side chains determines their tendencies to be found at the surface of globular proteins. A rolling sphere model, proposed by Lee and Richards (*7*), provides a reasonable semiquantitative basis for determining the exposure to solvent of the individual atoms of a protein whose configuration has been determined by exact structural methods. Applying this method to 12 crystalline proteins, Chothia (*8*) has calculated the number of residues of each amino acid that are inaccessible to solvent over 95 percent of their individual surface areas, expressed as a fraction of the total number of residues of that amino acid, which are present in all 12 proteins taken together. In Fig. 2, this fraction is plotted as a function of the hydration potentials in Table 1. Amino acid residues falling on the lower half of one scale, also fall on the lower half of the other. There are no exceptions (*9*).

This close relationship led us to question whether there might be any correspondence between the hydration potential of the various side chains and the nucleic acids that serve as their genetic determinants. The amino acid code (*10*) has been found to be moderately degenerate at the first position of the codon and highly degenerate at the third position of the codon, in the sense that several alternative bases serve to code for the same amino acid at these positions; but each amino acid is uniquely associated with the presence of a single base at the second position of the codon except serine (two alternative bases). In our study, the 18 side chains, ranked in order of increasing hydration potential, can be divided into two equal groups (Table 2). It is evident from inspection that the observed distribution of code letters is nonrandom. Thymine, for example, serves as the second code letter for seven amino acids. Every one of them has a hydrophilic side chain, and all are clustered near the origin of Fig. 2. Estimated conservatively (*11*), the probability that this might occur by chance is in the neighborhood of .0045.

The present scale of hydration potentials illustrates the probable importance of amino acid side chain interactions with solvent water as a factor in determining the overall configurations of proteins. It is not apparent that any simple physicochemical basis exists for the observed correlations with the modern genetic code, but it seems natural to suppose that coding similarities between amino acids with similar physical properties may have tended to offset the disruptive effects of mutation on the structural stability of proteins during their evolution (*12*). Figure 2 provides quantitative support for the widely held view that mutations that would result in the introduction of hydrophilic amino acids, at interior locations that were previously hydrophobic, are expected to be especially damaging. The observed distribution of code letters appears to minimize the likelihood of these events.

R. V. WOLFENDEN
P. M. CULLIS
C. C. F. SOUTHGATE
Department of Biochemistry, University of North Carolina, Chapel Hill 27514

References and Notes

1. J. A. V. Butler, *Trans. Faraday Soc.* **33**, 229 (1937); J. Hine and P. K. Mookerjee, *J. Org. Chem.* **40**, 292 (1975).
2. R. Wolfenden, *Biochemistry* **17**, 201 (1978).
3. P. Kebarle, in *Environmental Effects on Molecular Structure and Properties*, B. Pullman, Ed. (Reidel, Dordrecht, 1976), pp. 81–94.
4. W. Kauzmann, A. Bodansky, J. Rasper, *J. Am. Chem. Soc.* **84**, 1777 (1962).
5. The absorption spectrum observed for 3-methylindole in the vapor phase was almost identical with its spectrum in cyclohexane. Its concentration in the vapor phase was therefore estimated from its extinction coefficient (ϵ) determined in cyclohexane in separate experiments: $\epsilon_{267} = 6.74 \times 10^3$.
6. W. Kauzmann, *Adv. Protein Chem.* **16**, 1 (1959); M. Perutz, *J. Mol. Biol.* **13**, 646 (1965).
7. B. Lee and F. M. Richards, *J. Mol. Biol.* **55**, 379 (1971).
8. C. Chothia, *ibid.* **105**, 1 (1976).
9. The coefficient for linear correlation, for the 18 amino acids included in Fig. 2, is 0.90 ($P \leq .00001$). An exact value for the hydration potential of the side chain of Arg remains to be determined. Preliminary results indicate that it is even more negative than that of Asp, consistent with its extreme tendency to be exposed at the surface of globular proteins in which it is found (*8*). Earlier scales of hydrophobicity that were based on free energies of transfer of amino acids near their isoelectric points to ethanol or dioxan [Y. Nozaki and C. Tanford, *J. Biol. Chem.* **246**, 2211 (1971)], or to the surface of their aqueous solutions [H. B. Bull and K. Breese, *Arch. Biochem. Biophys.* **161**, 665 (1974)], when used as abscissas in plots similar to that of Fig. 2, exhibit correlation coefficients of 0.20 and 0.58, respectively. Some of the differences between these various scales are almost certainly due to the special solvation properties of zwitterionic amino acids.
10. M. W. Nirenberg, O. W. Jones, P. Leder, F. C. Clark, W. S. Sly, S. Petska, *Cold Spring Harbor Symp. Quant. Biol.* **28**, 549 (1963). The incidence of similar code words for similar amino acids, noted in a qualitative sense by these authors, has been a subject of commentary and speculation [see, for example, F. H. C. Crick, *J. Mol. Biol.* **38**, 367 (1968); C. Woese, *ibid.* **43**, 235 (1969)].
11. Comparing the dichotomy hydrophilic/not-hydrophilic with the dichotomy T/not-T, the significance level by Fisher's exact test is .0011. One such test was performed with each of the four bases, so that we estimate that the real significance is about four times larger. Regarding the overall pattern of the distribution in Table 2, including all four bases, the nonrandomness of the distribution was confirmed by the Kruskal-Wallis test: the P value was approximately .005. We thank Drs. R. C. Grimson and R. C. Elston, Department of Biostatistics, University of North Carolina, for advice in this matter.
12. R. M. Sonneborn, in *Evolving Genes and Proteins*, V. Bryson and H. J. Vogel, Eds. (Academic Press, New York, 1965), pp. 377–397.
13. J. T. Edsall and J. Wyman, *Biophysical Chemistry* (Academic Press, New York, 1958).
14. Supported by NIH grant GM-18325 and NSF grant PCM-7823016.

