did not synthesize it.

Other reports of stimulation of fungal growth by autoclaved media include that of Wilson and Lilly (1958) on the growth of some species of Ceratocystis, and Papavizas and Ayers (1964) with Aphanomyces euteiches. Neither papers discussed characterization of the stimulatory components.

Stimulating factors produced upon heating of the growth media have also been studied in mammalian cells. Stimulation of mammalian epithelial cells cultured in vitro was demonstrated by the addition to culture medium of a concentrate obtained from heated solutions of glucose and phosphate (Sergeant and Smith, 1960). A decrease in cell generation time was noted. The stimulatory effect was most pronounced when small inocula were used. The concentrate was prepared by autoclaving a solution containing glucose and phosphate, adsorbing with acid-washed activated charcoal for 12 hr, and washing the charcoal with water and methanol until the filtrate was colorless. The active factor was then eluted from the charcoal with a pyridine:water (40:60) solution and the dark brown eluate was resuspended in water and filter-sterilized. Sergeant and Smith suggested that the active factor was active in the concentration range of 10 to 250 µg/ml, and was probably a carboxylic acid with at least one hydroxyl group which supplemented or partially substituted for some component of the horse serum. The biological manifestation of activity was such that it promoted early and firm attachment of cells to glass.

Both inhibitory and stimulatory substances produced upon autoclaving

glucose and phosphate were noted in the growth of <u>Clostridium</u> <u>botulinum</u> (Bowers and Williams, 1962). The inhibitory substance was extractable with activated charcoal, and it increased with increased storage of the medium. The stimulatory substance acted by decreasing the time required for the appearance of turbidity.

The interrelationship between glucose and phosphate, aside from that seen in autoclaved media, has long been known to be involved in the "Pasteur effect," in which a limiting amount of phosphate in the presence of oxygen inhibits glycolysis almost universally. This occurs presumably by inhibiting the activity of the enzyme phosphofructokinase, which catalyzes the reaction converting fructose-6-phosphate to fructose-1,6-diphosphate (Mazur and Harrow, 1971). Respiration, on the other hand, can be inhibited, particularly in malignant tissue, by the addition of glucose, resulting in a high rate of aerobic glycolysis often seen in tumor cells. This phenomenon is known as the "Crabtree effect" (Mazur and Harrow, 1971). The "Crabtree effect" was seen to repress respiration by many yeasts when the glucose concentration was high and phosphate was limiting (DeDeken, 1966). However, reports of inhibition of microorganisms due to the combination of <u>both</u> high glucose and high phosphate concentrations have not been seen in the literature. In fact, the concentrations of sugar and phosphate which inhibited growth of <u>Trichophyton</u> were within the range of concentrations often used in microbiological media for both bacteria and fungi in order to elucidate metabolic pathways, to develop defined media, to compare clinical specimens, and to characterize enzymes.

With regard to work with microbial metabolism, Barnett (1972) developed a procedure for testing the ability of yeasts to utilize

sugars anaerobically, which involved the use of 0.2 M sugar solutions, and 0.1 M KH_2PO_4. Blumenthal et al. (1954) suggested the use of 0.1 M phosphate buffer for cell suspensions in a procedure to determine the relative extent of participation of the Embden-Meyerhof pathway and the hexose monophosphate shunt in the yeasts Saccharomyces cerevisiae and Torulopsis utilis. Pathways of glucose metabolism in the dermatophyte Trichophyton rubrum were elucidated by Chandra and Banerjee (1972, 1973) using 0.1 M phosphate.

In clinical work with the highly pathogenic fungus Cryptococcus neoformans, cell wall differences of clinical and soil isolates of the fungus were compared using growth media containing 0.2 M phosphate buffer (Cook et al., 1970). Inhibition of another pathogenic fungus, Coccidioides immitis, with the antibiotic amphotericin B was observed by measuring respiration of the organism in media containing 0.33 M phosphate buffer (Sippel et al., 1970).

Sabouraud medium, one of the most common commercial media for fungal growth, contains 0.2 M glucose, although the potassium phosphate concentration in Sabouraud is rather low, generally less than 0.02 M, depending upon the type of peptone used (Difco Manual).

These concentrations of glucose and phosphate have been used in bacterial growth media too. A growth medium containing 0.07 M potassium phosphate was suggested for nutrition of Streptococcus bovis (Barnes et al., 1961), and a solid lactose medium for Agrobacterium was buffered with phosphate concentrations of up to 0.2 M (Bernaerts, 1967). A 0.1 M phosphate buffer was also used to suspend species of Lactobacillus for tests determining the formation of stereospecific lactic dehydrogenases.

These are but a few examples of the variety of uses of media

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containing the ranges of sugar and phosphate found to inhibit T. mentagrophytes.

The purposes of this study were to elucidate the conditions under which inhibition due to high concentrations of glucose and phosphate occurs in Trichophyton mentagrophytes, to determine whether inhibition of this nature occurs in other organisms (some bacteria and various other fungi), and to partially characterize the compound(s) produced, upon autoclaving, which inhibit(s) growth of T. mentagrophytes.

II. MATERIALS AND METHODS

Organism. Trichophyton mentagrophytes ATCC 26323 was used throughout
this study.

Maintenance of the organism. Stock cultures of the organism were
maintained at room temperature on Sabouraud dextrose agar (Difco).
Granular type colonies undergo pleimorphic changes with time. To
avoid usage of the nongranular forms which develop, granular colonies
were transferred periodically to fresh Sabouraud agar.

Preparation and purification of the microconidial suspension. Sabouraud
dextrose agar plates were inoculated with spores from a single colony
and incubated at room temperature for seven days. The microconidia
were harvested from the agar surface by means of a glass rod covered at
one end with a piece of Tygon tubing, and emulsified in distilled water
(Hashimoto et al., 1972). The emulsion was then filtered through 10 to
12 layers of sterile cheese cloth to remove the mycelia, and the microco-
nidia collected in the filtrate were washed with cold distilled water by
repeated centrifugations until a microconidial suspension became free from
hyphal contamination, as observed by phase contrast microscopy. The
harvesting procedure and subsequent filtration and centrifugation were
carried out aseptically to avoid bacterial contamination of the suspension.
Wherever possible, the purification was carried out in the cold in order
to prevent germination of the dormant microconidia in the presence of
residual nutrients. In a purified microconidial suspension such as
that depicted in Fig. 1, a correlation was shown to exist between

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Fig. 1. Phase contrast micrograph of pure suspension of <u>T.</u>

mentagrophytes microconidia, free from hyphal and

bacterial contamination, harvested from an agar

surface with a glass rod covered with a piece of

Tygon tubing, and purified as described in the text.

(Micrograph generously donated by C. Wu, Department

of Microbiology, Loyola University Medical Center).
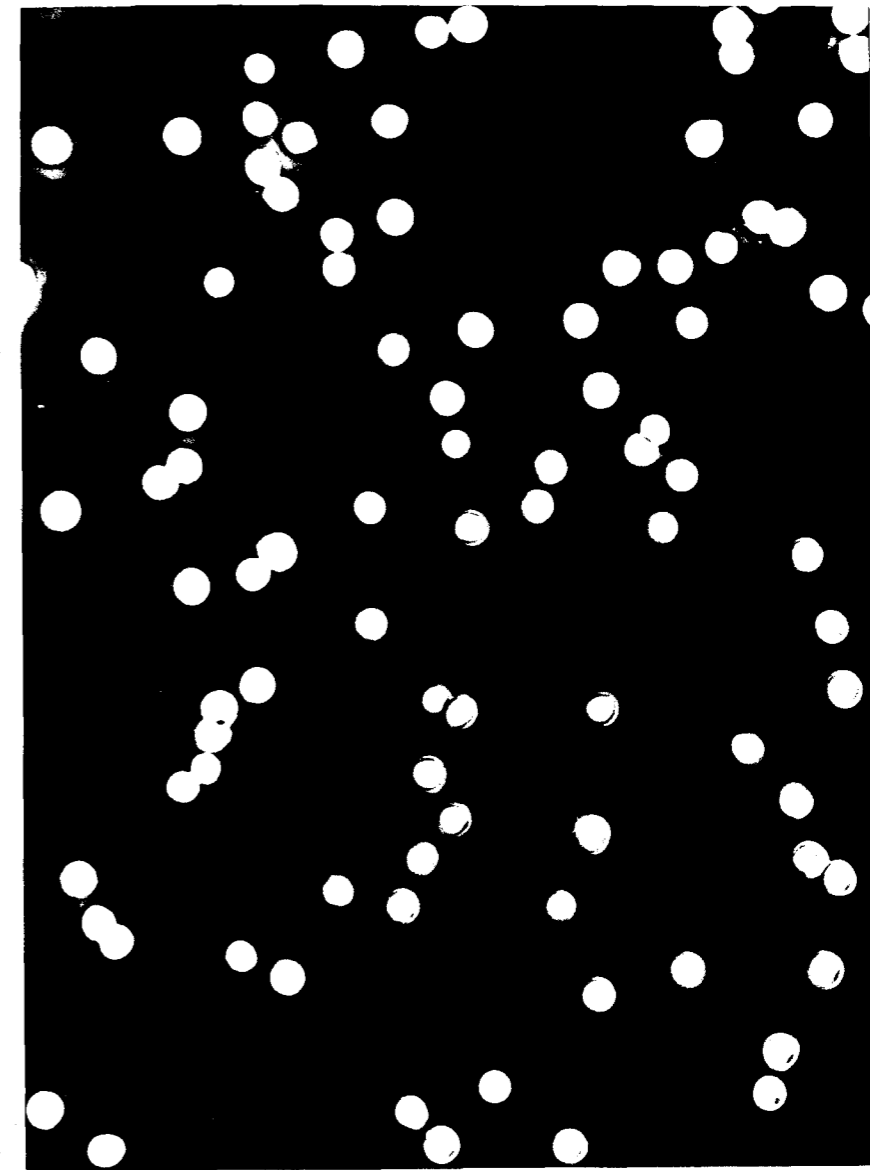
turbidity and number of microconidia, in the turbidity range of 100 to 200 Klett units (Wu, 1973). Therefore, by adjusting the turbidity of the microconidial suspension used in inocula, a predetermined number of microconidia could be consistently inoculated in each experiment.

Culture media. Two types of medium were used, a synthetic medium and a complex medium. The synthetic medium consisted of the following: $MgSO_4$, 2 mM; $FeCl_3$, 0.7 μM; $ZnSO_4$, 0.7 μM; $MnSO_4$, 0.6 μM; L-leucine, 15 mM; L-asparagine, 15 mM; glucose, 0.056 to 0.22 M (1 to 4%). The pH was adjusted to 6.5 with sodium phosphate buffer, which was added in the concentration range of 0.002 to 0.1 M. (When a single amino acid was added, L-leucine or L-alanine was used in the concentration of 15 mM). The complex medium contained 1% Bacto-peptone, to which were added glucose and phosphate as described for synthetic medium. For solid media, Bacto-agar was added to 1.5%.

Inhibition of fungal growth.

A. Germination. Dormant microconidia were inoculated into 125 ml flasks (Erlenmeyer) containing 50 ml of synthetic medium, and incubated with aeration on a New Brunswick gyratory shaker (250 r.p.m.). Aliquots were taken with a Pasteur pipette and fixed in 5% glutaraldehyde. Germination was determined by loss of refractility under the phase contrast microscope (Hashimoto et al., 1972) using an oil immersion objective (Nikon, DM 100X). Germination percentages were determined by randomly scoring 100 to 200 spores.

B. Vegetative growth.

1. Surface growth. The technique described by Mandels (1965) for measuring surface growth was modified as follows: Agar plates were

inoculated in the center of the plate with a circular piece of sterile filter paper (6 mm in diameter) which had been soaked in a microconidial suspension. The plates were incubated at 30 C and colony diameter was measured with a cm ruler in two perpendicular directions. The mean diameter of spreading growth was determined by averaging results from 5 to 10 plates, and the mean as well as the interval of standard deviation were plotted against time.

2. Total dry weight of growth in liquid media. To correlate spreading growth with a measurement of the total amount of fungus produced, dry weight determinations were performed as follows: A pre-determined number of dormant microconidia were inoculated into 250 ml flasks (Erlenmeyer) containing 50 ml of medium and incubated with aeration (250 r.p.m.) at 37 C. Cells were then harvested on a millipore filter (pore size 0.45 µm), washed with distilled water, and dried in an 85 C oven to a constant weight. Experimental flasks were prepared in duplicate or triplicate and the results were averaged, to compensate for inherent limitations of this technique. Unless otherwise indicated, 10^6 spores were inoculated into 50 ml of medium and incubated for 3 days.

3. Germ tube elongation. Dormant microconidia were inoculated into Sabouraud dextrose broth (Difco) and incubated with aeration (250 r.p.m.) until the mean germ tube length was approximately 3 µm (3 to 4 hr). The microconidia were then collected on a millipore filter, washed with distilled water, inoculated into 125 ml flasks containing 50 ml synthetic medium, and incubated at 37 C. Aliquots were taken and fixed with glutar-aldehyde as described above for determination of germination, and germ tube length was measured with an ocular micrometer in the microscope. Mean germ tube length was determined on the basis of 100 microconidia

randomly counted under the phase contrast microscope. Mean germ tube lengths and intervals of standard deviation were plotted against time.

Reversibility of inhibition. To determine whether inhibition of vegetative growth could be reversed, microconidia were germinated in Sabouraud dextrose broth until mean germ tube lengths approximated 3 μm. The microconidia were then collected on a millipore filter, washed, and resuspended in synthetic medium containing inhibitory concentrations of glucose and phosphate. Germ tube lengths were measured as described above. At specified intervals, the cells incubated in inhibitory medium were again collected on a millipore, washed with distilled water, resuspended in control medium, and germ tube length was again noted, and compared both with cells which were incubated in control medium and with those incubated in inhibitory medium for the entire time.

Characterization of the inhibitory substance(s).

A. Extraction with activated charcoal. A 250 ml Erlenmeyer flask containing 100 ml synthetic medium with the amino acids L-leucine and L-asparagine, and high concentrations of glucose and phosphate (0.22 and 0.1 M, respectively) was autoclaved for one hr (at 121 C, 15 lbs/inch^2). A 5 g quantity of Norit A was added to the medium which was then shaken intermittently for one hr at room temperature, and filtered through one layer of Whatman No. 1 filter paper. The charcoal-extracted medium was assayed biologically as described below.

B. Elution of the inhibitor(s). After first washing the adsorbed Norit with distilled water to remove residual water-soluble materials, the inhibitory substance was eluted one time from the Norit with 250 ml cold absolute ethanol, which was then evaporated over boiling water until

only a few ml remained. This was placed in an evaporating dish in a
vacuum desiccator until dry, and the residue was weighed and resuspended
in a known volume of distilled water.

