The Metabolism of Alicyclic Acids and Phenylpropanoids by Cereal Rust Uredospores and the Folic Acid Components of Wheat Stem Rust Uredospores

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Finally, I dedicate this thesis to my parents for their encouragement, advice and understanding.

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ABSTRACT

Uredospores of wheat leaf rust did not take up or metabolize exogenous supplies of quinate-U-^{14}C, shikimate-U-^{14}C, p-coumarate-α-^{14}C, or ferulate-α-^{14}C. Caffeate-α-^{14}C was converted to an unidentified ether-insoluble component which was recovered from the germination medium.

Phenylalanine-U-^{14}C and tyrosine-U-^{14}C were readily taken up by uredospores and were recovered from the solvent-soluble and insoluble fractions. No evidence for phenylalanine/tyrosine interconversion was obtained. Activity was not incorporated into glycosides or free and bound esters of phenolic acids when phenylalanine-U-^{14}C or tyrosine-U-^{14}C were used as precursors. Activity was not detected in free phenolic acids when uredospores were fed phenylalanine-U-^{14}C but when tyrosine-U-^{14}C was used as a precursor, radioactive p-coumarate was detected in the germination medium kept at 20°C, and radioactive p- coumarate and caffeate were recovered from the germination medium kept at 30°C.

Wheat stem rust and wheat leaf rust uredospore extracts

contained shikimate dehydrogenase and tyrosine ammonia-lyase activity. Quinate dehydrogenase and phenylalanine ammonia-lyase activities were not detected.

Extracts of ungerminated uredospores and uredospores germinated six and twelve hours were assayed with Lactobacillus casei, Streptococcus faecalis and Pediococcus cerevisiae. The assay organisms did not respond to the extracts in the absence of conjugase treatment indicating that most of the folates were present in conjugated forms with more than three glutamic acid moieties per molecule. During the six and twelve hour germination periods the total content of L. casei growth factors declined to 70.9 and 46.0% of the initial levels in ungerminated uredospores. In this same period, the content of S. faecalis growth factors declined to 54.5 and 15.2% of the initial levels indicating increases in the proportion of methylated folates during uredospore germination.

This trend was confirmed in a detailed analysis of folate components after fractionation of the extracts on DEAE-cellulose columns. The folate profiles consisted of 5 peak fractions. Two peak fractions present in profiles from ungerminated spores contained mostly formylated folates and were absent or greatly reduced in profiles from germinated spores. A peak fraction composed of 5-methyl-$H_4PteGlu_n$ was not observed in profiles of ungerminated spores but was predominant in those from spores germinated 12 hours.

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LIST OF ABBREVIATIONS

Ci	Curie
DEAE-Cellulose	Cellulose - N, N-Diethylaminoethyl ether
$H_4PteGlu$	Tetrahydrofolic acid
$H_4PteGlu_n$	Glutamic acid conjugates of Tetrahydrofolic acid
NAD	Nicotinamide-adenine dinucleotide
NADH	Reduced nicotinamide-adenine dinucleotide
NADP	Nicotinamide-adenine dinucleotide phosphate
NADPH	Reduced nicotinamide-adenine dinucleotide phosphate
RNA	Ribonucleic acid
m-RNA	Messenger-ribonucleic acid
t-RNA	Transfer-ribonucleic acid
Tris	Tris (hydroxymethyl) aminomethane

SECTION ONE

PREAMBLE

INTRODUCTION

Uredospores are the most abundant form of the rust fungi obtainable in the absence of the host plant and for this reason have been used in the majority of studies on the physiology of these parasites. These studies have usually been conducted with three objectives in mind. These objectives are to determine the biochemical differences resulting in the specificity of different physiological races of the fungus, the contribution of the fungus to the biochemical changes observed in the infected plant and the biochemical reasons for the obligate nature of the rusts. These studies have shown that uredospores are capable of a variety of metabolic processes and much of the current research on the physiology of the rusts is now directed towards attempts to grow the rust fungi in axenic culture and to determine the lesion(s) resulting in the low rates of protein synthesis in germinating rust uredospores.

In the sections to follow, the most pertinent literature related to the physiology of the rusts is briefly reviewed. Comprehensive reviews on this subject have recently been published by Shaw (34) and Staples and Wynn (46).

REVIEW OF LITERATURE

Lipid Metabolism

Uredospores are known to contain a variety of lipid storage products (1, 15, 34, 46) in amounts approximating 20% of the spore weight (34). During uredospore germination, these endogenous lipids decrease rapidly as the glycerides are degraded and converted to intermediary metabolic products (5, 7, 24, 42). Exogenous supplies of short chain fatty acids have been shown to be rapidly degraded by mechanisms similar to those used by other microorganisms (24, 25, 26, 27, 29). These studies demonstrate that uredospores utilize storage fats as a major source of energy during germination and are able to transform acetate released by oxidation of fatty acids into many cellular constituents. The metabolism of these constituents will be considered in the following sections.

Organic Acid Metabolism

Uredospores contain a variety of organic acids, with citrate and malonate predominating (40). Except for isocitrate, all organic acids necessary for a functional citric acid cycle have been isolated from uredospores. However, isotope precursor studies indicate that intermediates of the cycle are labelled in a manner consistent with the presence of isocitrate (24, 26, 27, 29, 42, 48, 49). All enzymes necessary for the functioning of the citric acid cycle have been isolated from uredospores except for α-ketoglutamic

dehydrogenase (5, 44, 56). These results are consistent with a functional citric acid cycle and more detailed evidence for the activity of this cycle has been presented elsewhere (24, 46).

There is considerable evidence that uredospores can bypass the citric acid cycle via the glyoxylate pathway. The enzymes necessary for this cycle, isocitrase and malate synthetase, have been isolated from uredospores of Melampsora (10), Uromyces and Puccinia (5, 12). Staples (4) suggested that 20 $\pm$ 10 percent of the ^{14}C labelled isocitrate formed from radioactive acetate was metabolized by isocitric dehydrogenase and that the remaining 80 $\pm$ 10 percent of the activity was converted to the glyoxylate pathway by isocitrase. However, other workers (24, 34) have suggested that the citric acid cycle has higher rates of turnover. On the basis of activity incorporated into C-1 and C-5 of glutamic acid under steady state conditions, Reisener (34) argues that the citric acid cycle is quantitatively more important than the glyoxylate pathway in germinating spores although the percentage of isocitrate metabolized via the glyoxylate pathway increases during germination.

Uredospores have been shown to fix carbon dioxide in the dark. Staples and Weinstein (45) suggested that the internal distribution of ^{14}C in malate resulted from the action of phosphoenolpyruvate carboxylase but they could not isolate this enzyme from uredospores. Mirocha and Rick (30) were not

able to isolate phosphoenolpyruvate carboxylase from uredospores of bean rust but were able to isolate the malic enzyme from bean and cereal rust uredospores. The enzyme activity was shown to decline during uredospore germination and the reaction sequence was postulated to mediate the anapleurotic replenishment of four carbon units in uredospores (30).

Carbohydrate Metabolism

During uredospore germination, carbohydrates are probably synthesized in a manner similar to carbohydrate synthesis in germinating oil seed plants. Uredospores are able to synthesize carbohydrates from exogenously supplied precursors (5, 15, 49) suggesting that reversal of glycolysis occurs in uredospores. In addition to the synthesis of carbohydrates from non-carbohydrate precursors, a variety of endogenous carbohydrates including arabinose, fructose, mannose, ribose, xylose, trehalose, glyceratol, arabatol, mannitol and glucosamine have been isolated from uredospores (34, 46). Uredospores are able to metabolize these carbohydrates and have been shown to possess a number of enzymes for carbohydrates metabolism. All enzymes necessary for the Embden - Meyerhoff - Paranas pathway except pyruvic dehydrogenase have been isolated from stem rust and bean rust uredospores (4). Hexose - monophosphate pathway enzymes have also been demonstrated in uredospores (4, 36, 44). Low C_6/C_1 ratios in uredospores (35, 37) suggest that the hexose - monophosphate pathway predominates

in uredospores and decreases in the C_6/C_1 ratio in rust-infected plants (33) are suggestive of a shift towards the hexose - monophosphate pathway after rust infection. Uredospores have also been reported to metabolize a variety of exogenously supplied sugars in a manner consistent with the operation of these pathways (21, 37). The utilization of exogenous supplies of carbohydrates has previously been thought to be low (1, 34, 46) but recent studies by Daly, Knoche and Wiese (7) indicate that higher carbohydrate concentrations are more effectively metabolized.

