UREASE TESTING AND YEAST TAXONOMY

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UREASE TESTING AND YEAST TAXONOMY

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PREFACE

This thesis contains two chapters plus appendixes. Chapter II is comprised of the manuscript entitled "Urease Testing and Yeast Taxonomy", which has been accepted for publication in the refereed international journal, the Canadian Journal of Microbiology. Although the manuscript is complete of itself without additional supporting material, a review of the current literature is presented in Chapter I, and appendixes describing experiments not contained in the published manuscript are also presented.

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The Phoenix is alive and well.

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CHAPTER I

LITERATURE REVIEW

The Taxonomic Significance of Urease

Within the yeasts of the Deuteromycotina, urea hydrolysis is useful in differentiating the form-classes, the Ascoblastomycetes (urease negative) from the Basidioblastomycetes (urease positive). The exception to this are the ascomycetous yeasts of the Schizosaccharomycetaceae, the genus Schizosaccharomyces, (Barnett et al., 1983) which are delineated by their distinctive morphology (Kreger-van Rij, 1984). Urea hydrolysis is also a useful charater in the clinical identification and differentiation (usually at the generic level) of medically important yeasts.

The taxonomic significance of urease has been examined by a number of investigators. Sen and Komagata (1979) briefly discussed the historical basis of urease as a taxonomically significant enzyme, citing Sakaguchi and Shizume (1937) as the first reference of urease testing used in the taxonomy and identification of yeasts. According to Sen and Komagata, Sakaguchi and Shizume tested 60 strains including 3 "Torula", 1 Schizosaccharomyces, and 1 unidentified strain which were urease positive.

Seeliger (1956) described the use of urease tests, performed on Christensen's urea agar (Christensen, 1946) to provide an easier and more definitive means of identifying clinical fungal isolates, especi-

ally Cryptococcus spp. Seeliger noted that in "earlier investigations" capsulated and red pigmented yeasts hydrolysed urea on Christensen's urea agar, whereas candida strains and saccharomycetes did not. Seeliger's results showed that: 1) The urease test could provide a "clear-cut" distinction between several genera of yeasts and yeast-like organisms, and that the "non-fermenting" genera Cryptococcus, Rhodotorula, Sporobolomyces, Pullularia, Trichosporon, and Candida were urease-positive; 2) The various species and their varieties of the genus Cryptococcus could not be distinguished from each other on the basis of the urease test; 3) The then current controversy concerning the the relationship between the gerera Cryptococcus and Lipomyces could be resolved on the basis of the urease test. and 4) The urease test may prove useful in determining the phylogenetic relationships amoung the black dematiaceous fungi Pullularia, Cladosporium, and Torula. Seeliger also noted that urea hydrolysis was not previously used as a means of differentiating yeast genera or species by Lodder and Kreger-van Rij ("The yeasts: a taxonomic study", 1952), and that the urease reaction on Christensen's urea agar is different from the assimilation tests for urea that Wickerham (1946) and others were performing.

Roberts, et al. (1978), who developed a rapid urea hydrolysis broth test based on a modification of Christensen's urea agar, stated that "urea hydrolysis has been a prominent point at which dichotomy occurs in the classification of yeasts", citing Adams and Cooper (1974) and Seeliger (1956) as referances. Roberts et al. also stated that urea hydrolysis (along with other tests) has been used to separate the medically important yeasts, Cryptococcus spp., Trichosporon spp., and

Rhodotorula spp., from yeasts which are unable to produce urease. Zimmer and Roberts (1979) reiterated the utility of urease tests in differentiating clinically important yeasts. They described a rapid test procedure by which clinical isolates of Cryptococcus neoformans can be differentiated from the other clinically important yeasts which are also hydrolyse urea, Rhodotorula, Trichosporon, and less frequently, Candida krusei. Of the Cr. neoformans strains tested, 99.6% produced a positive reaction within 15 min.

In describing the taxonomic significance of urea hydrolysis, van der Walt (1970) cited Seeliger (1956) as providing evidence that in general ascosporogenous yeasts fail to hydrolyze urea, and that, therefore, the primary utility of urease testing is as a confirmatory test for the anascosporogenous (ie., basidiomycetous) yeasts. For anamorphic yeasts (ie., those yeasts lacking a sexual state, or for which a mating type has not yet been discovered), Kreger-van Rij (1984) stated that the inability to hydrolyze urea in conjunction with the absence of xylose in their cell walls can distinguish ascosporogenous yeasts from other (ie., basidiomycetous) yeasts.

In their handbook cataloging yeast characteristics, Barnett, Payne and Yarrow (1983) stated that the characteristics used to identify yeasts are: 1) the microscopical appearance; 2) the mode of sexual reproduction; 3) certain physiological activities (ie., nutritional requirements); and 4) certain biochemical features (e.g., chemical composition of the cell wall). With respect to physiological activ- ities, the authors mentioned urea hydrolysis in addition to nine other tests, and stated that these tests are used chiefly for differentiating between species, but that "some genera" are distinguished by

these features.

Sen and Komagata (1979) used urease, tested for by growth on Christensen's urea agar, in combination with extracellular deoxyribonuclease (DNAse) to provide an indication (in connection with other characteristics) of taxonomic assignment. They noted that basidiomycetous yeasts and "related species" produced urease and extracellular DNAse, and that many ascosporogenous and asporogenous yeasts failed to produce either enzyme. Sen and Komagata also pointed out that in general urease positive strains show higher G+C DNA base composition than urease negative strains and cited Nakase and Komagata (1968 and 1971) as references for this observation.

The correlation of urease, extracellular RNAse and DNAse with yeast taxonomy was studied by Kocková-Kratochvílová (1982). Summarizing the conclusions of Sen and Komagata (1979) which her results supported, she noted the utility of using DNAse (and RNAse) in connection with urease and G+C mole% at high hierarchic levels. Kocková-Kratochvílová, like numerous other authors, based her conclusions on the "unquestioned" taxonomic significance of urease in discriminating between ascomycetous and basidiomycetous yeasts.

There are several characteristics other than urease production which can be used to delineate the form-classes. Miller, *et al.* (1976) in a paper describing the the establishment of a new anamorphic genus, *Phaffia*, discussed the criteria which they considered useful for differentiating the ascomycetous and basidiomycetous yeasts. These criteria are: 1) the ability to form ballistospores; 2) the ability to synthesize carotenoid pigments; 3) the mole% base composition of DNA; 4) the cell wall structure and mode of bud formation; and 5) the com-

position of the capsular envelope in those species which have capsules. For their classification of Phaffia, the authors stated the the "most conclusive evidence" of phylogenetic orign is the presence of a multi-layered cell wall near the area of repeated bud formation.

In his paper describing an emendation of fungal taxonomy, Moore (1980) described the characters on which his modifications are based. Moore stated that "the evidence presently available...points to all the higher fungi being either ascomycetous or basidiomycetous", and goes on to give seven form-class "affinity indicators" other than the characters of the septum type and the occurence of clamp connections. These indicators are: 1) The structure of the cell wall. Basidiomycetous yeasts have layered cell walls which becomes frayed at the site of bud formation, while ascomycetous yeasts have homogeneous cell walls; 2) Resistance to certain enzyme treatments which cause the cells to become protoplasts. Basidiomycetous yeasts are resistant and ascomycetous yeasts are not. 3) The empirical Diazonium blue B test. Basidiomycetes give a red color to the reagent, and Ascomycetes do not. 4) The ability to hydrolyse urea. Basidiomycetes are urease positive, and the Ascomycetes are urease negative. 5) The type of cytochrome Q (= coenzyme Q or ubiquinone). Cytochrome Q_{10} is limited to basidiomycetes, and they may also contain Q_8 and Q_9, whereas, Ascomycetes contain Q_6 and Q_7. 6) The mole% DNA base composition. While the G+C mole% may overlap, generally, Ascomycetes values range between 30-40 mole% with some as high as 50%. Conversely, basidiomycetes range between 50-70 mole% with some having values in the area of 45 mole%. 7) The location of somatic nuclear division. Nuclear division in basidiomycetes occurs within the bud, while division in the Ascomycetes

occurs within the parent cell.

In an effort to standardize the testing procedure, Hagler and Ahearn (1981) described a method for performing the Diazonium Blue B (DBB) test. The test differentiates basidiomycetous from ascomycetous yeasts. Repeating Moore (1980), the authors cited several characters which can be used to differentiate anamorphic basidiomycetous and ascomycetous yeast. These are: cell wall structure, enzyme induced release of protoplasts, DNA mole% base composition, oxidation and/or fermentation, extracellular urease, extracellular DNAse, the type of coenzyme Q, and reaction to DBB test. The DBB test involves the alkaline hydrolysis of cells followed by addition of buffered DBB (*ortho*-dianisidine, tetraazotized). Cells which are basidiomycetous in nature usually produce a red to violet positive reaction within a few seconds. Once a positive reaction is observed, 95% ethanol is added to prevent dissappearance of the chromogen.

Although urea hydrolysis has been accepted and widely used as a means of identifying and classifying yeasts, the variety of available test media, and the lack of standardized test procedures has resulted in disagreement in the reported urease reactions for some yeasts. Examples of disagreement in urease results can be found in Sen and Komagata (1979), whose results differ from those in Barnett, *et al*. (1983) with regard to *Hansenula bimundalis* which is reported as weakly positive. This discrepancy is not cited in the manuscript "Urease testing and yeast taxonomy" (see Chapter II) due to other more recent examples. The authors agree that *Saccharomycopsis lipolytica* (*Yarrowia* in Barnett, *et al*., 1983) is urease positive. The results of Kocková-Kratochvílová (1982) also differ from those reported in Barnett, *et al*.

(1983) for Saccharomycopsis lipolytica and Schizosaccharomyces pombe, which were both reported urease negative. Like Barnett, et al. (1983), Kreger-van Rij (1984) reported the former species as urease positive. Vishniac and Hempfling (1979), Baharaeen and Vishniac (1982), and Vishniac and Baharaeen (1982), the authors of several species of anamorphic yeasts isolated from the Dry Valleys of Antarctica, reported these unique yeasts as urease negative. This result conflicted with the otherwise basidiomycetous characteristics of these yeasts. Barnett, et al. (1983) reported Cryptococcus lupi as urease variable and Cr. vishniacii as urease positive.

Methods for Determination and/or Assay of Urease

Methods for detecting or assaying urease activity have involved the detection and measurement of either the reaction end products, NH_3 and CO_2, or the initial substrate, urea. Detection of ammonia has been especially useful for urease kinetics assays, as well as in detecting urease activity in bacteria and fungi. For fungal and bacterial cultures, the most frequently used approach involves the inclusion of pH indicators in buffered test media supplemented with urea. The ammonia produced from urea hydrolysis leads to alkalinization of the test medium causing the pH indicator to change color. A medium of this type was formulated by Christensen (1946) as an agar that would facilitate the growth of predominantly enteric bacteria while allowing for the detection of urease activity without the constraint that the oganism utilize the ammonia derived from the urea. The composition of the agar is: peptone, 0.1%; NaCl, 0.5%; KH_2PO_4, 0.2%, glucose, 0.1%; urea, 2%; phenol red, 0.0012%; agar2.0%. The glucose and urea are

filtered sterile and added aseptically. The agar is then inoculated and the organism allowed to grow for up to 7 days. As with all test media containing phenol red, a positive reaction is indicated by a deep red or magenta color in the agar. This medium has since been used widely to determine urease activity in yeasts, and has served as the foundation from which other urease tests have been constructed.

A rapid urease test broth was designed by Roberts, et al. (1978) to allow rapid screening of yeasts isolated from clinical specimens. The test was based on the composition of Christensen's urea agar and testing was performed in multi-well titration plates In Roberts' rapid test, cells from one large yeast colony (ca. 10 mm dia.) or from 3-4 small colonies (ca. 1-4 mm dia.) are scooped up on the end of a wooden applicator stick, thoroughly mixed in 0.2 mL of the test broth, and incubated at 37°C for up to 4 hours. The experimental results led to the following composition for rapid urea test broth: urea, 2%; phosphate buffer, 10^{-2} M; glucose, 0.1%; peptone, 0.1%; NaCl, 0.5%; and phenol red, 0.0012%.

The optimal conditions for rapid urease testing with yeasts were studied by Roberts, et al. (1978). The optimal concentration of phosphate buffer was 10^{-2} M. This concentration removed the false positive reactions seen when less than 10^{-3} M phosphate was used, and only slightly decreased the fastest reaction times seen when 3×10^{-3} to 10^{-3} M phosphate were used. Roberts, et al. noted that false positives resulting from low buffer concentration occured more frequently with older isolates (5 or more days old) grown in rich, peptone containing medium (Sabauroud's Dextrose Agar), and could have resulted from "nonspecific" indicator changes due to the natural alkalinity of

an older colony. The authors stated that the alkalinity of an older colony reflected the accumulation of ammonia during extended growth on peptone. Urea concentrations of 0.02 to 2% did not influence a positive reaction, but concentrations less than or equal to 0.002% allowed false negative reactions. For the strains examined by Roberts, et al., the optimal incubation temperature for rapid urea hydrolysis was 37°C. Higher temperatures led to both false positive and false negative results, and lower temperatures slowed the reaction rate. Roberts, et al. stated that the enzyme activity at 37°C seemed independent of incubation temperature because isolates unable to grow at 37°C showed "competent" urease production when tested at 37°C. The observation of specific enzyme activity in the absence of yeast growth was referenced to Hopkins and Land (1977). Roberts, et al. went on to state that this phenomenon is suggestive of a temperature-insensitive, constitutive enzyme.

Zimmer and Roberts (1979) described a procedure by which clinical isolates of Cryptococcus neoformans can be differentiated from the other clinically important yeasts. The test takes advantage of the ablilty of Cr. neoformans to overcome the low pH, and high urea concentration of the test broth, producing a positive urease reaction within 15 min. Christensen's urea agar base (Difco) was prepared to 5x strength, the pH of the broth adjusted to 5.5 $\pm$ 0.05, and filter sterilized. The broth was used to saturate cotton swabs, which were then lyophilized. To test an isolate, cells from several colonies were swabbed onto the impregnated cotton swab and placed into a test tube containing ca. 3 drops of 1% benzalkalonium (Zephiran) chloride (pH 4.86 $\pm$ 0.01). Tests were incubated at 45°C for up to 18 hours.

Difco Bacto Urea R Broth was used to assess the ability of 473 yeast species to hydrolyse urea by Barnett, Payne and Yarrow (1983). To test their isolates, a loopful of cells from a one or two day old culture was suspended in 0.5 mL broth, incubated at 37°C (regardless of the yeasts optimal growth temperature), and examined every half hour for up to 4 hours. The composition of Urea R Broth is: yeast extract, 0.01%; monopotassium phosphate, 0.0091%; disodium phosphate, 0.0095%; urea, 2.0%; and phenol red, 0.001% (Difco Manual 10th ed., 1984). This broth differs from Christensen's urea agar and its modifications described by Roberts, et al. (1978) and Zimmer and Roberts (1979) in that peptone is replaced by yeast extract, no glucose has been added, NaCl has been omitted, and the total phosphate buffer concentration is 1.34×10^{-3} M compared to 1.47×10^{-2} M and 1.0×10^{-2} M for Christensen's urea agar and Roberts' rapid broth.

The alkalinity resulting from ammonia production has also been used to follow the kinetics of urea hydrolysis by Stefanac, et al. (1969). In the assay system, differential spectrophotometry was used to follow the activity of urease in a buffered solution containing urea and phenol red pH indicator. The reaction was carried out in cuvettes: the absorbance at 560 nm of the mixture to which urease enzyme was added as zeroed to a cuvette containing only the urea-phenol red solution was read at one minute intervals. The µmoles of ammonia produced during a given time interval was determined from a standard curve of A_{560} vs. µmoles NH_3 generated by titrating the buffered urea-phenol red solution with ammonium carbonate. The specific activity of urease was calculated as: (mean increment of absorbance per minute) X 10 X (µmoles NH_3 corresponding to 0.1 increment of absorbance)/(final enzyme

concentration in mg/mL). The authors stated that the results obtained by this method "agree well" with the results of urease activities using other methods; that the procedure has the benefit of allowing continuous measurements of enzyme activity over an indeterminate time span, whereas, most other methods of assaying urease are of the "fixed interval" type in which the reaction is stopped after a predetermined time; and that the method is suitable for use in studying deaminase reactions of all types.

Urease activity in bacteria and fungi has been detected with the Berthelot color reaction (Bergquist and Searcy, 1963; Paliwal and Randhawa, 1977). Unlike the methods described above in which the ammonia end product is detected by a rise in the alkalinity of the test medium, the Berthelot reaction, or phenate-hypochlorite method, involves the reaction of ammonia (or aromatic amines) with an alkaline solution of phenol and hypochlorite in the presence of the catalyst, sodium nitroferricyanide dihydrate (= nitroprusside), to produce the blue chromagen indophenol (Kaplan, 1969). According to Kaplan, the Berthelot reaction is capable of reliably detecting microgram amounts of ammonia, and that this feature along with the formation of a stable blue chromagen with a maximum absorbance at 625 nm, allows the Berthelot reaction to be used in colorimetric kinetics assays of urease and deaminase enzymes.

The Berthelot test described by Bergquist and Searcy (1963) is used for differentiating the bacterium, Proteus, from other members of the Enterobacteriaceae. Tests were conducted as follows: the urea needed for testing was rehydrated in 5.0 ml distilled water from glass fiber strips (5 x 30 mm) which were impregnated with 50 µL of a 15%

urea solution and subsequently dried. A single colony of the test organism was used to inoculate the urea solution. Following incubation for 5 minutes at 37°C, 1.0 mL sodium hypochlorite and 1.0 mL sodium phenate-sodium nitroprusside reagent (Hyland UN-Test kit, Hyland Laboritories, Los Angeles) were added to the cell suspension. Urease positive strains developed a positve, blue Berthelot color reaction within 5 minutes on incubation at 37°C.

Noting the important connection between urea hydrolysis and yeast taxonomy, Paliwal and Randhawa (1976) designed a variant of the Bergquist and Searcy method which employed the Berthelot reaction to detect urea hydrolysis in yeasts. The test was conducted by suspending a loopful of cells grown for 3-4 days at 25°C on Sabouraud's dextrose agar in 4.0 mL deionized water, and adding 1.0 mL freshly prepared 1.6% urea solution. The suspension was incubated for 20 min. at 25°C, following which 3.0 mL sodium nitroprusside (0.1%), 2.0 mL sodium phenate (2.5%), and 3.0 mL sodium hypochlorite (ca. 0.06 N) were added in rapid succession. On further incubation at 37°C in the dark, urease positive strains produced a blue color within 30 min.

Paliwal and Randhawa compared the results of their Berthelot test with the results of yeasts grown on Christensen's urea agar. Of the 92 known urease-positive (basidiomycetous) yeast strains tested, including 77 *Cryptococcus neoformans* strains, urea hydrolysis was detected by both methods equally well. No urease activity was detected with either method for the 49 known urease-negative (acsomycetous) yeast strains tested.

The advantages of the Berthelot test of Paliwal and Randhawa are that it allows visualization of positive urease test results within

30-50 min., whereas, growth on Christensen's urea agar required as long as 72 hours until positive results could be seen. Roberts, et al. (1978) who briefly critiqued the method of Paliwal and Randhawa, gave three disadvantages to the approach: 1) preparation of the reagents is difficult and time consuming, 2) the reagents must be used the same day they are prepared, and 3) the inoculum of cells needed requires several days of pregrowth, which negates the efficiency of the faster Berthelot test.

Potentiometric methods involving the use of an ammonia electrode to measure the evolution of ammonia have also been used to assess urease activity and kinetics (Scism, 1983; Shapiro, 1976). Using this technique Shapiro was able to detect urease activity in powdered lichen extracts, and compared the activity seen in the lichens to the urease activity seen in soybean seeds. Shapiro noted the linear relation of ammonia concentration to millivolts, and that therefore, the equation of a straight line, $y = a + bx$, where $y = E$ in millivolts, $x = -\log[NH_4]$ and a and b were determined experimentally from known samples of NH_4Cl ($a = 5.208$ and $b = 0.905$) can be used. Shapiro stated that the primary utility of the potentiometric method was that it permited analysis of lichens rich in phenolic compounds which otherwise interfered with the reactions involved in titrametric and spectrophotometric methods, and that uninterrupted observation of enzyme activity over any length of time were possible.

A variety of methods for quantifying the ammonia liberated during urea hydrolysis were briefly reviewed by Kaplan (1969). The techniques mentioned are: coupling the reaction with L-glutamate: NAD oxido-reductase [deaminating EC 1.4.1.3.]; a coulometric titration of the

ammonia with hypobromite; the microKjeldahl and Conway diffusion techniques and other colorimetric methods (Kaplan cites Jacobs, S. 1965. Methods of Biochem. Anal. Vol. 13. D. Glick, ed. Interscience, New York, p.241); and titrimetric determinations of ammonia (Kaplan cites Bessman in Advances in Clin. Chem. Vol. 2, Sobotka and Stewart, eds. Academic Press, N.Y. 1959. p.135).

A unique method employing a pH-stat device to determine urease kinetics in responce to metal ion inhibitors is described by Toren and Burger (1968). This approach was chosen in order to conduct a continuous assay which simultaneously avoided the possibility of metal ion inhibition to an enzyme-coupled assay system. In the pH-stat method buffers were not required to maintain a constant pH, because as the concentration of ammonia increases, acid is automatically added to the reaction, and the progress of the reaction was followed by the amount of standardized perchloric acid required to maintain a constant pH.