C. Dialysis of crude extract. A 0.9 ml sample of the crude
ethanol extract was dialyzed in the cold against 250 ml distilled water.
The dialysate and non-dialyzable material were evaporated to dryness and
resuspended in a known volume of distilled water.

D. Assay for biological activity. A 5 ml sample of 150 to 200 Klett
microconidial suspension was inoculated into 50 ml Sabouraud dextrose
broth and incubated for 4 hr. The microconidia were then collected,
washed, and resuspended in 0.4 ml distilled water; 0.1 ml of the resulting
suspension were then inoculated into 1 ml of incubation mixture, which
consisted of Norit-extracted synthetic medium, or control medium to
which was added a known amount of inhibitor. (Control medium contained
0.02 M sodium phosphate buffer, pH 6.5, and 0.056 M glucose autoclaved
together in the synthetic medium described above). The incubation
mixture was incubated for 7 hr at 37 C, and germ tube lengths at that
time were determined as previously described.

Protoplast formation. A common method for isolating yeast protoplasts
is that described by Eddy and Williamson (1957), in which the yeast cells
are suspended in a solution containing buffer, enzyme from the intestine
of the snail Helix pomatia, and osmotic stabilizer in the concentration
of 0.55 M. To determine whether protoplasts could be prepared from
Trichophyton in this manner, 10^8 microconidia were inoculated into 50 ml
of Sabouraud dextrose broth and incubated with aeration at 37 C, until
the first sign of germ tube formation was evident (2 to 2½ hr). At that

point, the cells were collected by centrifugation at 10,000 r.p.m.
(IEC B-20 centrifuge) for 10 min at room temperature, washed with
distilled water, and resuspended in the incubation mixture, which consisted
of the following: osmotic stabilizer (sorbitol, mannitol, sucrose,
NaCl, $MgSO_4$, or $MgCl_2$), 0.3 to 0.5 M; $MgCl_2$, 0.02 M; glusulase (snail
enzyme), 10 to 20% (v/v); sodium citrate-sodium phosphate buffer,
pH 5.5, 0.005 M; H_2O to 1 ml. When indicated, 1% mercaptoethanol was
added to the incubation mixture (Davies and Elvin, 1964).

Chemicals. Bacto-agar, Bacto-peptone, and Sabouraud dextrose agar and
broth were obtained from Difco. Amino acids used in this study were
obtained from Sigma (L-leucine and L-alanine) and from Calbiochem
(L-asparagine). Glucose was obtained from Difco (Bacto-dextrose),
and all other sugars were obtained from Nutritional Biochemicals,
as were the sugar alcohols. Norit A was purchased from Sigma.
Snail enzyme (glusulase) was obtained from Endo Laboratories.

A. Inhibition of growth of T. mentagrophytes with high-GP medium.

Inhibition of growth of T. mentagrophytes was initially seen in
colony growth on solid media. Fig. 2 illustrates increasing inhibition
in high glucose medium (0.2 M) as the concentration of phosphate is
increased to 0.1 M. Inhibition of colony growth was measured
quantitatively as described above. Spreading growth on complex medium
containing higher concentrations of glucose and phosphate was inhibited
2- to 3-fold when compared with controls whether or not the glucose
was sterilized separately (Fig. 3). Inhibition was seen to the same
degree on synthetic medium (Fig. 4), although maximum growth yields
on synthetic medium were only about half those of growth on peptone.
The results of these experiments clearly showed that media containing
high concentrations of glucose and phosphate (high-GP medium) were
inhibitory to the surface growth of T. mentagrophytes.

Growth in liquid media was also inhibited by high concentrations
of glucose and phosphate. Table 1 illustrates the marked inhibition
of total dry weight of growth in high-GP medium as compared to controls.

Early vegetative growth in synthetic liquid media was determined
by measurement of germ tube elongation, as described previously. High
concentrations of glucose and phosphate inhibited germ tube elongation
in media in which glucose had been autoclaved together with the rest of
the components (glucose-autoclaved media), as seen in Fig. 5.

These data suggested that inhibition varied both with regard to the
manner of autoclaving, as well as the stage of the growth cycle which

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Fig. 2. Effect of high concentrations of glucose and phosphate
on the growth of T. mentagrophytes as demonstrated
on Sabouraud agar. All media were prepared in sodium
phosphate buffers (pH 6.5) and the concentrations of
glucose and phosphate for each medium are specified.
Each plate was inoculated with approximately 70
microconidia and incubated at 37 C for 7 days. Note
that essentially no colonies are observed when the
medium contains 0.1 M phosphate and 0.2 M (3.8%)
glucose. (Photograph generously donated by Dr. T.
Hashimoto, Department of Microbiology, Loyola
University Medical Center).

Fig. 3. Colony diameter (mean and one standard deviation) of
T. mentagrophytes grown at 30 C on solid complex
media (1% peptone) containing varied concentrations
of glucose and phosphate. A) Glucose autoclaved
separately. B) Glucose autoclaved together with media.
The difference between values for ▲ vs. ● is
significant at a level p < 0.001, as determined by
t test. Symbols: ▲, 0.22 M glucose and 0.1 M phosphate;
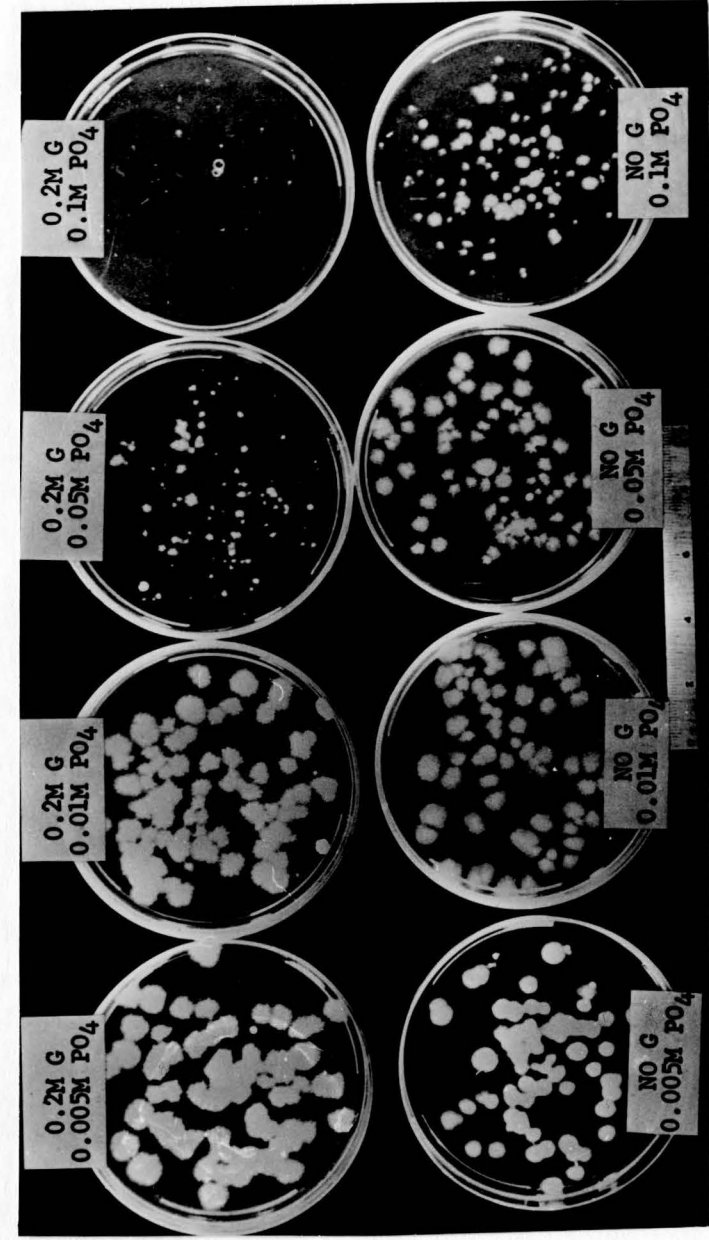●, 0.056 M glucose and 0.02 M phosphate; ✕, 0.011 M
glucose and 0.002 M phosphate.
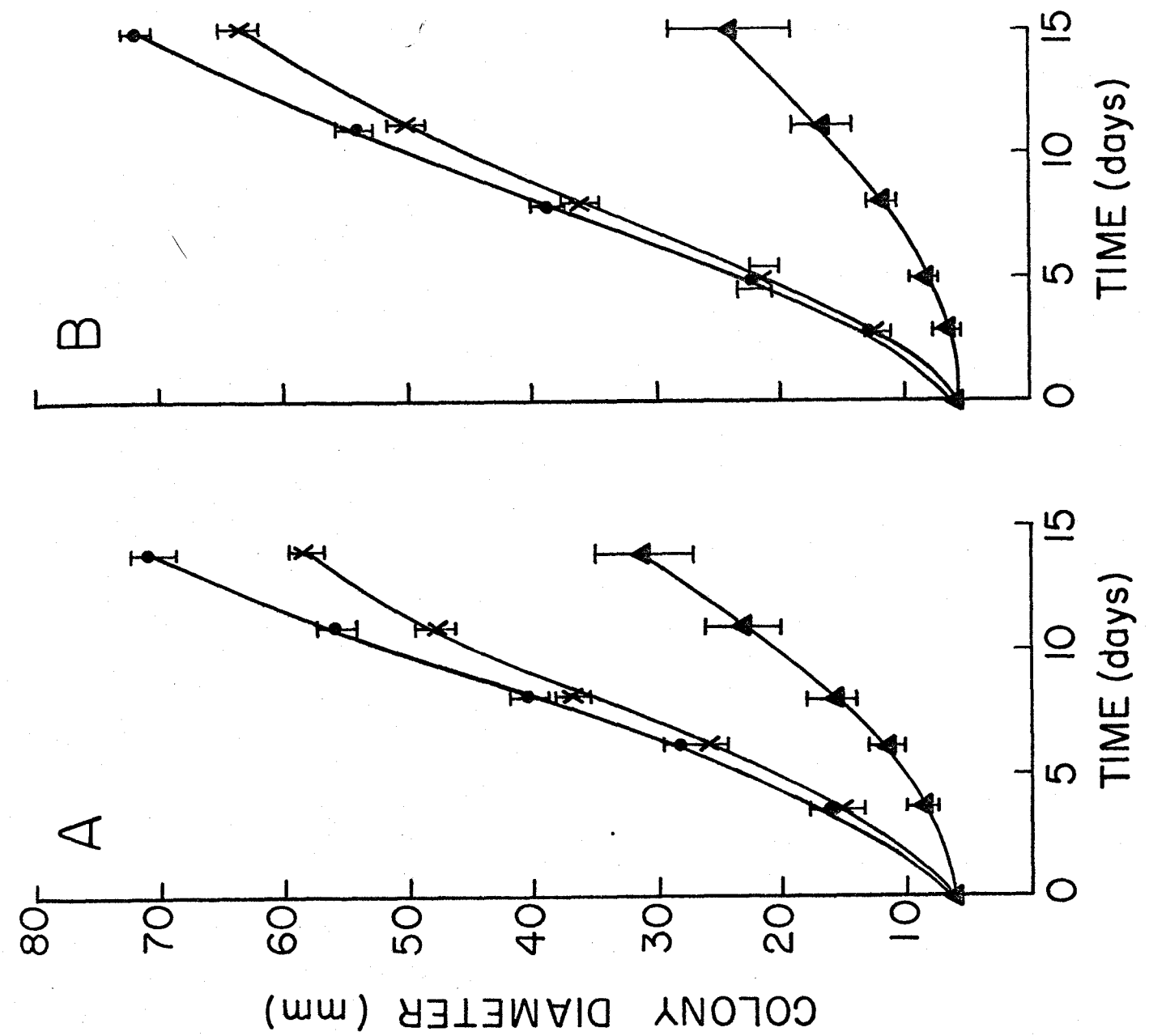
Fig. 4. Colony diameter (mean and one standard deviation) of
T. mentagrophytes grown at 30 C on solid synthetic
media containing a single amino acid (L-leucine) and
varied concentrations of glucose and phosphate.
A) Glucose autoclaved separately. B) Glucose auto-
claved together with media. The difference between
values for ▲ vs. ● is significant at a level of
$p < 0.001$, as determined by t test. Symbols: ▲, 0.22 M
glucose and 0.1 M phosphate; ●, 0.056 M glucose and
0.02 M phosphate; X, 0.011 M glucose and 0.002 M
phosphate.
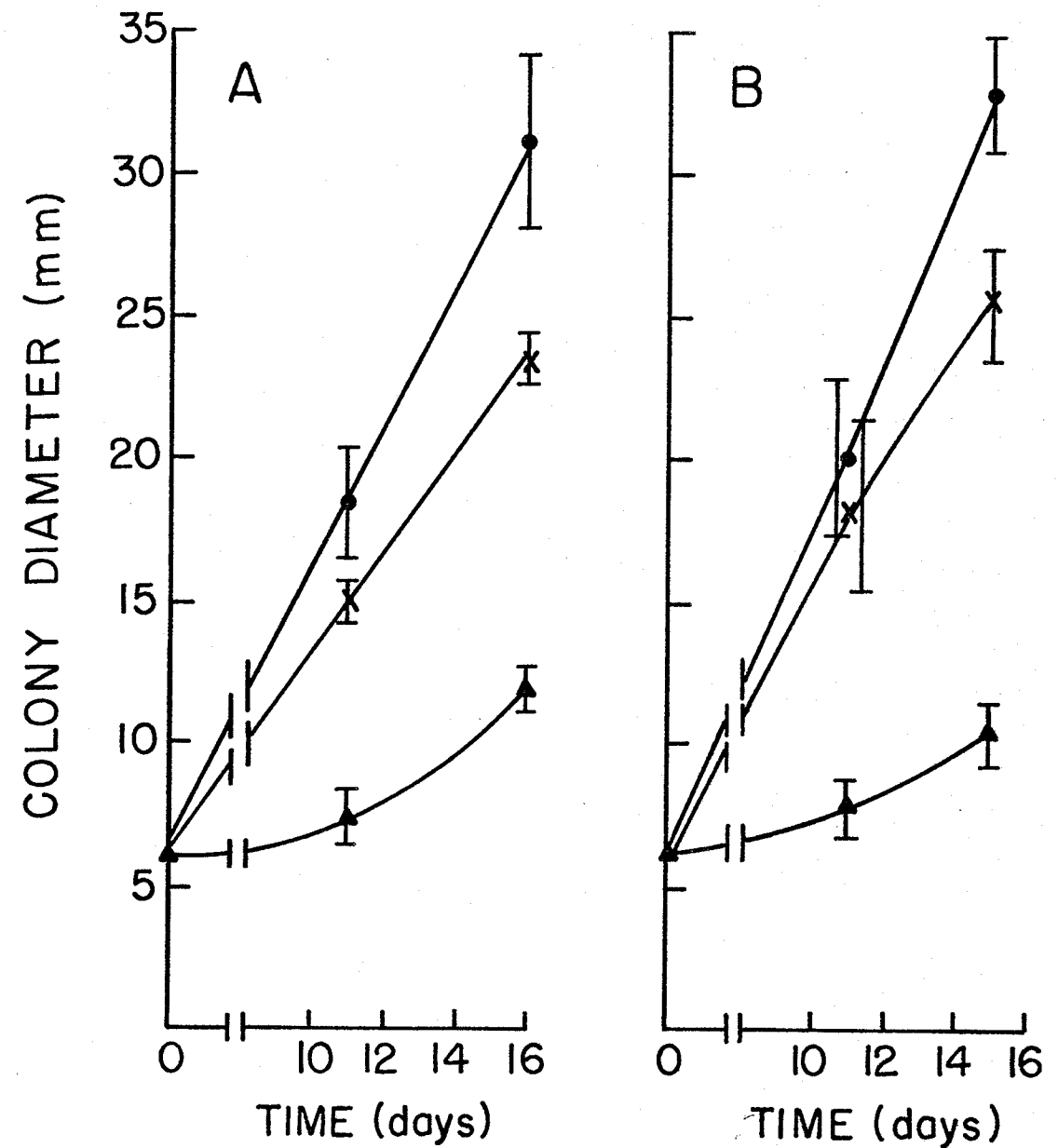
TABLE 1.