5 June 1979; revised 15 August 1979

Candidiasis: Detection by Gas-Liquid Chromatography of D-Arabinitol, a Fungal Metabolite, in Human Serum

Abstract. *D-Arabinitol was identified as a major metabolite of* Candida *species in human subjects. Gas-liquid chromatography was used to measure the concentration of D-arabinitol in serum. The study included subjects who were healthy and cancer patients who had proven invasive candidiasis or were colonized with* Candida. *D-Arabinitol concentrations greater than 1.0 microgram per milliliter were found in serum from patients with invasive infection. This technique may prove valuable in the diagnosis of invasive candidiasis.*

Invasive candidiasis is a common, life-threatening infection in immunosuppressed hosts, such as transplant recipients or cancer patients (*1*); however, it is difficult to diagnose invasive infections caused by *Candida* species. These yeasts are commonly isolated from various body sites and secretions of subjects who have no evidence of invasive disease (*2*). Yet many patients with invasive candidiasis do not have positive blood cultures (*3*). Also, recovery of the yeast from positive blood cultures is often delayed (*4*). Because a positive blood culture may not reflect invasive disease, patients may recover from transient fungemia without specific antifungal therapy. Similarly, detection of agglutinating and precipitating antibodies does not correlate consistently with invasive disease, especially in heavily immunosuppressed patients whose immunoglobulin function or levels may be depressed (*5*).

An alternative to standard culture and serologic diagnosis of invasive fungal infections involves chemical or immunologic detection of fungal cell metabolites. For example, the latex agglutination test for cryptococcal capsular polysaccharide is used widely to diagnose cryptococcal infection by the detection of antigen in blood and cerebrospinal fluid (*6*). Crossed electrophoresis (*7*), enzyme-linked immunosorbent assay (*8*), and passive hemagglutination inhibition (*9*) have been used to diagnose invasive candidiasis by detecting the circulating antigen. These reports suggest that antigen can be detected in the serum of some pa-

0036-8075/79/1102-0577$00.75/0

tients with fungal infection. Serum samples from healthy subjects and patients with bacteremia, candidemia, invasive candidiasis, or fungal colonization (*10*) have been analyzed by gas-liquid chromatography. The serum from candidemic patients contained higher concentrations of a compound tentatively identified as mannose, which may be derived from mannan, a major yeast cell wall component.