The carbon dioxide produced by hydrolysis of ^{14}C-labelled urea has been used to assess urease activity. In radiometric assays of this type, hydrolysis of ^{14}C-urea leads to the formation of ^{14}C-CO_2 which is then collected and its radioactivity measured. The advantages of ^{14}C-CO_2 assays over methods involving quantitation of ammonia or pH changes are that they can be used to measure low levels of urease activity while negating the production of endogenous ammonia encountered with crude extracts and in physiological studies, and as the radiometric assay is both specific and sensitive, it can be used with the low substrate concentrations required in kinetic studies (McDonald, _et al_. 1972).

Methods incorporating ^{14}C-urea vary chiefly in the manner by

which the $^{14}CO_2$ is quantitatively collected. Rabinowitz, et al. (1956) incorporated a complicated system in which the ^{14}C-CO_2 was driven from the reaction solution with nitrogen gas and bubbled through tubes containing a 4% solution of barium hydroxide layered with toluene. The resultant $BaCO_3$ precipitate was then formed into uniform pellets with a pilling machine, and the radioactivity of the pellets was determined with a mica-window Geiger-Müller counter.

To study the urease activity of leaf homogenates, Zhuravlev, et al. (1972) used a closed-loop system with an inline air pump to collect the ^{14}C-CO_2 as $BaCO_3$. The assay was stopped and the ^{14}C-CO_2 was driven from solution by adding 2 mL of a semi-saturated oxalic acid solution to the reaction vessel. The $BaCO_3$ precipitate was filtered from the collection vessel, washed, and the radioactivity was determined on the filter. The authors said that oxalic acid should be used because both sulfuric and hydrochloric acid increase the spontaneous decomposition of urea, leading to increased background radioactivity and lowered accuracy.

Davies and Shih (1984) described a unique method employing phenylethylamine to trap the ^{14}C-CO_2 produced by leaf homogenates. In their assay system, a disposable hypodermic needle was inserted completely through the cap of a 12 x 75 mm disposable polypropylene test tube; four filter paper discs saturated with 50 µL phenylethylamine were placed on the end of the needle; the assay mixture was added to the tube (0.210 mL total volume), and the cap was added, sealing the system and placing the filter disks above the reaction mixture. Following incubation for 40 minutes at 30°C, 0.5 mL of 0.4 M HCl was injected down the needle to terminate the reaction and evolve ^{14}C-CO_2.

After 45 minutes of further incubation at 30°C, the disks were transferred to scintillation fluid for liquid scintillation counting. According to the authors, phenylethylamine allowed immediate scintillation counting without chemiluminescence of the samples.

To examine the reaction stoichiometry, Davies and Shih (1984) compared the results of their ^{14}C-CO_2 assay with the results of ammonia evolution coupled to glutamate dehydrogenase. In parallel experiments, they found that commercial jack bean urease and maize and soy bean leaf extracts produced three times more ammonia than carbon dioxide. They noted that the reaction stoichiometry should have been 2 NH_3 to 1 CO_2; however, they were unable to adequately explain the anomalous results.

McDonald, et al. (1971) described a versatile and specific assay system in which the ^{14}C-CO_2 liberated during ^{14}C-urea hydrolysis was collected in a solution of hyamine hydroxide (methylbenzethonium hydroxide. See Windholz, et al. eds. 1983), and measured via liquid scintillation counting. The reaction and collection system consisted of a reaction flask (Kontes Glass Co. K-88230) or 10 or 25 mL thick-walled Erlenmyer flasks fitted with a rubber septum stopper through which a polypropylene centerwell was depended. The assay was conducted as follows: buffered urease solution was added to the reaction flask; the centerwell support rod was inserted through the a hole in the rubber septum; 0.2 mL of hyamine hydroxide (*p*-[diisobutyl-cresoxy-ethoxyethyl] dimethyl benzyl ammonium hydroxide) 1.0 M methanolic solution was added to the centerwell; the reaction was started by adding 0.1 mL 0.5 M ^{14}C-urea (total assay volume = 1.0 mL); the septum-centerwell assembly was fitted over the reaction flask, and the centerwell was slid down into the center of the reaction flask. The

sealed system was incubated with slow agitation at 30°C for varying times. The reaction was stopped and CO_2 driven from solution by injecting 1.0 mL 5 N sulfuric acid through the rubber septum with a hypodermic syringe, followed by an additional 30 minute incubation with agitation after which the apparatus was disassembled. To determine ^{14}C-CO_2, the centerwell was cut from its plastic support rod, placed in a vial containing 10 mL scintillation fluid, and counted by liquid scintillation.

To assess their ^{14}C-CO_2 assay, McDonald, et al. (1971) studied the effect of volatilization time on the recovery of ^{14}C-CO_2, the capacity of the hyamine trapping system, the effect of pH on the non-enzyme catalyzed hydrolysis of ^{14}C-urea, and compared the results of their ^{14}C-CO_2 assay with the results of ammonia evolution. The recovery of ^{14}C-CO_2 after volalitilization for 10 minutes was about 90%, reached 100% (ie., quantitative recovery) after 30 minutes, and continued so at 60 minutes, the longest time tested. Based on results using ^{14}C-sodium bicarbonate, the capacity of the hyamine hydroxide trapping system at 98% efficiency was 20 µmoles $^{14}CO_2$ / 30 min. volatilization period / 0.2 mL hyamine hydroxide. Over the pH range 6.0 to 9.5, the nonenzymatic hydrolysis of urea was "negligible", with the highest control (less urease) giving only 23 dpm over background when 5×10^5 dpm substrate was added. The formation of ^{14}C-CO_2 was within 1% of the amount expected from ammonia production as measured in parallel microdiffusion and Nesslerization assays. Duplicate determinations were usually within 2%, credited to routine pipetting error.

A similar method for collecting ^{14}C-CO_2 was described by Nasu, et al. (1892) as an assay for D-aspartate oxidase. The reaction mixture

(0.5 mL total volume) was placed in a 21 X 70 mm sample vial connected by 1/8 inch thick rubber tubing to an inverted polyethylene scintillation vial containing a 1 x 25 cm circularized strip of Whatman #1 filter paper saturated hyamin hydroxide. The connecting tube formed an airtight seal between the two vials. Following incubation, the reaction was stopped and dissolved $C0_2$ driven off by injecting 0.2 mL, 2 N HCl into the sample vial. Immediately after the HCl is injected, Nasu, et al. added phthalate buffer and aluminium sulfate to selectively convert the desired product to CO_2. The system was then disassembled, scintillation cocktail was added directly to the $C0_2$ collection vial, and radioactivity determined by liquid scintillation counting.

Urease assays which follow the decreasing concentration of urea include a radiometric approach in which the labelled carbon dioxide produced by the hydrolysis of ^{14}C-urea is driven of and the radioactivity if the remaining labelled urea is measured (McDonald, et al. 1972). However, frequently the diacetyl-monoxime assay is employed (Kaplan, 1969). Though the reactions of urea with diacetyl-monoxime are not well understood, the resultant chromogen can be readily measured at 520 nm. Kaplan noted that the best diacetyl-monoxime methods were not as sensitive as the Berthelot reaction, and that the method has the added disadvantages of an unstable, photosensitive chromagen and a bad reagent odor. However, the diacetyl-monoxime method has the advantage that the volatile end product, ammonia, need not be followed which Kaplan said is of "definite" advantage for automated assay methods.

The direct localization of urease within plant and bacterial

cells has been accomplished in a variety of ways. Murry and Knox (1977) developed a method in which fluorescein labelled antiurease rabbit antibodies were used to localize urease sites within germinating jack bean (Canavalia ensiformis) seeds via light microscopy. Granick (1937) isolated the sites of urease activity in soy bean and jack bean seeds using pH dependent haematoxylin stain which reacted with the ammonia produced from urea. A technique for localizing urease in electron microscope preparations was developed by McLean, et al. (1984). The ammonia produced by urease activity was precipitated with tetraphenylboron and then displaced with electron dense silver nitrate to allow visualization of urease sites via transmission electron microscopy.

The Isotope Effect on the Hydrolysis of 14C-Urea

When isotopes are used for chemical or biological experiments, the first assumption is that the isotope and its stable counterpart may well behave identically. However, the chemical behavior of an isotope will be different than that of its stable counterpart, because chemical reaction rates are influenced partly by the mass of the reactants and the mass of an isotope and its stable counterpart are different (Wolf, 1964). This difference in behavior is called the isotope effect. Generally, molecules containing the lighter isotopes of an element will react faster than those containing the heavier isotope (Wolf, 1964; Chase and Rabinowitz, 1959). The isotope effect is more pronounced for the lighter elements, like the isotopes of hydrogen, but, according to Wolf (1964), it is generally assumed to be negligible for the isotopes of carbon because the difference in reaction rates depend on the ratio

of the atomic weights, which is close to unity for ^{12}C and ^{14}C (14/12).

The carbon isotope effect on the urease enzyme catalyzed and acid hydrolysis of urea was studied by Schmitt and Daniels (1953) and Schmitt, *et al*. (1952). The enzymatic hydrolysis was conducted in pH 5.0 acetate buffer at 30°C (Schmitt, *et al*. 1952), and the acid hydrolysis was conducted in pH 5.0 phosphate buffer at 100°C (Schmitt and Daniels 1953). Schmitt and Daniels noted that 100°C incubation was required to give similar reaction rates between the enzyme and non-enzyme catalyzed reactions. In both cases the CO_2 was collected as $BaCO_3$ and subjected to mass spectrophotometry. The ratio of reaction rate constants for the enzymatic hydrolysis of urea were reported to be $k12_C/k14_C = 1.032$ and $k12_C/k13_C = 1.010$ (Schmitt, *et al*. 1952), and $k12_C/k14_C = 1.101$ and $k12_C/k13_C = 1.055$ for the acid hydrolysis (Schmitt and Daniels 1953). These results showed that enzymatic hydrolysis is affected much less by carbon isotope effects than the acid hydrolysis, and that the order of reaction rates for the isotopes was $^{12}C > {}^{13}C > {}^{14}C$. Using these ratios, the enzymatic hydrolysis has a 3.1% (1/1.032 X 100) greater affinity of hydrolysis for ^{12}C-urea than for ^{14}C-urea, and the acid hydrolysis yields 9.2% (1/1.101 X 100) difference in the affinity of hydrolysis.

Rabinowitz, *et al*. (1956) assessed the carbon isotope effects over time for the urease reaction by comparing the amount of specific radioactivity released as $^{14}CO_2$ to the amount of ^{12}C-urea plus ^{14}C-urea present initially. The reaction was carried out at pH 5.0 in acetate buffer at 37°C, and the $^{14}CO_2$ was collected and its radioactivity measured as described above. Rabinowitz, *et al*. reported that the greatest differences in specific activities of the ^{14}C-CO_2 were during

the initial phase of the reaction. At reaction lengths of 10 to 20 minutes an average maximum deficit of 10 $\pm$ 2% yield of ^{14}C-CO_2 was observed. Subsequently the effect diminished with 30 to 40% yield occuring between 120 and 150 minutes reaction time. The isotope effect was considered "negligible" as early as 50 to 80 minutes with the latter per cent values above corresponding to complete conversion of the ^{14}C-urea to ^{14}C-CO_2.

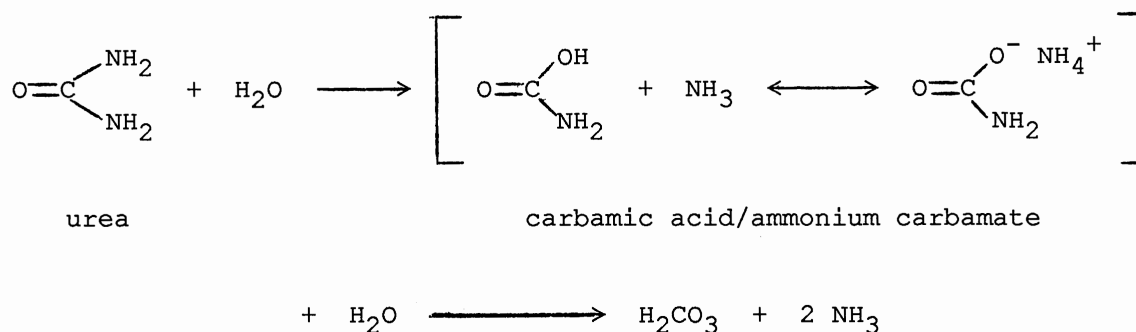
Properties of Urease

The properties of urease were most recently reviewed by Reithel (1971). Reithel discussed the enzyme's physical and chemical properties including molecular weight determination and subunit interactions, chemical composition, and miscellaneous physical properties; the catalytic characteristics including reaction mechanism, the active site, substrate specificity and kinetics; and ureases isolated from various organisms.

Ureases are defined by the reaction they catalyze. Ureases are hydrolases acting on the non-peptide C-N bonds of linear amides, and therefore, are given the systematic name urea aminohydrolase and an Enzyme Commission listing of EC 3.5.1.5. Other enzymes in this group include glutaminase, formamidase and formyltetrahydrofolate deformylase. Urease from jack bean (_Canavalis ensiformis_) meal was the first enzyme obtained in purified, crystalline form (Sumner, 1926). According to Reithel, ureases (plural) constitute many protein species with urease (singular) generally used to indicate that particular enzyme first crystallized by Sumner.

The stoichiometry of the urease reaction is: urea + 2 H_2O -> CO_2

+ 2 NH_3. The reaction is known to occur in two steps. The initial hydrolysis produces NH_3 and the intermediate, carbamate, and the second hydrolysis acts on the carbamate to yield CO_2 (as carbonic acid) and a second NH_3. The reaction is diagramed below:



$$+ \ H_2O \longrightarrow H_2CO_3 \ + \ 2\ NH_3$$

The nature of the active site was suggested by three types of studies: kinetic studies, titration of sulfhydryl groups, and inhibitor binding studies. K_m and V_{max} vs. pH experiments yield molecular ionization constants which suggest a histidine, pK_b 5.8, an alpha-ammonium group, pK_a 7, and a sulfhydryl group, pK_a 8.3, are the key moieties of the active site. The ammonium and sulfhydryl groups are involved in formation of enzyme-substrate complex and the histidine group is involved in the reaction catalysis. Titration of the "essential" sulfhydryl groups revealed the presence of eight active sites per 480,000 MW enzyme. Reithel (1971) pointed out that the titration could have led to structural changes, but that the retention of hydrolytic activity in 8M urea strongly suggests that the above conclusion is valid. Inhibitor binding studies indicate that there are two active sites per 16n enzyme (n = 480,000 MW). Urease (16n) which had lost its catalytic abilities was found to contain 2 moles of hydroxamic acid, a competitive inhibitor. Reithel pointed out that although there seem to be fewer active sites than there are 1n sub-

units, there is fair evidence for eight active sites per molecule of crystalline enzyme which is equivalent to one active site in a 2n enzyme.

Urease is notable in its specificty for urea, but many inhibitors have been described. These include hydroxamic acids, hydroxyurea, hydroxylamine, dihydroxyurea, thiourea and dimethyl sulfoxide. Reithel (1971) said that hydroxyurea could serve as a substrate, but the rate of its hydrolysis decreased as the reaction progressed, and when both hydroxyurea and urea were added to urease solutions, the extent of inhibition depended on the order of the additions and the length of time the enzyme was exposed to the inhibitor. Dihydroxyurea was also a substrate when present at high concentrations, but that like hydroxyurea, hydroxylamine and some hydroxamic acids, dihydroxyurea is a noncompetitive inhibitor. He added that it is unknown whether these compounds bind at or near the active site.

Kinetic studies indicated that reaction rates varied with temperature, pH, extent of reaction, ionic strength, concentration of reactants, type of buffer, and age of the enzyme preparation. Reithel (1971) reported the following K_m values for jack bean urease: in tris-HCl buffer pH 7.4, 25°C K_m = 4 mM, at pH 7.0, 38°C K_m = 3.28 mM; in maleate buffer pH 5.0, 21°C K_m = 4.0 mM, pH 7.0, 21°C K_m = 4.9 mM; and in tris-H_2SO_4 buffer pH 7.0, 21°C K_m = 5.1 mM, pH 9.0, 21°C K_m = 2.7. By using an ammonia electrode, Scism (1983) determined the K_m of a commercial jack bean urease preparation to be 5.4 ± 0.7 mM.

Inhibition of urease by various metals and the effect of buffers and chelating agents have been studied. Potentiometric data showed that Cu^{2+} and Zn^{2+} inhibit urease noncompetitively. Another potentio-

metric study determined 50% inhibition by Ag^{1+}, Hg^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, and Ni^{2+} (in decreasing inhibition), with Pb^{2+} being less than Cu^{2+}. Toren and Burger (1968), using the pH-stat method described above, found that the order of metal inhibition was $Ag^{1+} > Hg^{2+} > Cu^{2+} > Cd^{2+} > Mn^{2+} = Co^{2+} > Pb^{2+} > Ni^{2+}$. According to Reithel, urease inhibition was due to metal substitution of the sulfhydryl group within the active site. Phosphate has been shown to increase the urease reaction rate, whereas, EDTA (ethylenediamine tetraacetic acid) decreased urease activity. EDTA was also found to prevent inhibition by 1µM Cu^{2+} and Pb^{2+} but not Hg^{2+} and Ag^{2+}.

Determinations of the molecular weight of urease stimulated interest in using urease as a model for studying subunit interactions. Molecular weight determinations by Svedberg gave a value of 483,000 daltons which is substantially the same as the more recent value of 489,000. The preparative molecule weight of 489,000 daltons appears to result from the systematic arrangement of 16 single polypeptide chain subunits of 30,000 daltons.

Centrifugation studies showed the presence of 28 S and 36 S species with 4 S to 6 S and 12 S "impurities" also present. This strongly suggests the presence of enzyme oligomers corresponding to n, 2n and 3n where n = the 12S species. Further studies using the three predominant species isolated from jack bean urease using sephadex G-150 and 200 chromatography allowed the calculation of Stokes radii of 61, 79, 92, and 122 angstroms. When the first three values were assumed to have S values of 19, 28 and 36 the resultant molecular weight ratios were determined to be 1.0, 1.9, and 2.9.

These low molecular weight forms (less than 17,000 daltons)

exhibited urease activity. Sucrose gradient density ultracentrifugation was used to demonstrate that an active 12 S form had about 5% of the total activity. Gel electrophoresis allowed the visualization of activity in bands corresponding to n, 2n, 3n, 4n, 5n, and 6n (where n = 480,000 daltons). The bands were designated as "polymeric isozymes". Other bands with molecular weight of 240,000, 600,000, and 800,000 daltons were termed "epiisozymes", and were assumed to consist of multiple active assemblies of a single active subunit.

The demonstration of many active forms of urease with disparate mobilities points to a complex association-dissociation process and Reithel (1971) said that if an equilibrium exists between the forms, the kinetic constants are small. There is no definitive evidence about either the identity of the subunits or the groups involved in their interactions. Since it is possible to discern the dissociation of urease in 4 discrete steps to 8n, 4n, 2n, and n subunits, it seems likely that at least 4 binding arrangements are present, and since the dissociations are symmetrical the subunits are probably identical, at least down to 60,000 daltons. According to Reithel (1971), there is no compelling evidence for inter- or intrachain disulfide bonding in the 16n species. However, disulfide bonds do form in the 32n and 16n species, but such bonding is not mandatory for the formation of oligomers.

As might be expected, urease is remarkably resistant to unfolding in urea. There is no change in optical rotary dispersion or specific activity when the enzyme is subjected to 8 M urea. Unfolding occurs between 8 and 9 M urea, and the molecular weight drops from 489,000 d to 60,000 d; however, some activity persists.

The chemical composition of urease has also been studied. Reithel stated that both methionine and asparagine have been reported as N-terminal amino acids, a single C-terminal sequence of Tyr-Leu-Phe has been reported, and that the antigenic and catalytic sites are not identical since antiantibody-urease precipitates remained active. Sakaguchi, et al. (1984) reported a sequence of Phe-Glu-Pro-Gly-Asp-Cys-Asn-Ser-Thr-Phe-Lys around the active sulfhydryl (cysteine) center of jack bean urease. Dixon, et al. (1975) discovered that urease is a nickel metalloenzyme containing two gram-atoms of nickel per mole of active center, and proposed this as evidence of a biological role for nickel.

Some physical characteristics of urease listed by Reithel (1971) include: diffusion constant at 20°C in sodium sulfite = 3.46 X 10^{-7} cm^2 sec^{-1}; partial specific volume = 0.73; absorbance maximum at 278.5 nm; A_{280}/A_{260} ratio = 1.8 to 1.9; and isoelectric point via isoelectric focusing = 4.8.

Ureases from a wide spectrum of biological material have been studied. The ureases of Proteus rettgeri and Azotobacter vinelandii were shown to be inducible. Menyes and Fritsche (1972) have shown that one basidioblastomycetous yeast, Rhodotorula mucilaginosa, has a low level of constitutive urease production which upon exhaustion of ammonium or nitrate-N is succeeded, in the absence of cycloheximide, by a strong increase in activity, indicating derepression of an inducible enzyme.

Proteus mirabilis urease molecular weight was reported to be 151 Kd. Corynebacterium renale urease appeared to be the same as jack bean urease, as did that of Bacillus pasteurii. However, the 12 S jack bean

urease did not hybridize with the *B. pasteurii* enzyme. *Sarcina ureae* was found to produce urease as an exoenzyme. Cyanobacteria in pure culture displayed urease activity. Due to the extensive urease activity of intestinal bacteria, gastric urease is probably bacterial in origin. Urease activity has been demonstrated in soil samples over 8000 years old. Hilgenberg and Halling (1984) studied the urease activity of the zygomycetous fungus, *Phycomyces blakesleeanus*. They reported that their isolate was unable to utilize urea only in the presence of high amounts of zinc.