Effects of glucose and phosphate concentrations on the growth

of T. mentagrophytes in complex liquid media.

| | Growth (mg dry weight/50 ml) | |
Medium	Glucose autoclaved separately	Glucose autoclaved together
High[a]	18.8	17.2
Control[b]	27.7	52.9

[a] 0.1 M phosphate and 0.22 M glucose.

[b] 0.02 M phosphate and 0.056 M glucose.

Fig. 5. Germ tube elongation in synthetic media containing
the amino acids L-leucine and L-asparagine and varied
concentrations of glucose and phosphate. All
components were autoclaved together. Each point
represents the mean of germ tube lengths of 50
microconidia. The difference between the two
curves at 7 hr is significant at a level $p < 0.001$,
as determined by t test. Symbols: ▲, 0.22 M glucose
and 0.1 M phosphate; ●, 0.056 M glucose and 0.02 M
phosphate.
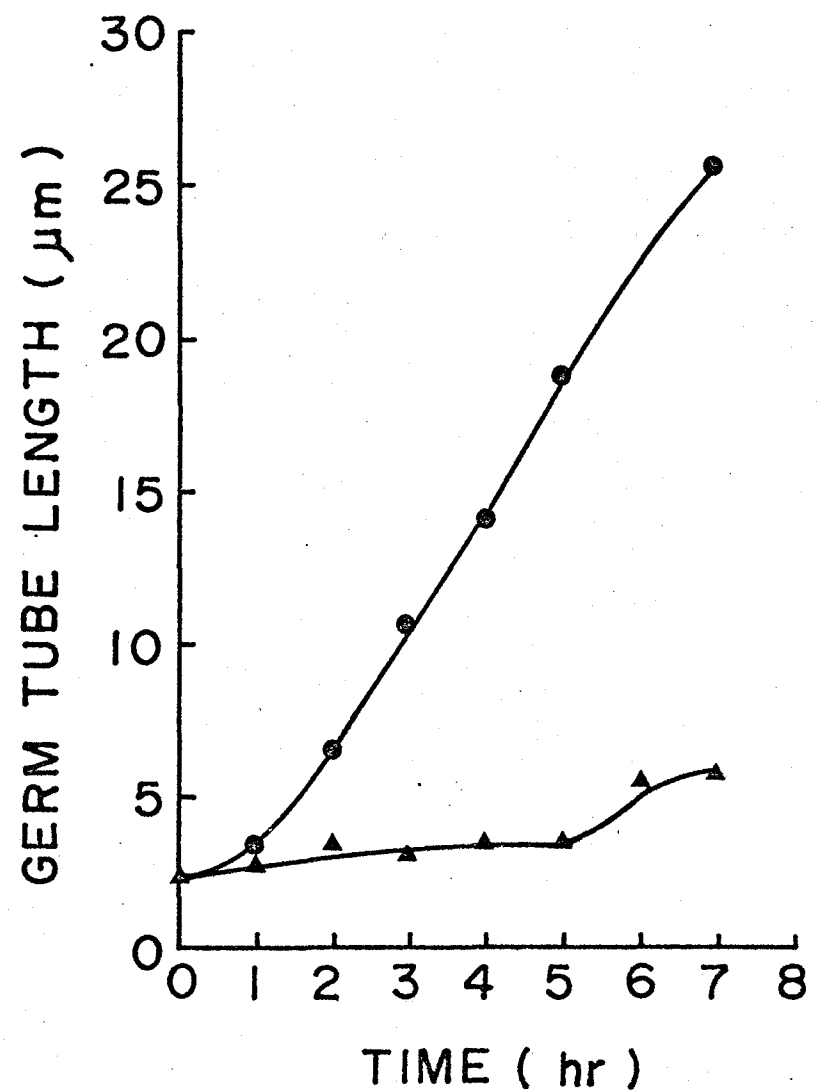
was measured. Inhibition at specific stages of the growth cycle was
then determined.

B. Determination of the stage of the growth cycle most susceptible to
high-GP inhibition.

Growth of the dermatophyte T. mentagrophytes is characterized by
a number of stages. Dormant microconidia first undergo a process of
germination when inoculated into germination medium. Germination of
microconidia is characterized by loss of refractility when viewed under
the phase contrast microscope (Hashimoto et al., 1972). A period of
outgrowth then follows, which is initiated with the emergence of a germ
tube from the non-refractile spore. The germ tube elongates into a
vegetative hypha, which eventually septates and branches out to form
intertwining hyphal masses called mycelia. Growth in the early stages
of the cycle, i.e., germination and early germ tube elongation, can
be measured quantitatively by the method described above (see "Materials
and Methods"). Germination and germ tube growth in high-GP media were
observed in this manner.

In a synthetic medium, the time required for at least 80% of dormant
spores to germinate is 4 to 5 hr. Fig. 6 depicts germination of
dormant spores in synthetic medium with high and control concentrations
of glucose and phosphate. In separately autoclaved media there was no
significant difference between germination in high-GP medium and germina-
tion in controls. This is in striking contrast to germination in media
in which glucose was autoclaved with the rest of the components.

Similar results were seen in the early stages of vegetative growth
following germination (Fig. 7). Although high concentrations of glucose
and phosphate in the media did not inhibit early germ tube elongation if

Fig. 6. Germination of $\underline{T}$. $\underline{mentagrophytes}$ microconidia in synthetic media containing amino acids L-leucine and L-asparagine, and varied concentrations of glucose and phosphate. Each point represents the percentage germination of 200 microconidia. A) Glucose autoclaved separately. B) Glucose autoclaved together with media. The difference between the curves in B at 5 hr is significant at a level $p < 0.001$, as determined by the test of χ^2. There was no significant difference between the curves in A. Symbols: ▲, 0.22 M glucose and 0.1 M phosphate; ●, 0.056 M glucose and 0.02 M phosphate.
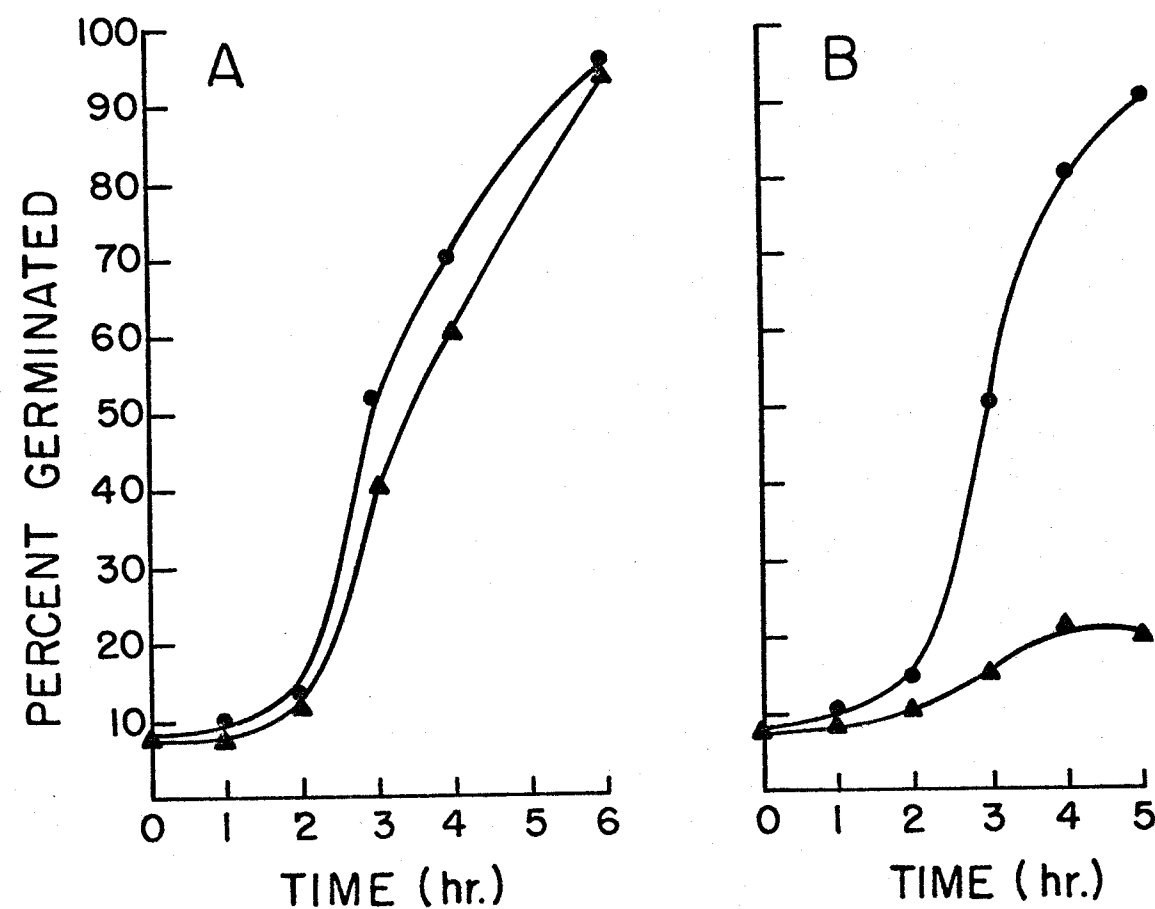
Fig. 7. Germ tube elongation of germinated microconidia of

T. mentagrophytes in synthetic media containing a

single amino acid (L-alanine) and varied concentrations

of glucose and phosphate. Each point represents the

mean and one standard deviation of germ tube lengths

of 50 microconidia. A) Glucose autoclaved separately.

B) Glucose autoclaved together with media. The

difference between the curves in B at 7 hr is

significant at a level $p < 0.001$, as determined by

t test. There was no significant difference between

the curves in A. Symbols: ▲, 0.22 M glucose and 0.1 M

phosphate; ■, 0.011 M glucose and 0.002 M phosphate.

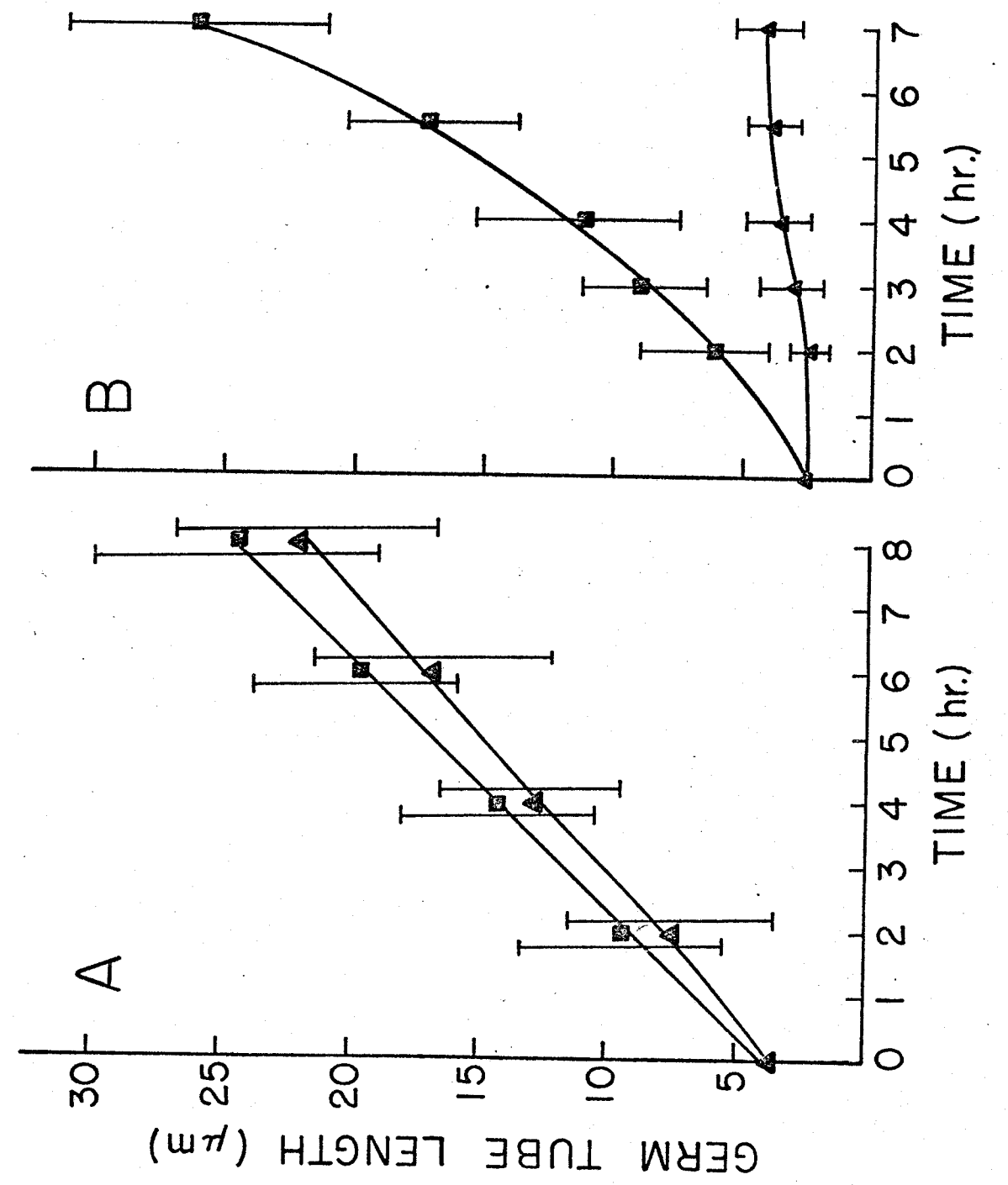the glucose was separately autoclaved, there was a distinct inhibition of elongation in high-GP medium in which all the components were autoclaved together.