In this report we describe the use of GLC and other methods to identify D-arabinitol as a major metabolite of *Candida* species and the use of GLC to detect this metabolite in the serum of patients with invasive candidiasis. Fourteen cultures of *Candida albicans* and 11 of *Candida tropicalis* were inoculated separately into 10 ml of 1 percent (weight to volume) yeast nitrogen base (Difco) containing glucose (2.5 g/liter) as the sole carbon source. Cultures were incubated for 24 hours at 37°C and then centrifuged at 1500*g* for 5 minutes. The supernatant was filtered through a 0.45-μm membrane filter (Millipore) and stored at −20°C before GLC analysis. The harvested cells were washed with sterile saline, resuspended in sterile distilled water, and sonicated for 2 hours in a Branson sonifier (100 W, Heat Systems, Plainview, New York). The resulting suspension was filtered through a 0.45-μm membrane filter, and the filtrate was stored at −20°C prior to analysis by GLC.

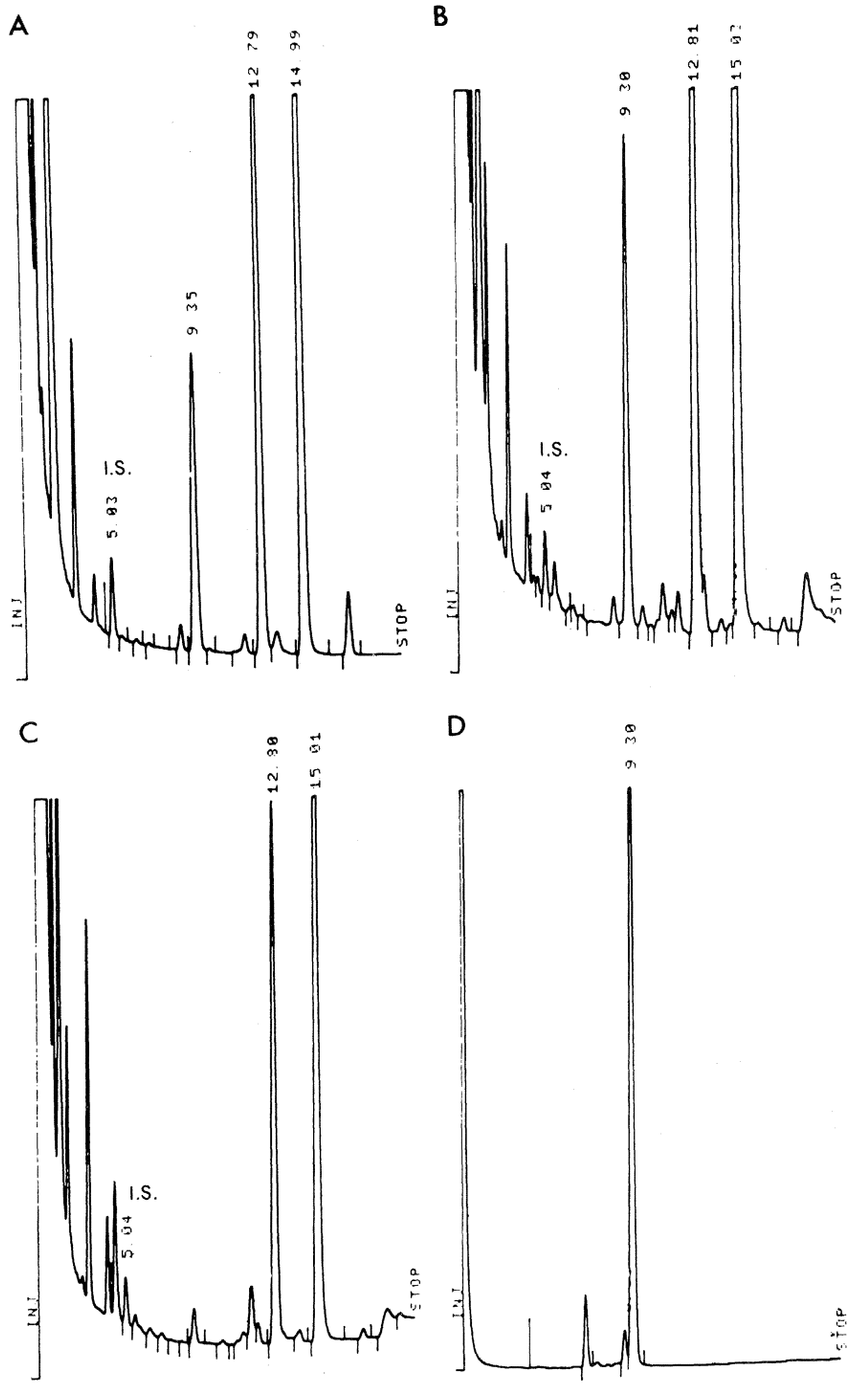

Fig. 1. Gas chromatographs of (A) culture filtrate of *Candida albicans* grown in a yeast nitrogen base with glucose as the sole carbon source, (B) serum from patient with *C. albicans* pneumonia, (C) serum from healthy laboratory worker, and (D) sample of authentic D-arabinitol. A 3-μl portion of the trimethylsilyl ether derivative was injected onto a 6-foot glass column packed with 3 percent SE-30 on 80/100 mesh Gas Chrom Q. The oven temperature was set to increase from 140° to 220°C at 4° per minute. Absolute retention times (in minutes) appear above the peaks of interest. Meso-erythritol, the internal standard (I.S., concentration, 10.0 μg/ml), eluted at 5.03 minutes. The peaks at 12.8 and 15.0 minutes are the derivatives of the α and β anomers of glucose.

Meso-erythritol or α-methyl-D-mannopyranoside (*11*), the internal standards, were dissolved in sterile distilled water at 5.0 μg/ml. Each solution was diluted in acetone (*12*) to a final concentration of 5.0 μg/ml. A 200-μl portion of an internal standard solution was then combined with 100 μl of culture filtrate, sonicated cell suspension, or patient serum. The mixture was