Many fungi have been shown to form sugar alcohols (39) and large sugar alcohol pools composed predominantly of mannitol and aribitol have been demonstrated in rust uredospores (7, 12, 28, 38). The uredospore enzymes capable of reduction of sugars to sugar alcohols are NADH specific (13, 56) and could conceivably function in the oxidation of NADH formed by fat utilization during uredospore germination (31). This, however, does not appear to be the case as sugar alcohols are rapidly utilized during uredospore germination (7). Therefore, the high levels of sugar alcohols in uredospores appear to serve primarily as storage reservoirs of carbohydrates.

High concentrations of other sugars such as trehalose may provide a carbohydrate pool which is available for use during uredospore germination. Ungerminating uredospores of stem rust and bean rust have been shown to convert glucose into glucomannan polysaccharides (58). Bean rust uredospores

are able to convert carbohydrates into α-linked glucomannan proteins during the first two hours of germination (57). This material decreases later in germination and appears to form a temporary carbohydrate reservoir for use during later stages of germination.

During germination of uredospores deposition of new cell wall material is evident and large increases in chitin have been observed in germinated uredospores (38). In the initial stages of germination the soluble carbohydrate pools present in uredospores probably provide precursors for chitin synthesis and for pentose polymers present in germ tube cell walls (17). Synthesis of the chitin precursor, glucosamine, from ^{14}C labelled precursors has been reported in germinating uredospores (24, 49). Uridine diphosphate-N-acetylglucosamine is known to participate in cell wall synthesis in fungi (6) and is the predominate nucleotide in uredospores of stem rust and flax rust (9, 23). This component is not present in uninfected wheat leaves but increases steadily after rust infection (23) and probably reflects increasing fungal cell wall synthesis as infection progresses.

Electron Transport

Oxidation of fatty acids is known to result in production of NADH and the formation of large amounts of this pyridine nucleotide would be expected as the result of degradation of lipid reserves during uredospore germination. However, Rohringer

(31) has found that the ratios of NADH/NAD are similar in uredospores prior to and after 6 hours germination. Since NAD levels did not increase, uredospores must oxidize NADH formed during germination.

Allen (2) first identified cytochrome oxidase in uredospore extracts. Subsequent studies have substantiated this report and electron transport systems capable of oxidizing pyridine nucleotides have been isolated from uredospores of bean rust (4) and stem rust (4, 56). After germination the activities of cytochrome oxidase, succinic cytochrome C-reductase and $NADH_2$ cytochrome C-reductase increased (56). From cell free studies, White and Ledingham (56) suggested that uredospores have an electron transport system similar to that of other fungi and the stability of NADH/NAD ratios during germination (31) indicates the operation of an efficient system in uredospores.

Protein and Nucleic Acid Metabolism

Studies comparing germinating uredospores with a number of saprophytic fungi have indicated that protein synthesis does not occur to the same extent in uredospores as in conidia of saprophytic fungi (24, 25, 41). Although there is little protein synthesis, pectinase and mucilaginase may be induced in uredospores germinating in the presence of appropriate substrates (54). Further studies have demonstrated alterations in the isozymic composition of acid phosphatase,

cytochrome oxidase, malate dehydrogenase and succinic dehydrogenase during uredospore germination and showed that isozymes of acid phosphatase increased while other enzymes decreased (44). Such synthesis is apparently at the expense of pre-existing protein and results in protein turnover but net protein synthesis is not detected (24).

Failure of uredospores to synthesize protein may be only symptomatic of other deficiencies. For instance, Reisener (24) has shown that synthesis of a number of amino acids is sluggish in uredospores. He suggests that extremely low synthetic rates of one or more amino acids may restrict protein synthesis.

Staples (41) suggested that inhibited protein synthesis of uredospores may be the result of aberrent nucleic acid synthesis. Uredospores of bean rust have ribosomes with sedimentation properties similar to ribosomes of yeast and higher plants (47) and these ribosomes have been shown to function in in vitro protein synthesizing systems (50, 60). Uredospores also appear to possess activating enzymes (43), t-RNA and m-RNA and ribosomes have also been isolated from bean rust uredospores and shown to be capable of protein synthesis in the absence of synthetic m-RNA (47). Ribosomes that appeared to be membrane bound were also isolated from ungerminated uredospores (47).

The in vitro activity of m-RNA and ribosomes isolated from uredospores remains constant during the first four hours of uredospore germination but activity in both fractions de-

creases rapidly after this time (41). Further studies have shown that the ribosomal levels follow similar trends (60). These studies also showed that levels of monosomes and polyribosomes decreased at similar rates. The ability of microsomes to direct in vitro protein synthesis doubled during 16 hours of uredospore hydration, remained stable for two hours after germination and slowly declined (60).

These studies leave little doubt that uredospores have a full protein synthesizing complement. They also imply that internal controls of protein synthesis are functional in uredospores. Lower levels of ribosomes and decreases in the ability of m-RNA to program protein synthesis suggests that undifferentiated uredospores may not synthesize nucleic acid.

In recent years, a number of externally supplied stimuli have been shown to induce development of infection structures in rust uredospores (18, 34). Nuclei of uredospores germinating on collodion films have been observed to divide just prior to and during the process of development of infection structures. This strongly suggests that nucleic acid synthesis occurs during development of infection structures. Studies of the effects of RNA and protein synthesis inhibitors on stem rust uredospores suggests that RNA and protein synthesis is necessary for formation of infection structures (8). The differential effects of these inhibitors also suggests that RNA synthesis precedes protein synthesis and is initiated as the result of external environmental stimuli.

Protein synthesis by uredospores therefore appears to be internally regulated. Once these regulatory mechanisms are understood, the rusts may possibly be grown in culture with little difficulty. The practical implications of *in vitro* culture of the rusts are obvious and it is largely to this end that many physiological studies of rust uredospores have been directed for the past 15 years.

RESEARCH OBJECTIVES

The present study considers two different aspects of uredospore physiology. The aromatic metabolism of uredospores of leaf rust and stem rust was investigated in one phase of the study. In the other phase, the folate constituents of wheat stem rust uredospores were identified.

The metabolism of aromatic compounds in the host-parasite relationship has been studied by Rohringer et al (32) and by Fuchs et al (11) but the metabolism of these compounds by the rust fungus was not investigated. The aromatic metabolism of uredospores has received scant attention and nothing is known of the synthesis and degradations of these compounds by rust fungi. Van Sumere et al (55) characterized the coumarins and phenolic acids of uredospores of stem rust, but, the levels recovered from the spores were so low that quantitative determinations were not made. Four benzoic acids, five cinnamic acids, and five coumarins were identified and found to be present in the free form and/or bound as glycosides. The present studies on aromatic metabolism were thus undertaken to obtain information on the metabolism of aromatic compounds by the rusts and to assess the role of the parasite in the metabolism of aromatic compounds by rust infected wheat.

The varied metabolic activities of the rust fungi were discussed in the literature review. Uredospores are capable of a variety of biosynthetic processes and even contain a functional

system for protein synthesis. This may be true of many obligate parasites, for instance, the malarial parasites, Plasmodium spp., appear to have similar biochemical properties (3). However, the malarial parasites appear to be unable to form coenzyme A or folic acid coenzymes and it has been suggested that these defects together with permeability defects may account for the obligate nature of these organisms (3).