Germination and early vegetative growth, in summary, were both inhibited by high-GP glucose-autoclaved media, but not by high concentrations of glucose and phosphate in separately-autoclaved media (Figs. 6 and 7), whereas colony growth and total dry weight of growth in liquid media were inhibited by high-GP medium even when the glucose was separately autoclaved (Figs. 3-5).

C. Parameters affecting inhibition.

1. Spore concentration. The degree of inhibition was dependent upon the concentration of microconidia in the inoculum (Table 2). The degree of inhibition in separately autoclaved media was less pronounced than in glucose-autoclaved media, and when the inoculum size was increased to 2×10^7 spores per 50 ml medium, growth in high-GP medium exceeded that of controls. However, it must be noted that glucose-autoclaved control media was much more suitable for growth than separately autoclaved control media. This raised the possibility that autoclaving glucose-containing media may produce both inhibitory and stimulatory substances, as was noted by Bowers and Williams (1962) for growth of Clostridium botulinum.

Table 2 also shows that addition of heat-killed spores to a small inoculum of live Trichophyton spores had no effect on overcoming inhibition.

2. Incubation temperature. More pronounced inhibition of growth in high-GP medium, as determined by total dry weight, was observed at 29 C than at 37 C (Table 3). This seemed to be true regardless of the

TABLE 2.

Effect of inoculum size on degree of high-GP inhibition of
growth of T. mentagrophytes in complex liquid media.

Inoculum Size[a]	Medium	Glucose separate		Glucose together	
		Dry weight (mg/50 ml)	% Control	Dry weight (mg/50 ml)	% Control
1×10^5	High[b]	----	----	13.8	36
	Control[c]	----	----	37.9	100
1×10^6	High	18.8	67	17.2	33
	Control	27.7	100	52.9	100
1×10^7	High	44.4	88	54.5	44
	Control	49.9	100	122.6	100
2×10^7	High	69.2	133	77.2	60
	Control	52.0	100	128.2	100
10^5 live 2×10^7 dead[d]	High	----	----	13.1	29
	Control	----	----	44.6	100

TABLE 2. (continued)

[a]Number of spores inoculated into 50 ml medium.

[b]1% peptone plus 0.22 M glucose and 0.1 M PO_4.

[c]1% peptone plus 0.056 M glucose and 0.02 M PO_4.

[d]2×10^7 spores were heat-killed by autoclaving for 15 min and added to the inoculum of 10^5 live spores.

TABLE 3.

Effect of incubation temperature on degree of high-GP inhibition
of growth of <u>T. mentagrophytes</u> in complex liquid media.

Temperature (C)	Media	Glucose autoclaved separately		Glucose autoclaved together	
		Dry weight (mg/50 ml)	% Control	Dry weight (mg/50 ml)	% Control
29	High[a]	28.7	53	25.4	37
	Control[b]	54.3	100	68.7	100
37	High	16.9	69	14.4	57
	Control	24.4	100	25.2	100

[a]1% peptone plus 0.22 M glucose and 0.1 M PO_4.

[b]1% peptone plus 0.056 M glucose and 0.02 M PO_4.

manner of autoclaving. The temperature factor was less obvious when spreading growth was observed (Fig. 8). At each temperature, spreading growth on control media was approximately 3-fold greater than growth on high-GP media.

3. <u>Concentration of glucose and phosphate</u>. Very low concentrations of glucose and phosphate (0.011 and 0.002 M, respectively) were optimal for germ tube elongation, although greater concentrations were necessary for dry weight control media (0.056 and 0.02 M glucose and phosphate, respectively), as dry weight experiments were generally incubated for three days.

Fig. 9 shows that low glucose-high phosphate medium, and high glucose-low phosphate medium were both suitable for germ tube elongation, although somewhat less so than controls. In contrast, high-GP medium afforded almost no germ tube elongation in the 7 hour time period.

Subsequent hyphal growth, as measured by total dry weight of growth in varying glucose and phosphate concentrations is seen in Fig. 10 and 11. In high glucose (0.22 M) medium, growth decreased with increasing concentrations of phosphate (Fig. 10). Similarly, in media containing 0.1 M phosphate, growth decreased with increasing concentrations of glucose (Fig. 11). High concentrations of phosphate alone resulted in somewhat less growth than media containing high glucose but low phosphate. Although this was not the case when germ tube elongation was measured, colony growth dramatically emphasized this sensitivity to phosphate (Fig. 12).

The difference in degree of inhibition and sensitivity to phosphate in colony growth versus germination, germ tube elongation, and growth

Fig. 8. Colony growth of T. mentagrophytes on solid complex media with varied concentrations of glucose and phosphate at room temperature (A), 30 C (B), 35 C (C), and 37 C (D). Symbols: ▲ , 0.22 M glucose and 0.1 M phosphate, glucose autoclaved with medium; ● , 0.056 M glucose and 0.02 M phosphate, glucose autoclaved with medium; △ , 0.22 M glucose and 0.1 M phosphate, glucose autoclaved separately; ○ , 0.056 M glucose and 0.02 M phosphate, glucose autoclaved separately.
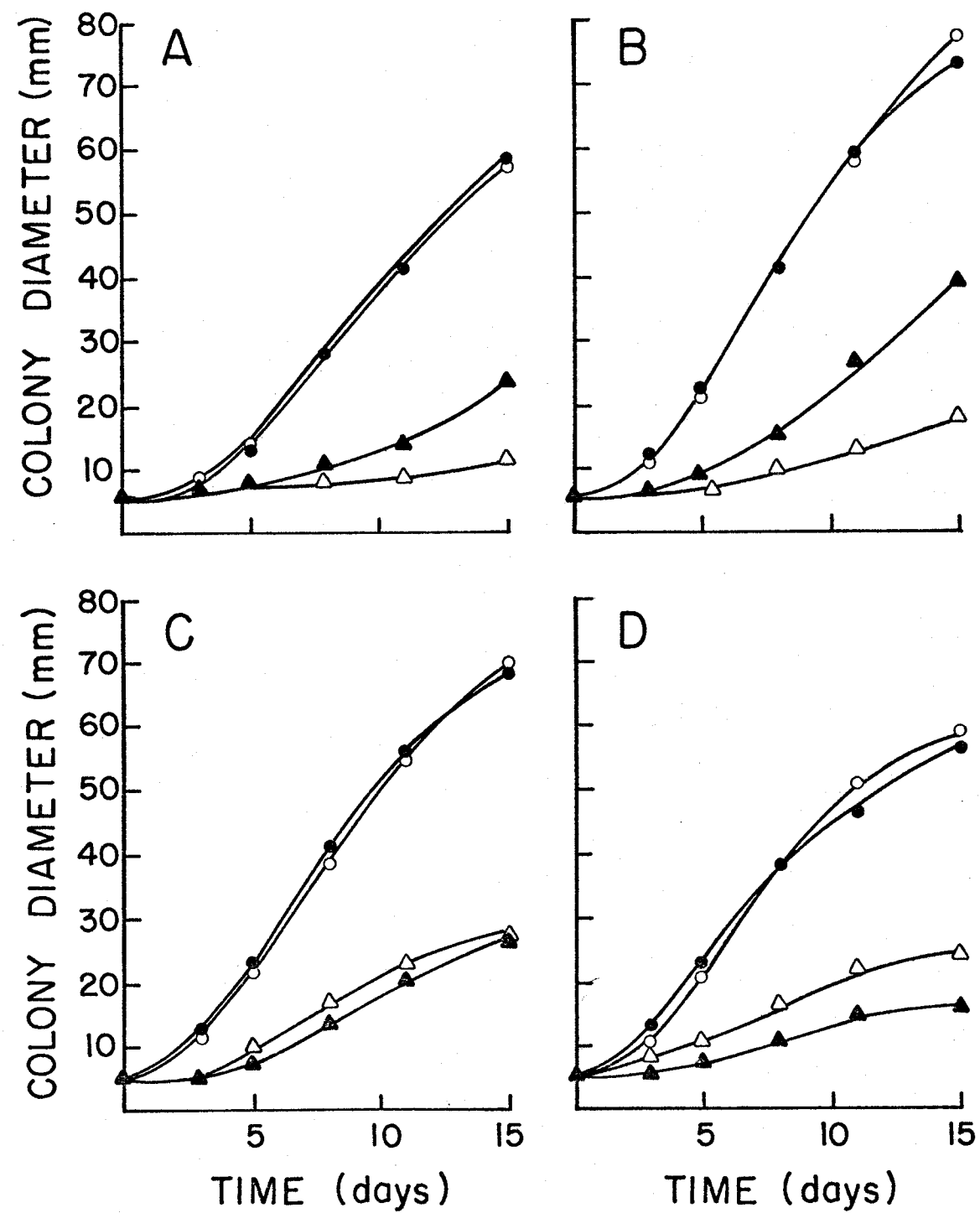
Fig. 9. Germ tube elongation in varied concentrations of glucose and phosphate in glucose-autoclaved synthetic media. Each point represents the mean and one standard deviation (where indicated) of germ tube lengths of 50 microconidia. Symbols: X, 0.011 M glucose and 0.002 M phosphate; ●, 0.056 M glucose and 0.02 M phosphate; △, 0.001 M glucose and 0.1 M phosphate (low glucose, high phosphate); O, 0.22 M glucose and 0.002 M phosphate (high glucose, low phosphate); ▲, 0.22 M glucose and 0.1 M phosphate.
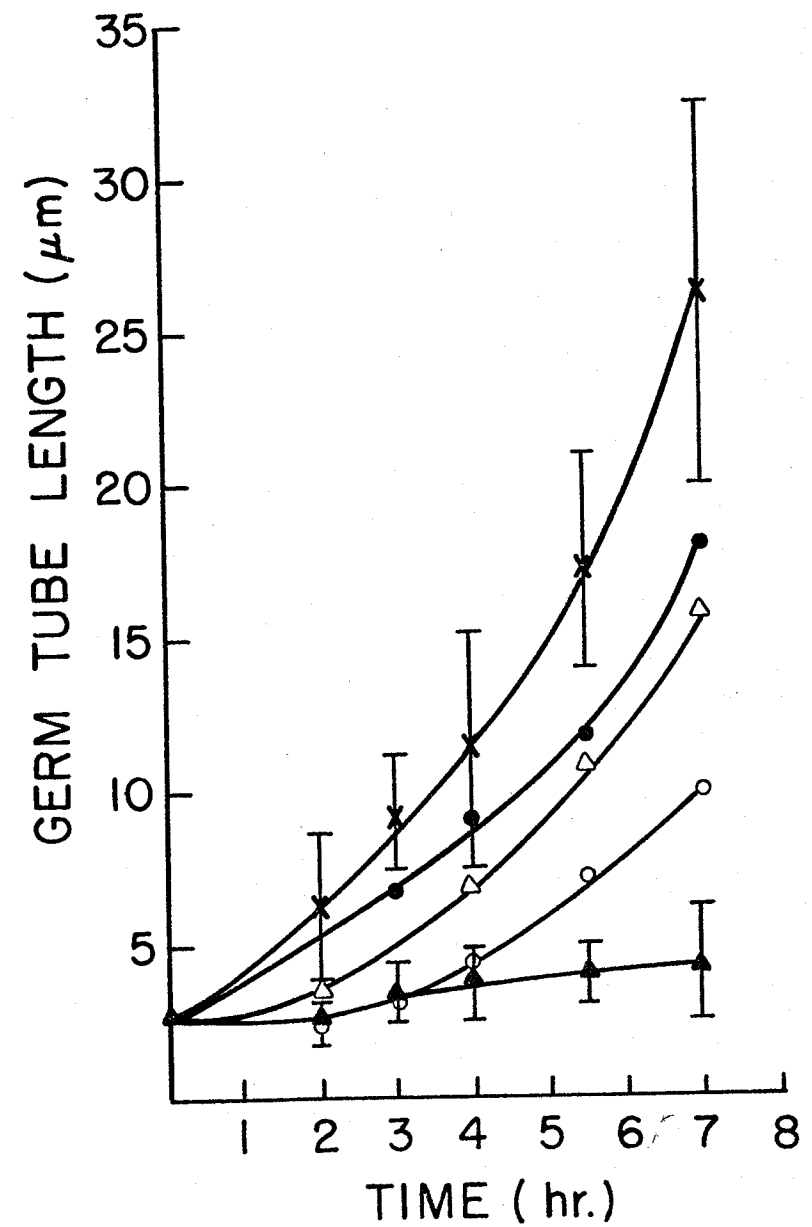
Fig. 10. Effect of increased phosphate concentration on dry
weight of growth of T. mentagrophytes in complex
liquid media containing 0.22 M glucose (separately
autoclaved).
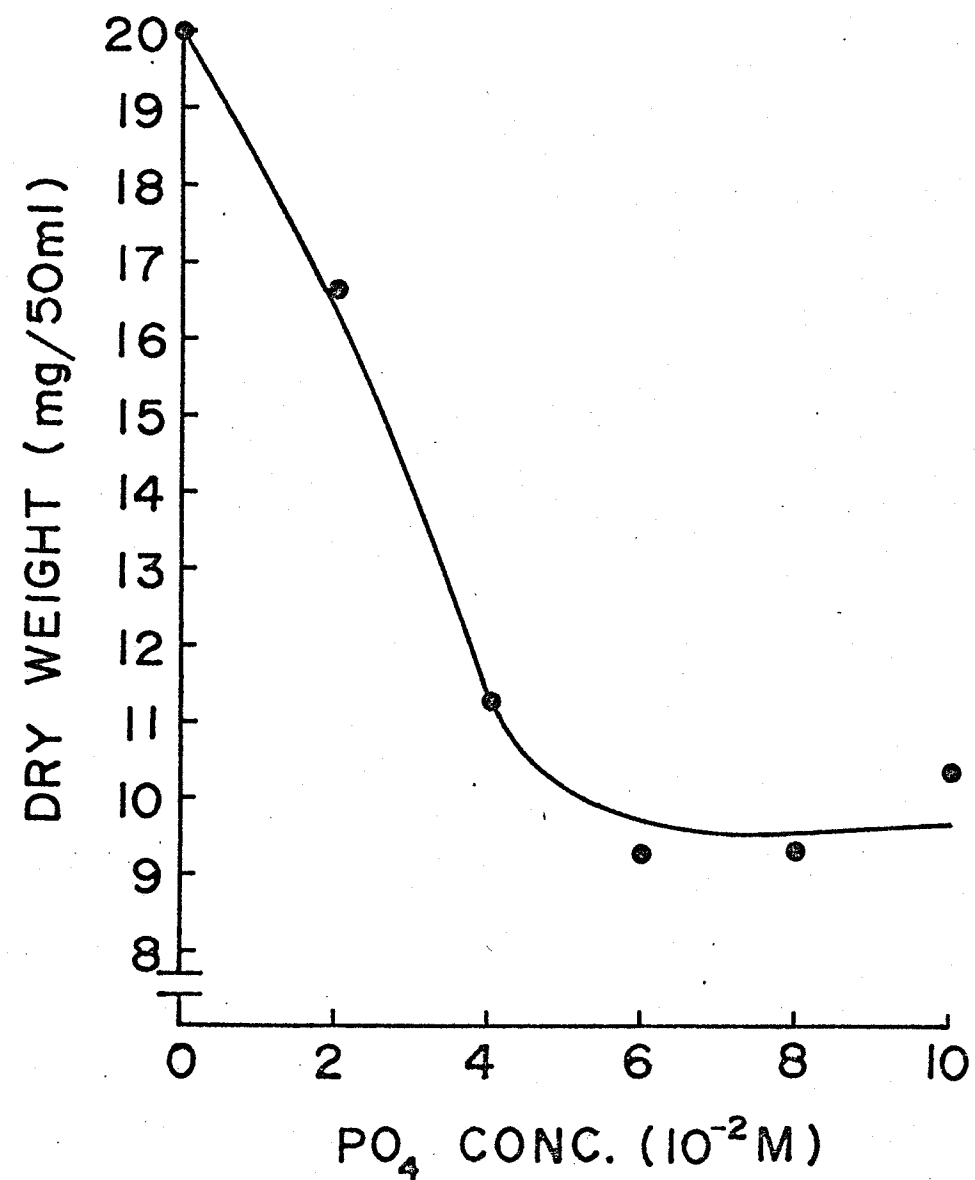
Fig. 11. Effect of increased glucose concentration on dry
weight of growth of T. mentagrophytes in complex
liquid media containing 0.1 M phosphate. (Glucose
separately autoclaved).
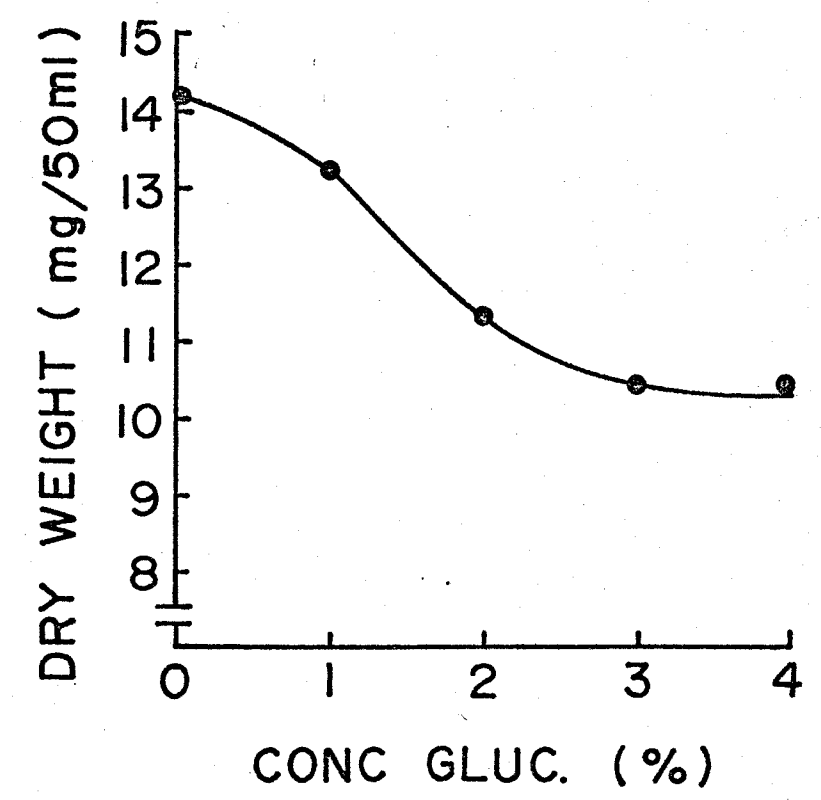
Fig. 12. Effect of concentrations of phosphate and glucose on colony growth of T. mentagrophytes on complex glucose-autoclaved solid media. A) 0.22 M glucose. Symbols: △, 0.02 M PO_4; O, 0.05 M PO_4; □, 0.08 M PO_4; ▲, 0.1 M PO_4; ●, control, 0.056 M glucose and 0.02 M PO_4. B) 0.1 M PO_4. Symbols: △, 0.056 M glucose; O, 0.112 M glucose; □, 0.168 M glucose; ▲, 0.22 M glucose; ●, control, 0.056 M glucose and 0.02 M PO_4.
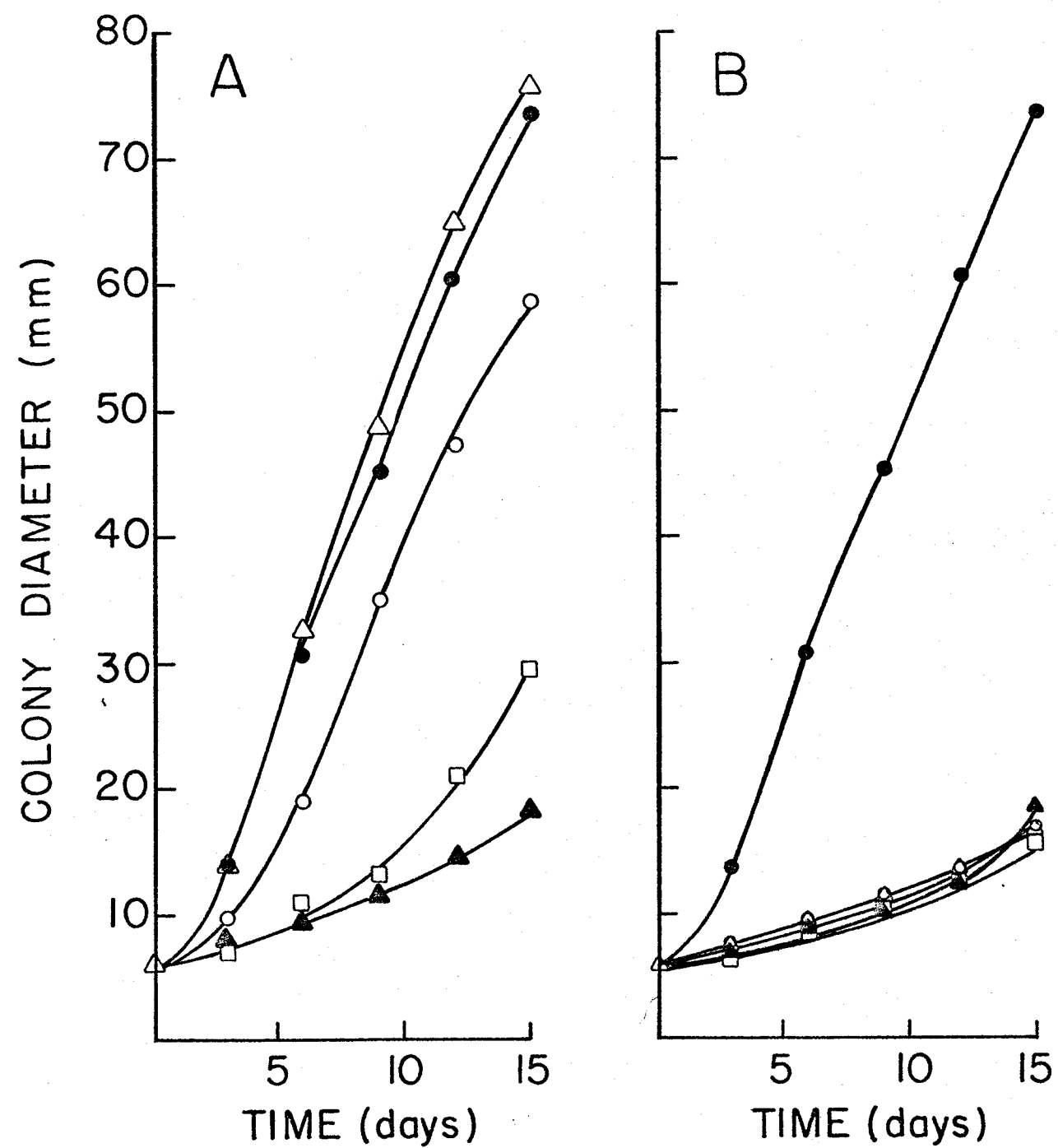
in liquid media may be a function of aerial conidiation on agar and its effect on peripheral growth, and is explained further in the discussion section.