Differentiation of *Cryptococcus neoformans* varieties

As a causative agent of several serious mycological diseases of man, the identification of the varieties of *Cryptococcus neoformans* (the anamorphic state of *Filobasidiella neoformans*) motivated a number of attempts at "biochemical serogrouping". Biochemical serogrouping is the identification by physiological properties associated with the serogroups used in the primary definition of varieties. Unfortunately, biochemical serogrouping has not to date been completely successful in separating the anamorphs *Cr. neoformans* var. *neoformans* (serotypes A and D) from *Cr. neoformans* var. *gattii* (serotypes B and C).

Classification of the varieties of *Cryptococcus neoformans* by assignment to the teleomorph based on sexual compatibility has been used. However, this approach is impractical because of the time required, and because some isolates fail to respond to tester strains even though sexual isolation is incomplete.

Bowman and Ahearn (1977) studied the ecology and epidemiology of *Cryptococcus neoformans* strains. Historically *Cr. neoformans* has been

recognized as the etiological agent of pulmonary and meningitic infections in man. Over 330 cases occur annually in the United States. The occurence of _Cr_. _neoformans_ occurs in pigeon excreta and there is a possibility of a rare saprophytic symbiosis with man. Matings of the isolates from pigeon droppings with _F_. _neoformans_ mating-types _a_ and _alpha_, serotype D (NIH 3502) revealed that 49% (23/47) of the isolates failed to produce morphological evidence of mating (ie., clamp connections, substrate and aerial hyphae, basidia and viable, sexually competent basidiospores). Matings of human isolates with both _F_. _neoformans_ mating-types _a_ and _alpha_, serotype D (NIH 3502) and _F_. _bacillispora_ mating-type _a_ serotype C (NIH 191) and _alpha_, serotype B (NIH 444) showed that 55% (44/80) of the isolates failed to give evidence of a sexual stage (ie., neither mating-type _a_ or _alpha_). The authors also reported that normal clamp connections, haustoroid structures, mature basidia and basidiospores were "occasionally" produced when the _alpha_ mating-type of _F_. _neoformans_ and the _a_ mating-type of _F_. _bacillispora_ were combined; however, the viability of the resultant basidiospores was not determined.

Bennett, _et al_. (1978) found that _Cr_. _neoformans_ var. _gattii_ (serotypes B and C) could be distinguished from _Cr_. _neoformans_ var. _neoformans_ (serotypes A and D) by differences in their assimilation of _l_-malic acid, and that the assimilation of fumaric and succinic acids, greening of _Guiztia_ seed agar and slow assimilation of creatinine were also useful, but to a lesser degree. Auxanograms were conducted by inoculating Yeast Nitrogen base (Difco) with a 1:200 dilution of an $O.D._{600nm} = 0.2$ cell suspension and then placing about 5-8 mm^3 of crystals of l-malic, succinic or fumaric acid on the plate. The

results showed that isolates of serotypes B and C assimilated all three acids faster and more often than isolates of serotypes of A and D, with l-malic acid showing the greatest difference in that growth was heavier than with fumaric acid and the pattern changed less with time than with succinic acid.

Kwon-Chung, et al. (1978) developed a creatinine-dextrose-bromthymol blue test agar (CDB) and using this separated Cr. neoformans serotypes B and C from serotypes A and D, establishing a new species, Cr. bacillispora. The differential medium contained per liter: creatinine, 1 g; dextrose, 0.5 g; KH_2PO_4, 1 g; $MgSO_4$-$7H_2O$, 0.5 g; thiamine solution (Bejectal), 1 drop; bromthymol blue solution (0.4%), 20 mL; and agar, 10.0 g. The pH of the medium was adjusted to 5.7, giving a gold color to the agar. Isolates assimilating creatinine, thereby generating ammonia which increased the alkalinity of the agar, turned the agar greenish-blue to deep blue. To test an isolate, a loopful of a 2 day- or 2 week-old culture grown on either MEA or modified SDA was streaked on either a slant or plate of the test medium and incubated at 25°C for 1 week. All isolates of serotypes B and C produced a positive reaction within 48 hours, with all but one being positive within 24 hours. Only 3.3% of the serotype A isolates, and 8.0% of the serotype D isolates were positive at 48 hours: none were positive at 24 hours.

Creatinine metabolism in Cr. neoformans and Cr. bacillisporus was examined further by Polacheck and Kwon-Chung (1980). Creatinine was utilized by both organisms as a nitrogen but not a carbon source. The enzyme responsible, creatinine deiminase, catalyzed the single-step reaction of creatinine to methylhydantoin and ammonia, and enzyme pro-

duction in both species was found to be induced by creatinine. For Cr. neoformans, enzyme production was repressed by the accumulation of ammonia, whereas, in Cr. bacillisporus ammonia had no repressive effect. The authors stated that this difference provides a biochemical explanation for the different reactions seen in the diagnostic creatinine-dextrose-bromthymol blue test. The authors postulated that the variation in enzyme repression is probably based on the ecological differences between the two species: Cr. neoformans (serotypes A and D) are frequently found in pigeon droppings which are rich in the nitrogenous waste product creatinine; whereas, Cr. bacillisporus (serotypes B and C) are not found in pigeon droppings.

Muchmore, et al. (1980) incorporated the creatinine-dextrose-bromthymol blue test of Kwon-Chung, et al. in an epidemiological study of 108 Cr. neoformans isolates obtained from infected patients and soil samples in Oklahoma. The genus and species of the isolates was confirmed by the production of a brown pigment on Staib's birdseed agar, mouse virulence, and by physiological and biochemical analysis. The results of the CDB tests showed that 68 (85%) of the isolates were sereotype A-D, and that 12 (15%) were serotype B-C. The authors noted that their percentage of B-C isolates was much higher than in a previous study in which only 6% (13/208) of the isolates were serotype B-C.

Schmeding, et al. (1981) examined the sexual compatibility between serotypes of 37 Filobasidiella (Cr.) neoformans strains in an effort to evaluate the validity of differentiating Cr. neoformans, Cr. bacillispora, F. neoformans, and F. bacillispora. In their introduction, the authors note the importance of cryptcoccosis as one of the

"world's major systemic mycoses both in incidence and prevalence". They also provide a brief history about the classification of Cr. neoformans: They state that according to Kwong-Chung, Cr. neoformans produces two morphologically and physically distinct perfect states, Filobasidiella neoformans which is produced when compatible strains of Cr. neoformans serotypes A and D mate, and F. bacillispora which is produced when strains of Cr. neoformans serotypes B and C mate. On this basis, Kwong-Chung, et al. (1978) established Cr. bacillispora to accomodate serotypes B and C. The results of Schmeding's crosses showed that basidia and basidiospores were produced when sixteen strains were crossed with a mating-type strains, and when 4 strains were crossed with alpha mating-type strains. Five of the strains tested were self-fertile. However, 12 of the 37 (32%) failed to mate with any strain and were not self-fertile (ie., neither mating-type a or alpha). Schmeding, et al. also found that 9 alpha mating-type strains of serotypes B and C and that 7 alpha mating-type strains of serotypes A and D mated equally well with the type cultures of F. neoformans and F. bacillispora, but the viability of the basidiospores was not determined. Based on their results, the authors concluded that Cr. neoformans (Sanfelice) Vuillemin = Cr. neoformans var. gattii Vanbreuseghem et Takashio = Cr. bacillispora Kwon-Chung et Bennett and that Filobasidiella neoformans Kwong-Chung = F. bacillispora Kwong-Chung.

In assessing the taxonomy of Filobasidiella (Cr.) species, Kwon-Chung, et al. (1982a) discovered that the species she described as Cr. bacillisporus was synonymous with the previously described Filobasidiella anamorph, Cr. neoformans var. gattii. (Vanbreuseghem and

Takashio. 1970. Ann. Soc. Belg. Med. Trop. 50: 695-702). The taxonomic assessment was based on the results of test crosses, the assimilation of l-malic acid, the production of ammonia on CDB agar, the morphology of the yeast cells from agar and from infected mice, and by growth rate at 37°C. Indicating conspecificity, test crosses between the type culture of Cr. neoformans var. gattii (ATCC 32269, mating-type alpha) and Cr. neoformans var. neoformans (ATCC 34874, mating-type a) produced basidiospores with between 25-30% viability, and crosses of the type culture of Cr. bacillisporus (ATCC 32609, mating-type alpha) and Cr. neoformans var. neoformans (ATCC 34874, mating-type a) produced basidiospores with up to 70% viability. However, the cross of Cr. neoformans var. gattii (ATCC 32269, mating-type alpha) with Cr. bacillisporus (ATCC 32608, mating-type a) produced only 30% viable basidiospores, a value which seemed low for a "self" cross. The authors noted that despite the low frequency of viable basidiospores resulting from these crosses, the observed genetic markers were meiotically recombined. They stated further that since Cr. neoformans var. neoformans and Cr. neoformans var. gattii do not share the same ecological niche, the possibility of of an intercross occuring in nature was "highly unlikely".

Both Cr. neoformans var. gattii and Cr. bacillisporus assimilated l-malic acid and produced a positive CDB test within 48 hours equally well. Also both produced similar, distinctive morphologies on agar and in mouse cerebral tissue: from agar, cells were lemon- to tear-shaped, oblong, globose or sometimes spindle shaped and produced a large extracellular capsule causing the colonies to be very mucoid. Budding, globose cells produced a large isthmus which was not seen in Cr.

neoformans var. neoformans. From mouse cerebral tissue stained with Gomori's methenamine-silver, cells appeared globose to oblong, often numerous swollen cells measuring up to 13 μm were seen, and less frequently cells forming finger-like projections were seen. Cr. neoformans var. neoformans did not assimilate l-malic acid nor produce a positive CDB test within 48 hours. The authors stated that Cr. neoformans var. neoformans was also differentiated from Cr. neoformans var. gattii and Cr. bacillisporus by its ability to grow more vigorously at 37°C and by its higher degree of virulence when injected intravenously in mice. However, temperature sensitivity by Cr. neoformans var. gattii was not completely dependable because only 87% of the Cr. neoformans var. neoformans strains and over 5% of the Cr. neoformans var. gattii strains tested grew consistantly well at 37°C.

Citing the poor performance of earlier differential tests used for biochemical serogrouping, Kwon-Chung, et al. (1982b) devised an agar containing L-canavanine, glycine, and bromthymol blue. Having obtained and serotyped the 12 Cr. neoformans isolates previously determined by Muchmore, et al. (1980) via the creatinine-dextrose-bromthymol blue test to be serotypes B and C, Kwon-Chung, et al. found that one isolate was serotype C and the remaining 11 were serotype A.

Kwon-Chung, et al. also cited the work of Salkin and Hurd (1982) who developed yet another test medium to differentiate the two varieties. Salkin and Hurd's medium contained 1.6 μg/mL cycloheximide, 1.0% glycine and phenol red. Salkin and Hurd found that 88% of the serotype B-C isolates assimilated glycine as a sole carbon source, only 20% of the serotype A isolates, none of the D serotypes assimilated glycine, and that the A serotype isolates which did assimilate glycine

were sensitive to cycloheximide at the concentration used, 1.6 µg/mL. However, Kwon-Chung *et al*. stated that they had found cycloheximide sensitivity was not a dependable means of differentiating the two varieties, and, although the results of the Salkin and Hurd test were less ambiguous than the results of the creatinine-dextrose-bromthymol blue test, the percentage of false positive results (ie., a positive reaction by serotypes A and D) were equally high with both tests.

The medium devised by Kwon-Chung *et al*. contained 1% glycine as the sole carbon and nitrogen source, 3% L-canavanine as a selective agent inhibiting the growth of serotypes A and D, and 0.008% bromthymol blue. A positive test occurs when the color of the agar changes from greenish-yellow (initial pH 5.8) to deep blue (pH 7.0). Of the 143 *Cr*. *neoformans* var. *neoformans* (serotypes A and D) isolates tested none produced a positive reaction after 5 days growth at 25-30°C. Whereas, 76% (53/70) of the *Cr*. *neoformans* var. *gattii* (serotypes B and C) were positive by 2 days, and all 70 gave a positive reaction after 5 days. Kwon-Chung, *et al*. noted the importance of incubating the inoculated medium at 25-30°C, and that the age and size of the inoculum was critical if false positive reactions were to be avoided. Both old cultures which would contain many moribund cells, or large inocula which could smother otherwise healthy cells led to alkalinization of the test medium, causing false positive results by *Cr*. *neoformans* var. *neoformans* (serotypes A and D).

Schmeding *et al*. (1983) evaluated the biochemical variation of 97 *Cr*. *neoformans* and *Cr*. *bacillisporus* strains, and with the aid of a computerized numerical taxonomy program generated a dendrogram revealing the isolates clustered at the 86% level of similarity. The

dendrogram was based on M-similarity and was calculated as the number of positive matches plus the number of negative matches divided by the total number of tests times 100. The 44 characters used were weighted equally. The ability to assimilate l-malic acid, creatinine, and growth at 37°C were among the 44 physiological capabilities tested. Test crosses between the strains were also conducted.

The results of the biochemical tests showed that all 97 isolates were capable of growing at 37°C on YM slants. Twenty-two percent (21/97) of the isolates utilized creatinine, and of the 37 strains for which serotype data were avaliable, all the 13 serotypes B and C were creatinine positive and the 24 serotypes A and D were creatinine negative. L-malic acid was utilized by 35% (34/97) of the strains with 10 of the 13 serotypes B and C and 11 of the 24 serotypes A and D growing on this substrate. L-malic acid was used by 15 of the 21 strains that utilized creatinine and 19 of the 76 strains that failed to utilize creatinine. According to Schmeding et al., based on statistical evaluation (Chi-squared test; alpha value = 0.05) the two characters, l-malic acid and serotype, appeared to be independent of each other; whereas, l-malic acid and creatinine utilization were not independent. The results of test crosses showed that 48 of the 97 strains produced the teleomorphic, Filobasidiella state either in self-crosses or when crossed with a strain of compatible mating-type, but that F. neoformans serotypes A and D crossed with compatible mating-types of F. bacillispora serotypes B and C were infertile.

In analyzing their dendrogram data, Schmeding et al. stated that similarity values of 80-85% are used as the limits of species in studies of bacterial strains or clones by the ATCC, and studies

involving fungal isolates in which the percent similarity corresponding to species ranged from 77 to 85% were also cited. The dendrogram generated by the authors' computer program showed that the 97 strains clustered at the 86% similarity level. Thus on the basis of their results, Schmeding et al. stated that F. neoformans and F. bacillispora are the same species.

In discussing their conclusions Schmeding et al. noted that the Cr. neoformans serotypes are based on capsular antigens which differ in the number and arrangement of D-xylose and D-glucuronic acid side residues on a mannose backbone, and that the arrangement of these side residues in the B and C serotypes seems to more conducive to the nutritive transport of creatinine, l-malic acid, l-sorbose, meso-erythritol, and inulin.

Schmeding et al. summarized the problems inherent in the taxonomy of anamorphic yeasts in the following paragraph:

> The concept of imperfect species is not clear. Species are generally considered to be populations of similar phenotypes and genotypes. If mutation leads to an altered gene complement, and a distinctive change in morphology or physiology is recognized, the altered population may be considered a new species. Radical mutations leading to new phenotypes are more likely to survive in asexual organisms than in sexual organisms where radical change might interfere with proteins essential for syngamy or meiosis.

Wood, et al. (S. Wood, H. J. Shadomy, S. Shadomy, W. E. Dismukes, R. Y. Chau, and the NIAID Mycosis Study Group, Newsletter, U. S. Fed. for Culture Collections 15: 20.) used glycine-cycloheximide-phenol red agar (GCP), GCP agar without cycloheximide, and glycine-L-canavanine-bromthymol blue agar to biochemically serotype 308 Cr. neoformans isolates. According to the authors, despite the subjective interpre-

tation of the color reactions on the 3 media, "definite" A/D or B/C strains were identifiable, along with "intermediates". Wood, et al. stated that these results demonstrated the difficulties of using biochemical tests for serotyping.

Bottone, et al. (1986) described the isolation and identification of a highly encapsulated strain of Cr. neoformans from human lung tissue. The authors used glycine-cycloheximide-phenol red and glycine-canavanine-bromthymol blue agars, as well as a slide agglutination test to serotype the isolate. All 3 tests indicated that the isolate was serotype B. Citing the presence of large capsules which are anomalous in Cr. neoformans pulmonary infections and the lack of epidemiologic knowledge about the serotypes involved in cryptococcosis, Bottone, et al. recommended the use of the two media as a part of standard clinical identification procedures.

Bibliography

Baharaeen, S., and H. S. Vishniac. 1982. Cryptococcus lupi sp. nov., an Antarctic basidioblastomycete. Int. J. Syst. Bacteriol. 32: 229-232.

Baharaeen, S., and H. S. Vishniac. 1984. 25S ribosomal RNA homologies of basidiomycetous yeasts: taxonomic and phylogenetic implications. Can. J. Microbiol. 30: 613-621.

Barnett, J. A., R. W. Payne, and D. Yarrow. 1983. Yeasts: characteristics and identification. Cambridge Univ. Press, Cambridge. 811 pp.

Bennett, J. W., K. J. Kwon-Chung, and T. S. Theodore. 1978. Biochemical differences between serotypes of Cryptococcus. Sabouraudia 16: 167-174.

Bergquist, L. M. and R. L. Searcy. 1963. Application of the Berthelot reaction for rapid detection or urea hydrolysis by Proteus strains. J. Bacteriol. 85: 954-955.

Bottone, E. J., P. A. Kirschner, and I. F. Salkin. 1986. Isolation of highly encapsulated Cryptococcus neoformans serotype B from a patient in New York City. J. Clinical Microbiol. 23: 186-188.

Bowman, P. I., and D. G. Ahearn. 1977. Ecology of Cryptococcus neoformans in Georgia. Proc. Fourth Int. Conf. on the Mycoses, PAHO Scientific Pub. No. 356: 258-268.

Chase, G. D., and J. L. Rabinowitz. 1959. In Principles of Radioisotope Methodology. Burgess Pub. Co., New York, p. 311.

Christensen, W. B. 1946. Urea decomposition as a means of differentiating Proteus and paracolon cultures from each other and from Salmonella and Shigella types. J. Bacteriol. 52: 461-466.

Davies, H. M., and L. M. Shih. 1984. Urease from leaves of Glycine max and Zea mays. Phytochem. 23: 2741-2745.

Difco Manual, 10th ed. 1984. Difco Laboratories Inc., Detroit, Michigan. pp. 1039-1045.

Dixon, N. E., C. Gazzola, J. J. Walters, R. L. Blakeley, and B. Zerner. 1975. Jack bean urease (EC 3.5.1.5). A metalloenzyme. A simple biological role for nickel? J. Am. Chem. Soc. 97: 4131-4133.

Granick, S. 1937. Urease distribution in plants: general methods. Plant Physiol. 12: 471-486.

Hagler, A. N., and D. G. Ahearn. 1981. Rapid diazonium blue B test to detect basidiomycetous yeasts. Int. J. Syst. Bacteriol. 31: 204-208.

Hilgenberg, W., and H. J. Halling. 1984. Urea content and urease activity of Phycomyces blakesleeanus under different culture condition. Biochem. Physiol. Pflanzen 179:369-379.

Hopkins, J. M., and G. A. Land. 1977. Rapid method for determining nitrate utilazation in yeasts. J. Clin. Microbiol. 5: 497-500.

Kaplan, A. 1969. The determination of urea, ammonia, and urease. Methods Biochem. Anal. 17: 311-324.

Kocková-Kratochvílová, A. 1982. Urease and extracellular nucleases of yeasts. Folia Microbiol. 27: 404-412.

Kreger-van Rij, N. J. W. 1984. Principles of classification, pp. 15-42. In The yeasts a taxonomic study, third revised and enlarged edition. Elsevier Science Pub., Amsterdam.

Kwon-Chung, K. J., J. E. Bennett, and T. S. Theodore. 1978. Cryptococcus bacillisporus sp. nov.: Serotype B-C of Cryptococcus neoformans. Int. J. Syst. Bacteriol. 28: 616-620.

Kwon-Chung, K. J., J. E. Bennett, and J. C. Rhodes. 1982a. Taxonomic studies on Filobasidiella species and their anamorphs. Antonie van Leeuwenhoek 48: 25-38.

Kwon-Chung, K. J., I. Polacheck, and J. E. Bennett. 1982b. Improved diagnostic medium for separation of Cryptococcus neoformans (serotypes A and D) and Cryptococcus neoformans var. gattii (serotypes B and C). J. Clinical Microbiol. 15: 535-537.

McDonald, J. A., K. V. Speeg, Jr., and J. W. Campbell. 1972. Urease: A sensitive and specific radiometric assay. Enzymologica 42: 1-9.

McLean, R. J. C., K.-J. Cheng, W. D. Gould, and J. W. Costerton. 1985. Cytochemical localization of urease in a rumen Staphylococcus sp. by electron microscopy. Appl. and Environ. Microbiol. 49: 253-255.

Menyes, H., and W. Fritsche. 1972. Regulation der Urease Synthese in Rhodotorula mucilaginosa. Zeitschr. Allg. Mikrobiol. 12: 219-229.

Miller, M. W., M. Yoneyama, and M. Soneda. 1976. Phaffia, a new yeast genus in the Deuteromycotina (Blastomycetes). Int. J. Syst. Bacteriol. 26: 286-291.