The rapid degradation of lipids during uredospore germination and the high activity of the citric acid cycle suggests that coenzyme A is functional at least during initial stages of uredospore germination. Leaching of spore constituents from uredospores is known to occur (7, 16) but is paralleled by leakiness of spores of saprophytic fungi (1). Uredospores of leaf rust are known to have levels of folic acid similar to the amounts found in wheat tissue (14), however, the folate composition of uredospores is not known. Studies on the identity of the folate constituents of uredospores of stem rust and changes occurring in the folate composition during uredospore germination were initiated in order to help evaluate the role of the folic acid coenzymes in the rusts and the contribution of rust folates to the folate profiles observed in the host-parasite complex.

The results of these studies are presented in the following two sections. Because of publication costs and limited journal space concise writing and brevity of form is becoming increasingly essential. A major criticism of

graduate programs is that the young professional scientists graduating from these programs have not received sufficient training to present scientific papers in an acceptable form. For this reason, these studies are presented in essentially the same form as that required for submission to a scientific journal. In addition, a speculative discussion of the type encouraged in a thesis is presented in the general discussion.

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SECTION TWO

METABOLISM OF ALICYCLIC ACIDS AND PHENYLPROPANOIDS BY WHEAT STEM RUST AND WHEAT LEAF RUST UREDOSPORES

ABSTRACT

Uredospores of wheat leaf rust did not take up or metabolize exogenous supplies of quinate-U-^{14}C, shikimate-U-^{14}C, p-coumarate-α-^{14}C, or ferulate-α-^{14}C. Caffeate-α-^{14}C was converted to an unidentified ether-insoluble component which was recovered from the germination medium.

Phenylalanine-U-^{14}C and tyrosine-U-^{14}C were readily taken up by uredospores and were recovered from the solvent-soluble and insoluble fractions. No evidence for phenylalanine/tyrosine interconversion was obtained. Activity was not incorporated into glycosides or free and bound esters of phenolic acids when phenylalanine-U-^{14}C or tyrosine-U-^{14}C were used as precursors. Activity was not detected in free phenolic acids when uredospores were fed phenylalanine-U-^{14}C but when tyrosine-U-^{14}C was used as a precursor, radioactive p-coumarate was detected in the germination medium kept at 20°C, and radioactive p-coumarate and caffeate were recovered from the germination medium kept at 30°C.

Wheat stem rust and wheat leaf rust uredospore extracts contained shikimate dehydrogenase and tyrosine ammonia-lyase activity. Quinate dehydrogenase and phenylalanine ammonia-lyase activities were not detected.

INTRODUCTION

Although the pathway of aromatic biosynthesis (5) and degradation (26) is well established in certain fungi, little is known of the synthesis and interconversion of aromatic compounds in rust fungi. Ungerminated uredospores of wheat leaf rust contain small amounts of shikimate (6) and germinating uredospores of stem rust can utilize glucose-U-^{14}C as a precursor for the synthesis of phenylalanine and tyrosine (16). This suggests that uredospores can synthesize aromatic compounds via the shikimate pathway.

A number of phenolic acids and coumarins have been isolated in the free form and as bound glycosides from ungerminated uredospores of wheat stem rust (28). However, nothing is known of the processes by which uredospores metabolize phenolic compounds.

This study was undertaken to obtain further information on the metabolism of aromatic compounds by the rust fungus. Since uredospores are the most readily available source of the fungus, the study was conducted by administering radioactive substrates to germinating uredospores and by determination of enzyme activities in cell free systems.

MATERIALS AND METHODS

Rust Fungi and Feeding of Radioactive Material

Uredospores of race 56 of stem rust (*Puccinia graminis* Pers. f. sp. *tritici* Erikss. & Henn.) and race 15 of leaf rust (*P. recondita* Rob. ex. Desm. f. sp. *tritici*) were collected from greenhouse grown plants. The spores were subsequently sieved through an 80 mesh screen and stored at 3°C for periods not exceeding 4 months.

Before feeding of radioactive materials, the uredospores were hydrated for 12 hours in a water saturated atmosphere and 250 mg lots of uredospores were suspended in 10 ml of 0.1% Tween 20 and centrifuged (22). The supernatant was decanted and the procedure repeated twice with sterile distilled water. This procedure effectively completed hydration of uredospores, minimized aggregation of spores and removed endogenous germination inhibitors.

Immediately after the washing procedure, the uredospores were suspended in 100 ml of sterile water containing coumarin (0.5mg/ml) and placed in 19 cm diameter dishes. Radioactive substrates were then added, the dishes were sealed with parafilm sheets and agitated in a rotary shaker (0.75" off center) at 55 rpm for 6 hours in darkness at either 20°C or 30°C. Under these conditions 60% germination occurred at 20°C but less than 10% germination occurred at 30°C.

Extraction and Fractionation

After the 6 hour metabolic period, the suspensions were mixed with 2 g of Celite analytical filter aid (Canadian Johns-Manville Co., Port Credit, Ontario) and filtered to separate the uredospores from the germination medium. The uredospores were rinsed 4 times with 25 ml aliquots of distilled water, the rinse water was combined with the germination medium and the combined extracts taken to dryness at 40°C. After rinsing, the uredospores were placed in a 50 ml homogenization flask containing 10 gm of 0.95 - 1.00 mm glass beads and 10 ml of cold acetone. The flask was immediately placed in a Braun M.S.K. homogenizer (Bronwill Scientific, Rochester, N.Y.), and shaken at 4,000 oscillations/min for 5 min at -20°C as described by Rick et al (17).

The contents of the flask were centrifuged and the insoluble material was washed by successive centrifugations with acetone, cold 90% aqueous methanol, cold 80% ethanol, and 80% aqueous ethanol at 78°C. All supernatants were combined and concentrated in vacuo together with 2 g Celite analytical filter aid. This fraction was resuspended in water and an aqueous extract of the solvent soluble components was made as previously described (18).

The fractionation procedure outlined by Fuchs et al (4) was used to separate sugars, organic acids, amino acids, free phenolic acids, soluble esters and glycosides present in the germination medium and the solvent-soluble fraction obtained

from uredospores fed phenylalanine-U-^{14}C and tyrosine-U-^{14}C. The insoluble material was also fractionated as previously described (4) to recover insoluble esters and bound phenylalanine and tyrosine. In experiments where $^{14}CO_2$ was collected, a center well containing 0.2 ml of 20% KOH was added to the feeding dish. The dish was then sealed with lanolin and a 20 cm watch glass.

The insoluble material was combusted by the van Slyke and Folch wet ashing procedure (18) or with perchloric acid and hydrogen peroxide (9). Radioactivity in the combusted fractions was then determined with a Nuclear Chicago Scintillation Spectrometer with Toluene Triton-X-100 as a scintillator fluid (14).

Paper and Thin-Layer Chromatography

Unidimensional ascending paper chromatography on Whatman #1 paper with the solvent system n-butanol/acetic acid/water (4:1:5) was used to separate amino acids. The phenolic acids were chromatographed in a descending system using benzene/acetic acid/water (125:72:3) as described by Steck (23). Thin layer chromatography on plates prepared with silica gel G and cellulose powder (MN-300, purchased from Macherey, Nagel and Co., Duren, Germany) according to the procedure of van Sumere (29) was also used to separate phenolic acids. Solvent systems used were benzene/acetic acid/water (125:72:3), n-butanol/pyridine/water (14:3:3), chloroform/ethyl acetate/

formic acid (2:1:1), chloroform/acetic acid/water (4:1:1) and toluene/ethyl formate/formic acid (5:4:1). Quinate and shikimate were separated on 250 μ MN-cellulose layers with benzyl alcohol/tertiary butanol/isopropanol/water/formic acid (3:1:1:1:0.07) as the solvent system or on Whatman #1 paper with n-butanol/acetic acid/water (40:20:22). The n-butanol extractives were chromatographed two-dimensionally as previously described (18).