4. Other sugars. Three pentoses and six hexoses were tested for their ability to inhibit growth of Trichophyton in high-GP medium. Table 4 shows that all but sorbose resulted in inhibition when used in the concentration of 0.22 M and autoclaved separately from the rest of the medium. As with glucose, inhibition was much more pronounced when the sugars were autoclaved together with the rest of the medium, in which case all, including sorbose, resulted in inhibition when used in higher concentration.

These results are seen quantitatively in Table 5 for fructose, mannose, galactose, and rhamnose. Inhibition with high sugar concentrations in separately sterilized media was considerable for fructose and rhamnose, and only slightly apparent for galactose, while no inhibition could be seen in high mannose-high phosphate medium. When the sugars were sterilized with the medium, all showed greater dry weight yields in controls, as compared to high sugar-high phosphate media. Here too, sugar-sterilized control media generally resulted in greater dry weight yields than did separately-sterilized controls, further suggesting the possibility that in addition to inhibitory substances produced upon autoclaving, an enhancement factor may also be present.

D. Reversibility of inhibition.

Were inhibition of germination and germ tube elongation due to a substance or substances arising upon autoclaving glucose in phosphate-containing media, the possibility of reversing inhibition by removing

TABLE 4.

Growth[a] of T. mentagrophytes in complex liquid media
containing high concentrations of phosphate and various sugars

| Sugar | Concentration | Growth (3 days) | |
		Sugar sterilized separately	Sugar sterilized together
D-Xylose	High[b]	+/-	-
	Control[c]	+	++
D-Arabinose	High	++	+
	Control	+++	+++
D-Ribose	High	++	-
	Control	++++	+++
L-Sorbose	High	++	-
	Control	++	++
D-Fructose	High	+++	+
	Control	++++	+++
D-Mannose	High	+++	+
	Control	++++	+++
D-Galactose	High	+++	+
	Control	++++	++++
D-Glucose	High	++	++
	Control	+++	++++
D-Mannitol	High	++	++
	Control	+++	+++
L-Rhamnose	High		+
	Control		+++

TABLE 4 (continued)

[a]One drop of spore suspension (approximately 10^6 spores/ml) was inoculated into 1 ml medium in disposable capillary tubes which were incubated for 3 days at which time growth was rated according to the following scale: -, no visible growth; +/- to ++++, very faint to very dense growth. Samples were prepared in triplicate.

[b]0.22 M sugar and 0.1 M phosphate.

[c]0.056 M sugar and 0.02 M phosphate.

TABLE 5.

Effect of high concentrations of phosphate and different sugars on growth of T. mentagrophytes in complex liquid media.

Sugar	Concentration	Sugar sterilized separately Dry weight (mg/50 ml)	Sugar sterilized together Dry weight (mg/50 ml)
D-Fructose	High[a]	17.0	16.1
	Control[b]	28.8	49.1
D-Mannose	High	25.4	14.9
	Control	21.1	38.9
D-Galactose	High	20.0	11.6
	Control	25.9	26.4
L-Rhamnose	High	9.3	18.4
	Control	20.1	32.0

[a] 0.22 M sugar and 0.1 M phosphate.

[b] 0.056 M sugar and 0.02 M phosphate.

the inhibitory substance(s) would seem likely. Fig. 13 illustrates
that cells which were incubated in inhibitory, high-GP medium for 3 hr
and were then collected, washed, and resuspended in control medium,
resumed germ tube elongation, indicating that there existed a strong ten-
dency toward reversal of inhibition upon removal of the inhibitor(s).

E. Effect of high-GP media on growth of other organisms.

   1. Bacteria. Growth of the bacteria Bacillus cereus T, Escherichia
coli (a clinical isolate), and Staphylococcus aureus Towler in unsupple-
mented 1% peptone is shown in Fig. 14, and in high-GP and low-GP media
in Fig. 15. Separately autoclaved high-GP medium resulted in the
shortest lag time and highest maximum growth for B. cereus. Glucose-
autoclaved high-GP medium seemed to prolong the lag phase, although
maximal turbidity at the stationary phase was the same as that of controls.

   Similarly, E. coli showed maximum growth in separately-autoclaved
high-GP medium. Growth in glucose-autoclaved high-GP medium was also
greater than in controls, although slightly less than in separately
autoclaved high-GP medium.

   Only S. aureus grew better in lower concentrations of glucose
and phosphate. Although growth in separately autoclaved high-GP
medium was approximately 60% as great as controls, glucose-autoclaved
high-GP medium dramatically inhibited growth of the organism.

   2. Other fungi. Four other fungi were inoculated in various
concentrations into high-GP and control media, and their total dry
weights after incubation for 3 days were noted. Cell numbers in the
inoculum could be estimated for the yeasts Saccharomyces cerevisiae and
Candida albicans from turbidity readings, as a correltaion was seen to

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Fig. 13. Reversibility of inhibition of germ tube elongation in synthetic medium (containing L-leucine and L-asparagine). Arrow indicates point at which microconidia were transferred to control medium. Symbols: ▲, microconidia incubated in 0.22 M glucose and 0.1 M phosphate throughout; ●, microconidia incubated in 0.056 M glucose and 0.02 M phosphate throughout; △, microconidia incubated in 0.22 M glucose and 0.1 M phosphate for 3 hr, and then transferred to 0.056 M glucose and 0.02 M phosphate.
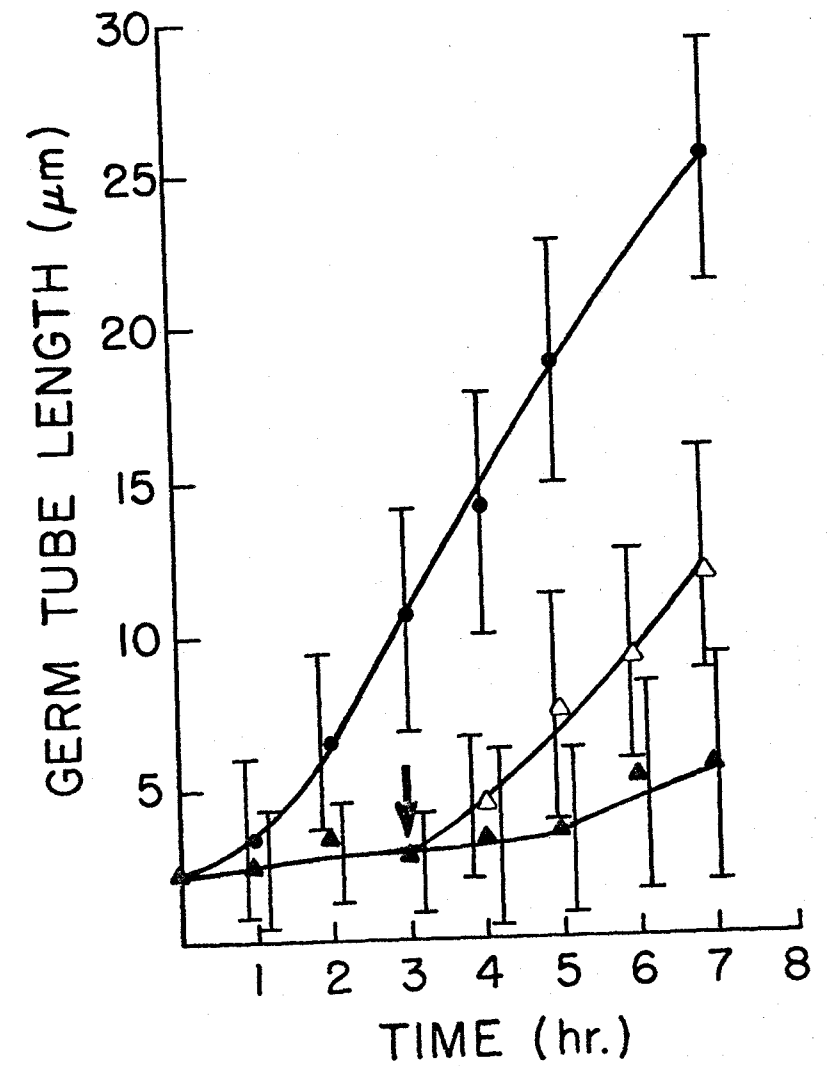
Fig. 14. Growth of bacteria in unsupplemented 1% peptone.