Moore, R. T. 1980. Taxonomic proposals for the classification of

marine yeasts and other yeast-like fungi including the smuts. Botanica Marina 23: 361-373.

Muchmore, H. G., E. N. Scott, F. G. Felton, and R. A. Fromtling. 1980. Cryptococcus neoformans serotype groups encountered in Oklahoma. Am. J. Epidemiol. 112: 32-38.

Murry, D. R., and R. B. Knox. 1977. Immunofluorescent localization of urease in the cotyledons of jack bean, Canavalia ensiformis. J. Cell Sci. 26: 9-18.

Nasu, S., F. D. Wicks, and R. K. Gholson. 1982. The mammalian enzyme which replaces B protein of E. coli quinolinate synthetase is D-aspartate oxidase. Biochim. Biophys. Acta 704: 240-252.

Paliwal, D. K., and H. S. Randhawa. 1977. Rapid method for detection of urea hydrolysis by yeasts. Applied and Environ. Microbiol. 33: 219-220.

Polacheck, I., and K. J. Kwon-Chung. 1980. Creatinine metabolism in Cryptococcus neoformans and Cryptococcus bacillisporus. J. Bacteriol. 142: 15-20.

Rabinowitz, J. L., T. Sall, J. N. Bierly, Jr., and O. Oleksyshyn. 1956. Carbon isotope effects in enzyme systems. I. Biochemical studies with urease. Arch. Biochem. Biophys. 63: 437-445.

Reithel, F. J. 1971. Ureases, p. 1-21. In P. D. Boyer (ed.), The Enzymes IV. Hydrolysis (3rd ed.). Academic Press, New York.

Roberts, G. D., C. D. Horstmeier, G. A. Land, and J. H. Foxworth. 1978. Rapid urea broth test for yeasts. J. Clinical Microbiol. 7: 584-588.

Robinson, R. A., and R. H. Stokes. 1959. Electrolyte Solutions, 2nd. ed. Academic Press, New York. p. 518.

Sakaguchi, K., K. Mitsui, N. Nakai, and K. Kobashi. 1984. Amino acid sequence around a cystein residue in the active center of jack bean urease. J. Biochem. 96: 73-79.

Salkin, I. F., and N. J. Hurd. 1982. New medium for differentiation of Cryptcoccus neoformans serotype pairs. J. Clin. Microbiol. 15: 169-171.

Schmeding, K. A., S. C. Jong, and R. Hugh. 1981. Sexual compatibility between serotypes of Filobasidiella neoformans (Cryptococcus neoformans). Curr. Microbiol. 5: 133-138.

Schmeding, K. A., S. C. Jong, and R. Hugh. 1983. Biochemical variation of Cryptococcus neoformans. Mycopathologia 84: 121-131.

Schmitt, J. A., and F. Daniels. 1953. The carbon isotope effect in the acid hydrolysis of urea. J. Am. Chem. Soc. 75: 3564-3566.

Schmitt, J. A., A. L. Myerson, and F. Daniels. 1952. Relative rates of hydrolysis of urea containing C^{14}, C^{13} and C^{12}. J. Phys. Chem. 56: 917-920.

Scism, A. J. 1983. Urease kinetics with an ammonia electrode. Trans. Missouri Acad. Sci. 17: 147-154.

Seeliger, H. P. R. 1956. Use of a urease test for the screening and identification of cryptococci. J. Bacteriol. 72: 127-131.

Sen, K., and K. Komagata. 1979. Distribution of urease and extracellular DNAse in yeast species. J. Gen. Appl. Microbiol. 25: 127-135.

Shapiro, I. A. 1976. Determination of urease activity in lichens by a direct potentiometric method. Sov. Plant Phisiol. 23: 712-714.

Stefanác, Z., M. Tomaskovíc, and Z. Rakovíc-Tresíc. 1969. Spectrophotometric method of assaying urease activity. Analytical Letters 2 (4): 197-210.

Sumner, J. B. 1926. The isolation and crystalization of the enzyme urease. J. Biol. Chem. 69: 435-441.

Toren, Jr., E. C., and F. J. Burger. 1968. Trace determination of metal ion inhibitors of the urea-urease system by a pH-stat kinetic method. Mikrochimica ACTA. pp. 1049-1058.

van der Walt, J. P. 1970. Criteria and methods used in classification, pp. 34-113. In J. Lodder (ed.), The Yeasts - A Taxonomic Study. North Holand Publishing Co., Amsterdam.

van Uden, N., P. Abranches, and C. Cabeça-Silva. 1968. Temperature functions of thermal death in yeasts and their relation to the maximum temperature for growth. Arch. fur Mikrobiol. 61: 381-393.

Windholz, M., S. Budavari, R. F. Blumetti, and E. S. Otterbein (eds.). 1983. The Merck Index, 10th Ed. Merck and Co., Inc., Rahway, NJ. pp. 153 & 865.

Vishniac, H. S. 1983. An enation system for the isolation of Antarctic yeasts inhibited by conventional media. Can. J. Microbiol. 29: 90-95.

Vishniac, H. S., and S. Baharaeen. 1982. Five new Basidioblastomycetous yeast species segregated from Cryptococcus vishniacii emend. auct., an Antarctic yeast species comprising four new varieties. Int. J. Syst. Bacteriol. 32: 437-445.

Vishniac, H. S., and W. P. Hempfling. 1979. Cryptococcus vishniacii sp. nov., an Antarctic yeast. Int. J. Syst. Bacteriol. 29: 153-158.

Vishniac, W. V., and M. Santer. 1957. The thiobacilli. Bacteriol. Rev. 21: 195-213.

Weast, R. C. (ed.). 1979. CRC Handbook of Chemistry and Physics. CRC Press, West Palm Beach. p. D-203.

Wolf, G. 1964. Isotopes in Biology. Academic Press Inc., New York. pp. 45-51.

Zhuravlev, Yu. N., T. I. Kramar, and L. D. Seletskaya. 1972. Determination of urease activity with the aid of C^{14}-urea. Sov. Plant Physiol.[Fizsiol. Rast] 19: 171-174.

Zimmer, B. L., and G. D. Roberts. 1979. Rapid selective urease test for the presumptive identification of Cryptococcus neoformans. J. Clinical Microbiol. 10:380-381.

CHAPTER II

UREASE TESTING AND YEAST TAXONOMY

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Abstract

When urease production was assayed by the hydrolysis of ^{14}C-urea, all basidiomycetous yeasts tested, including the *Cryptococcus vishniacii* complex (previously reported urease negative), produced significant amounts of ^{14}C-CO_2. The Schizosaccharomycetaceae were the only urease positive ascomycetous yeasts tested. *Yarrowia lipolytica* was urease negative. The stoichiometry of ^{14}C-urea hydrolysis paralleled by Roberts' rapid urea hydrolysis (RUH) test indicated that causes of anomalous results in conventional urease testing include acidification and alkalinization of the test medium by products of endogenous metabolism and autolysis rather than urease activity. Anomalous results also occurred when cells were grown on media containing the chelating agent ethylenediamine tetraacetic acid (EDTA) prior to RUH. The addition of EDTA to a complex natural medium inhibited urease production in all yeasts reportedly growing at 35°C (and all other yeasts tested) except *Filobasidiella* (*Cr.*) *neoformans* var. *neoformans* (NIH 12). The RUH test could differentiate at the

varietal level. Fil. (Cr.) neoformans var. neoformans was about 10 times more resistant to EDTA in media used for the growth of cells prior to RUH testing than was Fil. neoformans var. bacillispora (Cr. neoformans var. gattii) (NIH 191). Urease production by Fil. neoformans var. bacillispora was specifically restored to half maximal activity by the addition of 22 µM Ni^{+2} (as $NiCl_2$) to a growth medium containing 0.100 mM EDTA.

Introduction

The production of extracellular urease has been generally considered a universal character of basidiomycetous yeasts shared by very few yeasts with ascomycetous affinity (Hagler and Ahearn 1981; Kreger-van Rij 1984; Moore 1980). Although Sen and Komagata (1979) credit the first observation of taxonomic significance to Sakaguchi and Shizume (1937, cited from Sen and Komagata), the inaccessibility of Japanese literature has led western scientists to date such significance from Seeliger's (1956) application of the urease test of Christensen (1946) to a wide variety of yeast species. While urease activity can be assessed in a number of ways (see Kaplan 1969; Reithel 1971; Shapiro 1976; McLean et al. 1985), urease activity in yeasts has invariably been investigated using growth on Christensen's agar or the modification of Christensen's medium developed for rapid urea hydrolysis (RUH) in Roberts' laboratory (Roberts et al. 1978; Zimmer and Roberts 1979). These procedures depend, as do nearly all urease assays, on the production of ammonia. Any ammonia produced raises the pH of the assay medium, causing the indicator, phenol red, to become pink to magenta.

The results of urease tests conducted in different laboratories have not always agreed. Recent examples include disagreement as to the urease activity of the ascomycetes *Schizosaccharomyces pombe* and *Yarrowia* (*Saccharomycopsis*) *lipolytica*, and the basidiomycetous Antarctic yeasts of the *Cryptococcus vishniacii* complex. The major compendia of yeast characters (Barnett et al. 1983; Kreger-van Rij 1984) reported the ascomycetous yeasts urease positive; Kocková-Kratochvílová (1982) reported them urease negative. The yeasts of the *Cr*. *vishniacii* complex were characterized as urease negative by their authors (Vishniac and Hempfling 1979; Baharaeen and Vishniac 1982; Vishniac and Baharaeen 1982) but the strains examined by Yarrow were reportedly urease positive or variable (Barnett et al. 1983). These contradictory results led us to examine the correlation of ammonia production and the evolution of ^{14}C-CO_2 from ^{14}C-urea during RUH and the influence of growth medium on subsequent RUH. We have confirmed the production of urease by basidiomycetous yeasts and ascomycetous yeasts belonging to the Schizosaccharomycetaceae (the genus *Schizosaccharomyces*), but also confirmed the variability of growth and RUH tests for urease activity. We have found that the varieties of *Filobasidiella* (*Cryptococcus*) *neoformans* can be distinguished by RUH after growth on chelated media.

Materials and Methods

Strains

The strains examined (Table I) were maintained on yeast culture agar (YCA, Vishniac 1983; MYSW collection, personal collection of H. S. Vishniac) or yeast malt agar (YM; all other strains), transferred at 3

month intervals, and refrigerated on transfer (psychrophiles) or after growth at room temperature. YCA was developed for culturing certain Antarctic yeasts which are inhibited by conventional yeast media, and contained per liter: glucose, 4.0 g; sodium glutamate, pH 6.0, 2.0 mM; NH_4Cl, 2.0 mM; NaCl, 50.0 mM; $MgSO_4$-$7H_2O$, 0.2mM; potassium phosphate buffer, pH 6.0, 5.0 mM; the trace metals solution of Vishniac and Santer (1957), 1.0 mL (1/10 the recommended concentration); Wickerham's vitamin mix (from Kreger-van Rij 1984); yeast extract 0.5 g; and agar, 18 g.

Modifications of conventional urease tests

Urease activity was examined on Christensen's urea agar (1946) and by RUH (Roberts et al. 1978). Roberts' procedure was modified in order to quantify ammonia production: equal volumes of double strength RUH broth and a freshly prepared cell suspension (in sterile glass-distilled water) adjusted to $O.D._{650\ nm} = 40$ were combined in a 16 X 125 mm screw-capped borosilicate culture tube, and incubated in a New Brunswick Scientific Co. Model G-76 Gyrotory water bath shaker (180-200 rpm) coupled with a New Brunswick Scientific Co. Model RF-10 Frigidflow coolant circulator. Following incubation, the reaction was stopped by placing the tubes in ice before sedimenting the cells in an IEC Model Cl clinical centrifuge at a setting of 6 for 5 minutes. The supernatant was placed on ice until the absorbance at 560 nm, determined to be the absorbance maximum for alkaline phenol red in RUH broth, was measured. Absorbances approaching 0.100 were observed in uninoculated controls and negative (*Saccharomyces cerevisiae*, Barnett et al. 1983) controls. Absorbances below 0.250 were negative to the naked eye;

TABLE I

THE STRAINS EXAMINED FOR UREASE PRODUCTION

Agaricostilbum palmicolum UBC 3-14

Bullera alba NRRL-Y-6655^T

**Candida acuta* ATCC 42713^T (assignable to *Vanrija*)

**C. antarctica* NRRL-Y-7808^T (assignable to *Vanrija*)

**C. buffoni* ATCC 18813^T (assignable to *Vanrija*)

**C. muscorum* ATCC 34886^T (recieved as *Rhodotorula*, assignable to *Vanrija*)

**C. podzolica* ATCC 34208^T (assignable to *Vanrija*)

**C. tsukubaensis* NRRL-Y-7792^T (assignable to *Vanrija*)

Chionosphaera apobasidialis UBC 6097-3 and 6097-8

Cryptococcus albidus var. *albidus* ATCC 10666^T

Cr. asgardensis MYSW 302Y310^T (= ATCC 46399)

Cr. baldrensis MYSW 302Y259^T (= ATCC 46400)

Cr. bhutansis ATCC 22461^T

Cr. consortionis MYSW A801-3aY92^T (= ATCC 56686)

Cr. friedmannii MYSW A801-133Y100^T (= ATCC 56687)

Cr. hempflingii MYSW 306Y212^T (= ATCC 46401)

Cr. heveanensis NRRL-Y-1510^T

Cr. himalayensis IAM 4963^T

Cr. laurentii ATCC 18803^T

Cr. lupi MYSW 202Y252^T (= ATCC 44529)

Cr. socialis MYSW A801Y3aY1^T (= ATCC 56685)

Cr. terreus NRRL-Y-4231^T

TABLE I (Continued)

Cr. tyrolensis MYSW 306Y366 (= ATCC 46405) and MYSW A812-34aY42

Cr. vishniacii var. vishniacii MYSW 304Y268^T (= ATCC 36649)

Cr. wrightensis MYSW 303Y206 (= ATCC 46406)

Cystofilobasidium bisporidiis ATCC 24496 (received as Rhodosporidium)

*Dioszegia hungarica NRRL-Y-6667^T (received as Cryptococcus)

Filobasidiella (Cr.) neoformans var. neoformans NIH 12^T (= ATCC 28957)

**Fil. neoformans var. bacillispora (Cr. neoformans var. gattii) NIH 191 (= ATCC 28908)

Filobasidium capsuligenum NRRL-Y-7098^T and ATCC 14437

F. uniguttulatum ATCC 24227^T

Leucosporidium frigidum ATCC 22029^T

L. nivalis ATCC 22031^T

L. scottii ATCC 22182^T

L. stokesii ATCC 22178^T

Phaffia rhodozyma ATCC 24202^T

Platygloea peniophora UBC 6233

Rhodosporidium diobovatum NRRL-Y-7196^T

Rhodosp. toruloides NRRL-Y-1091^T

Rhodotorula araucariae ATCC 22078^T

Rh. minuta NRRL-Y-1589^T

Rh. pilmanae NRRL-Y-1738^T

Saccharomyces cerevisiae (cloned from commercial preparation)

Schizosaccharomyces japonicus var. japonicus NRRL-Y-1026^T

Schiz. malidevorans NRRL-Y-12794^T

Schiz. octosporus ATCC 4206

TABLE I (Continued)

Schiz. pombe ATCC 2476

Sirobasidium magnum UBC 6077

Sporidiobolus johnsonii NRRL-Y-2259[T]

Sporid. salmonicolor ATCC 623

Sporobolomyces albo-rubescens NRRL-Y-12931[T]

Sterigmatomyces elviae NRRL-Y-7504[T]

Trichosporon aquatile NRRL-Y-7051[T]

Tr. beigelii NRRL-Y-1490[T]

Tr. brassicae ATCC 24124[T]

*Vanrija aquatica ATCC 18806[T] (received as Candida)

*V. curvata NRRL-Y-1511[T] (received as Candida)

*V. fujisanensis NRRL-Y-4824[T] (received as Candida)

*V. humicola ATCC 14438[T] (received as Apiotrichum)

*V. ingeniosa ATCC 22993[T] (received as Rhodotorula)

*Yarrowia lipolytica NRRL-YB-423[T]

ATCC, American Type Culture Collection, Rockville, MD, U.S.A.; IAM, Institute of Applied Microbiology, University of Tokyo, Japan; MYSW, personal collection of junior author; NIH, National Institutes of Health, Bethesda, MD, U.S.A.; NRRL, Northern Regional Research Laboratory USDA, Peoria, IL, U.S.A.; UBC, University of British Columbia, Vancouver, BC, Canada.

[T] Type culture of strain.

* The generic assignment or epithet is disputed. We consider Moore's (1980) Vanrija most appropiate for basidioblastomycetes of uncertain relationship to Cryptococcus or Rhodotorula (treated as Candida, Cryptococcus, or Rhodotorula in cited handbooks), as well as for those colorless "Candida" species known to lack close rRNA homology to these genera (Baharaeen and Vishniac 1984). Dioszegia is placed in Cryptococcus in cited handbooks: While we concur that the sole species of this genus lacks generic distinction, the grounds for this

TABLE I (Continued)

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assignment appear inadequate. *Yarrowia* is retained in *Saccharomycopsis* in Kreger-van Rij (1984).

** type of *Filobasidiella* (*Cr*.) *Bacillispora*: This taxon is now given varietal status as *Fil*. *neoformans* var. *bacillispora* (Kwon-Chung et al. 1982a). The previously described anamorph, *Cryptcoccus* *neoformans* var. *gattii*, is differentially typified.

those between 0.250 and 0.500 usually appeared more or less pink to rose ("weak"); while absorbances above 0.500 were unambigu- ously positive, approaching magenta to the naked eye. Absorbance and optical density were measured with a Bausch and Lomb Spectronic 70 spectrophotometer using 1 cm or 0.5 cm path length cuvettes, with the readings being corrected to 1 cm path length.

RUH broth, containing per liter yeast extract (Difco) 0.1 g, monobasic potassium phosphate (Mallinckrodt) 0.91 g, dibasic sodium phosphate (Sigma) 0.95 g, phenol red (Sigma) 0.01 g, and urea (Sigma) 20.0 g, was prepared as a concentrate, filter sterilized, and kept frozen until use. Phenol red was omitted from the RUH broth used in $^{14}C\text{-}CO_2$ assays, but the broth was otherwise prepared and stored identically. The phosphate concentration, 1.34×10^{-2} M, is that recommended by Roberts et al. (1978) for the avoidance of false positive reactions and is tenfold that used by Yarrow (Barnett et al. 1983).

The cell concentration needed to saturate the RUH test system was determined experimentally for _Fil_. (_Cr_) _neoformans_ var. _neoformans_ (NIH 12) at 37°C for 1 h and for _Cryptococcus_ _albidus_ at 37°C for 1 h and 20°C for 2 h. Accordingly, cell suspensions yielding a final concentration of $O.D._{650\ nm} = 20$ were used. This concentration was equivalent to that used in the laboratories of Roberts and of Yarrow.

Time and temperature of growth and RUH incubations were chosen after considering convenience and preliminary data. Cells were grown in a Precision Model 815 low temperature incubator. Except when examining the effect of growth medium on RUH, cells were grown on YM. The length of incubation on YM required to produce urease positive

cells during RUH at physiological temperatures was a function of growth temperature and strain. *Leucosporidium scottii* (a psychrophile strongly urease positive in growth tests) gave a weakly positive RUH at 20°C for 1 h after 4 days incubation at 10°C, was fully positive after 10 days, and continued so for at least 32 days. The mesophilic (in this context, unable to grow under refrigeration) *Cryptococcus albidus* reacted in essentially the same way to 10°C incubation. *Cryptococcus vishniacii* var. *vishniacii* reacted variably during determination of a time at temperature curve for 10°C. Additional time at temperature curves were constructed for *Cr. albidus* and *Filobasidiella* (*Cr.*) *neoformans* var. *neoformans*, representing mesophiles incapable and capable of 36°C growth. Strains capable of 10°C growth were tested in RUH at 20°C for 2h after 11-14.5 days at 10°C on YM and after 7 or 17 days at 17°C. Cultures of mesophiles growing poorly or failing to grow at 36°C were tested at 25°C for 2 h after growth at 25°C for 72 to 74 h, or 28°C for 68 h. Mesophiles growing well at 36°C were tested at 37°C after growth for 40-45 h at 36°C.

^{14}C-urea hydrolysis and determination of stoichiometry

Urease activity was measured as ^{14}C-CO_2 evolution from ^{14}C-urea, with ammonia production determined in parallel RUH tests. CO_2 was collected by the method of Nasu et al. (1982). One mL of yeast suspension (prepared as above) was placed in a 21 X 70 mm sample vial closely connected by 1/8" wall, rubber tubing to an inverted polyethylene scintillation vial containing a 1 X 25 cm circularized strip of Whatman #1 filter paper saturated with 0.5 mL methylbenzethonium hydroxide (hyamin hydroxide) 1 M in methanol (Sigma). One mL of

double strength RUH broth including 1 μCi ^{14}C-urea (4.5 μCi/mmole; New England Nuclear) was injected into the sample vial immediately before incubation for a standard 2 h period at 20, 25, or 37°C in the shaking water bath. Exceptions to the 2 h test period included strains tested only at 37°C in Table 2, and the reported 4 h test of *Yarrowia lipolytica*. The reaction was stopped and dissolved $C0_2$ driven off by injecting 1.0 mL of 2 N HCl into the sample vial, then shaking for 2 h at 37°C. The system was then disassembled. Scintillation cocktail (Fisher Scinti Verse II) was added directly to the $C0_2$ collection vial and a 0.300 mL aliquot of the reaction mixture was placed in another vial with cocktail. Vials were held overnight in the dark before counting in a Beckman Model LS 7500 microprocessor controlled liquid scintillation counter. Within the limits of pipetting error, all radioactivity was recovered.