Enzyme Assays

Uredospores were disrupted in acetone with the Braun mechanical cell homogenizer as previously described and an acetone powder was prepared. This powder was then suspended in an appropriate buffer for enzyme assay and the buffer insoluble materials were removed by centrifugation. Acetone powders were also prepared from primary leaves of 7-day old greenhouse grown wheat seedlings (var. Little Club) and enzyme extracts were prepared as described for rust uredospores. Aliquots of the unfractionated enzyme extracts were stored in liquid nitrogen until needed. To determine whether enzyme inactivation occurred during extraction, unfractionated uredospore extracts were assayed for malate dehydrogenase activity. Activity in the extracts was comparable to published data (17), indicating that malate dehydrogenase and presumably other enzyme systems of similar stability survived the extraction procedure.

Shikimate dehydrogenase was assayed by measuring the increase in optical density at 340 mμ (27). The assay system contained 9.0 μ moles of shikimate, 1.0 μ mole of NADP, 160 μ moles of Tris-HCl buffer and the enzyme preparation in a final volume of 3.0 ml. The extracts were tested for activity at pH 7.5, 8.5 and 9.5 at 20°C. Quinate dehydrogenase was assayed at pH 9.5 in a similar system, except that quinate was substituted for shikimate and NAD was substituted for NADP.

Phenylalanine and tyrosine ammonia lyase activity was measured by determining the utilization of radioactive precursors (24). The assay systems contained 360 μ moles of Na-borate buffer (pH 8.8) or 100 μ moles of Tris-HCl (pH 8.8), 200 nanomoles of reduced glutathione and 1 μCi (2.0 nanomoles) of L-phenylalanine-U-^{14}C or 1 μCi (3.5 nanomoles) of L-tyrosine-U-^{14}C, and enzyme, in a final volume of 4.0 mls. After 6 hours incubation at 40°C the reaction mixture was adjusted to pH 2.0 with HCl and extracted with peroxide free ether. The ether phase was concentrated, the radioactivity in the fraction determined, and products were identified chromatographically as previously described. Tyrosine ammonia lyase activity was also determined spectrophotometrically as described by Neish (13). The enzyme system consisted of 5 μ moles of tyrosine, 100 μ moles of Tris-HCl (pH 8.8), 200 nanomoles of reduced glutathione and enzyme extract in a final volume of 4.0 ml. The mixture was incubated at 37°C for 6 hours and extracted with ether. The ether soluble materials were concentrated

and taken up in 0.05 N NaOH. The identity of the product was confirmed by comparing the absorption spectrum of the ether soluble component with the absorption spectrum of authentic p-coumarate. Quantitative determination of the p-coumarate was made by measuring the optical density at 333 mμ.

In order to determine the amount of bacterial contamination, wheat leaf rust uredospores were washed as previously described, diluted to appropriate levels and placed on a nutrient medium composed of 2% sucrose, 0.5% yeast extract, 2% agar in 1 liter of aqueous extract from 200 g of boiled potatoes. After seeding with uredospores, the media were incubated at 30°C for 24 hours and the number of bacterial colonies were recorded. Bacterial buildup during uredospore germination was determined in the same way by incubating uredospores germinated under the conditions used during feeding of radioactive substrates.

Chemicals and radiochemicals

Quinate-U-^{14}C (5.0 mCi/mM) and shikimate-U-^{14}C (8.4 mCi/mM) were purchased from New England Nuclear Corporation, Boston, Mass., U.S.A.

Purchases of L-phenylalanine-U-^{14}C (504 mCi/mM) and L-tyrosine-U-^{14}C (288 mCi/mM) were made from the Radiochemical Center, Amersham, England. Approximately 1% of the activity of the L-phenylalanine-U-^{14}C preparation was present as ether-soluble contaminants. When applicable, appropriate corrections

were made to account for these radiochemical impurities.

p-Coumarate-α-^{14}C (950 μCi/mM) caffeate-α-^{14}C (850 μCi/mM) and feruliate-α-^{14}C (630 μCi/mM) were kindly provided by Drs. W. Steck and L.R. Wetter (Prairie Regional Laboratory, Saskatoon, Saskatchewan). All other chemicals were of the best quality commercially available.

RESULTS

Quinate and Shikimate Feeding

When wheat stem rust uredospores were germinated in the presence of 1 μCi of shikimate-U-^{14}C at 20°C, over 99% of the radioactivity was recovered in the germination medium. Similar results were observed when wheat leaf rust uredospores were fed 0.5 μCi of quinate-U-^{14}C or shikimate-U-^{14}C. Paper and thin layer chromatography of the germination media indicated that interconversion of shikimate and quinate did not occur. Activity in phenylalanine or tyrosine was not detected.

Quinate and Shikimate Dehydrogenase Activity

Quinate dehydrogenase activity was not detected in either uredospore extracts or extracts from primary leaves of wheat. However, extracts from uredospores of leaf rust and stem rust catalyzed the conversion of 7.5 and 6.0 nanomoles, respectively, of shikimate to dehydroshikimate per minute per mg of protein at pH 9.5. Extracts of primary leaves of wheat contained a more active shikimate dehydrogenase (43.8 nanomoles per mg protein) than uredospores. Inhibitors of shikimate dehydrogenase were not present in the uredospores since uredospore extracts did not inhibit the shikimate dehydrogenase activity of the leaf extracts. $NADPH_2$ oxidase activity was not detected in uredospore extracts nor was reduction of NADP observed in the absence of shikimate. Shikimate dehydrogenase activity of uredospore extracts was similar at pH 9.5 and pH 8.5

but activity decreased by 50% at pH 7.5.

Distribution of Radioactivity Derived from L-phenylalanine-U-^{14}C and L-tyrosine-U-^{14}C

Table I summarizes the activities recovered from the germination medium, solvent-soluble and insoluble fractions of wheat leaf rust uredospores fed 2 μCi of L-phenylalanine-U-^{14}C and L-tyrosine-U-^{14}C. These data indicate that significant uptake of both phenylalanine-U-^{14}C and tyrosine-U-^{14}C occurred during the six hour germination period. Comparisons of uptake of the two amino acids were not made from the data presented in Table I because of the presence of metabolic products of the administered amino acids in the germination medium. Free phenolic acids accounted for 22.7% of the activity recovered in the germination medium of uredospores fed tyrosine-U-^{14}C for 6 hours at 30°C. Activity in this fraction may indicate leaching of tyrosine-U-^{14}C derived phenolic acids from the spores or, alternatively, it may indicate extracellular metabolism of tyrosine-U-^{14}C.

When uredospores germinated at 15°C were fed phenylalanine-U-^{14}C and harvested at 2, 4 and 6 hours, 8.8, 40.0 and 76.9% respectively of the total activity was taken up from the germination medium.

The distribution of activity recovered from germination media is presented in Table II. The amino acid fractions contained most of the activity and all of this resided in un-

metabolized precursor. Feeding of tyrosine-U-^{14}C did, but feeding with phenylalanine-U-^{14}C did not give rise to activity in the fraction of free phenolic acids. The germination medium from spores fed tyrosine-U-^{14}C held at 20°C contained p-coumarate as the only labelled phenolic acid. When held at 30°C during the germination period, it also contained caffeate. Identity of these components was determined by cochromatography with authentic compounds in 5 solvent systems.

Most of the radioactivity in the uredospore solvent-soluble and hydrolized insoluble fractions was isolated as phenylalanine and tyrosine. The major portion of the activity in the insoluble fraction of uredospores fed phenylalanine-U-^{14}C was released after pronase treatment, as well as by acid hydrolysis, suggesting that activity in the insoluble fraction was bound as protein.

When uredospores were fed tyrosine-U-^{14}C at 1°C more than than 99% of the activity was recovered from the germination medium after 6 hours. When uredospores were germinated for 4 hours or 7.5 hours at 20°C and subsequently exposed to phenylalanine-U-^{14}C at 3°C for 3.5 hours 86% and 95% respectively of the total activity fed was recovered from the germination medium. These results suggest that phenylalanine and tyrosine are not appreciably absorbed to uredospores or germ tubes and that uptake of these amino acids is an energy requiring process.