centrifuged at 1500*g* for 5 minutes to separate precipitated proteins. The supernatant was transferred to a glass centrifuge tube, heated in a 40°C water bath, and dried under a nitrogen stream (Organomation Associates, Inc., North Borough, Massachusetts). Trimethylsilyl (TMS) ether derivatives were prepared according to the method of Sweeley *et al.* (*13*). The residue was treated, in a glass-stoppered tube, with 0.1 ml of the silylating agent (pyridine, hexamethyldisilazane, and TMS, 6:4:2) (*14*). The reaction proceeded at an ambient temperature for at least 20 minutes.

A 3-μl portion of the silylated sample was analyzed with a Hewlett-Packard 5730A gas chromatograph equipped with a flame ionization detector and 3380A integrator. For analysis of TMS ethers the instrument was fitted with dual 6-foot coiled glass columns (1/4-inch in diameter) packed with 3 percent SE-30 on 80/100 mesh Gas-Chrom Q (Hewlett-Packard). The nitrogen carrier gas was set at a flow rate of 40 ml/min, and the oven temperature was programmed to rise from 140° to 220°C at 4° per minute. All peaks with retention times between 4 and 20 minutes were integrated. Absolute peak areas were reported, and the concentra-

tions of components were calculated from the known concentration of the internal standard by simple proportion.

A compound, eluting with a retention time of 9.35 minutes (retention time relative to α-glucose, 0.73), was found by GLC to be a major component of *C. albicans* and *C. tropicalis* culture filtrates and sonicated cell suspensions. Twenty-five cultures of *Candida* species produced this compound with an efficiency of 4 to 17 percent (based on glucose consumed) when grown in a yeast nitrogen base with glucose. Fig. 1A shows the chromatogram of a *C. albicans* culture filtrate. The peak at 9.35 minutes represents the component of interest; the peaks with the retention times of 12.79 and 14.99 minutes are the α and β anomers of glucose; meso-erythritol appears at 5.03 minutes. The compound was also found in the serum of a patient with *C. albicans* pneumonia (Fig. 1B), but it was not seen in the serum of a healthy laboratory worker (Fig. 1C).