The stoichiometry of RUH was assessed for those data sets in which the phenol red endpoint was not reached. The $C0_2$ produced was calculated as (dpm ^{14}C-$C0_2$ X 666 μmoles urea) / dpm ^{14}C-urea. The ammonia produced was calculated from the fraction protonated at the test temperature and indicated pH of the experiment (using $k_b$values from Weast 1979), assuming the fraction of protonated ammonia (NH_4^+) was the sum of μeq NaOH required to reach pH plus the protons available from dissociation of dissolved $C0_2$ produced by urease activity (k_{a1} at test temperatures, calculated from Robinson and Stokes, 1959). The values for pH and μeq NaOH were determined from the A_{560} of parallel RUH tests by interpolation from titration of RUH broth with standard 1.0 and 0.5 N NaOH (Sigma). For example, *Cr. albidus*, tested at 25°C, produced an A_{560} = 0.227 $\pm$ 0.009. Titration data indicated that this

A_{560} was reached when 3.71 μmoles/mL of NaOH had raised the pH to 7.50. It follows that (3.71 μeq/mL X 2.0 mL) 7.42 μeq NH_4^+ were produced. In addition, ammonia protonated by the first dissociation of H_2CO_3 (but present as ammonium carbonate, without affecting the pH) was produced. The proton contribution of H_2CO_3 dissociation was 6.90 μeq, since k_{a1}, 25°C, = 4.30 X 10^{-7}, $[H^+]$ at pH 7.50 = 3.162 X 10^{-8}, $C0_2$ (23.46 X 10^3 dpm X 666 μmoles / 2,107.75 X 10^3 dpm) = 7.41 μmoles. k_{a1} = $[H^+]$ $[HC0_2^-]$ / $[C0_2]$ or k_{a1} / $[H^+]$ = $[HC0_3^-]$ / $[C0_2]$. The ratio of the ionized species to total $C0_2$ produced is, therefore, 13.60/14.60 (93.14% dissociation), yielding the figure given. Therefore, the total NH_4^+ = 14.32 μeq. Since $[OH^-]$ at pH 7.50 = 3.162 X 10^{-7}, and k_b, 25°C, = 1.774 X 10^{-5}, the ratio of dissociated to undissociated hydrated ammonia is 56.1/57.1 (98.26% dissociation) and the total ammonia produced was 14.57 μmoles. The stoichiometry of this experiment (14.57 μmoles NH_3 / 7.41 μmoles $C0_2$) was 1.97.

Jack bean urease (EC 3.5.1.5, Sigma Type IX) produced in 40 minutes 16.36 μmoles NH_3 and 6.96 μmoles $C0_2$ (corrected for controls lacking urease), a ratio of 2.35 to 1. When these figures were corrected for the maximum isotope effect (Rabinowitz et al. 1956) the ratio of 15.60 μmoles NH_3 to 7.73 μmoles $C0_2$ was 2.02, a figure not significantly different from the theoretical ratio of 2.00. The ratios after 80 minutes of urease activity were 2.47 (uncorrected) and 2.21 (corrected for isotope effect). The phenomenon of high ratios has been noted by others (Davies and Shih 1984) but is unexplained. Because the isotope effect varies with time and yield (Rabinowitz et al. 1956) and has not been investigated under the conditions of these experiments, the data presented have not been corrected.

Effects of growth medium

Cells used to survey the effect of medium on subsequent RUH were grown on YCA, YM, (Difco) Sabouraud's dextrose agar (SDA), (Difco) malt extract agar (MEA), or peptone-yeast-glucose agar (PYG). PYG contained per liter: peptone (Difco) 5.0 g, yeast extract (Difco) 3.0 g, glucose (Mallinckrodt) 5.0 g, and (Difco) agar 18.0 g. RUH tests reported in Table III were conducted for 1 h at 37°C or 2 h at 25°C, using cells grown on PYG $\pm$ the addition of 0.2 mM $MgSO_4 \cdot 7H_2O$ and trace metal solution (TMS, Vishniac and Santer 1957). The final concentrations in TMS are: ethylenediamine tetraacetic acid (EDTA) 171 µM, Ca^{+2} 49.9 µM, Co^{+2} 4.9 µM, Cu^{+2} 7.5 µM, Fe^{+2} 18 µM, Mn^{+2} 25.6 µM, Mo^{+6} 6.2 µM, and Zn^{+2} 76.5 µM. Cells used to assess the effects of chelation and divalent cations on subsequent RUH were transferred to the growth medium and harvested from such plates with glass tools. Contact with metal was avoided when possible during medium preparation. Reagent grade chemicals (Sigma) were used except as otherwise specified. RUH was performed at 37°C unless otherwise specified, for the times given. The experiments on sensitivity to EDTA shown in Figure 2 were performed with cells heavily inoculated onto PYG $\pm$ the specified concentrations of EDTA. An inoculum of about 10 OD units (OD X mL) per 100 X 15 mm petri dish will produce cells with the sensitivities shown; an inoculum of 0.5 OD units will produce cells about twice as sensitive. In experiments on the relief of EDTA inhibition, metal ions were supplied as $CoCl_2 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $NiCl_2 \cdot 6H_2O$, and $ZnSO_4 \cdot 7H_2O$.

Results

Yeasts strongly positive in growth tests on Christensen's agar were also strongly positive in RUH tests. Yeasts reacting weakly or variably on Christensen's agar reacted variably in RUH. Low growth temperatures appeared to slow rather than suppress urease production, but increased the probability of negative RUH (as well as growth tests) for weak urease producers. The strongly positive mesophile Cryptococcus albidus and psychrophiles Agaricostilbum palmicolum, Bullera alba, Chionosphaera apobasidialis UBC 6078-8, Cystofilobasidium bisporidiis, Leucosporidium scottii, Phaffia rhodozyma, Platygloea peniophora, Sirobasidium magnum, Vanrija fujisanensis, V. ingeniosa, and V. muscorum were RUH positive after growth at 10°C. Psychrophiles reacting weakly or variably during growth testing reacted variably to RUH. Although positive RUH reactions were recorded for Candida podzolica, Cr. consortionis, Cr. hempflingii, Cr. lupi, Cr. tyrolensis (type), Cr. vishniacii var. vishniacii, Cr. wrightensis, Dioszegia hungarica, and Leucosporidium stokesii, negative reactions were recorded for Chionosphaera apobasidialis UBC 6078-3, Cr. asgardensis, Cr. baldrensis, Cr. bhutanensis, Cr. friedmannii, Cr. himalayensis, Cr. socialis, Cr. terreus, Cr. tyrolensis (alternate strain), Leucosporidium frigidum, L. nivalis, and Vanrija aquatica, as well as for the mesophiles Cr. laurentii and V. curvata, after 10°C growth. When the negative strains were retested after growth at 17°C, positive RUH was recorded for all.

The urease activity of selected strains during hydrolysis of ^{14}C-urea is shown in Table II. These strains represent differences in taxonomic group, temperature response, and degree of urease activity in

growth and RUH tests. All basidiomycetous yeasts, regardless of the temperature of RUH testing, produced $^{14}C-CO_2$ clearly in excess of uninoculated controls and of the ascomycete Saccharomyces cerevisiae (negative control). All recognized species in the ascomycete family Schizosaccharomycetaceae were urease positive. The ascomycete Yarrowia lipolytica was urease negative. Although test temperatures above the physiological limits of growth may result in the death of psychrophiles, the urease activity of both mesophiles and psychrophiles was greater at 37°C than at 20°C, justifying the practise (Barnett et al. 1983; Roberts et al. 1978) of conducting RUH at 37°C.

The stoichiometry observed in 2 h ^{14}C-urea assays is plotted in Figure 1, as µmoles of NH_3 versus µmoles CO_2 produced. The mean ratio (± standard deviation) of NH_3 to CO_2 at 20°C was 1.90 ± 0.14 (N = 12); at 25°C, 1.92 ± 0.11 (N = 6); at 37°C, 1.89 ± 0.19 (N = 12). These means do not significantly differ from each other or from the mean ratio of RUH for 0.5 h at 37°C, 1.83 ± 0.05 (N = 5). Ratios with Z scores greater than 2 were omitted from the calculations as follows: L. stokesii (20°C), 2.84; L. stokesii (37°C), 2.50; V. humicola (20°C), 1.44; V. humicola (25°C), 1.39; V. humicola (37°C), 1.40. The mean ratio of all 2 h RUH was 1.90 ± 0.15 (N = 30).

The sensitivity of ^{14}C-urea hydrolysis was greater than that of RUH determined spectrophotometrically. Urease negative A_{560} values were paralleled by significant $^{14}C-CO_2$ production when strong urease producers were tested for very short periods or after growth at suboptimal temperatures. Fil. (Cr.) neoformans var. neoformans cells tested by RUH at 37°C for 5 minutes produced an A_{560} of 0.063 (negative), but $^{14}C-CO_2$ gave 12.32 x 10^3 dpm (positive). L. scottii

TABLE II

DPM $^{14}C-CO_2$ EVOLVED BY SELECTED YEAST STRAINS TESTED IN RAPID UREA HYDROLYSIS BROTH CONTAINING 1 μCi ^{14}C-UREA

		dpm $^{14}C-CO_2$ evolved X 10^3 at:		
Strain	*Character	37°C	25°C	20°C
Cryptococcus albidus	B/M		33.575	
Cr. asgardensis	B/P	36.749		26.314
Cr. baldrensis	B/P	23.213		13.625
Cr. bhutanensis	B/P	46.506		28.369
Cr. friedmannii	B/P	17.360		9.530
Cr. heveanensis	B/M		17.798	
Cr. lupi	B/P	50.181		30.719
Cr. socialis	B/P	49.896		30.971
Cr. tyrolensis (not type)	B/P	61.193		48.503
Cr. vishniacii var. *vishniacii*	B/P	60.711		38.529
Filobasidiella neoformans var. *neoformans*	B/T	87.757		
Filobasidium capsuligenum	B/M		42.490	
F. capsuligenum (not type)	B/M		35.924	
Leucosporidium frigidum	B/P	40.838		24.734
L. nivalis	B/P	28.394		22.646
L. scottii	B/P	45.718		22.187
L. stokesii	B/P	24.620		19.925
Saccharomyces cerevisiae	A/T	0.957		0.799
Schizosaccharomyces japonicus var. *japonicus*	A/M		72.234	

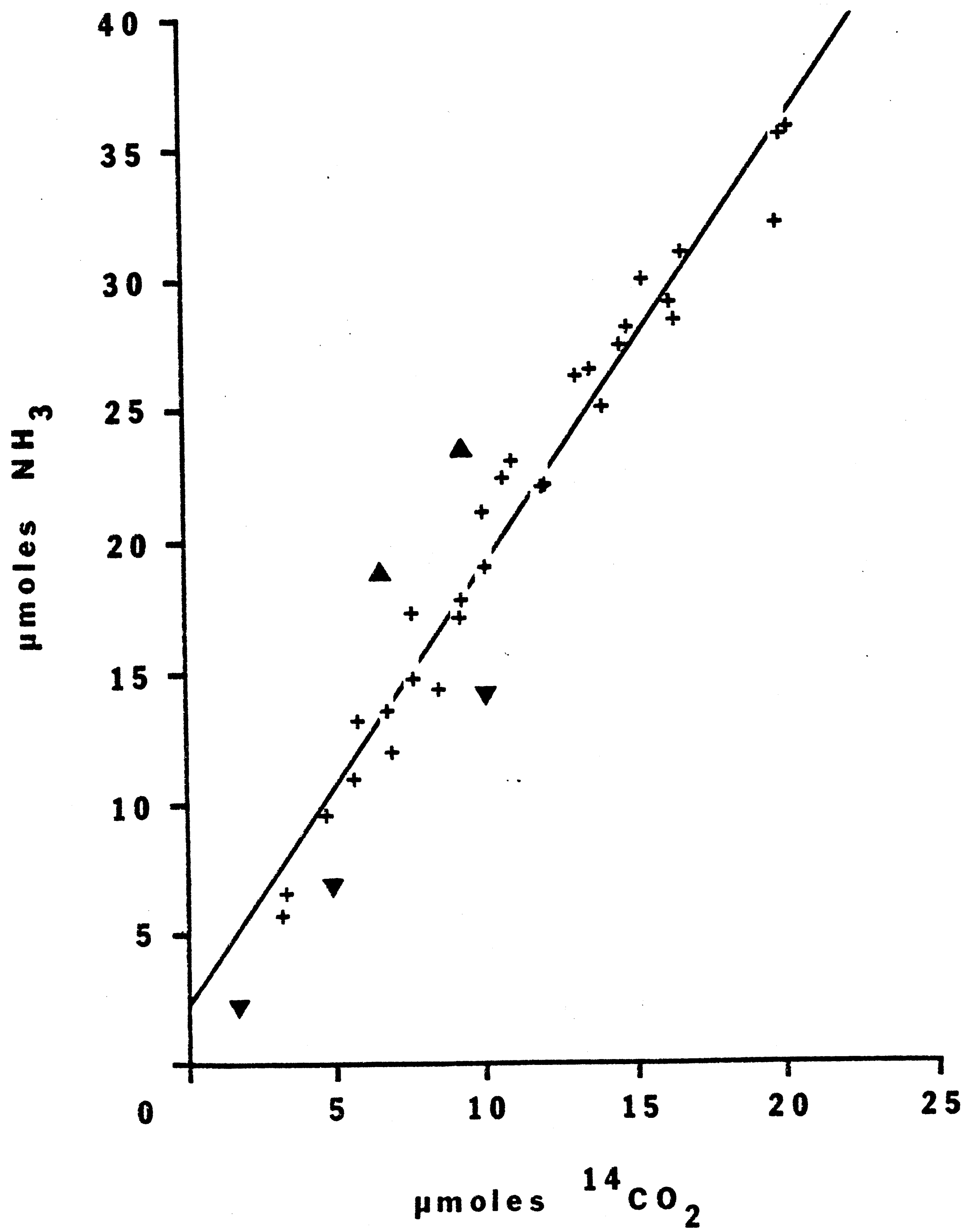
TABLE II (Continued)

Schiz. malidevorans	A/T	37.48		
Schiz. octosporus	A/T	51.423		
Schiz. pombe	A/T	46.155		
Sterigmatomyces elviae	B/M		44.148	
Trichosporon aquatile	B/T	36.512		
Tr. beigelii	B/M		21.497	
Vanrija aquatica	B/P	60.493		19.925
V. curvata	B/M	36.479		5.399
V. humicola	B/M		15.807	
Yarrowia lipolytica	A/T	0.800[a]		
Negative control:				
No cells added		0.251	0.917	0.210
		0.466		0.247
		0.416		

* A = ascomycetous; B = basidiomycetous; P = good growth at 4°C; M = growth poor or none at 4°C and at 36°C; T = good growth at 36°C.

[a] 4 hours at 37°C produced 1.587×10^3 dpm; this value is considered not significantly in excess of negative controls.

Figure 1. Stoichiometry of 2 hour Rapid Urea Hydrolysis using ^{14}C-urea. **+**, data points yielding linear regression of $y = 2.09 + 1.69x$ ($N = 30$; $r = 0.98$). ▲, Leucosporidium stokesii, excluded data points with significantly high stoichiometry. ▼, Vanrija humicola, excluded data points with significantly low stoichiometry.


40
35
30
25
20
15
10
5
0
5
10
15
20
25
μmoles NH_3
μmoles $^{14}CO_2$

cells grown for 7 days at 10°C produced, on one occasion, an A_{560} of 0.064 with $^{14}C-CO_2$ giving 11.03 x 10^3 dpm.

The medium used for cell growth affected RUH response. Cells harvested from acid agars (SDA, MEA) occasionally carried over some of the medium acidity into the RUH broth, resulting in false negative reactions. Nearly all cells grown on YCA were urease negative. When the trace metal solution component of YCA was included in otherwise suitable media, YM and PYG, RUH by harvested cells was invariably decreased (Table III). None of the other ingredients of YCA produced similar effects. The addition of peptone or yeast extract to YCA did not confer urease activity.

The most notable retention of urease activity after growth on PYG plus TMS was that of Fil. (Cr.)neoformans var. neoformans (NIH 12). Resistance to TMS differentiated this more virulent variety (Kwon-Chung et al. 1982a) not only from Fil. neoformans var. bacillispora (Cr. neoformans var. gattii) (NIH 191) but from all other similar yeasts reported capable of 35°C growth (Barnett et al. 1983). Of 35°C-growing yeasts, Table III omits Sarcinosporon inkin, Trichosporon loubieri, Rhodotorula glutinis, and Rh. mucilaginosa (Rh. rubra). Sarcinosporon inkin ATCC 18020^{T} was not examined because of very poor growth at 35°C. Trichosporon loubieri is synomymous with Tr. cutaneum (Tr. beigelii) (Kreger-van Rij, 1984). The Rhodotorula spp. omitted are composites of which any appropriate teleomorphs were examined; they differ notably from Fil. (Cr.) neoformans in colony color.

The inhibitory component of TMS proved to be the chelating agent, EDTA. The near 10 fold difference between Fil. (Cr.) neoformans var. neoformans and Fil. neoformans var. bacillispora (Cr. neoformans var.

gattii) in sensitivity to EDTA is shown in Figure 2. Growth yields were not noticeably affected by the EDTA concentrations used in these experiments. EDTA inhibition was specifically reversed, in both varieties, by the addition of Ni^{+2} (Table IV). Dose response data are presented in Figure 3 and Table V. RUH (0.5 h) activity of Fil. neoformans var. bacillispora grown on PYG + 0.1 mM EDTA + 20 μM Ni^{+2} produced an A_{560} (± S.D.) of 0.392 ± 0.024 in one experiment performed in triplicate, 0.378 ± 0.122 to 0.387 ± 0.124 when five independent experiments were averaged. The half maximal nickel requirement was calculated to be 22 μM. The nickel requirement for urease activity of Fil. (Cr.) neoformans var. neoformans grown with 1.00 mM EDTA is substantially similar. The addition of Ni^{+2} to YCA restored urease activity to Fil. neoformans var. bacillispora and to Cr. albidus grown on this medium, but the Ni^{+2} requirement was more critically balanced between toxicity and inadequacy than in PYG. 50 μM Ni^{+2} was toxic. Although yeast extract (0.3%) and peptone (0.5%) contained insignificant Ni^{+2} on assay in YCA, yeast extract appeared to potentiate the effect of 10 μM Ni^{+2}, increasing the A_{560} of Fil. neoformans var. bacillispora 0.234 ± 0.151 over Ni^{+2} alone.

Discussion

Since the RUH test procedure is simple, rapid, and economical, it is likely to remain in use both for identification and for classification as part of the formal description of new species of yeasts. The sensitivity and precision of RUH is adequate to reliably detect urease activity at temperatures from 20° to 37°C in most yeast species capable of producing $^{14}C\text{-}CO_2$ from ^{14}C-urea.