Conversion of phenylalanine to tyrosine could not be detected indicating that tyrosine is not formed via hydroxyla-

tion of phenylalanine in germinating uredospores.

Wheat stem rust uredospores were fed phenylalanine-U-^{14}C and tyrosine-U-^{14}C at 20°C as previously described. The uredospores were disrupted and extracted with hot water (90°C). Results indicated that uptake and conversion of the amino acids was similar to those in wheat leaf rust uredospores.

Phenylalanine and Tyrosine Ammonia Lyase Activity

When wheat leaf rust and wheat stem rust uredospore extracts were assayed for ammonia-lyase activity, tyrosine ammonia-lyase but not phenylalanine ammonia-lyase was detected. Leaf rust and stem rust tyrosine ammonia-lyase activity was 2.3 and 4.5 milli-units (an enzyme unit was defined as 0.01 uM p-coumaric acid formed per hour per mg protein). Purification of the extracts would have been desirable to determine if low phenylalanine ammonia-lyase activity was associated with the tyrosine ammonia-lyase but fractionation was impractical because of the quantity of uredospores required for purification of an enzyme of such low activity.

Levels of Uredospore Bacterial Contaminants and the Contribution of these Bacteria to the Metabolism of Tyrosine-U-^{14}C

Uncertainties regarding the level of bacterial contamination of uredospores raised doubts as to whether the results of the amino acid feedings were due to uredospore or bacterial metabolism. Counts of leaf rust uredospore bacterial contaminants indicated that the level of contamination did not

increase throughout the six hour germination period and was not greater than 2.0×10^4 bacteria per mg of leaf rust uredospores. Two hundred and fifty mg lots of uredospores were suspended in 0.1% Tween 20 (100 ml) and a bacterial suspension was obtained by filtration. The filtrate was added to autoclaved or disrupted uredospores in the presence of tyrosine-U-^{14}C for 6 hours as previously described. Uptake and conversion of tyrosine-U-^{14}C to free phenolic acids could not be detected. When large numbers of rapidly growing bacteria (O.D. = 0.10 at 660 mμ) were allowed to metabolize tyrosine-U-^{14}C in a nutrient medium for 6 hours at 30°C, uptake of tyrosine-U-^{14}C was observed but conversion of this amino acid to p-coumarate or caffeate could not be detected. This leaves little doubt that the effects observed in feeding experiments with tyrosine-U-^{14}C were caused by uredospores and not by bacteria associated with them.

Distribution of Radioactivity Derived from p-Coumarate-α-^{14}C, Caffeate-α-^{14}C and Ferulate-α-^{14}C

Distribution of activity in the various fractions from wheat leaf rust uredospores germinating at 30°C and fed 0.4 μCi of p-coumarate-α-^{14}C, caffeate-α-^{14}C and ferulate-α-^{14}C is presented in Table III. The activity from p-coumarate and ferulate feeding was recovered as unmetabolized precursor when the germination medium was extracted with ether. These results show that the uredospores did not take up these compounds or

convert them to other products with extra cellular enzymes.

Distribution of activity after feeding of caffeate differed from that observed after feeding p-coumarate and ferulate. After ether extraction of the germination medium, 75% of the activity remained in the aqueous phase (Table III). When uredospores were germinated at 20°C equal amounts of activity were present in the aqueous and ether phase.

Results similar to those obtained with germinating uredospores at 30°C were observed when homogenized uredospores were incubated with caffeate-α-^{14}C under similar conditions. In contrast, when autoclaved uredospores were exposed to caffeate, 90% of the activity fed was recovered in the ether fraction (Table IV).

The radioactive compounds in the aqueous phase were insoluble in n-butanol indicating that they are not glycosidic. The activity also did not appear to reside in ester components because the radioactive metabolites were stable to alkaline hydrolysis. The radioactive components were absorbed on columns of Amberlite IR-120 and could not be removed with NH_4OH at concentrations as high as 5N. The radioactive compounds were not mobile in electrophoretic systems at pH 2.0 or 7.0 or in chromatographic systems using benzene/acetic acid/water or chloroform/ethyl acetate/formic acid as solvents. The unknown material may consist of condensation polymers of caffeate moeties. However, such polymers must be relatively small because they are dialyzable.

DISCUSSION

Because of technical difficulties sterile spores have seldom been used in physiological studies of the rusts. Levels of bacterial contaminants vary with uredospores from various sources (3) and have been shown to convert uredospore metabolic products to other substances (19). Thus the results of studies where the contribution of bacterial contaminants was not known must be regarded with some doubt. For this reason, the levels of bacteria associated with uredospores were investigated and were demonstrated not to contribute to the effects observed in this study.

Germinating uredospores of stem rust are known to synthesize phenylalanine and tyrosine from glucose (16). In most microorganisms (5, 8) and higher plants (30) these amino acids are synthesized via the shikimate pathway. Limited amounts of shikimate have been isolated from uredospores (6) and, in the present study, shikimate dehydrogenase was detected in extracts from uredospores. Quinate dehydrogenase was not detected in extracts from uredospores but this enzyme may be very labile since it has not been found in wheat (25). While the evidence is not conclusive it does suggest that the shikimate pathway is operative in uredospores.

The inability of rust uredospores to take up exogenously supplied shikimate-U-^{14}C or quinate-U-^{14}C precluded isotope tracer studies of synthesis of aromatic compounds from

these alicyclic acids. In addition, the low levels of shikimate isolated from ungerminated uredospores of leaf rust (6) may indicate that this compound is not readily taken up by rust mycelium in the infected host. Poor shikimate uptake, however, is not restricted to the rusts, as some Streptomyces isolates are unable to take up this compound (1). Ungerminated uredospores of stem rust also contain very low levels of phenolic acids (28) and also failed to take up exogenously supplied radioactive phenolic acids.

Both phenylalanine-U-^{14}C and tyrosine-U-^{14}C were readily taken up by uredospores and most of the activity taken up was present in the insoluble fraction. It is possible that these amino acids were incorporated into protein but the data must be interpreted with caution because incorporation of other exogenously supplied amino acids into uredospore protein is known to be low (22) and protein synthesis is restrained by mechanisms not yet explained (21). Shu et al (20) reported that compared to other amino acids phenylalanine-U-^{14}C was readily taken up and incorporated into spore carbon by uredospores of wheat stem rust but the values reported were much lower than those observed in the present study. These differences can be attributed to differing environmental conditions and to widely varying specific activities of precursors.

Inability of rust uredospores to hydroxylate phenylalanine is consistent with the results of Fuchs et al (4) for rust-infected wheat leaves. Conversion of phenylalanine to

tyrosine has been reported for a number of microorganisms including bacteria, phycomycetes, ascomycetes and fungi imperfecti but was not detected in yeasts and basidiomycetes and may be rare in these organisms (2).

Higher plants synthesize cinnamic acid derivatives from phenylalanine and tyrosine but bacteria, algae and animals generally degrade phenylalanine and tyrosine via phenylpyruvate and homogentisate (25). Some basidiomycetes, unlike most microorganisms, are able to degrade phenylalanine to cinnamate derivatives (9, 11, 14) but of three basidiomycetes examined, only Sporobolomyces was able to convert both phenylalanine and tyrosine to such compounds (10). The Uredinales share with other basidiomycetes the ability to metabolize p-coumarate but they are the only group of fungi known to degrade tyrosine but not phenylalanine to cinnamate derivatives and to possess tyrosine ammonia-lyase but not phenylalanine ammonia-lyase.