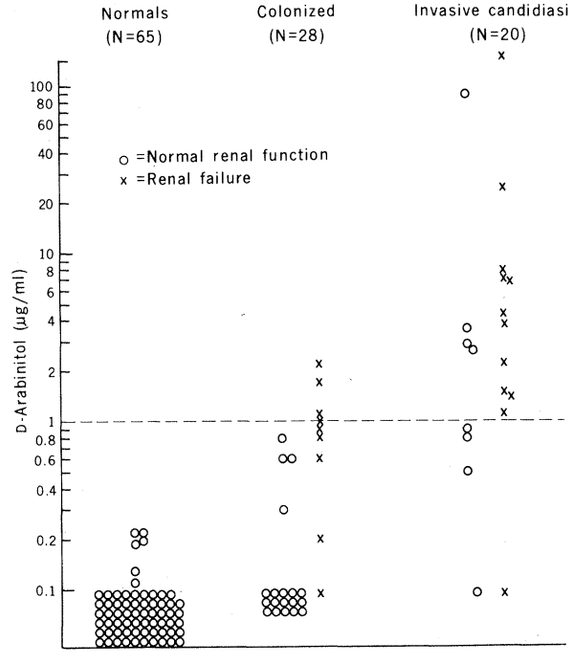

Fig. 2. Maximum concentrations of serum D-arabinitol were detected in the study of 113 people; serum from 11 of the normal subjects showed trace amounts of D-arabinitol. Colonized patients had at least two positive yeast cultures but no evidence of invasive candidiasis. The diagnosis of invasive candidiasis was established by autopsy or biopsy. Renal function status was determined in each patient.

The compound from the culture filtrate was identified by mass spectrometry and by comparison with known compounds. Samples were collected from the effluent stream of the chromatograph, the condensate was rechromatographed to demonstrate purity, and mass spectrometry was performed on a portion of the condensate (*15*). Melting points were determined on a Thomas-Hoover apparatus. Peracetate derivatives of sample components were prepared by adding acetone and internal standard, as described above. The supernatant was evaporated, and the residue was treated with a solution of acetic anhydride and pyridine (5:1) at 50°C for 4 hours (*16*). Derivatives were recovered by evaporating the reaction mixture and taking up the residue in chloroform. Peracetates were chromatographed on two columns with different liquid phases. The SE-30 column, a nonpolar silicone, was operated at 160°C with the nitrogen carrier flow rate at 40 ml/min. A second column incorporated a polar polyether phase: 10 percent Carbowax (20M) on 80/100 Supelcoport (Supelco), with 6-foot coiled glass (1/4 inch in diameter); nitrogen carrier flow rate was 100 ml/min; oven temperature, 240°C. Two peaks were considered to have the same absolute retention time when they eluted from the column at times differing by less than 1.0 percent.

Mass spectrometry was performed on a sample of the unidentified compound collected from a sonicated suspension of *C. albicans*. Chemical ionization mass spectrometry (isobutane) of the TMS ether and an underivatized specimen yielded pseudomolecular ions at mass to charge ratios of 513 and 153, respectively. The latter specimen was then taken up in deuterium oxide. This process resulted in the appearance of a new ion with an *m/e* ratio of 158, which suggested the presence of five exchangeable protons. These findings and the analysis of major ion fragments identified the compound as a five-carbon unbranched polyol or sugar alcohol (pentitol).

When this compound was compared by GLC with TMS and with peracetate derivatives prepared from authentic samples of the pentitols, the compound cochromatographed with D-arabinitol. The chromatogram of the TMS ether of D-arabinitol is shown in Fig. 1D. To further prove the compound's structure, we prepared the peracetate derivative from a concentrate of *C. albicans* culture filtrate. The recrystallized material, a sample of authentic D-arabinitol peracetate, and a mixture of the two, melted at 76°C.

D-Arabinitol was thus identified as a major cellular constitutent or metabolic product of *Candida* species. We then sought to determine the relationship between serum arabinitol concentrations and the nature and degree of *Candida* infection. We assembled 118 serum specimens from 20 patients with invasive candidiasis confirmed by autopsy or biopsy, 46 serums from 28 patients who were colonized with yeast, and one serum specimen each from 65 healthy hospital employees.