TABLE III

THE EFFECT OF TRACE METALS SOLUTION ADDED TO GROWTH MEDIUM ON SUBSEQUENT RAPID UREA HYDROLYSIS

Strain	Urease reaction after growth on:		ΔA_{560}
	a) PYG	b) PYG + TMS	(b - a)
1. Grown at 36°C			
Candida acuta	+	-	-0.636
C. antarctica	+	-	-0.906
Filobasidiella neoformans			
var. *neoformans*	+	+	-0.044
Fil. neoformans			
var. *bacillispora*	+	weak	-0.536
Rhodosporidium toruloides	+	-	-0.910
Rhodotorula minuta	+	-	-0.334
Schizosaccharomyces malidevorans	+	-	-0.834
Schiz. octosporus	+	-	-0.774
Schiz. pombe	+	weak	-0.548
Sporidiobolus johnsonii	+	-	-0.710
Sporobolomyces albo-rubescens	+	-	-0.740
2. Grown at 25°C			
Candida buffoni	+	weak	-0.456
C. tsukubaensis	+	+	-0.310
Cryptococcus albidus	+	-	-0.336
Cr. heveanensis	weak	-	-0.144
Cr. laurentii	+	-	-0.424

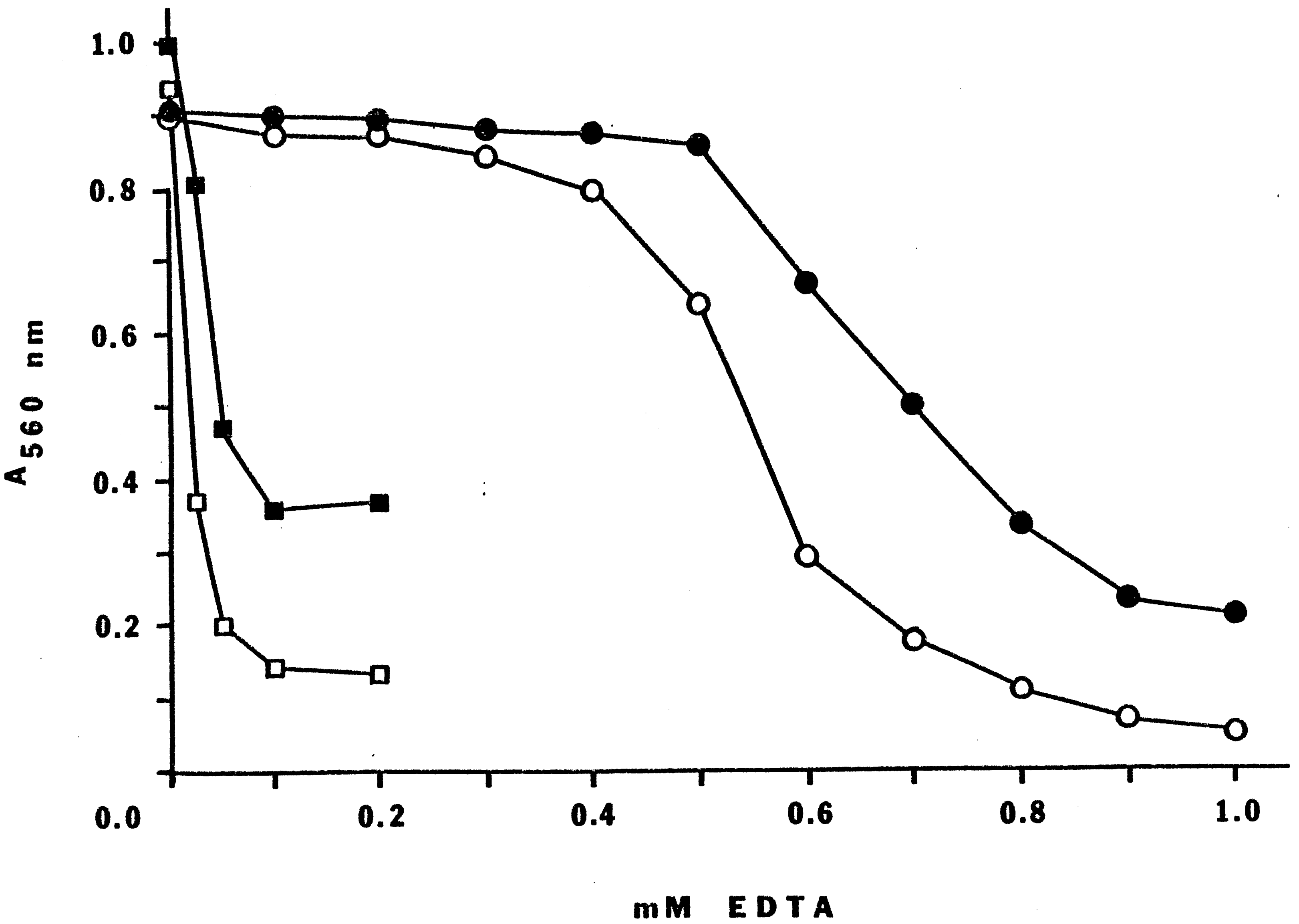
TABLE III (Continued)

Filobasidium capsuligenum	+	-	-0.600
F. uniguttulatum	+	-	-0.268
Rhodosporidium diobovatum	weak	-	-0.246
Rhodotorula araucariae	+	-	-0.508
Rh. pilmanae	weak	-	-0.382
Schizosaccharomyces japonicus			
var. *japonicus*	weak	-	-0.296
Sterigmatomyces elviae	+	-	-0.388
Trichosporon aquatile	- *	-	NA
Tr. beigelii	weak	-	-0.280
Tr. brassicae	+	weak	-0.462
Vanrija humicola	- *	-	NA

* An example of variable RUH: These strains gave positive RUH tests as well as $^{14}C\text{-}CO_2$ evolution in the experiment reported in Table II.

NA = Not Applicable.

Figure 2. The effect of EDTA concentration in Peptone-Yeast extract-Glucose agar growth medium on subsequent Rapid Urea Hydrolysis. *Filobasidiella neoformans* var. *bacillispora* (*Cr*. *neoformans* var. *gattii*) NIH 191 tested for 1 h, □ and 2 h, ■. *Fil*. (*Cr*.) *neoformans* var. *neoformans* NIH 12 tested for 1 h, ○ and 2 h, ●. RUH was conducted at 37°C.



A_{560} nm
1.0
0.8
0.6
0.4
0.2
0.0
0.2
0.4
0.6
0.8
1.0
mM EDTA

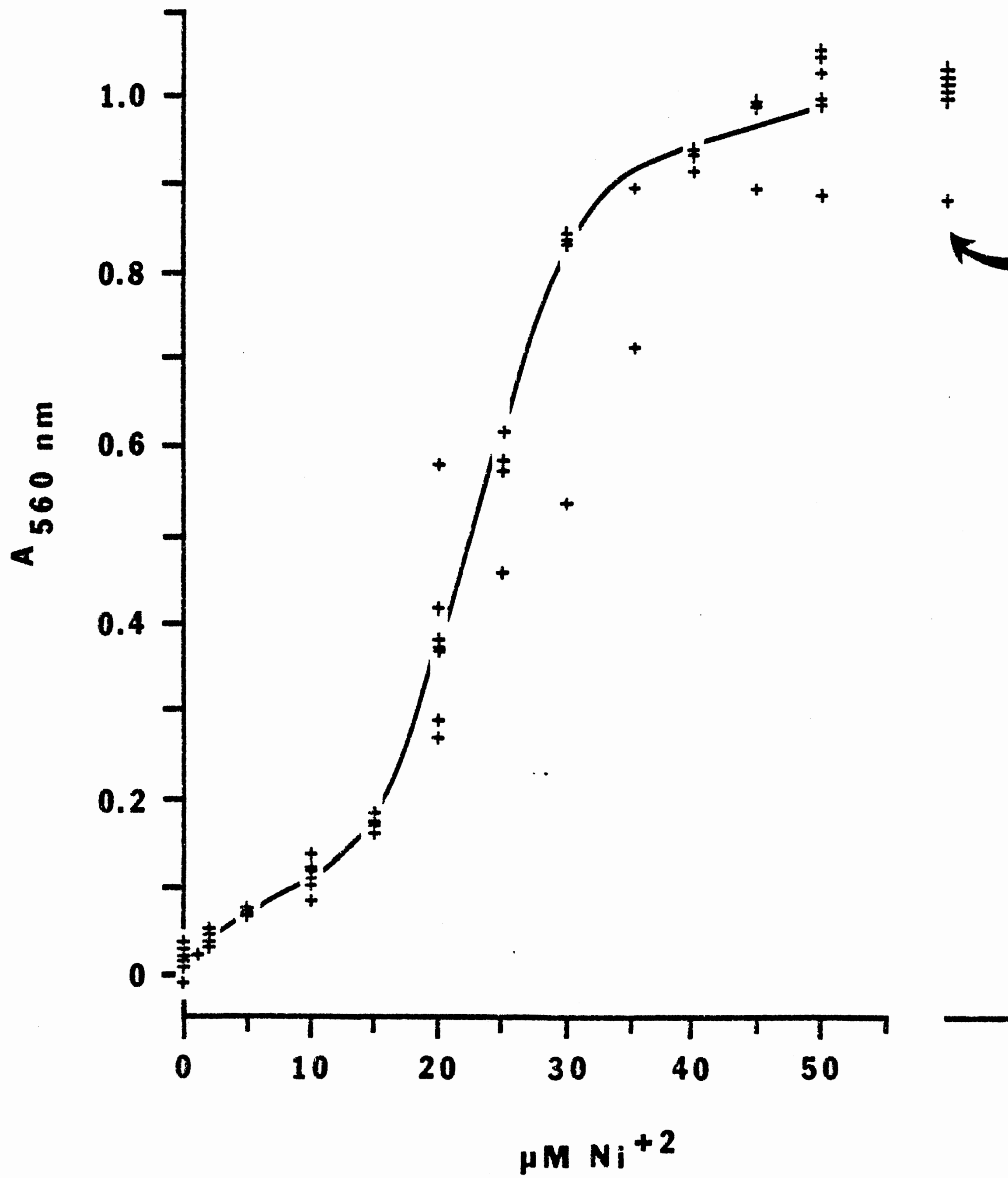
TABLE IV

RELIEF OF THE INHIBITION CAUSED BY ETHYLENEDIAMINE TETRAACETIC ACID BY THE DIVALENT METALS PRESENT IN TRACE METALS SOLUTION*

Strain	Medium	A_{560} after:	
		0.5 h	1.0 h
Filobasidiella neoforman var. bacillispora			
	PYG agar	1.002	1.034
	" + 0.100 mM EDTA	0.030	0.230
	" " + 20 µM Co^{+2}	0.020	0.198
	" " " Cu^{+2}	0.024	0.144
	" " " Fe^{+2}	0.030	0.150
	" " " Ni^{+2}	0.272	0.754
	" " " Mn^{+2}	0.026	0.142
	" " " Zn^{+2}	0.014	0.114
Filobasidiella neoformans var. neoformans			
	PYG agar	1.017 ± 0.015 (S.D.)	
	" + 1.00 mM EDTA	0.020 ± 0.002	
	" " + 20 µM Co^{+2}	0.005 ± 0.022	
	" " " Cu^{+2}	0.021 ± 0.002	
	" " " Fe^{+2}	0.036 ± 0.023	
	" " " Ni^{+2}	0.527 ± 0.053	
	" " " Mn^{+2}	0.035 ± 0.007	
	" " " Zn^{+2}	0.040 ± 0.026	

* Ni^{+2} (added as $NiCl_2$) was not originally an ingredient of Trace Metals Solution.

Figure 3. The effect of Ni^{+2} (as $NiCl_2$) concentration in Peptone-Yeast extract-Glucose agar growth medium containing 0.100 mM EDTA on subsequent RUH by *Filobasidiella neoformans* var. *bacillispora* (*Cr. neoformans* var. *gattii*) NIH 191. RUH was conducted for 30 min. at 37°C. The arrow indicates Rapid Urea Hydrolysis responses of cells grown on unamended Peptone-Yeast extract-Glucose agar. The results of 5 independent experiments are shown. The curve, drawn with the aid of a cubic spline fitting program, is based on the results of a single experiment conducted in triplicate.



1.0
0.8
0.6
0.4
0.2
0
$A_{560\ nm}$
0
10
20
30
40
50
μM Ni^{+2}

TABLE V

RESPONSE TO NICKEL DURING RAPID UREA HYDROLYSIS TESTING BY *Cryptococcus neoformans* var. *neoformans*

Growth Medium	A_{560} ± S.D.
PYG agar	1.045 ± 0.006
" + EDTA, 1.00 mM	0.026 ± 0.006
" " + Ni^{+2}, 10 µM	0.188 ± 0.014
" " " 20 µM	0.405 ± 0.004
" " " 50 µM	0.906 ± 0.006

RUH tests are not reliable when urease production is weak. False negatives result when endogenous metabolism produces an excess of hydrogen ions. We have observed acidification of Christensen's agar and of RUH broth, as well as the false negative reactions presented in Table 3. The difference between the mean stoichiometry of yeast RUH and that of jack bean urease indicates that yeast cells typically do produce hydrogen ions during RUH. When urease activity was weak, as in V. humicola, this production of endogenous CO_2 and/or organic acids was sufficient to significantly distort the observed stoichiometry.

False positive tests result when urease activity is not the major alkalizing factor. Since psychrophiles may die rapidly in RUH broth at 37°C, the most likely cause of urease stoichiometries exceeding that of jack bean urease is proteolysis. Although psychrophilic yeasts survive supraoptimal temperatures well in several non-nutritive media (van Uden et al. 1968; unpublished data), the loss of viability of Cr. vishniacii var. vishniacii cells in RUH broth at 37°C for 2 h approached 100% (unpublished data). The very high stoichiometry observed in L. stokesii at 37°C is presumed to have arisen in this way. The discrepancies which we cited in the introduction are evidence that these problems do not vanish when the testing period is prolonged to 4 h and/or the phosphate buffer concentration lowered to 1.34×10^{-3} M.

The results presented suggest that urease activity would be strengthened by the inclusion of nickel, which is not a recognized ingredient of any published yeast medium and yeast extract which is not an ingredient of Christensen's agar, in growth media. Urease is known to be a nickel-containing metalloenzyme (Dixon et al. 1975), although Hilgenberg and Halling (1984), found that Phycomyces blakesleeanus (a

zygomycetous fungus) utilized urea as sole N source only when zinc, rather than nickel, was added to their medium. We have not pursued this further because urease is a redundant character at the class level in yeast systematics. The "most conclusive" (Miller et al. 1976) indication of basidiomycetous affinity in anamorphic yeasts is the more laborious detection of a layered cell wall and collared bud scar by electron microscopy. The empirical diazonium blue B test (by the method of Hagler and Ahearn 1981) is rapid, economical, and has shown better correlation with wall and bud scar characters (both in published reports and in unpublished data of this laboratory) than the conventional urease tests.

The use of RUH at the varietal level, to differentiate Filobasidiella (Cr.) neoformans var. neoformans from Fil. neoformans var. bacillispora (Cr. neoformans var. gattii), is of potential clinical as well as taxonomic value. Dr. Kwon-Chung, in cooperation with our laboratory, has examined a number of strains of both varieties, with results to be reported elsewhere. The identification of the varieties of Cryptococcus neoformans (the anamorphic state) has been sufficient a problem to motivate a number of attempts at "biochemical serogrouping", i.e. identification by physiological properties associated with the serogroups used in the primary definition of varieties. Assignment to the teleomorph by sexual compatibility is impractical not only because of the time required but because, although sexual isolation is incomplete, 32 to 55% of isolates failed to respond to tester strains used (Bowman and Ahearn 1977; Schmeding et al. 1981). Unfortunately, biochemical serogrouping has not to date been completely successful in separating Cr. neoformans

var. neoformans (serotypes A and D) from Cr. neoformans var. gattii (serotypes B and C). The assimilation of l-malic acid by Cr. neoformans var. gattii (Bennett et al. 1978; Kwon-Chung et al. 1982a) was reported by Schmeding et al. (1983) to occur in Cr. neoformans var. neoformans as well. The rapid creatinine utilization of Cr. neoformans var. neoformans (Kwon-Chung et al. 1978; Polacheck and Kwon-Chung 1980; Muchmore et al. 1980) also crossed varietal borders (Kwon-Chung et al. 1982b; Schmeding et al. 1983). Rapid growth at 37°C, characteristic of Cr. neoformans var. neoformans, was also variable (Kwon-Chung et al. 1982a). Although some clinical investigators (Bottone et al. 1986) currently recommend the use of glycine-cycloheximide-phenol red agar (GCP, Salkin and Hurd 1982) and glycine-canavanine-bromthymol blue agar (GCB, Kwon-Chung et al. 1982b), Shadomy's laboratory found "intermediates" during the biochemical serogrouping of 304 isolates with these tests (S. Wood, H. J. Shadomy, S. Shadomy, W. E. Dismukes, R. Y. Chau, and the NIAID Mycoses Study Group, Newsletter, U.S. Fed. for Culture Collections 15: 20). The strains which we have used differ very markedly in their ability to compete with the chelating power of EDTA for the nickel required for urease synthesis.

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Bibliography

Baharaeen, S., and H. S. Vishniac. 1982. Cryptococcus lupi sp. nov., an Antarctic basidioblastomycete. Int. J. Syst. Bacteriol. 32:229-232.

Baharaeen, S., and H. S. Vishniac. 1984. 25S ribosomal RNA homologies of basidiomycetous yeasts: taxonomic and phylogenetic implications. Can. J. Microbiol. 30:613-621.

Barnett, J. A., R. W. Payne, and D. Yarrow. 1983. Yeasts: characteristics and identification. Cambridge Univ. Press, Cambridge. 811 pp.

Bennett, J. W., K. J. Kwon-Chung, and T. S. Theodore. 1978. Biochemical differences between serotypes of Cryptococcus. Sabouraudia 16:167-174.

Bottone, E. J., P. A. Kirschner, and I. F. Salkin. 1986. Isolation of highly encapsulated Cryptococcus neoformans serotype B from a patient in New York City. J. Clinical Microbiol. 23: 186-188.

Bowman, P. I., and D. G. Ahearn. 1977. Ecology of Cryptococcus neoformans in Georgia. Proc. Fourth Int. Conf. on the Mycoses, PAHO Scientific Pub. No.356: 258-268.

Christensen, W. B. 1946. Urea decomposition as a means of differentiating Proteus and paracolon cultures from each other and from Salmonella and Shigella types. J. Bacteriol. 52: 461-466.

Davies, H. M., and L.-M. Shih. 1984. Urease from leaves of Glycine max and Zea mays. Phytochem. 23:2741-2745.

Dixon, N. E., C. Gazzola, J. J. Walters, R. L. Blakeley, and B. Zerner. 1975. Jack bean urease (EC 3.5.1.5). A metalloenzyme. A simple biological role for nickel? J. Am. Chem. Soc. 97:4131-4133.

Hagler, A. N., and D. G. Ahearn. 1981. Rapid diazonium blue B test to detect basidiomycetous yeasts. Int. J. Syst. Bacteriol. 31:204-208.

Hilgenberg, W., and H. J. Halling. 1984. Urea content and urease activity of Phycomyces blakesleeanus under different culture conditions. Biochem. Physiol. Pflanzen 179:369-379.

Kaplan, A. 1969. The determination of urea, ammonia, and urease. Methods Biochem. Anal. 17:311-324.

Kocková-Kratochvílová, A. 1982. Urease and extracellular nucleases of yeasts. Folia Microbiol. 27:404-412.

Kreger-van Rij, N. J. W. (ed.) 1984. The yeasts a taxonomic study, third revised and enlarged edition. Elsevier Science Pub., Amsterdam. 1082 pp.

Kwon-Chung, K. J., J. E. Bennett, and T. S. Theodore. 1978. Cryptococcus bacillisporus sp. nov.: Serotype B-C of Cryptococcus neoformans. Int. J. Syst. Bacteriol. 28:616-620.

Kwon-Chung, K. J., J. E. Bennett, and J. C. Rhodes. 1982a. Taxonomic studies on Filobasidiella species and their anamorphs. Antonie van Leeuwenhoek 48:25-38.

Kwon-Chung, K. J., I. Polacheck, and J. E. Bennett. 1982b. Improved diagnostic medium for separation of Cryptococcus neoformans (serotypes A and D) and Cryptococcus neoformans var. gattii (serotypes B and C). J. Clinical Microbiol. 15:535-537.

McLean, R. J. C., K.-J. Cheng, W. D. Gould, and J. W. Costerton. 1985. Cytochemical localization of urease in a rumen Staphylococcus sp. by electron microscopy. Appl. and Environ. Microbiol. 49:253-255.

Miller, M. W., M. Yoneyama, and M. Soneda. 1976. Phaffia, a new yeast genus in the Deuteromycotina (Blastomycetes). Int. J. Syst. Bacteriol. 26:286-291.

Moore, R. T. 1980. Taxonomic proposals for the classification of marine yeasts and other yeast-like fungi including the smuts. Botanica Marina 23:361-373.

Muchmore, H. G., E. N. Scott, F. G. Felton, and R. A. Fromtling. 1980. Cryptococcus neoformans serotype groups encountered in Oklahoma. Am. J. Epidemiol. 112: 32-38.

Nasu, S., F. D. Wicks, and R. K. Gholson. 1982. The mammalian enzyme which replaces B protein of E. coli quinolinate synthetase is D-aspartate oxidase. Biochim. Biophys. Acta 704:240-252.

Polacheck, I., and K. J. Kwon-Chung. 1980. Creatinine metabolism in Cryptococcus neoformans and Cryptococcus bacillisporus. J. Bacteriol. 142:15-20.

Rabinowitz, J. L., T. Sall, J. N. Bierly, Jr., and O. Oleksyshyn. 1956. Carbon isotope effects in enzyme systems. I. Biochemical studies with urease. Arch. Biochem. Biophys. 63:437-445.

Reithel, F. J. 1971. Ureases, p. 1-21. In P. D. Boyer (ed.), The Enzymes IV. Hydrolysis (3rd ed.). Academic Press, New York.

Roberts, G. D., C. D. Horstmeier, G. A. Land, and J. H. Foxworth. 1978. Rapid urea broth test for yeasts. J. Clinical Microbiol.

7:584-588.

Robinson, R. A., and R. H. Stokes. 1959. Electrolyte Solutions, 2nd. ed. Academic Press, New York. p.518.

Salkin, I. F., and N. J. Hurd. 1982. New medium for differentiation of Cryptococcus neoformans serotype pairs. J. Clinical Microbiol. 15:169-171.

Schmeding, K. A., S. C. Jong, and R. Hugh. 1981. Sexual compatibility between serotypes of Filobasidiella neoformans (Cryptococcus neoformans). Curr. Microbiol. 5:133-138.

Schmeding, K. A., S. C. Jong, and R. Hugh. 1983. Biochemical variation of Cryptococcus neoformans. Mycopathologia 84:121-131.

Sen, K., and K. Komagata. 1979. Distribution of urease and extracellular DNAse in yeast species. J. Gen. Appl. Microbiol. 25:127-135.

Shapiro, I. A. 1976. Determination of urease activity in lichens by a direct potentiometric method. Sov. Plant Phisiol. 23:712-714.

van Uden, N., P. Abranches, and C. Cabeça-Silva. 1968. Temperature functions of thermal death in yeasts and their relation to the maximum temperature for growth. Arch. fur Mikrobiol. 61:381-393.

Vishniac, H. S. 1983. An enation system for the isolation of Antarctic yeasts inhibited by conventional media. Can. J. Microbiol. 29:90-95.

Vishniac, H. S., and S. Baharaeen. 1982. Five new Basidioblasto-mycetous yeast species segregated from Cryptococcus vishniacii emend. auct., an Antarctic yeast species comprising four new varieties. Int. J. Syst. Bacteriol. 32:437-445.

Vishniac, H. S., and W. P. Hempfling. 1979. Cryptococcus vishniacii sp. nov., an Antarctic yeast. Int. J. Syst. Bacteriol. 29:153-158.

Vishniac, W. V., and M. Santer. 1957. The thiobacilli. Bacteriol. Rev. 21:195-213.