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Table I

Distribution of activity among fractions after feeding of L-phenylalanine-U-^{14}C and L-tyrosine-U-^{14}C to wheat leaf rust uredospores. Germination and feeding period was 6 hours (Data expressed as percentage of activity fed)

Administered Precursor and germination temperature	Germination medium	Uredospore		
		Solvent-Soluble	Insoluble	Total
L-Phenylalanine-U-^{14}C				
20°C	5.4	10.7	75.3	91.4
30°C	24.1	28.4	40.5	93.0
L-Tyrosine-U-^{14}C				
20°C	14.6	32.8	45.0	92.4
30°C	28.0	20.0	42.8	90.8

Table II

Distribution of radioactivity in the germination medium of wheat leaf rust uredospores fed L-phenylalanine-U-^{14}C and L-tyrosine-U-^{14}C for 6 hours (Data expressed as percentage of activity fed)

Administered precursor and germination temperature	Sugars and Organic acids	Amino Acids	Free Phenolic Acids	Soluble Esters	Total
L-Phenylalanine-U-^{14}C					
20°C	0.2	2.8	-*	0.2	4.3
30°C	0.3	23.1	-	0.1	23.5
L-Tyrosine-U-^{14}C					
20°C	0.8	12.4	2.1	0.3	15.6
30°C	0.2	18.6	6.3	0.3	25.4

*Corrected for free phenolics present as contaminants in phenylalanine-U-^{14}C

Table III

Distribution of activity among fractions after feeding* p-coumarate-α-^{14}C, caffeate-α-^{14}C and ferulate-α-^{14}C to wheat leaf rust uredospores

(Data expressed as percentage of activity fed)

Substrate	Germination medium		Uredospore solvent-soluble	Total
	Aqueous Phase	Ether Phase		
p-Coumarate-α-^{14}C	0.6	86.0	3.6	90.2
Caffeate-α-^{14}C	75.0	16.7	0.5	92.2
Ferulate-α-^{14}C	3.5	91.0	1.5	96.0

*germination and feeding period 6 h at 30°C

Table IV

Activity in ether-soluble and ether-insoluble compounds after feeding of caffeate-α-^{14}C to germinating uredospores, homogenized uredospores, or autoclaved uredospores of the wheat leaf rust fungus for 6 hours (Data expressed as percentage of activity fed)

Treatment	Aqueous Phase	Ether Phase	Total
Uredospores			
germinated at 20°C	47.4	46.4	93.8
germinated at 30°C	75.0	16.7	91.7
Homogenized uredospores			
30°C	69.5	25.0	94.5
Autoclaved uredospores			
30°C	0.9	95.3	96.2

SECTION THREE

FOLATE DERIVATIVES IN UNGERMINATED AND GERMINATED WHEAT STEM RUST UREDOSPORES

ABSTRACT

Extracts of ungerminated uredospores and uredospores germinated six and twelve hours were assayed with Lactobacillus casei, Streptococcus faecalis and Pediococcus cerevisiae. The assay organisms did not respond to the extracts in the absence of conjugase treatment indicating that most of the folates were present in conjugated forms with more than three glutamic acid moieties per molecule. During the six and twelve hour germination periods the total content of L. casei growth factors declined to 70.9 and 46.0% of the initial levels in ungerminated uredospores. In this same period, the content of S. faecalis growth factors declined to 54.5 and 15.2% of the initial levels indicating increases in the proportion of methylated folates during uredospore germination.

This trend was confirmed in a detailed analysis of folate components after fractionation of the extracts on DEAE-cellulose columns. The folate profiles consisted of 5 peak fractions. Two peak fractions present in profiles from ungerminated spores contained mostly formylated folates and were absent or greatly reduced in profiles from germinated spores. A peak fraction composed of 5-methyl-$H_4PteGlu_n$ was not observed in profiles of ungerminated spores but was predominant in those from spores germinated 12 hours.

INTRODUCTION

The limited saprophytic growth of the rust fungi could be due to an inability to synthesize some essential metabolite in the absence of the host. Comparative studies have shown that protein and nucleic acid synthesis does not occur to the same extent in uredospores as in conidia of saprophytic fungi (28). Although protein synthesis is sluggish, pectinase and mucilaginase may be induced in uredospores germinating in the presence of appropriate substrates (31). Further studies have demonstrated alterations in the isozymic composition of acid phosphatase, cytochrome oxidase, malate dehydrogenase and succinic dehydrogenase during uredospore germination. The isozymes of acid phosphatase increased while other isozymes decreased (27). Such synthesis is apparently at the expense of preexisting protein and results in protein turnover but no net protein synthesis (18). Isotope tracer studies under steady state conditions showed that the limited turnover rates decline during uredospore germination (19).

Staples (26) suggested that inhibition of protein synthesis may result from an inability of the germinating uredospore to maintain the synthesis and utilization of messenger RNA. Additionally, extremely low synthetic rates of amino acids such as serine may also restrict protein synthesis (19).

Folate compounds are known to be essential co-factors in the synthesis of purines, thymine, methionine and also

in the serine-glycine interconversion (30). Of particular interest to the present work are the reports on folate-mediated synthesis of N-formyl-methionyl-tRNA and the function of this compound in initiation of protein synthesis (16). None of these reactions have been investigated in uredospore material but it is possible that one of the folate-mediated reactions is not operating in rust spores prior to contact with the host and that as a consequence uredospores are unable to initiate or maintain synthesis of nucleic acids and proteins. Rohringer et al (22) have reported the distribution and identities of folates in healthy and rust-infected wheat leaves. Total folates in ungerminated wheat leaf rust uredospores were reported by Hollomon et al (7). In the present study the identity and concentration of folates in ungerminated and germinated wheat stem rust uredospores has been determined.

Designations and abbreviations of folate derivatives are essentially as recommended by the IUPAC Commission for the Nomenclature of Biological Chemistry (J. Biol. Chem. 241, 2991-2992 (1966)).

MATERIALS AND METHODS

Plant Material

Wheat stem rust (*Puccinia graminis* (Pers.) f. sp. *tritici* Erikss, & Henn.) uredospores, race 56, were collected from greenhouse-grown wheat (*Triticum aestivum* L.). The uredospores were either used immediately or stored in liquid nitrogen until needed.

Uredospore Germination

Uredospores were removed from liquid nitrogen storage and their germinability restored by exposure to a temperature shock similar to that described by Loegering and Harmon (14). The uredospores (400 mg) were then evenly dispersed on the surface of 4 liters of sterile distilled water in a 18 x 36" sink. The sink was sealed with a glass cover to prevent atmospheric exchange and the uredospores were exposed to vapors from a 1 liter solution of 10^{-4}M nonal aldehyde (21). Light was excluded from the sink for the duration of the germination period in order to allow maximum uredospore germination. After 1.5 hours at 20°C, germination was observed to be in excess of 99%.

Extraction

Ungerminated and germinated uredospores were extracted by the method of Silverman *et al* (25). The uredospores (400 mg) were transferred to a 50 ml homogenization flask containing

10 ml of 1% potassium ascorbate solution (pH 6.0) and 20 g of glass beads (0.95 - 1.00 mm diameter). The homogenization flask was immediately transferred to a Braun M.S.K. mechanical cell homogenizer (Bronwill Scientific, Rochester, N.Y.) and homogenized at 0°C as described by Rick et al (20). Visual observation showed that more than 99% of the uredospores were disrupted after this treatment. The homogenate was subsequently transferred to a flask containing 2.5 g washed Celite analytical filter aid and aqueous extracts were obtained as previously described (22). In experiments with germinated uredospores, the germination medium was also assayed. The germination medium (800 ml) was concentrated with a flash evaporator and made to a final volume of 10 ml with 1% potassium ascorbate (pH 6.0). All extracts were stored in liquid nitrogen until assayed for folate derivatives.

Assay Procedure

Lactobacillus casei (ATCC 7469), Streptococcus faecalis (ATCC 8043) and Pediococcus cerevisiae (ATCC 8081) were used to characterize the folates present in uredospore extracts. Stock culture maintenance, assay procedure and DEAE-cellulose chromatography procedures were the same as previously described (22).

RESULTS

Folate Levels

Table I shows the growth responses of the assay organisms, L. casei, S. faecalis and P. cerevisiae to unfractionated extracts of wheat stem rust uredospores. Only traces of growth promoting activity were present prior to chicken pancreas conjugase treatment, indicating that most folates were present in conjugated forms with more than three glutamic acid moieties per molecule.