In this study, patients who had at least two positive yeast cultures from throat or sputum, urinary tract, stool, or wound but no evidence of invasive candidiasis, are termed "colonized." Seventeen of 20 patients with invasive candidiasis and 27 of 28 colonized patients had an underlying neoplastic disease. The status of renal function in each patient was determined from serum creatinine and blood urea nitrogen values, and, if available, creatinine clearance. Patients with serum creatinine values greater than 1.7 mg/dl were considered to have renal failure. The specimens were coded; TMS derivatives were prepared; and the samples were chromatographed on the SE-30 column. Assay standards were made by adding known amounts of D-arabinitol to pooled human serum. Standards containing 10.0, 5.0, 1.0, and 0.5 μg/ml were prepared and derivatized in duplicate; each duplicate was assayed four times. The highest coefficient of variation at any dilution was 0.19 μg/ml; the average was 0.15 μg/ml. The greatest difference between observed and expected values was 20 percent of the expected value; the average was 10 percent.

Meso-erythritol was originally used as the internal standard. However, Pitkanen (*17*) reported, and our experience confirmed, that this compound is present in the serum of patients in renal failure. The serums of all colonized patients with detectable D-arabinitol and of all patients with invasive candidiasis were retested with α-methyl-D-mannopyranoside as the internal standard.

Figure 2 shows the maximum serum D-

arabinitol concentrations detected in the study of 113 people. D-Arabinitol was not found in serum from 54 of the 65 normal controls, and serum from the remaining 11 normal subjects had only trace amounts of D-arabinitol, with the highest concentration at 0.2 μg/ml. Three colonized patients with renal failure had serum D-arabinitol concentrations greater than 1.0 μg/ml: the highest concentration was 2.2 μg/ml. No other colonized patient, including six others in renal failure, had serum D-arabinitol concentrations greater than 1.0 μg/ml. Maximum serum D-arabinitol concentrations exceeded 1.0 μg/ml in 15 of the 20 patients with invasive candidiasis, that is, in 11 of the 12 with renal failure and in four of the eight with normal renal function.

To provide additional data for support of the peak eluting at 9.35 minutes found in the serum of patients with invasive candidiasis, we prepared peracetate derivatives of serums from four patients with high D-arabinitol levels. These derivatives and D-arabinitol peracetate were chromatographed on two columns with different liquid phases on SE-30 and Carbowax (20M). The peak of interest in each of the serum samples cochromatographed with the D-arabinitol derivative. The retention time on Carbowax at 240°C was 13.92 minutes and on SE-30 at 160° it was 11.61 minutes.

Accurate diagnosis of invasive candidiasis is essential for effective treatment. Our results show that D-arabinitol, a metabolite of *Candida* species, can be detected in serum by GLC at concentrations greater than 1.0 μg/ml in most patients with proved invasive candidiasis.

TIMOTHY E. KIEHN*
EDWARD M. BERNARD
JONATHAN W. M. GOLD
DONALD ARMSTRONG
Diagnostic Microbiology Laboratory and Infectious Disease Service, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York 10021

References and Notes

1. M. Cline, R. P. Gale, E. R. Stiehm, G. Opelz, L. S. Young, S. A. Feig, J. L. Fahey, *Ann. Intern. Med.* **83**, 691 (1975); J. A. Krick and J. S. Remington, *Clin. Haematol.* **5**, 249 (1976); D. Armstrong, *Transplant. Proc.* **5**, 1245 (1973).
2. P. Toala, S. A. Schroeder, A. K. Daly, M. Finland, *Arch. Intern. Med.* **126**, 983 (1970).
3. P. D. Hart, E. Russell, Jr., J. S. Remington, *J. Infect. Dis.* **120**, 169 (1969); C. L. Taschdjian, P. J. Kozzinn, E. F. Toni, *Ann. N.Y. Acad. Sci.* **174**, 606 (1970).
4. G. D. Roberts and J. A. Washington II, *J. Clin. Microbiol.* **1**, 309 (1975).
5. G. Filice, B. Yu, D. Armstrong, *J. Infect. Dis.* **135**, 349 (1977).
6. N. Bloomfield, M. A. Gordon, D. F. Elmendorf, Jr., *Proc. Soc. Exp. Biol. Med.* **114**, 64 (1963).
7. N. H. Axelsen and C. H. Kirkpatrick, *J. Immun. Meth.* **2**, 245 (1973).
8. R. C. Warren, A. Bartlett, D. E. Bidwell, M. D. Richardson, A. Voller, L. O. White, *Brit. Med. J.* **1**, 1183 (1977).
9. M. H. Weiner and W. J. Yount, *J. Clin. Invest.* **58**, 1045 (1976).
10. G. G. Miller, M. W. Witwer, A. I. Braude, C. E. Davis, *J. Clin. Invest.* **54**, 1235 (1974).
11. Chemical standards were purchased from Applied Science Laboratories, State College, Pa.
12. All solvents were of spectral grade.
13. C. Sweeley, R. Bentley, M. Makita, W. Wells, *J. Amer. Chem. Soc.* **89**, 2497 (1963).
14. Reagents purchased from Aldrich Chemical Co., Milwaukee, Wis.
15. Mass spectrometry was performed by V. Parmakovitch, Chemistry Department, Columbia University, New York.
16. W. Niechermeir, *Anal. Biochem.* **40**, 466 (1971).
17. E. Pitkanen, *Clin. Chim. Acta* **38**, 221 (1972).
18. Supported by a Memorial Hospital Biomedical Research Support Grant.
* Send reprint requests to T.E.K.