Weast, R. C. (ed.). 1979. CRC Handbook of Chemistry and Physics. CRC Press, West Palm Beach. p.D-203.

Zimmer, B. L., and G. D. Roberts. 1979. Rapid selective urease test for the presumptive identification of Cryptococcus neoformans. J. Clinical Microbiol. 10:380-381.

APPENDIX A

VIABILITY OF *Cryptococcus vishniacii* UNDER THE STANDARD CONDITIONS OF RAPID UREA HYDROLYSIS TESTING

Introduction

The urea hydrolysis tests most frequently used to assess urease activity in yeasts are rapid methods which involve incubating a dense suspention of pregrown cells in 0.33 M urea at 37°C for up to 4 hours (Roberts, *et al*., 1978; Barnett, *et al*., 1983). The results of urease tests conducted in different laboratories have not always agreed. The psychrophilic, Antarctic yeasts of the basidioblastomycetous *Cryptococcus vishniacii* complex when grown on Christensen's urea agar were characterized as urease negative by their authors (Vishniac and Hempfling, 1979; Baharaeen and Vishniac, 1982; Vishniac and Baharaeen, 1982), but the strains examined by Barnett, *et al*. (1983) were reported as urease positive or variable. These contradictory results led us to examine the viability of the psychrophilic yeast *Cr*. *vishniacii* var. *vishniacii* when exposed to the standard conditions of rapid urea hydrolysis (RUH) testing.

Materials and Methods

Stock cultures of *Cr*. *vishniacii* var. *vishniacii* (ATCC 36649) were maintained on YC agar slants at 4°C, and transferred to fresh

slants at monthly intervals. Working cultures were maintained on either YCA or YM agar slants in a Precision Model 815 low temperature incubator at 10°C. Cells for experimental use were grown through secondary broth culture at 10°C with 160 rpm agitation in a New Brunswick Scientific Co. Model G-76 Gyrotory water bath shaker coupled with a New Brunswick Model RF-10 Frigidflow coolant circulator. The cultures were taken from the water bath and kept on ice prior to harvesting and testing. The cells were harvested by centifugation at 3000x g for 5 minutes at 4°C in a Sorvall Model RC-2B refrigerated centrifuge, washed in 100 mL sterile E-Base, and resedimented by the same procedure. Testing at 37°C was conducted in a second New Brunswick Model G-76 Gyrotory water bath shaker with 160 rpm agitation. Determination of colony forming units per milliliter (cfu/mL) were made from spread plates of YC agar inoc- ulated in triplicate with 0.1 mL aliquots from dilutions prepared in sterile E-Base. The plates were incubated in a Precision Model 815 low temperature incubator at 10°C. The composition of the growth and test media are described in Appendix C.

The optical density (O.D.) at 650 nm of cell suspensions and the absorbance (A) at 560 nm, determined to be the absorbance maximum for alkaline phenol red in RUH broth, were measured in Ultra-Vu polystyrene disposable cuvettes, 4.5 mL, 1.0 cm path length and 2.9 mL, 0.5 cm path length (readings corrected to 1 cm path), respectively, with a Bauch and Lomb Spectronic 70 spectrophotometer. Measurements of pH were made with an Orion Research Model 910300 semi-micro combination pH electrode and Orion Research Model 701A digital Ionanalyzer.

For determination of $A_{560\ nm}$ of RUH broth a 2 mL aliquot of the

cell suspension in RUH broth was removed to a 16 X 125 mm screw-capped culture tube, the urease reaction stopped by placing the cells on ice, and the RUH broth supernatent was obtained by sedimentinging the cells with an IEC Model Cl clinical centrifuge at a setting of 6 for 5 minutes. The response to RUH testing was judged according to the following: absorbances below 0.250 were negative to the naked eye; those between 0.250 and 0.500 usually appeared more or less pink to rose ("weak"); while absorbances above 0.500 were unambiguously positive, approaching magenta to the naked eye.

Three independent experiments were conducted. In the first, cells were grown in YC broth to mid exponential phase (k = 0.094), harvested washed, and resedimented as described. The cells were resuspended in a 25 mL Erlenmyer flask to O.D. $_{650\ nm}$ = 30 with sterile E-Base, and immediately placed in the 37°C water bath shaker. At 0, 1, 2, 4, and 6 hours, 0.2 mL samples were removed, dilutions of 4 x 10^{-4}, $^{-5}$, $^{-6}$, and $^{-7}$ were prepared, and triplicate YC agar spread plates were inoculated. After 10 days incubation, the colonies were counted, and the cfu/mL was determined.

In the second experiment, cells were grown in YM broth to early stationary phase (k = -0.006), and harvested and washed as described. The harvested cells were then resuspended in RUH broth to O.D. $_{650\ nm}$ = 30 (20 mL total volume) in a 100 mL Erlenmyer flask, and immediately placed in the 37°C water bath shaker. At 0, 1, 2, 4, and 6 hours, 0.2 mL samples were removed to prepare dilutions for subsequent plating, a 2.0 mL aliquot was also removed to determine the absorbance of the RUH broth, and a microscope slide was prepared using methylene blue (0.15 mM in 0.13 M phosphate buffer pH 7.4). Spread plates were prepared as

described from the following dilutions: At 0 and 1hr., 8 x 10^{-7}, 1.6 x 10^{-7}, and 8 x 10^{-8}; at 2hr., 1.6 x 10 $^{-6}$, 8 x 10 $^{-7}$,and 1.6 x 10^{-7}; at 4 hr., 1.6 x 10 $^{-6}$, 8 x 10 $^{-7}$, and 8 x 10^{-7}; and at 6 hr., 8 x 10^{-5}, 8 x 10^{-6}, and 1.6 x 10^{-6}. The plates were incubated as described, and the colonies were counted after 19 days. The absorbance of the RUH broth was measured as described. The average percentage of cells which excluded methylene blue was determined by microscopic observation of three fields from the wet mount slides.

In the third experiment, Cr. vishniacii var. vishniacii cells were grown in YM broth to late exponential phase (k = 0.078), and harvested and washed as described. The cells were resuspended in sterile glass-distilled water to O.D.$_{650\ nm}$ = 40. For testing in E-Base, 5.0 mL of the O.D. = 40 cell suspention was mixed with an equal volume of double strength E-Base in a 25 mL Erlenmyer flask, and placed in the 37°C bath. At 0, 1, 2, 3 and 4 hours, 0.2 mL samples were removed, diluted, and YC agar plates were prepared and incubated as described. The dilutions used were: at 0, 1, and 2 hr., 8 x 10^{-6}, $^{-7}$ and $^{-8}$; and at 3hr., 8 x 10^{-7}, $^{-8}$ and $^{-9}$.

At the same time, testing in RUH broth was conducted by mixing 1.0 mL of double strength RUH broth and 1.0 mL of the O.D. = 40 cell suspension in a 16 X 125 mm screw-capped culture tube. One tube was prepared for each sample time, 0, 1, 2 and 3 hours, and all tubes were placed in the 37°C shaker bath simultaneously. Following incubation, 0.2 mL samples were removed for dilution and plating, and then the reaction was stopped by placing the tube in ice before sedimenting the cells in the clinical centrifuge. The aspirated supernate was placed on ice until the A $_{560\ nm}$ and pH were measured. YC agar plates were

spread from the following dilutions: at time = 0 hr., 8 x 10^{-6}, $^{-7}$ and $^{-8}$; at 1 hr., 4 x 10^{-6}, $^{-7}$ and $^{-8}$; at 2 hr., 1 x 10^{-2}, $^{-3}$, $^{-4}$, $^{-5}$ and $^{-6}$; and at 3 hr., 1 x 10^{-1}, $^{-2}$, $^{-3}$ and $^{-4}$. Both the E-Base and RUH plates were incubated as described and the colonies were counted after 20 days.

Results and Discussion

The viability of the psychrophilic yeast *Cr. vishniacii* var. *vishniacii* subjected to 37°C incubation in E-Base and RUH broth is shown in Figure 4 and Table VI. The results clearly show that the viability of *Cr. vishniacii* var. *vishniacii* is drastically reduced by incubation at 37°C in both E-Base and RUH broth. However, the cells survive 37°C testing more readily when incubated in E-Base, a non-nutritive mineral solution, than in RUH broth which contains 0.33 M urea. The ability of yeasts to survive supraoptimal temperatures when incubated in non-nutritive, mineral solution has been reported (van Uden, *et al*., 1968). The results of the RUH tests conducted here are variable; however whether the variable results represent physiological responses to temperature, growth phase, growth rate and/or cell suspension density or are artifacts resulting from experimental variation or errors cannot be determined due to the lack of intervening data points and of sufficient redundancy within and between experiments.

Cr. vishniacii var. *vishniacii* reacted variably to 37°C RUH testing (Table VI). The early stationary phase cells used in second experiment produced a weakly positive reaction by 2 hrs. (A_{560} = 0.443), and were strongly positive by 4 hrs. (A_{560} = 0.824). Whereas, the late exponential phase cells used in the third experiment

Figure 4. Survival of _Cr_. _vishniacii_ var. _vishniacii_ tested at 37°C in non-nutritive, mineral solution and in Rapid Urea Hydrolysis broth. E-Base plus mid-exponential cells, □; and plus late exponential cells, ■. RUH broth plus early stationary cells, △; and plus late exponential cells, ▲.

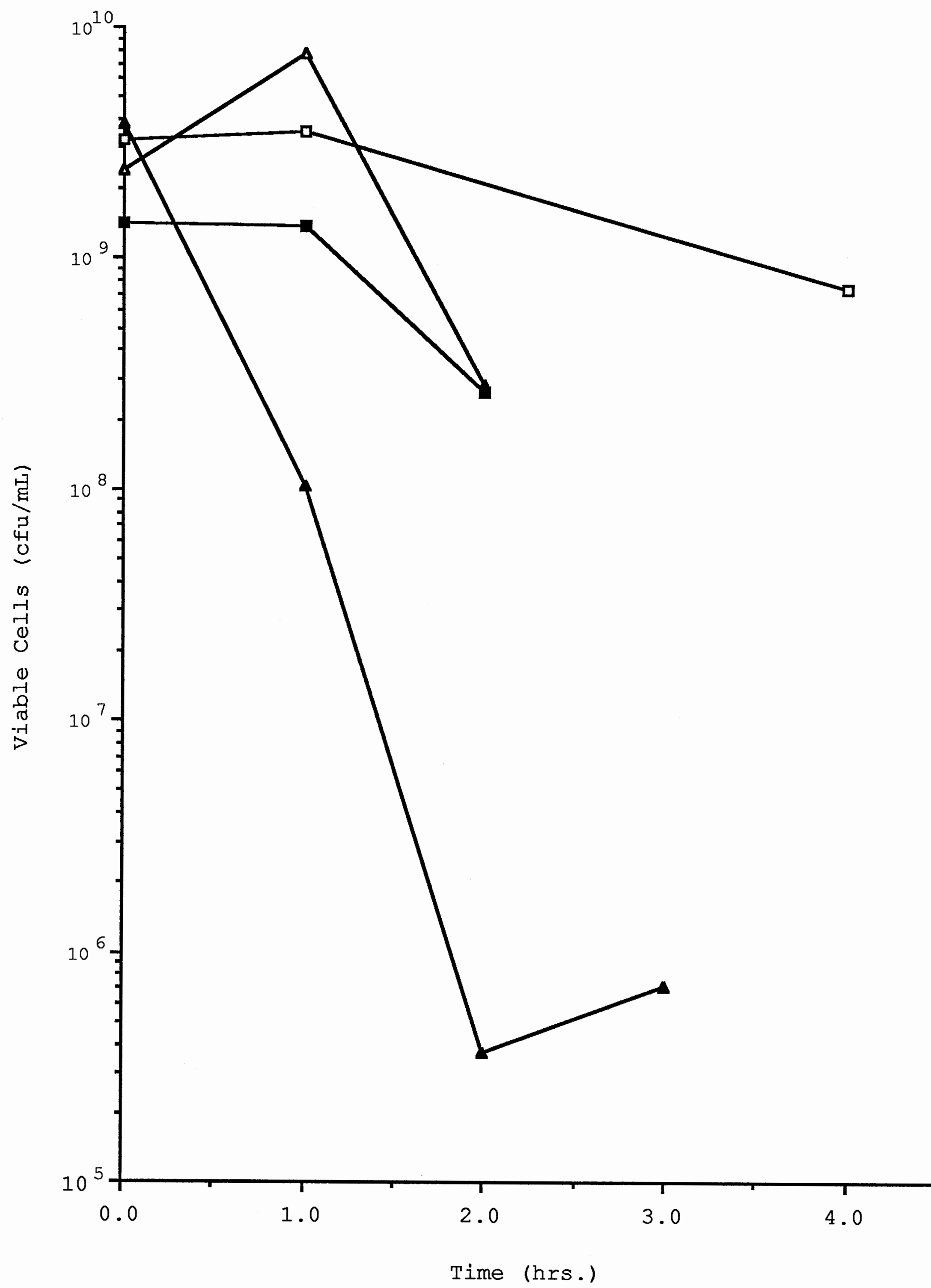

10^10
10^9
10^8
10^7
10^6
10^5
Viable Cells (cfu/mL)
0.0
1.0
2.0
3.0
4.0
Time (hrs.)

TABLE VI

VIABILITY AND PERFORMANCE OF *Cr. vishniacii* DURING 37°C TESTING IN MINERAL BASE AND RAPID UREA HYDROLYSIS BROTH

Time	Viability of cells tested in:			Performance:
	E-Base (%)	RUH broth (%)		$A_{560\ nm}$
Early Stationary Phase Cells:				
0 h	NA	2.41×10^9 (100)[a]	96.3 %[b]	0.073
1	"	7.83×10^9 (325)	78.3 %	0.172
2	"	2.84×10^8 (11.8)	77.7 %	0.443
4	"	T F T C	57.3 %	0.824
6	"	T F T C	35.4 %	1.200
Late Exponential Phase Cells:				
0 h	1.42×10^9 (100)	3.85×10^9 (100)		0.000
1	1.40×10^9 (98.6)	1.04×10^8 (2.7)		0.008
2	2.66×10^8 (18.7)	3.37×10^5 (0.01)		0.016
3	T F T C	7.26×10^5 (0.02)		0.020
Mid Exponential Phase Cells:				
0 h	3.26×10^9 (100)	NA		NA
1	3.58×10^9 (109.8)	"		"
2	T N T C	"		"
4	7.53×10^8 (23.1)	"		"
6	T N T C	"		"

NA = Not Applicable; T F T C = Too Few To Count; T N T C = Too Numerous To Count; [a] Cell count (cfu/mL); [b] Dye exclusion determined by light microscopy of cells stained with methylene blue.

failed to produce a positive reaction after 3 hrs incubation (A_{560} = 0.020). The pH of the RUH broth in the latter experiment mirrored the color reaction, rising to 6.92 from an initial of 6.89. A weakly positive reaction occurs between pH 7.4 to 7.8.

The growth rate in batch culture of the tested cells appeared to influence both the amount of cell death and the response to RUH testing. Of the cells tested in E-Base, those harvested in mid exponential phase (k = 0.094) and suspended to $O.D._{650}$ = 30 experienced less death than those harvested in late exponential phase (k = 0.078) and suspended to $O.D._{650}$ = 20. The results of RUH tests showed the opposite trend. The cells harvested in early stationary phase (k = -0.006) and suspended to $O.D._{650}$ = 30 produced a positive RUH reaction and experienced less cell death than the cells harvested in late exponential phase and suspended to $O.D._{650}$ = 20 which failed to produce a positive RUH response.

The cell suspension density may influence cell death and RUH activity. The cells used in the third experiment (k = 0.078; $O.D._{650}$ = 20) died more rapidly than the cells tested in the other experiments and also failed to produce a positive RUH reaction. A cell suspension density of $O.D._{650}$ = 20 did not limit the positive RUH reaction for _Cr_. _neoformans_ or _Cr_. _albidus_ (see Appendix B). Furthermore, _Cr_. _vishniacii_ var. _vishniacii_ was found to hydrolyze ^{14}C-urea to a greater extent then uninoculated and urease-negative controls (see Chapter II). That the 0 hour plate counts of the suspension used for RUH testing in the third experiment (3.85 x 10^9 cfu/mL) were higher than the counts of the other experiments ($O.D._{650}$ = 30) may explain the results, but is more indicative of the inherent differences between

cfu/mL and the O.D. of encapsulated yeasts.

Cell death may influence the results of RUH tests in three ways. Cell death may cause false positive RUH results: Cr. vishniacii var. vishniacii is a psychrophilic yeast, unable to grow at temperatures above 22°C (Vishniac and Hempfling, 1979), and as the current study shows these cells died rapidly during 37°C incubation. When cells die and subequently lyse, the internal enzymes are released to the test medium, usually resulting in alkalization. The occurence of false positive reactions in tests relying on alkalization via incubation at supraoptimal temperatures and by using aged cells has been reported (Kwon-Chung, et al., 1982b; Roberts, et al., 1978). Cell death may have little effect on RUH results: The external urease in yeasts may be a temperature insensitive, constituitive enzyme, and thus always present and functional regardless of the condition of the cell was postulated by Roberts, et al. (1978), and the observation of nitrate reductase activity in the absence of yeast growth has also been reported (Hopkins and Land, 1977). Cell death may cause false negative results. Some bacteria (Reithel, 1971) and the basidioblastomycetous yeast Rhodotorula mucilaginosa (Menyes and Fritsche, 1972) are known to possess inducible ureases. Thus, the lag time between exposure to urea and the release of a functional enzyme coupled with temperature shock and rapid cell death when psychrophilic cells are exposed to 37°C could account for false negative results.

Selected Bibliography

Baharaeen, S., and H. S. Vishniac. 1982. *Cryptococcus lupi* sp. nov., an Antarctic basidioblastomycete. Int. J. Syst. Bacteriol. 32: 229-232.

Barnett, J. A., R. W. Payne, and D. Yarrow. 1983. Yeasts: characteristics and identification. Cambridge Univ. Press, Cambridge. 811 pp.

Hopkins, J. M., and G. A. Land. 1977, Rapid method for determining nitrate utilization by yeasts. J. Clin. Microbiol. 5: 497-500.

Kwon-Chung, K. J., I. Polacheck, and J. E. Bennett. 1982. Improved diagnostic medium for separation of *Cryptococcus neoformans* (serotypes A and D) and *Cryptococcus neoformans* var. *gattii* (serotypes B and C). J. Clin. Microbiol. 15: 535-537.

Menyes, H. and W. Fritsche. 1972. Regulation der urease synthese in *Rhodotorula mucilaginosa*. Zeitschr. Allg. Mikrobiol. 12: 219-229.

Reithel, F. J. 1971. Ureases, p. 1-21. *In* P. D. Boyer (ed.), The Enzymes IV. Hydrolysis (3rd ed.). Academic Press, New York.

Roberts, G. D., C. D. Horstmeier, G. A. Land, and J. H. Foxworth. 1978. Rapid urea broth test for yeasts. J. Clin. Microbiol. 7: 584-588.

van Uden, N., P. Abranches, and C. Cabeça-Silva. 1968. Temperature functions of thermal death in yeasts and their relation to the maximum temperature for growth. Arch. fur Mikrobiol. 61: 381-393.

Vishniac, H. S., and S. Baharaeen. 1982. Five new Basidioblastomycetous yeast species segregated from *Cryptococcus vishniacii* emend. auct., an Antarctic yeast species comprising four new varieties. Int. J. Syst. Bacteriol. 32: 437-445.

Vishniac, H. S., and W. P. Hempfling. 1979. *Cryptococcus vishniacii* sp. nov., an Antarctic yeast. Int. J. Syst. Bacteriol. 29: 153-158.

APPENDIX B

DETERMINATION OF THE OPTIMAL CONDITIONS FOR RAPID UREA HYDROLYSIS TESTING

Introduction

The cell concentration needed to saturate the Rapid Urea Hydrolysis (RUH) test system and the time and temperature of growth and RUH incubations were not adequately quantified by Roberts, *et al.* (1978), who described a rapid urea hydrolysis test system, or by Barnett, *et al.* (1983), who employed Difco Urea R Broth to assess the urease activity of 473 yeast species. Thus, this appendix describes the procedures and discusses the results of experiments quantifying these parameters.

Materials and Methods

Stock cultures were maintained on YCA or YM agar slants at 4°C, and transferred to fresh slants at monthly intervals. The stock cultures of *Cryptococcus albidus* var. *albidus*, ATCC 10666, and *Filobasidiella* (*Cryptococcus*) *neoformans* var. *neoformans*, NIH 12 (=ATCC 28957), were incubated for 24 to 48 hours at room tepmerature (ie., 25°C to 28°C) prior to 4°C storage. Working cultures of the psychrophiles (ie. strains unable to grow above 25°C) *Cryptococcus vishniacii* var. *vishniacii*, ATCC 36649 and *Leucosporidium scottii*, ATCC 22182 and the mesophile *Cryptococcus albidus* var. *albidus* were incubated at 10°C on either slants or plates of YM agar in a Precision

Model 815 low temperature incubator. Working cultures of the mesophiles (ie. strains unable to grow at 4°C) Cryptococcus albidus var. albidus and Filobasidiella (Cr.) neoformans var. neoformans, representing strains incapable and capable of 36°C growth, were incubated on either plates or slants of YM agar at 25°C and 36°C in a Precision Model 6 incubator. Cultures incubated at 10°C were grown for 4 to 32 days; cultures incubated at 25°C and 36°C were grown for 1 to 4 days. The choice of growth and test temperature was based on the physiological optimum of the strains tested as reported in handbooks of yeast characteristics (Barnett, et al., 1983; and Kreger-van Rij, 1984). The composition of the growth media and test broth are described in Appendix C.