Fig. 1 shows drifts of folate concentration in uredospores during their germination. The responses of L. casei and S. faecalis are suggestive of shifts towards methylated folates during the germination interval. Since neither S. faecalis nor P. cerevisiae respond to methylated folates, the response of these two organisms would be expected to undergo similar changes unless, as is suggested by chromatographic data, methylated components were derived from P. cerevisiae inactive precursors.

Circumstantial evidence for a stoichometric turnover of S. faecalis and P. cerevisiae growth promoting folates to 5-methyl-$H_4PteGlu_n$ can be obtained if several assumptions are made. The growth promoting properties of 5-methyl-$H_4PteGlu$ for L. casei are only about 1/3 (39%) those of 5-formyl-$H_4PteGlu_n$ (22). Because of the similar response of L. casei and S. faecalis to extracts from ungerminated spores, only

insignificant amounts of the folate conjugates are assumed to be present as methylated derivatives before germination. If we further assume that the decrease of S. faecalis active growth factors during germination is entirely due to synthesis of methylated folates from S. faecalis active folates, the proportion of methylated derivatives in germinated spores can be calculated. According to this calculation methylated folates should increase from 44.5 to 84.8% of the total L. casei active folate during the 6 and 12 hour germination periods, respectively. The data in Table II show that this prediction is nearly correct, supporting the view that the spores synthesized methylated folates from non-methylated folates during germination and that the total folate content remained constant throughout the germination period. Thus the decline in the response of L. casei during germination (Table I) apparently results from a lower response to methylated folates rather than a decline in the total folate level.

In order to determine if folates leached from the uredospores during germination, the germination medium was concentrated and assayed with L. casei. Growth promoting activity was not detected but the possibility remained that compounds leached into the medium were converted to non-growth promoting substances. To investigate this possibility, a spore homogenate was diluted with distilled water, placed in darkness at 20°C for 12 hours and then concentrated and assayed for

folate content. Approximately 50% of the growth promoting activity initially present was recovered, suggesting that germinating spores did not release folate derivatives into the germination medium during the germination period.

Fractionation of Folates by DEAE-cellulose Column Chromatography

Uredospore extracts were chromatographed on DEAE-cellulose columns. Each column fractionation was repeated once and similar results were observed in duplicate runs. Folate recoveries were calculated by totalling the response of L. casei to all fractions. The recoveries were 92%, indicating that folate losses during chromatography were insignificant. Before assay with L. casei, S. faecalis and P. cerevisiae, each fraction was treated with chicken pancreas conjugase (22). The folate profiles from the column fractionation are shown in Fig. 2. Tentative identification of the components in each peak fraction relied mainly on the differential response obtained with the assay organisms. Chromatographic data have been reported for various folate derivatives (1, 5, 25, 32, 33) and they were also used as a guide in identification. They are, however, of limited value for this purpose because of uncertainties regarding the degree of conjugation.

Peak fraction I, the minor peak emerging at tube 11, elicited a growth response from S. faecalis but not from L. casei or P. cerevisiae. S. faecalis specific growth factors have not been reported to emerge at this position. The un-

known growth factor(s) elicited increased response of S. faecalis after conjugase treatment, but conjugates composed of glutamic acid moieties are not expected to appear so early in the elution sequence. This suggests that conjugase treatment released growth promoting activity other than by conjugase activity. Alternatively, the unknown growth factor may be a folate conjugate containing mono-carboxylic amino acids in place of glutamic acid. Such conjugates have been reported (34), and conjugates of this type would be expected to emerge earlier in the elution sequence than analogous conjugates containing glutamic acid.

Peak fraction II supported growth of all three assay organisms. It probably contained at least two components: a formyl-H_4PteGlu conjugate and a 5-methyl-H_4PteGlu conjugate. That this fraction contained two components can be recognized most easily if profiles from nongerminated spores are compared with those from spores germinated for 6 hours. Folates with similar chromatographic properties have been reported previously (5, 32).

Peak fraction III supported the growth of L. casei and S. faecalis but not that of P. cerevisiae. It probably contained conjugates of 5-methyl-H_4PteGlu as well as formylated folate conjugates oxidized in the pteridine ring. Previously published chromatographic data have indicated the presence of an unidentified conjugate of 5-methyl-H_4PteGlu which is eluted at this location (5) and oxidized formyl folate conjugates of a similar

nature may have been present in extracts of N. crassa (32).

Peak fraction IV probably contained a reduced formyl folate conjugate. Both formylated (1) and methylated (32) folate conjugates have been reported to emerge in this region but the responses of S. faecalis and P. cerevisiae do not suggest the presence of appreciable quantities of methylated derivatives.

The compound in peak fraction V has the characteristics of a 5-methyl-H_4PteGlu conjugate, probably with a higher level of conjugation than those in peak fractions II and III. A folate component with similar properties has been reported in extracts of N. crassa (32).

The tentative identification of folates in the peak fractions are summarized in Table III.

Shifts in folate composition during uredospore germination

Three major changes occurred during germination. The formylated folate derivatives emerging in peak fractions II, III and IV declined to low levels or disappeared (Fig. 2). The concentration of the methylated folate in peak fraction II also decreased. Concurrently, folate activity in peak fraction V appeared and the methylated folates in peak fractions IV and V became predominant. In summary, levels of formyl-H_4PteGlu$_n$ derivatives decreased and those of 5-methyl-H_4PteGlu$_n$ derivatives increased during germination. Stoichoimetric turnover of folate derivatives is suggested because the total folate

content in the spores probably did not change during uredospore germination and because spore-borne folates were not secreted into the medium.

DISCUSSION

Total folate levels from uredospores of stem rust were similar to those reported from uredospores of leaf rust (7) and mycelium of other fungi (24, 32). Leaf rust uredospores, however, were reported to contain only small amounts of conjugated folates (7). The differences in the degree of conjugation of folates reported for leaf rust and stem rust may be characteristic of the two species. But, it is more probable that the absence of conjugated folate derivatives in leaf rust uredospores is an artifact, possibly caused by autolysis of the derivatives during the lengthy disruption process used in the other study (7).

The high degree of conjugation of folates from uredospores of wheat stem rust, however, was comparable to that observed in other fungi (24, 32) but was in marked contrast to folates from healthy and rust-infected wheat leaves (22) and other higher plants (8, 9, 10, 23). Conjugated methylated folates increased after spore germination and increases in methylation were also noted in folates of rust-infected leaves (22). However the levels of conjugation of methylated folates from germinating uredospores was higher than those of infected leaves (22) suggesting that the uredospore folates do not contribute appreciably to the folates recovered from the host-parasite complex. This may be due to dilution of rust folates by host folates, differences in the composition of

folates in uredospores and mycelium or poor extraction of folates from rust tissue in the infected plant.

The predominance of conjugated folates and changes occurring in the folate composition during germination suggests that the conjugates are active participants in metabolic reactions rather than storage forms of folates. Polyglutamates from animal and bacterial sources are reported to be functionally active and in some cases absolute metabolic requirements (13). Wright (35) has reported that *in vitro* serine - glycine inter-conversion by serine - glycine hydroxymethyl transferase of *Clostridium* is completely dependent on polyglutamyl pteridine derivatives. Polyglutamate derivatives of 5-methyl-H_4PteGlu have also been demonstrated to function in the cobalamin-independent methyltransferase which functions in the synthesis of methionine in *Escherichia coli* (3, 11).