Symbols: O , B. cereus; ●, E. coli; ★, S. aureus.
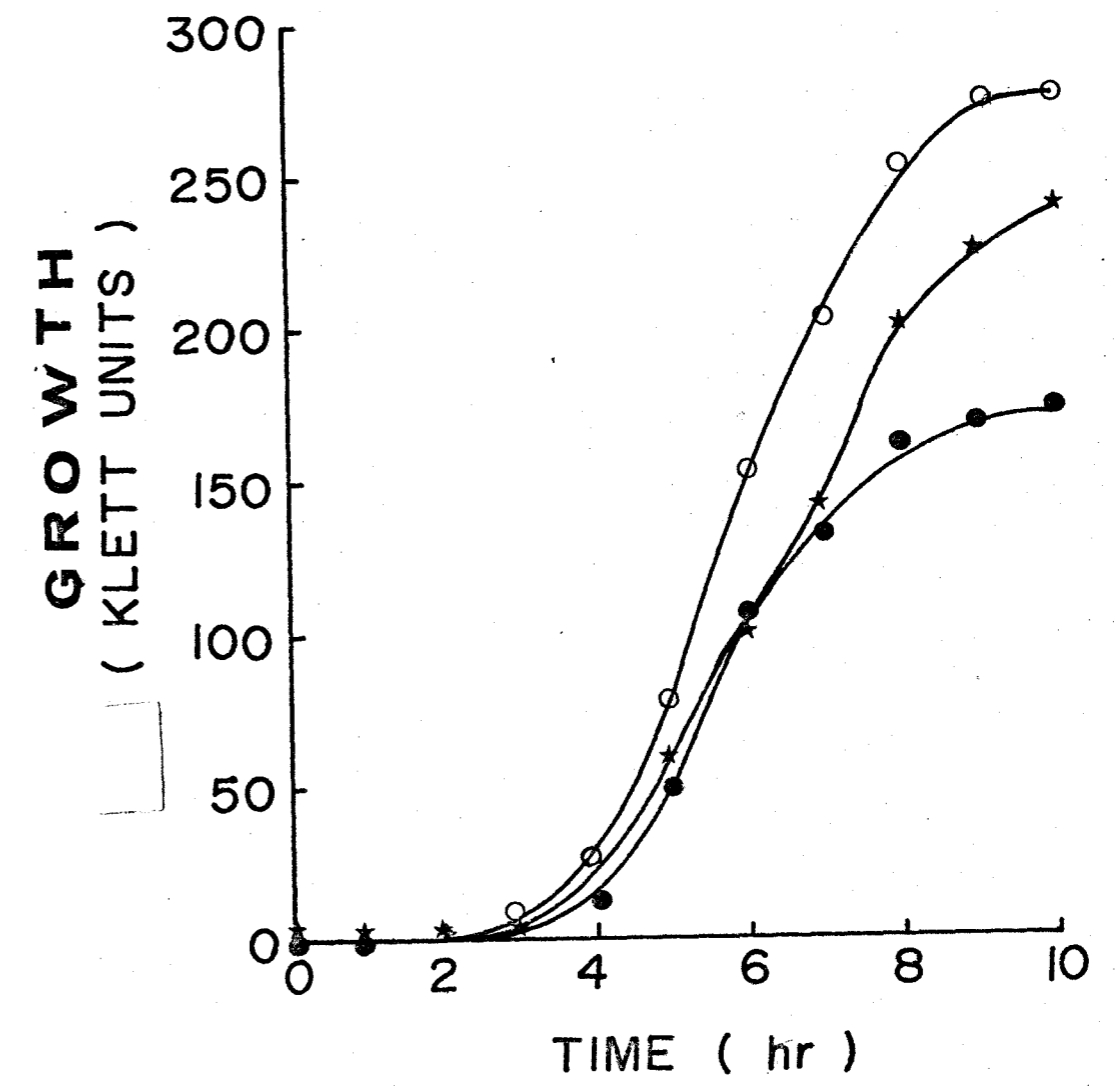
Fig. 15. Growth of B. cereus (A), E. coli (B), and S. aureus (C) in media containing 1% peptone and varied concentrations of glucose and phosphate. Symbols: ▲ , 0.22 M glucose and 0.1 M phosphate, glucose autoclaved with medium; ● , 0.056 M glucose and 0.02 M phosphate, glucose autoclaved with medium; △, 0.22 M glucose and 0.1 M phosphate, glucose autoclaved separately; ○ , 0.056 M glucose and 0.02 M phosphate, glucose autoclaved separately.
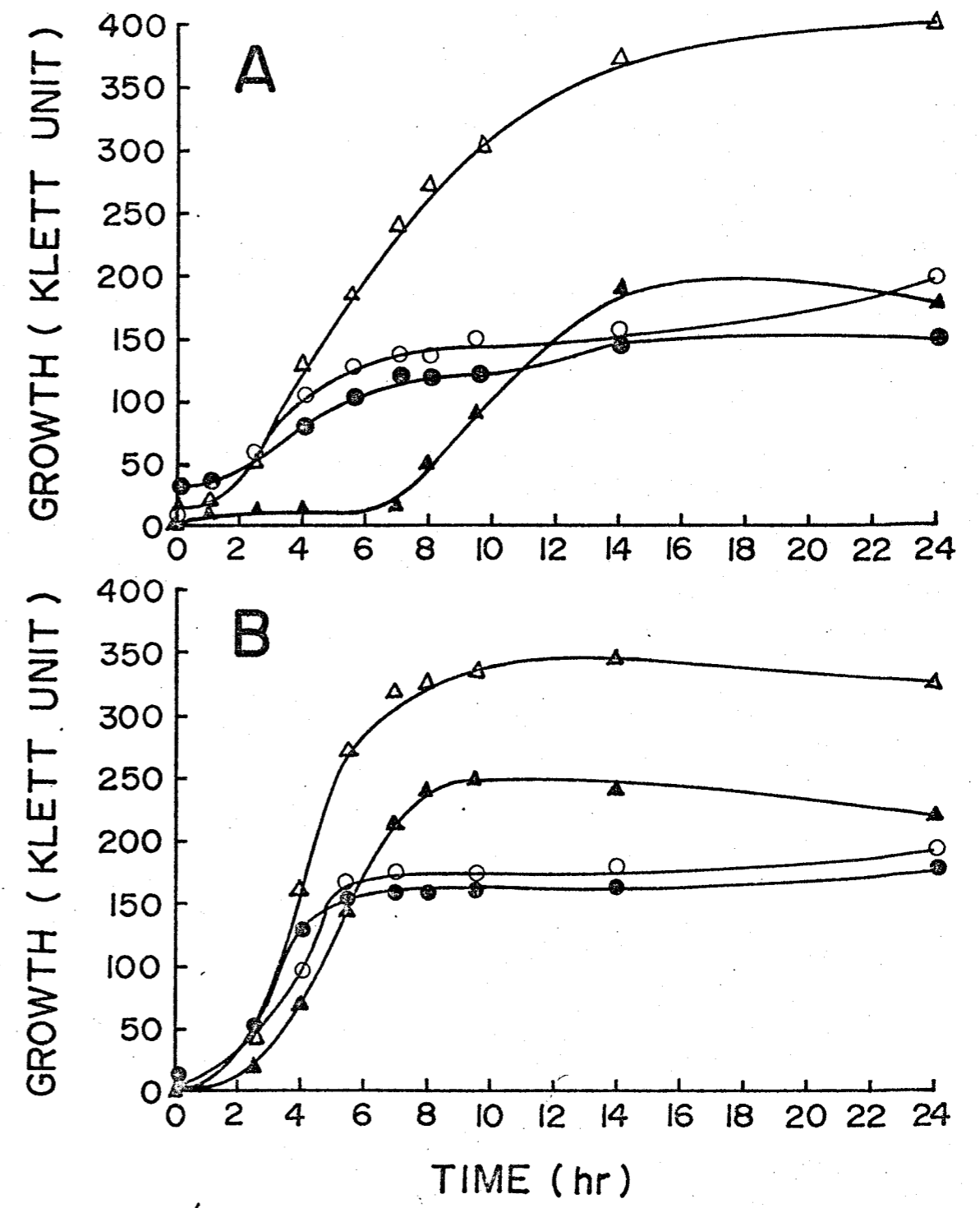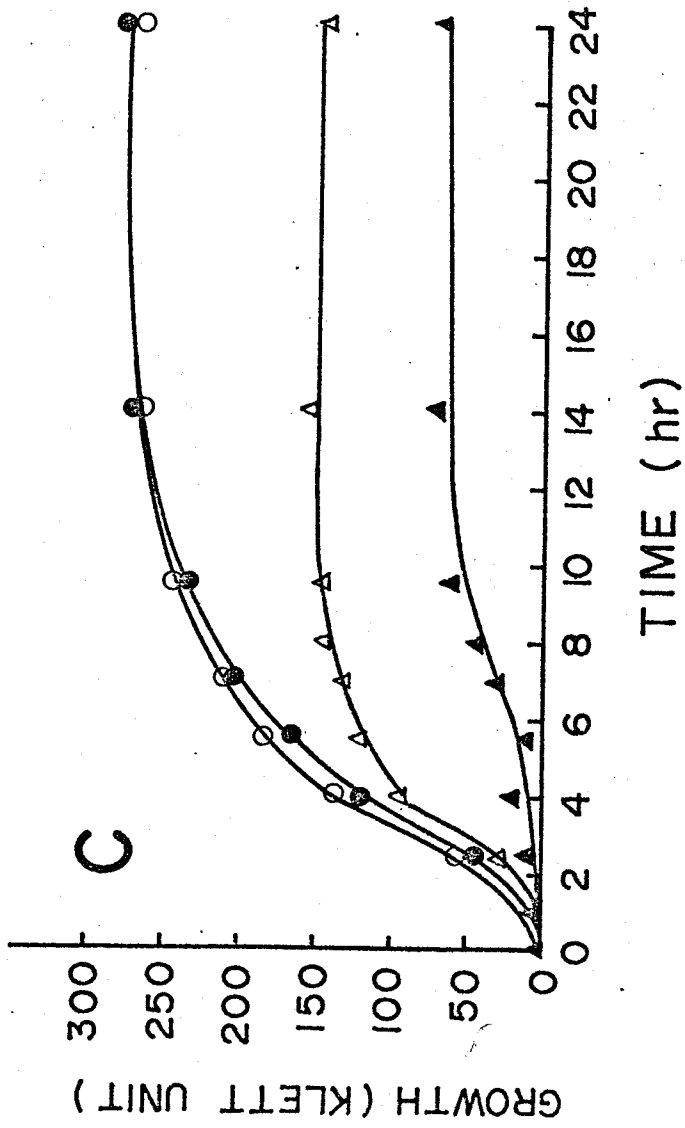
exist between turbidity and cell number for these yeasts (Fig. 16). Cell numbers for all four fungi, however, was confirmed by plate counts on Sabouraud dextrose agar.

None of the fungi tested were inhibited by high-GP media when inoculated in higher concentrations (Table 6), in marked contrast to Trichophyton, which was inhibited by glucose-autoclaved high-GP media even when the inoculum size was as great as 2×10^7 microconidia per 50 ml medium.

S. cerevisiae was inhibited by high-GP medium when the inoculum size was reduced to 6×10^4 cells in 50 ml medium, and Geotrichum candidum showed only slight inhibition with an inoculum size of 4×10^2 per 50 ml medium.

These results indicate that although some other fungi may be inhibited by glucose-autoclaved high-GP medium, they are very significantly less susceptible to inhibition than is Trichophyton, as evident from the large inoculum size necessary for Trichophyton to overcome the inhibition.

F. Preliminary isolation and characterization of the inhibitory substance(s).

As the data presented above confirmed earlier work showing that autoclaving glucose in phosphate-containing media resulted in the formation of some inhibitory product(s), as discussed above, a partial characterization of the product(s) thus formed which inhibited T. mentagrophytes was attempted.