9 April 1979; revised 4 June 1979

Microbodies in Germinating Fern Spores: Evidence for Glyoxysomal Activity

Abstract. *Enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase, are active during the germination of spores of the fern* Dryopteris filix-mas. *Increases in activity of both enzymes are correlated with the breakdown of lipid reserves. The occurrence of these enzymes suggests that the microbodies previously described in these spores are glyoxysomes.*

Fern spores are single-celled, haploid structures enveloped by a sculptured and impervious wall. Under appropriate conditions they can be induced to germinate and later develop into the prothallus or gametophyte. The morphological aspects of germination have been studied, and detailed descriptions of developmental events are available (*1*). Recently, however, research has been directed toward understanding the physiological and molecular mechanisms involved in spore germination (*2, 3*). The dry spore apparently contains stable messenger RNA's (mRNA's) that code for proteins essential to germination. When these stored messages are activated, hydrolytic enzymes are thought to be synthesized and to break down accumulated food reserves, thus providing the major source of carbon and energy for germination. Direct proof for the activity of hydrolytic enzymes during germination of fern spores and identity of the enzymes involved is lacking. However, in seeds of higher plants, enzymes involved in the degradation of stored materials are known, and their synthesis during germination depends on long-lived mRNA's previously transcribed during embryogenesis (*4*). Where lipid is the primary storage product the degradation of reserves in endosperm or cotyledons coincides with an increase in activity of glyoxylate cycle enzymes. These are compartmentalized in specialized microbodies, glyoxysomes, and participate in the catabolism of fatty acids and the generation of succinate that is converted to sucrose. This process of gluconeogenesis in seeds has been studied, and its importance as a primary metabolic pathway during germination is well known (*4, 5*).

Fern spores contain appreciable quantities of lipid, the amount in different species varying from 4 to 79 percent of the spore weight (*6*). The fatty acid composition of spore lipids generally is similar, qualitatively and quantitatively, to that of seed lipids. In addition, we reported the presence of microbodies in germinated spores of *Dryopteris filix-mas* (*7*), and these microbodies have been identified in spores of the fern *Polypodium vulgare* (*8*) and in the horsetail *Equisetum arvense* (*9*). The occurrence of these organelles in certain fern spores having appreciable quantities of lipid reserves suggested to us that glyoxysomal metabolism could be involved as a primary process in germination. Spore lipids might serve as food reserves, but no biochemical evidence has been presented for the involvement of the glyoxylate cycle or the participation of specific lipid-degrading enzymes. We report here that isocitrate lyase (E.C. 4.1.3.1) and malate synthase (E.C. 4.1.3.2), key enzymes of the glyoxylate cycle, are present during spore germination, and their activities coincide with the degradation of lipids.

Spores of *D. filix-mas* were collected in Hanover, New Hampshire, in July 1976 and 1977 and stored at 5°C. They were sifted through lens paper, divided into 100-mg lots, and germinated in petri dishes lined with filter paper moistened with 10 ml of water and one drop of wetting solution (Aerosol OT). Spores were maintained in a controlled room at 25°C and exposed daily to 12 hours of illumination (~ 2675 lu/m^2) provided by a combination of incandescent bulbs and Cool White fluorescent tubes. At various times germinated spores were collected, blotted between filter paper, and

0036-8075/79/1102-0580$00.50/0