Rapid urea hydrolysis testing was conducted by the following protocol: Cells were harvested using a wire loop and suspended in 2 or 5 mL sterile glass-distilled water; the optical density (O.D.) at 650 nm of the cell suspension was measured, and the volume of cell suspension needed to yield the appropriate final O.D. in 2 or 1.6 ml total volume was calculated from the equation, mL X O.D. = O.D.U. (optical density units). One or 0.8 mL of the adjusted cell suspension was combined with an equal volume of double strength RUH broth in a 16 X 125 mm screw-capped borosilicate culture tube and the tubes were immediately placed in a New Brunswick Scientific Co. Model G-76 Gyrotory water bath shaker coupled to a New Brunswick Scientific Co. Model RF-10 frigidflow coolant circulator operating at 180-200 rpm agitation for incubation at 20°C for 2 h, 25°C for 2 h or 37°C for 0.5 or 1 h.

Following incubation, the reaction was quenched by placing the

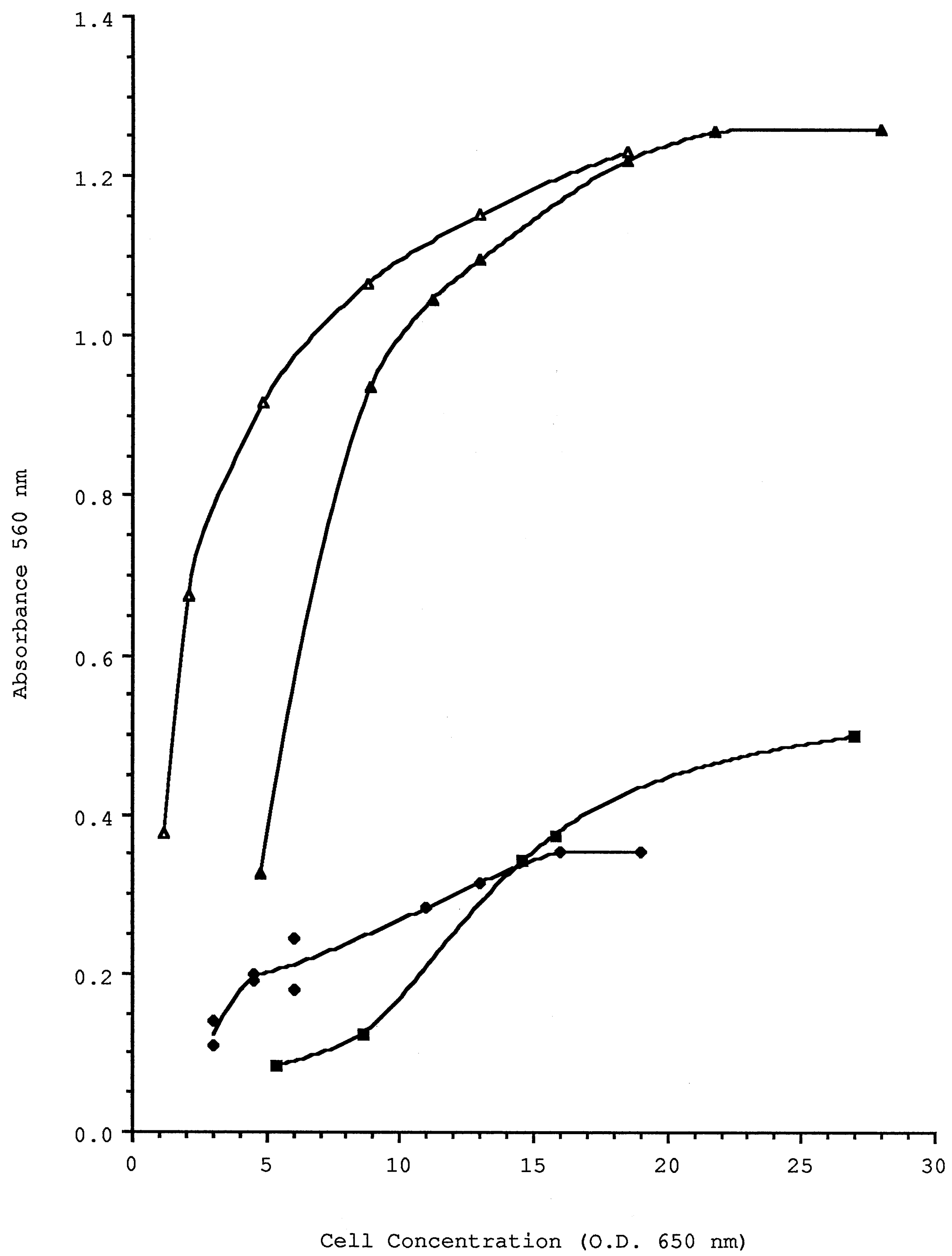
tubes on ice prior to sedimenting the cells in an IEC Model Cl clinical centrifuge at a setting of 6 for 5 minutes. The RUH broth supernatant was aspirated into a second test tube and kept on ice until the absorbance (A) at 560 nm, determined to be the absorbance maximum for alkaline phenol red in RUH broth, was measured. Absorbances below 0.250 were negative to the naked eye; those between 0.250 and 0.500 usually appeared more or less pink to rose (weakly positive); while absorbances above 0.500 were unambiguously positive, approaching magenta to the naked eye. The O.D. $_{650\ nm}$ of cell suspensions and the A $_{560\ nm}$ were measured in Ultra-Vu polystyrene disposable cuvettes, 4.5 mL, 1.0 cm path length and 2.9 mL, 0.5 cm path length (readings corrected to 1 cm path), respectively, using a Bauch and Lomb Spectronic 70 spectrophotometer.

Results and Discussion

Cell Concentrations

The effect of varying the cell concentration in RUH test broth on subsequent RUH activity is shown in Figure 5. The cell concentration providing optimal RUH activity occurred at about O.D. $_{650\ nm}$ = 20. After growth at room temperature for 36.17 and 71.04 hours, *Cr. albidus* cells tested for 2 hours at 20°C in broth containing 1.34 X 10^{-2} M phosphate displayed maximal RUH activity at suspension densities (O.D. $_{650\ nm}$) ranging from 16 to 27 (mean = 21.5). The maximum RUH response by *Fil*. (*Cr*.) *neoformans* var. *neoformans* grown at room temperature for 40.50 hours and tested at 37°C for 0.5 and 1 hour in broth containing 1.34 X 10^{-3} and 1.34 X 10^{-2} M phosphate was obtained at suspension densities of 18.5 and 21.8. The RUH response by both strains displayed

Figure 5. The effect of cell concentration (O.D. $_{650\ nm}$) in test broth on Rapid Urea Hydrolysis activity. *Cryptococcus albidus* var. *albidus* (ATCC 10666) grown at room temperature for 36.17 hours, ◆, and 71.04 hours, ■, and tested at 20°C for 2 hours. *Filobasidiella* (*Cryptococcus*) *neoformans* var. *neoformans* (NIH 12) grown at room temperature for 40.50 hours and tested at 37°C for 0.5 and 1 hour in broth containing 1.34 X 10^{-3}, Δ, and 1.34 X 10^{-2}, ▲, M phosphate.



Absorbance 560 nm
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
Cell Concentration (O.D. 650 nm)

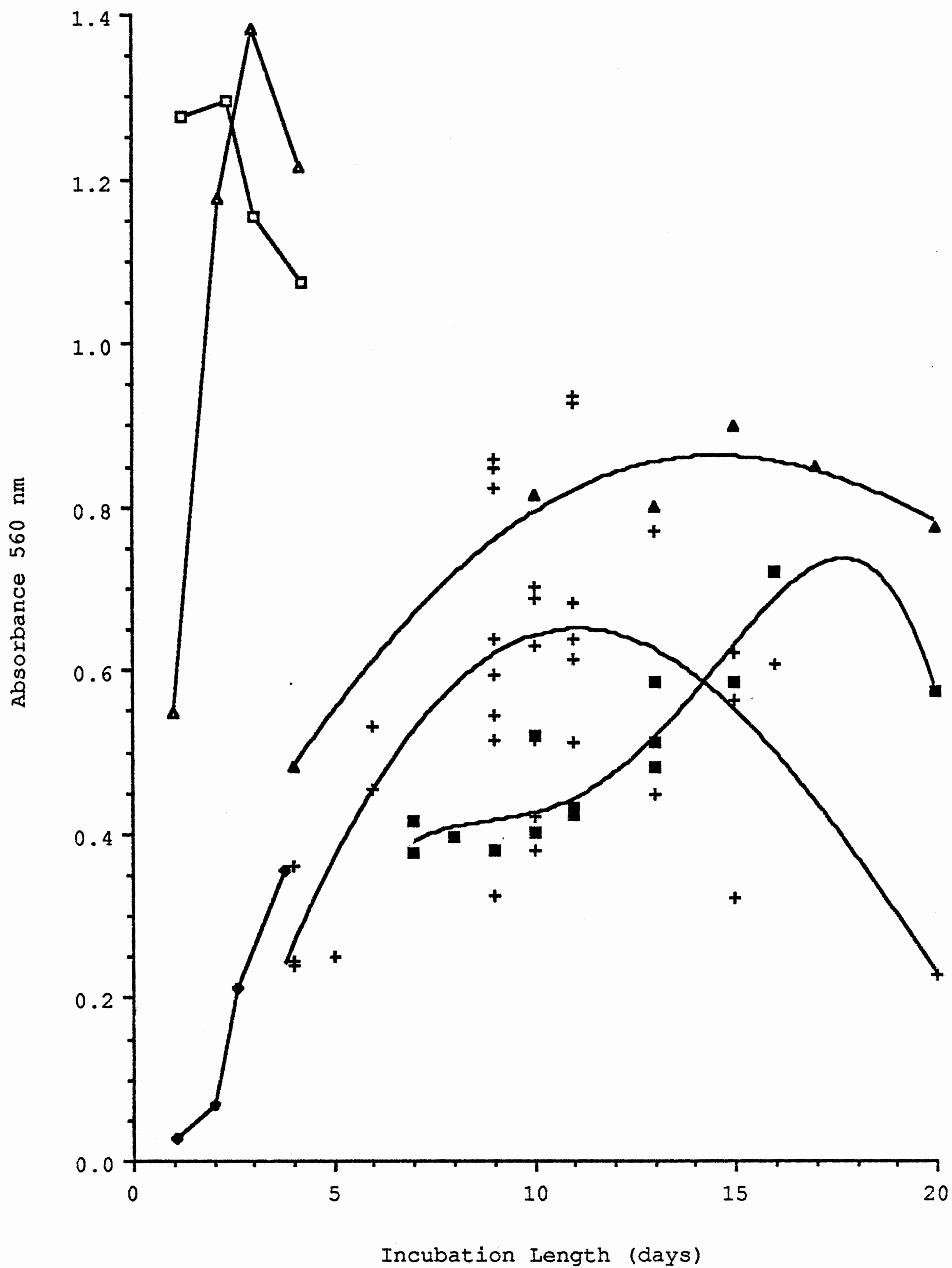
rapid flattening at about O.D. $_{650\ nm}$ = 16, and though both produced maximal responses at suspension densities of about O.D. $_{650\ nm}$ = 22, a density of O.D. $_{650\ nm}$ = 20 did not significantly inhibit RUH activity. Thus, in all subsequent RUH experiments a suspension density of O.D. $_{650\ nm}$ = 20 was used.

Incubation Lengths

RUH incubation lengths were based on the need to obtain comparative results between strains tested at the same and different temperatures. The incubation lengths needed for urease positive controls to develope a positive RUH reaction depended on the incubation temperature and strain. In a single experiment conducted in triplicate Fil. neoformans var. neoformans, a yeast strongly positive in RUH tests, tested at 37°C for 5, 10, 15, and 30 min. produced A_{560} ($\pm$ S.D.) values of 0.063 $\pm$ 0.003, 0.127 $\pm$ 0.009, 0.285 $\pm$ 0.017, and 0.797 $\pm$ 0.012. Based on this, cells tested at 20°C and 25°C were incubated for 2 hours, and cells tested at 37°C were incubated for 0.5 or 1 hour.

The RUH performance of yeasts grown for varying lengths of time at 10°C, room temperature, or 36°C on YM agar are graphed in Figure 6. The yeasts grown at 10°C and tested at 20°C for 2 hours displayed maximum RUH activity when grown between 11 and 18 days. The smooth curves in Figure 6 were based on a quadratic polynomial fit of the data and show that maximum RUH activity for Cr. albidus var. albidus grown at 10°C (N = 34, r = 0.67) occured at 11 days; that Leucosporidium scottii (N = 7, r = 0.98) produced a maximal response at 14.5 days; and that Cr. vishniacii var. vishniacii (N = 14, r = 0.92) displayed maximal RUH activity after 17.5 days incubation. Fil. neoformans var.

Figure 6. The effect of incubation length at physiological temperatures on subsequent Rapid Urea Hydrolysis activity. □, *Filobasidiella* (*Cr*.) *neoformans* var. *neoformans* grown at 36°C and tested at 37°C for 1 hour. △, *Fil*. (*Cr*.) *neoformans* var. *neoformans* grown at room temperature and tested at 37°C for 1 hour. ◆, *Cryptococcus albidus* grown at room temperature and tested at 20°C for 2 hours. +, *Cryptococcus albidus* var. *albidus* (N = 34, r = 0.67); ■, *Cryptococcus vishniacii* var. *vishniacii* (N = 14, r = 0.92); and ▲, *Leucosporidium scottii* (N = 7, r = 0.98) grown at 10°C and tested at 20°C for 2 hours. The smooth curves were computed from a quadradic polynomial fitting computer program.



1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Absorbance 560 nm
0
5
10
15
20
Incubation Length (days)

neoformans and *Cr. albidus* var. *albidus* grown at room temperature for 1-4 days and tested at 37°C for 1 hour or 20°C for 2 hours, respectively, displayed maximum RUH activities after 3 to 3.75 days (ie., 72 to 90 hours) incubation. *Fil. neoformans* var. *neoformans* grown at 36°C and tested at 37°C for 1 hour produced maximal RUH activity after 30 to 60 hours. Based on these results strains capable of 10°C growth were tested in RUH at 20°C for 2 hours after growth for 11-14.5 days at 10°C on YM. Cultures of mesophiles growing poorly or failing to grow at 36°C were tested at 25°C for 2 hours after growth at 25°C for 72 to 74 h, or 28°C for 68 h. Mesophiles growing well at 36°C were tested at 37°C for 0.5 or 1 hour after growth for 40-45 h at 36°C.

Figure 6 clearly shows that an initial lag period was produced by all the strains. This lag was especially pronounced for *Cr. vishniacii* var. *vishniacii* whose curve displays a pronounced shoulder. Also, after the incubation length providing maximum response at all growth and test temperatures, a rapid decrease in RUH acivity was seen. *L. scottii*. showed a less rapid decrease in RUH activity, producing an A_{560} of 0.712 after 32 days incubation (not graphed). However, on addition to RUH broth the visibly degenerating 32 day culture immediately turned the test broth pink, a strong indication of proteolytic activity within the culture. That the use of moribund cells may lead to false positive reactions in RUH tests was reported by Roberts, *et al*. (1978), who stated that the alkalinity of an older culture was caused by the accumulation of ammonia during extended growth on media containing peptone.

Selected Bibliography

Barnett, J. A., R. W. Payne, and D. Yarrow. 1983. Yeasts: characteristics and identification. Cambridge Univ. Press, Cambridge. p. 25.

op. cit. 811 pp.

Kreger-van Rij, N. J. W. (ed.) 1984. The yeasts a taxonomic study, third revised and enlarged edition. Elsevier Science Pub., Amsterdam. 1082 pp.

Roberts, G. D., C. D. Horstmeier, G. A. Land, and J. H. Foxworth. 1978. Rapid urea broth test for yeasts. J. Clinical Microbiol. 7: 584-588.

APPENDIX C

COMPOSITION OF GROWTH AND TEST MEDIA

Christensen's Urea Agar (CUA)

Christensen, W. B. 1946. Urea decomposition as a means of differentiating *Proteus* and paracolon cultures from each other and form *Salmonella* and *Shigella* types. J. Bacteriol. 52: 461-466.

Urea (filter sterilized/aseptically added)	20.0	gm/L
Glucose	1.0	gm/L
Peptone (bacto)	1.0	gm/L
NaCl	5.0	gm/L
KH_2PO_4	2.0	gm/L (14.7mM)
Phenol red	0.012	gm/L
Agar	20.0	gm/L

final pH 6.8 ± 0.2

E-Base

E-Base is a non-nutritive, mineral solution used to prepare "in house" media. The H_3BO_4 and KI were omitted from the E-Base used in the experiments described in Appendix A.

NaCl	50	mM
$MgSO_4$-$7H_2O$	0.2	mM
Potassium Phosphate Buffer (pH 6.0)	1.0	mM
Trace Metal Solution (TMS)	1.0	mL/L
H_3BO_4	50	µg/L
KI	50	mM

Malt Extract Agar (MEA)

Difco Manual, 10th ed. 1984. Difco Laboratories Inc., Detroit, Michigan. pp. 557-558.

Maltose (technical)	12.75	gm/L
Dextrin (Difco)	2.75	gm/L
Glycerol	2.35	gm/L
Peptone (bacto)	0.78	gm/L
Agar	15.0	gm/L

final pH 4.7 + 0.2

Peptone-Yeast Extract-Gluose Agar (PYG)
± Trace Metals Solution

Glucose	5.0	gm/L
Peptone (bacto)	5.0	gm/L
Yeast Extract	3.0	gm/L
Agar	18.0	gm/L

final pH 6.9 ± 0.2

When PYG is used to test the effect of Trace Metals Solution on subsequent urea hydrolysis, the following are added to the receipe above:

Trace Metals Solution (TMS)	1.0	mL/L
$MgSO_4$-$7H_2O$	0.2	mM

final pH 6.9 ± 0.2

Rapid Urea Hydrolysis Broth (RUH Broth)

RUH Broth was modified from Difco Urea R Broth (see below) according to the findings of Roberts, G. D., C. D. Horstmeier, G. A. Land, and J. Foxworth. 1978. Rapid urea broth test for yeasts. J. Clin. Microbiol. 7: 584-588.

Urea (filter sterilized/aseptically added)	20.0	gm/L

Yeast Extract	0.1	gm/L
KH_2PO_4	0.91	gm/L (6.67mM)
Na_2HPO_4	0.95	gm/L (6.67mM)
Phenol red	0.012	gm/L

final pH 6.9 ± 0.2

Sabouraud's Dextrose Agar (SDA)

Difco Manual, 10th ed. pp. 768-772.

Glucose	40.0	gm/L
Neopeptone (bacto)	10.0	gm/L
Agar	15.0	gm/L

final pH 5.6 ± 0.2

Trace Metals Solution (TMS)

The TMS stock solution described below is added to media at 1.0 mL/L which is 1/10 the strength recommended by the its authors, Vishniac, W. V., and M. Santer. 1957. The thiobacilli. Bacteriol. Rev. 21: 195-213.

Ethylenediamine tetraacetic acid (EDTA), disodium salt	171	mM
$CaCl_2$ (anhydrous)	49.9	mM
$CoCl_2$-$6H_2O$	4.9	mM
$CuSO_4$-$5H_2O$	7.5	mM
$FeSO_4$-$7H_2O$	18.0	mM
$MnCl_2$-$4H_2O$	25.6	mM
$(NH_4)_6Mo_7O_{24}$-$4H_2O$	6.2	mM
$ZnSO_4$-$7H_2O$	76.5	mM

Urea R Broth

Difco Manual, 10th ed. 1984. pp. 1039-1045.

Urea	20.0	gm/L
Yeast Extract	0.1	gm/L
KH_2PO_4	0.091	gm/L (0.67mM)
Na_2HPO_4	0.095	gm/L (0.67mM)
Phenol red	0.01	gm/L

final pH 6.9 ± 0.2

Wickerham's Vitamin Mix

van der Walt, J. P. 1970. Criteria and methods used in classification. In The yeasts - a taxonomic study. Edited by J. Lodder. North Holand Publishing Co., Amsterdam. pp.34-113.

Biotin	2.0	µg/L
Calcium Pantothenate	400	µg/L
Folic acid	2.0	µg/L
Insositol	2000	µg/L
Niacin	400	µg/L
Para-aminobenzoic acid	200	µg/L
Pyridoxine HCl	400	µg/L
Riboflavin	200	µg/L
Thiamin HCl	400	µg/L

Yeast Culture Agar (YCA)

Vishniac, H. S. 1983. An enation system for the isolation of Anarctic yeasts inhibited by conventional media. Can. J. Microbiol. 29: 90-95.

Glucose (aseptically added)	5.0	gm/L
Sodium Glutamate (pH 6.0)	2.0	mM
NH_4Cl	2.0	mM
NaCl	50	mM

$MgSO_4$-$7H_2O$	0.2	mM
Potassium Phosphate Buffer (pH 6.0)	1.0	mM
Trace Metals Solution (TMS)	1.0	mL/L
Wickerham's Vitamin Mix (100x stock)	1.0	mL/L
Yeast Extract	0.5	gm/L
Agar	18.0	gm/L

Yeast Malt Agar (YM)

Difco Manual, 10th ed. , pp. 1131-1133.

Glucose	10.0	gm/L
Peptone (bacto)	5.0	gm/L
Yeast Extract	3.0	gm/L
Malt Extract	3.0	gm/L
Agar	18.0	gm/L

final pH 6.2 ± 0.2

VITA

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