No significant change in the degree of inhibition was observed when autoclaved media were aged at room temperature up to 20 days before inoculation (Table 7), evidence which alludes to the stability of the inhibitor to storage. Preliminary experiments (not shown) suggested

Fig. 16. Standard curve correlating turbidity in Klett units with cell number for C. albicans (A) and S. cerevisiae (B).
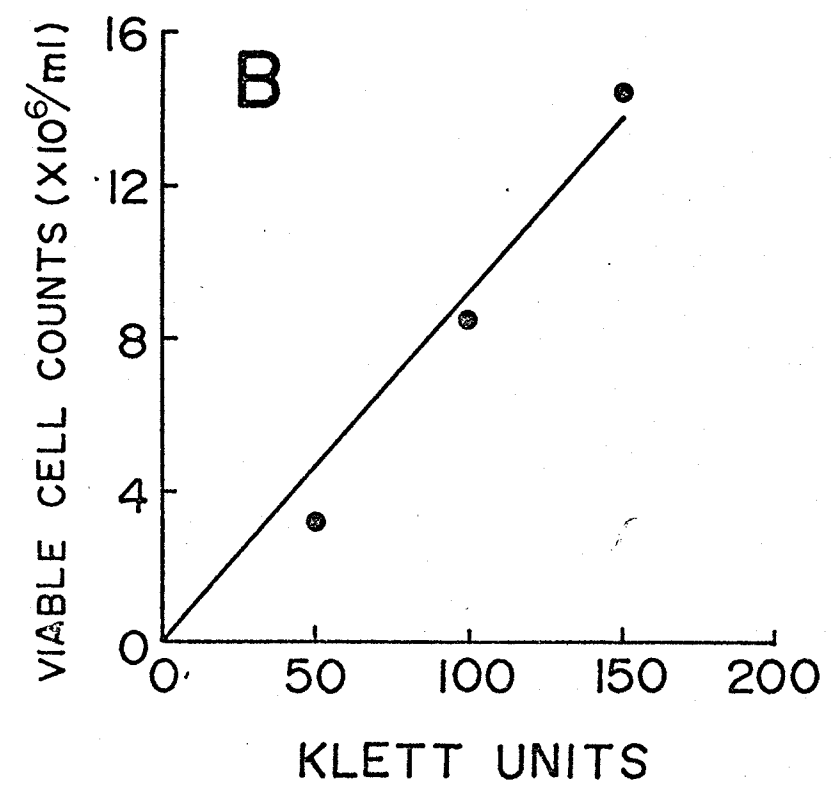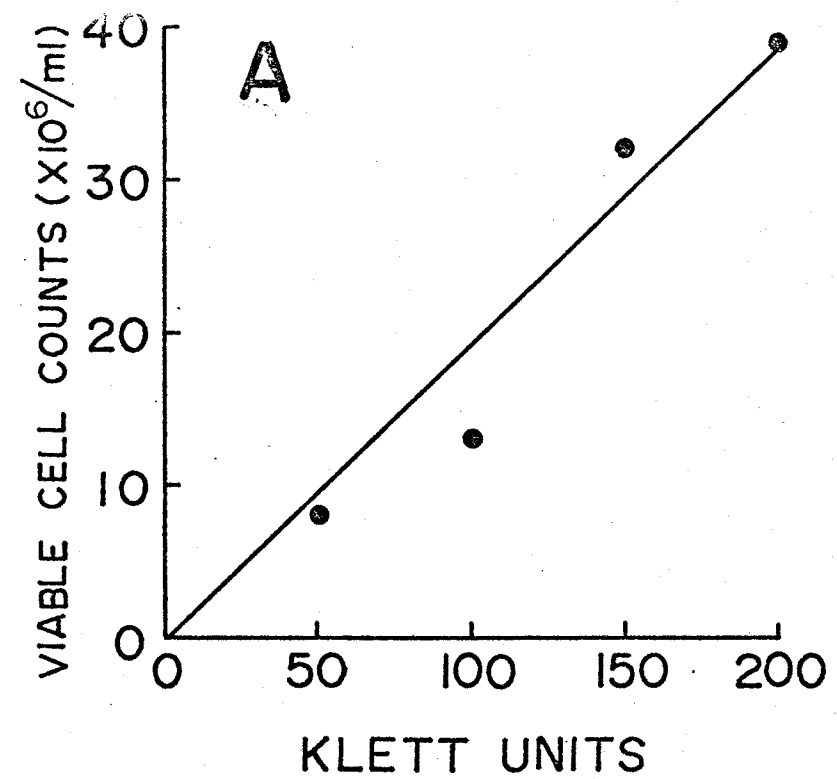
TABLE 6.

Effect of high-GP glucose-autoclaved, complex media
on growth of 4 fungi.

Organism	Inoculum[a] size	Medium	Dry weight[d] (mg/50 ml)	% Control
A. niger	5 x 10^5	High[b]	192	127
		Control[c]	151.4	100
	5 x 10^4	High	132.6	108
		Control	122.5	100
G. candidum	4 x 10^4	High	181.1	97
		Control	187.0	100
	4 x 10^2	High	138.6	74
		Control	186.9	100
S. cerevisiae	6 x 10^6	High	207.9	135
		Control	154.5	100
	6 x 10^4	High	54.1	39
		Control	138.8	100
C. albicans	2 x 10^7	High	361.5	171
		Control	211.2	100
	2 x 10^5	High	345.0	194
		Control	178	100

[a]Number of cells inoculated into 50 ml medium, determined by standard plating technique.

[b]0.22 M glucose and 0.1 M phosphate.

[c]0.056 M glucose and 0.02 M phosphate.

[d]After incubation for 3 days. Mean of duplicate flasks.

TABLE 7.

Age of medium[a] (Days)	Medium	Dry weight (mg/50 ml)	
		Glucose autoclaved separately	Glucose autoclaved together
20	High[b]	17.0	15.8
	Control[c]	29.3	44.9
10	High	15.2	15.0
	Control	29.0	40.2
Fresh	High	17.0	16.8
	Control	26.6	35.6

[a]Aged media were prepared 20 and 10 days prior to inoculation and stored at room temperature until inoculation. Duplicates prepared for each medium

[b]0.22 M glucose and 0.1 M phosphate.

[c]0.056 M glucose and 0.02 M phosphate.

that the amount of inhibitor present, as reflected in the degree of inhibition of growth, increased with prolonged autoclaving. For this reason, media from which the inhibitor was to be extracted were auto-claved for one hr before addition of activated charcoal. Table 8 shows the results of a germ tube elongation experiment in which spores were inoculated into charcoal-extracted media. Germ tube elongation in extracted media was significantly greater than in fresh, non-extracted media. After a 7 hr incubation period, germ tube elongation in charcoal-extracted high-GP medium was almost double that seen in fresh high-GP medium, indicating that the inhibitor could be extracted with activated charcoal.

The inhibitor was eluted from the charcoal with cold absolute ethanol, as described above, and assayed for biological activity, as shown in Table 9. When the crude inhibitor which had been extracted with ethanol was added in concentrations below 8 mg/ml, no significant decrease in germ tube length was evident after incubation for 7 hr. However, inhibition began to become apparent at a concentration of 8 mg/ml, and germ tube elongation was completely inhibited when the extract concentration reached approximately 60 mg/ml. This table also shows that most of the inhibitory activity of the crude ethanol extract could be found in the dialyzable material, and therefore con-ceivably in the size range of a glucose degradation product, as predicted in earlier work with other organisms.

TABLE 8.

Germ tube elongation in Norit-extracted synthetic medium

Incubation time (Hr)	Medium	Germ tube length (μm) (mean and standard deviation)	
		Norit extracted	Unextracted control
3.5	High[a]	11.7 $\pm$ 5.1	4.4 $\pm$ 3.1
	Control[b]	14.6 $\pm$ 5.7	10.3 $\pm$ 5.8
7	High	25.6 $\pm$ 5.5	10.4 $\pm$ 5.3
	Control	34.1 $\pm$ 7.0	21.3 $\pm$ 7.7

[a] 0.22 M glucose and 0.1 M phosphate.

[b] 0.056 M glucose and 0.02 M phosphate.

TABLE 9.

Germ tube elongation in the presence of inhibitor

Conc. inhibitor (mg/ml)		
Crude extract	Dialyzable material	Germ tube length[*] (µm)
0	--	21.7 ± 5.6
2.1	--	24.4 ± 5.5
4.3	--	19.8 ± 5.5
8.6	--	14.7 ± 4.7
61.6	--	2.5 ± 1.1
--	0	16.5 ± 4.7
--	62	2.8 ± 1.1

*Mean and standard deviation of germ tube length after 7 hr incubation. (Mean germ tube length at time zero was 2.2 ± 1.4 µm).

IV. DISCUSSION

A. Growth parameters used to determine inhibition.

Four different parameters of growth---germination, early germ tube elongation, colony growth, and dry weight of growth in liquid media---were measured in order to present a more complete picture of the effects of increased glucose and phosphate concentrations on growth of T. mentagrophytes. Germ tube elongation of T. mentagrophytes was measured differently from other, probably less reliable, applications of this technique. The germ tube specific growth rates of seven filamentous fungi on complex solid media were found to be substantially greater than the specific growth rates determined by total dry weight in liquid media (Trinci, 1971b). Trinci suggested that since germ tube elongation on complex solid media may reflect utilization of endogenous resources which would account for this parameter showing a maximum growth rate, perhaps germ tube growth rate should not be used to study the influence of nutrients upon growth. In contrast to Trinci's experiments, however, early germ tube elongation in this work (Figs. 5, 7, and 9) was a measure of elongation in liquid media, and is therefore probably a more valid parameter by which to measure growth.

Colony growth, on the other hand, is probably the least reliable parameter when taken by itself. Trinci showed (1971a) that, although the specific growth rates of hyphae in peripheral growth zones of some fungi appeared identical to the maximum specific growth rate in submerged culture regardless of variations in temperature and

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concentration of inhibitor, hyphal growth rates were seen to vary with changes in nutrient concentrations. In particular, changes in glucose concentrations may influence radial growth rates by influencing the rate of septation or occlusion of septation (Trinci, 1969). In these conditions, hyphal growth rates would not parallel the organisms' maximum specific growth rates. In Trichophyton, conditions which are suboptimal for colony growth, such as increased glucose and phosphate concentrations and higher growth temperatures (37 C), result in an increase in aerial hypha formation and microconidiation, in which case little if any evidence of young hyphae at the colonial periphery can be found. For this reason, inhibitory factors are manifested by severely inhibited growth at the colonial periphery, whereas these same factors applied to growth in liquid media might show less dramatic, albeit significant inhibition. In light of these considerations, determination of total dry weight of growth in liquid media probably serves as a more reliable indication of the maximum specific growth rate of T. mentagrophytes, and as Mandels suggests (1965), of the growth of filamentous fungi in general.

B. The nature of inhibition.

While the evidence presented above suggests that inhibition of growth of T. mentagrophytes was caused by increased concentrations of glucose and phosphate added to the media, the results must be seen in light of some additional factors. Osmotic pressure must be taken into account, as the addition of glucose and phosphate also increases the osmotic pressure of the medium. T. mentagrophytes was seen to be sensitive to relatively low concentrations of NaCl (Fig 17 and 18) as well as to certain other salts such as KNO_3 (data not shown).

Fig. 17. Colony growth of T. mentagrophytes on solid, glucose-
autoclaved, complex media containing 0.056 M glucose,
0.02 M phosphate, and increasing concentrations of
NaCl. Symbols: ●, control; △, 0.01 M NaCl; ✕, 0.05 M
NaCl; ■, 0.10 M NaCl; ○, 0.15 M NaCl; ▲, 0.20 M
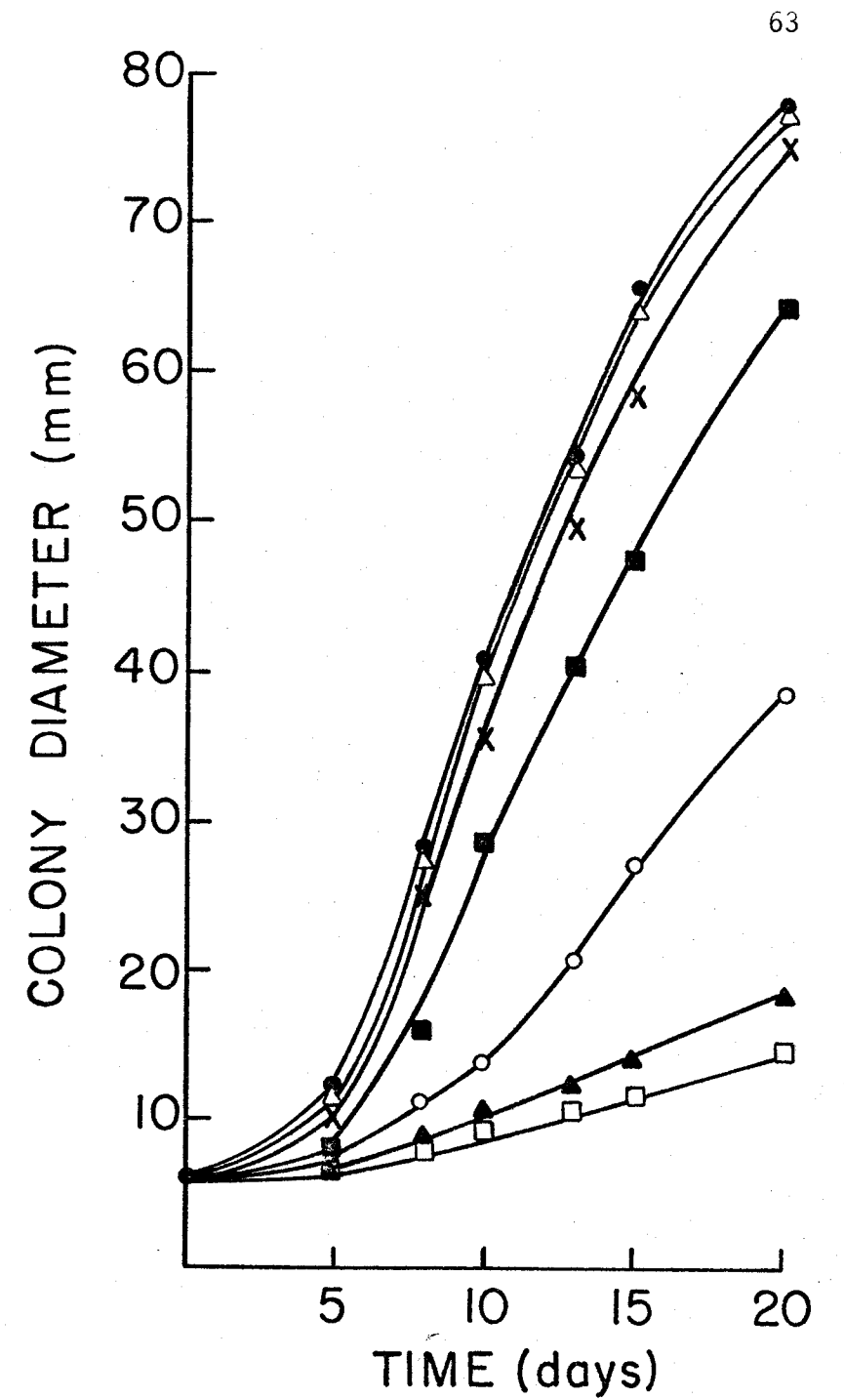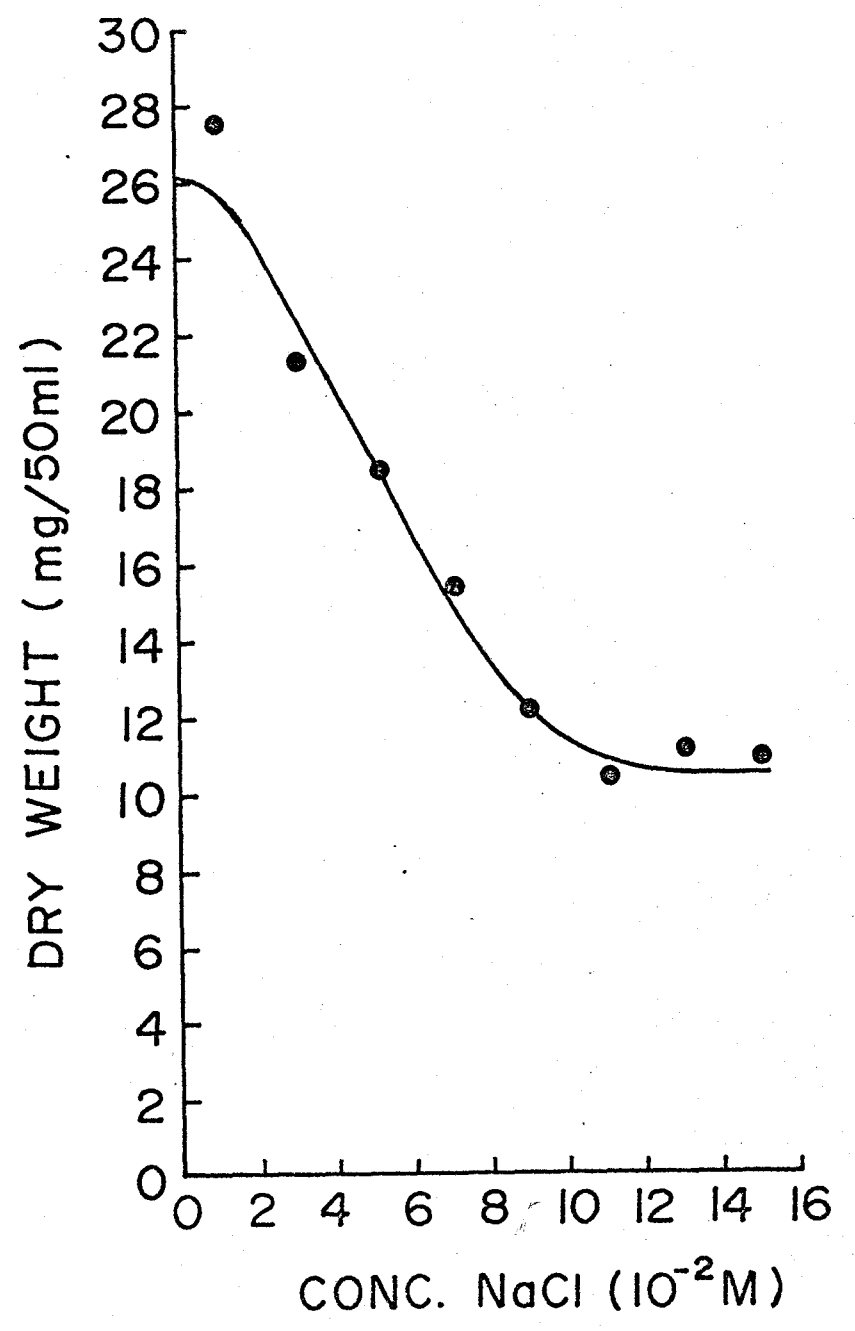NaCl; □, 0.25 M NaCl.

Fig. 18. Effect of NaCl on dry weight of growth of
T. mentagrophytes for 3 days in complex
liquid media containing 0.056 M glucose
and 0.02 M phosphate.

Sensitivity of this organism to a variety of osmotic stabilizers was seen in earlier unpublished studies not related to this thesis, in attempts to induce protoplast formation. A common technique used frequently to induce protoplast formation in fungal cells generally involves suspending the cells in a solution containing snail gut enzyme and 0.5 M osmotic stabilizer (Eddy and Williamson, 1957). However, sorbitol, mannitol, sucrose, NaCl, and $MgSO_4$ in concentrations of 0.3 to 0.5 M resulted in plasmoptysis of the germinating Trichophyton microconidia before the protoplast had emerged halfway from the cell wall. Shrunken protoplasts were seen even when 1% mercaptoethanol was added to the incubation mixture, as suggested for protoplast formation in Saccharomyces fragilis (Davies and Elvin, 1964), and with concentrations of snail enzyme which varied from 10 to 20%. Elimination of the centrifugation and washing steps did not relieve this condition.

The observations that separately autoclaved high-GP media did not significantly inhibit germination (Fig. 6A) or early germ tube elongation (Fig. 7A) suggest that the inhibitory effect in the early stages of growth is not due to increased osmotic pressure, and that marked inhibition occurs only in glucose-autoclaved high-GP media (Fig. 6B and 7B), probably due, primarily, to the actions of a heat-produced inhibitory substance.

Inhibition of growth in liquid media does occur when glucose is separately autoclaved (Tables 1 and 2), although to a lesser extent than in glucose-autoclaved media. Inhibition at this stage in separately autoclaved high-GP media may very well be due to both a specific inhibitory effect of high-GP media as well as to the increase in osmotic pressure caused by the addition of the glucose and phosphate.

Inhibition is more pronounced, however, in glucose-autoclaved media.

Both glucose and phosphate were necessary in higher concentrations in order to inhibit germ tube elongation in glucose-autoclaved media (Fig. 9), which is consistent with the assumption that inhibition at this stage is caused by a heat-formed product of glucose and phosphate. Other sugars, including pentoses, also inhibited growth (Tables 4 and 5) when used in the concentration of 0.22 M, suggesting that the inhibitory phenomenon is more generally due to high sugar-high phosphate media, rather than a more specific glucose-phosphate effect. The role of phosphate in inhibition as opposed to sodium ions in the buffer was affirmed as other phosphate buffers such as potassium phosphate also caused inhibition when used in the concentration of 0.1 M (Hashimoto, personal communication).

C. Mechanism of inhibition.

The heat-formed product of glucose and phosphate inhibits T. mentagrophytes primarily in the early stages of growth. In subsequent hyphal growth, inhibition is eventually overcome with increased incubation time (data not shown) suggesting that the inhibitor affects growth by prolonging the lag phase. Since dry weight experiments measured growth after incubation for 3 days, at which time the cells are still in the exponential phase of growth, the inoculum size is probably critical in the lag or early exponential phase of growth, and inhibition even of very small inocula may eventually be overcome by stationary phase.

Preliminary investigation showed that although glucose metabolism is not affected in inhibited cells, amino acid metabolism may be inhibited, since in high-GP synthetic medium containing ammonium sulfate

(rather than amino acids) as the sole source of nitrogen, growth
of germinated microconidia is uninhibited (Hashimoto, personal
communication). The inhibitor may then interfere with amino acid
uptake or with metabolic utilization of the amino acids, and may
exert greater inhibitory effects when the cells are more actively
metabolizing amino acids, which would explain the greater degree
of inhibition at 29 C than at 37 C (Table 3). It is also possible
that temperature may affect the chemical nature of the inhibitor
itself, or the ability of the fungal cells to interact with the
molecule. The nature of this interaction could be that of a loose
binding, which could easily be disrupted simply by washing the cells
with water, as evident from the illustration of reversibility of
inhibition of germ tube elongation (Fig. 13), suggesting that the
mechanism of inhibitory action could be a physical disruption of
normal amino acid uptake.

A number of explanations are possible for the effect of increasing
spore concentration. It is possible that certain cells in the
population are resistant to inhibition and that increasing the inoculum
size increases the concentration of resistant cells. Alternatively,
the cells which are sensitive to inhibition may produce a substance
which is capable of interacting with the inhibitor and thereby
overcoming its effect. Kauffmann (1974) proposed this mechanism to
explain the fact that the addition of a live or heat-killed suspension
of Pseudomonas aeruginosa to an inoculum of the organism grown on
media containing polymyxin resulted in an increase in the minimum
inhibitory concentration of the antibiotic necessary to inhibit growth.
The addition of heat-killed spores did not alter the degree of inhibition

in Trichophyton (Table 2), which would indicate that if there were a neutralizing substance, it would probably be thermolabile, or else absent from dormant microconidia. This latter possibility would imply that the mechanism of overcoming inhibition of exponential growth could not occur earlier, at the stages of germination and early germ tube elongation. This is in fact the case. In germination and germ tube elongation experiments, inocula significantly greater than 2×10^7 spores per 50 ml medium are used in order to carry out microscopic observations, yet even at this cell concentration inhibition is very pronounced. By the time the cells have reached exponential growth, possibly enough of the neutralizing material will have been produced to overcome the inhibitory effects and eventually reach the same maximum growth at stationary phase.

The concentration of inhibitor available to each spore may be a critical factor in the spore concentration effect, which would also account for the lesser degree of inhibition seen with larger inocula. This would be true especially if the amount of inhibitor formed upon autoclaving for 15 min were relatively small, in which case a greater concentration would be needed in order to inhibit a greater number of cells. The observation that growth inhibition in the exponential stage is decreased with larger inocula would also seem to argue against the possibility that the autoclave effect is that of decreasing the availability of certain nutrients by converting them to a form in which the fungi are incapable of utilization.

It is possible that the concentration of the inhibitory substance which is present in control media stimulates growth of T. mentagrophytes, whereas higher concentrations of both glucose and phosphate, when

autoclaved, produce greater amounts of the substance which results
in inhibition of growth. An alternative explanation for the stimula-
tion of growth seen in glucose-autoclaved control media as compared
to that seen in separately autoclaved control media is that autoclaving
the media results in the production of a number of substances,
possibly of both stimulatory and inhibitory nature.

D. The heat-formed inhibitory compound.

The heat-formed product which has been shown here to inhibit
growth of T. mentagrophytes could be extracted from the media by
adsorption to activated charcoal. Earlier workers showed that heat-
formed factors stimulatory to the growth of certain lactobacilli and
propionibacteria (Rogers et al., 1956), as well as to B. globigii
(Sergeant et al., 1957) were also charcoal-extractable. Similarly-
formed products which were inhibitory to Clostridium botulinum
and to Vibrio cholerae have also been shown to be charcoal-adsorbable
(Bowers and Williams, 1962; Finkelstein and Lankford, 1957). However,
Bowers and Williams found that the amount of inhibitor present seemed
to increase with storage, whereas storage of the media which was
inhibitory to Trichophyton had no effect on the degree of inhibition of
growth of the organism (Table 7).

Inhibition of Trichophyton growth in glucose-autoclaved media
was reduced as the inoculum size increased (Table 2). A similar cell
concentration effect was observed by Sijpesteijn (1949), in reducing
the lag in growth of Sporocytophaga myxococcoides in the presence of
high concentrations of glucose; and by Finkelstein and Lankford (1957),
with regard to growth of Vibrio cholerae in glucose-autoclaved media.

In certain instances where glucose-autoclaved media stimulated growth
of some microorganisms, i.e., in B. globigii and certain propioni-
bacteria, growth in filter-sterilized media could occur to some
extent only when large inocula were used (Sergeant et al., 1957;
Field and Lichstein, 1957).

Finally, the inhibitory substance in glucose-autoclaved
Trichophyton growth media could be eluted from the activated charcoal
with ethanol (Tables 8 and 9), as was the substance which
proved to be stimulatory to the growth of some of the propioni-
bacteria (Field and Lichstein, 1958).

It is evident that no definitive statement can be made as to
the nature of the component(s) arising from the autoclaving of sugar-
containing media. Autoclaving may result in inhibition or stimulation
in different organisms. The product(s) formed may arise from reactions
between glucose and phosphate, glucose and amino acids, glucose and
ammonium chloride, or any other combination of these substances.
Cell concentration appears to influence the extent of susceptibility
to inhibition or stimulation. Activated charcoal extraction seems to
remove the stimulant or inhibitor in some cases, but not in others.
Some workers claim that autoclaving inactivates some essential nutrients
in the media, while others suggest that activity of the autoclave-formed
compound is due not only to inactivation of some nutrients but in
addition causes the production of new, toxic products. The pH at which
autoclaving occurs also appears to affect the nature of the product formed.

In summary, autoclaving various media, particularly sugar-containing
media, may result in the degradation of some components and/or the

synthesis of new products which may have a variety of different effects on microorganisms. Glucose-autoclaved media are inhibitory to the growth of Trichophyton mentagrophytes when higher concentrations of glucose and phosphate are added. As discussed previously, the concentrations of 0.2 M glucose and 0.1 M phosphate which inhibited the growth of T. mentagrophytes were well within the range often used in microbiological media. In particular, the commercially available Sabouraud medium, which is used extensively for fungal growth and cultivation, contains 0.2 M glucose. Numerous examples of the use of 0.1 M phosphate in microbiological media have also been cited (see "Introduction"). In light of the results presented in this work, glucose and phosphate concentrations merit consideration in the interpretation of data involving growth of microorganisms in media in which these nutrients are contained.

V. SUMMARY

Inhibition of growth of the dermatophyte Trichophyton mentagrophytes has been demonstrated when the concentrations of both glucose and phosphate in the media were increased to 0.2 M glucose and 0.1 M phosphate. All four parameters of growth tested, germination, germ tube elongation, colony growth, and growth in liquid media, were inhibited in these concentrations when the glucose was autoclaved together with the medium. In separately autoclaved media, however, high concentrations of glucose and phosphate were not inhibitory to the organism in the early stages of growth.

In all parameters which tested growth in liquid media, elevated concentrations of both sugar and phosphate resulted in maximum inhibition, although T. mentagrophytes appeared to be somewhat more sensitive to high phosphate alone than to high glucose alone. Inhibition of total growth in liquid medium varied with the inoculum size, and was more pronounced at 29 C (a more favorable temperature for growth of the fungus) than at 37 C. Evidence suggested that the organism was inhibited by prolonging the lag phase of the growth cycle. Reversibility of inhibition was demonstrated at the early stages of germ tube elongation. Several hypotheses were presented to elucidate the mechanism by which inhibition occurs.

Of the three bacteria tested, only Staphylococcus aureus was inhibited to some extent under the conditions of high glucose and high phosphate; Escherichia coli and Bacillus cereus were not significantly affected. None of the other four fungi tested, Saccharomyces cerevisiae,

Candida albicans, Geotrichum candidum, and Aspergillus niger, were
as sensitive to high concentrations of glucose and phosphate as was
T. mentagrophytes, although it is suggested that if inoculated in low
enough concentrations, inhibition of these fungi may occur as well.

A partial characterization of the inhibitory substance indicated
that it was stable to storage at room temperature, adsorbable with
activated charcoal, and capable of being eluted from the charcoal
with cold absolute ethanol. When the eluate was dialyzed, most of
the inhibitory activity could be found in the dialyzable material.

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APPROVAL SHEET

The thesis submitted by Miriam L. Greenberg has been read
and approved by the members of the Advisory Committee listed below.

The final copies have been examined by the director of the
thesis and the signature which appears below verifies the fact that
any necessary changes have been incorporated and that the thesis is
now given final approval with reference to content and form.

The thesis is therefore accepted in partial fulfillment of
the requirements for the degree of Master of Science.

12-19/74

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