The rapid disappearance of formylated folate derivatives during uredospore germination could limit synthesis of purine nucleotides during germination. Staples (26) has reported decreases in template activity of m-RNA and *in vitro* amino acid incorporation into protein by ribosomes isolated from uredospores germinated for more than 4 hours. He suggested that germinating uredospores were unable to maintain synthesis of RNA for growth processes. Subsequent work by Yaniv and Staples (36) showed that the levels of ribosomes decrease between 4 and 8 hours germination to 42% of initial levels. Unavailability of soluble nucleotide could limit RNA synthesis

but this may not be a factor as large soluble nucleotide pools composed of uridine and adenosine derivatives have been recovered from wheat leaf rust uredospores (6) and from uredospores of wheat stem rust and flax rust (17). Significant reductions of the pool sizes in flax rust uredospores were not apparent after 14 hours germination (17) but critical evaluation of changes in individual nucleotides could not be made because of small sample sizes. The available data, thus, are not sufficient to correlate nucleic acid metabolism of the rusts with changes occurring in the folates during uredospore germination.

In yeast and E. coli the formation of N-formylmethionyl-tRNA has been demonstrated (15) and the importance of this derivative in the initiation of protein synthesis is well documented (16). The presence of 10-formyl-H_4PteGlu is a necessary requirement for synthesis of this component (4). Initiation of protein synthesis may not depend only on N-formylmethionyl-tRNA since Kim (12) has recently demonstrated the formation of N-formylseryl-tRNA in the presence of 10-formyl-H_4PteGlu or 5-formyl-H_4PteGlu. The possibility thus exists that an organism, such as a germinating uredospore, which has depleted its formyl folate reserves would not be able to effectively initiate protein synthesis.

Increases in the concentration of methylated folate derivatives in germinating uredospores might occur if methionine synthesis were blocked. Substantial amounts of methionine have been reported in the free amino acid pools of ungerminated

stem rust uredospores (2, 29) but changes in the methionine content during germination are not known. Activity from sodium acetate-2-^{14}C was incorporated into protein bound methionine of P. sorghi after 6 hours germination in the presence of the ^{14}C labelled compound (28) but activity in free methionine and rates of methionine synthesis were not reported. On the other hand, activity was not incorporated into bound methionine when glucose-U-^{14}C or valerate-U-^{14}C were fed to germinating stem rust uredospores (18). The immediate precursor of methionine, homocysteine, is detected only in trace amounts in wheat stem rust uredospores (2, 29). Again nothing is known of the synthesis of homocysteine in germinating uredospores but a deficiency of this compound could restrain methionine synthesis and might lead to the increases observed in methylated folate derivatives. These reports suggest that methionine is one of the amino acids with an extremely low rate of synthesis in stem rust uredospores. If this is true, unavailability of methionine could also inhibit protein synthesis in germinating spores.

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Table I

Growth promoting activity of unfractionated wheat stem rust uredospore extracts for *Lactobacillus casei*, *Streptococcus faecalis* and *Pediococcus cerevisiae*

	FOLATE CONTENT*					
	Ungerminated uredospores		Uredospores germinated for 6 hours		Uredospores germinated for 12 hours	
Test Organism	without conjugase	with conjugase	without conjugase	with conjugase	without conjugase	with conjugase
L. casei	0.92	24.50	0.25	17.38	0.48	11.27
S. faecalis	-**	25.93	-	14.13	-	3.93
P. cerevisiae	-	13.88	-	10.06	-	1.43

*µg 5-Formyl-H_4PteGlu (L-leucovorin) equivalents per gram uredospores.

**No detectable growth promoting activity.

Table II

Calculated and actual response of L. casei to extracts from wheat stem rust uredospores germinated for 6 and 12 hours

Germination period	Calculated Percentage of 5-methyl-$H_4PteGlu_n$*	Calculated L. casei response**	Actual L. casei response
6 hours	45.5	17.70	17.38
12 hours	84.8	11.82	11.27

*Calculated from the response of S. faecalis expressed in Fig. 2 (see text).

**Response expressed as µg 5-formyl-$H_4PteGlu$ (L-leucovorin) equivalents per gram uredospores.

Table III

Probable identity of folate derivatives in peak fractions obtained by DEAE-cellulose column chromatography of wheat stem rust uredospore extracts

Peak fraction	Folate derivative	References
I	Unidentified folate	-
II	?-Formyl-H_4PteGlu$_n$	23-33
	5-Methyl-H_4PteGlu$_n$	6
III	5-Methyl-H_4PteGlu$_n$	6
	?-Formyl-PteGlu$_n$	33
IV	?-Formyl-H_4PteGlu$_n$	1
V	5-Methyl-H_4PteGlu$_n$	1, 33

Fig. 1. Effect of germination on the total folate content of wheat stem rust uredospores. Calculations from the data in Table I are expressed as percentage response of each organism to extracts from ungerminated uredospores. Response of *L. casei* (————), *S. faecalis* (. . . . . .) and *P. cerevisiae* (- - - - - - - - -).

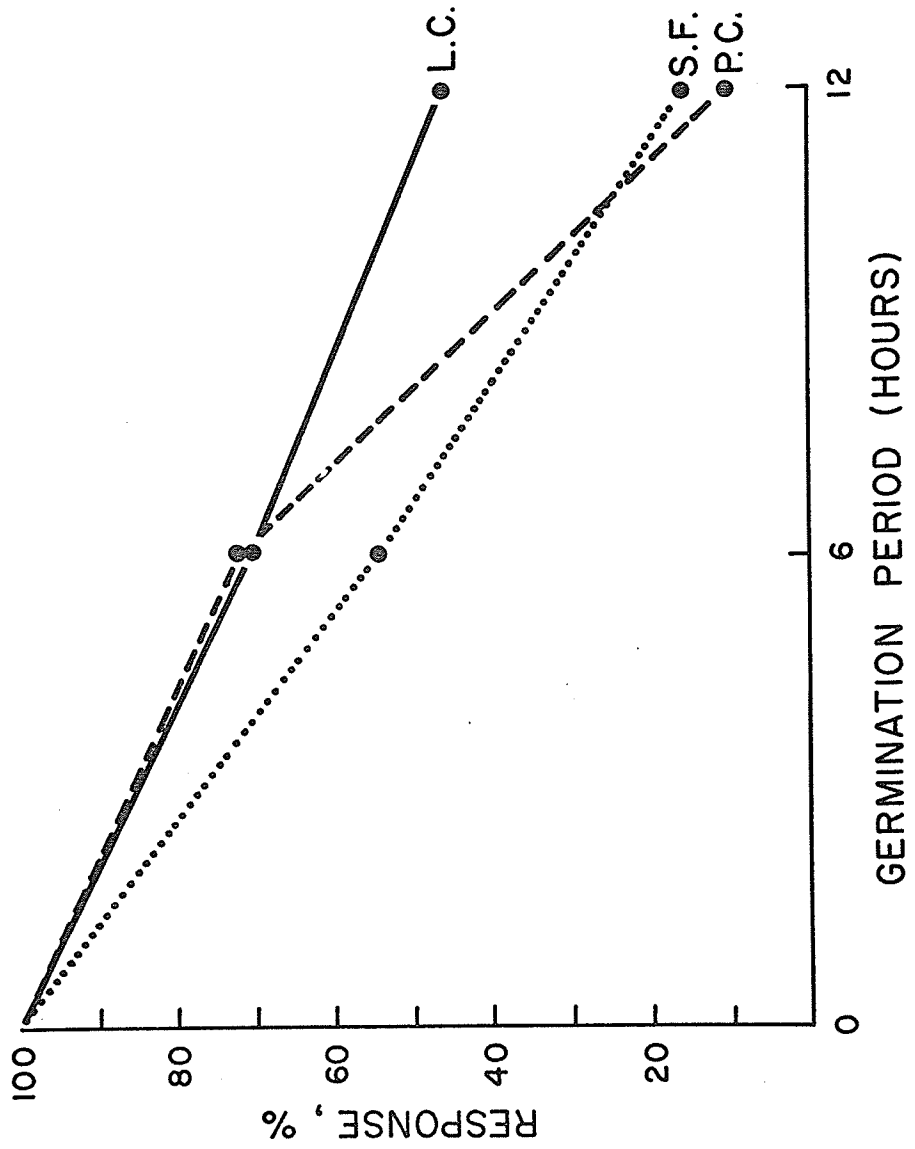
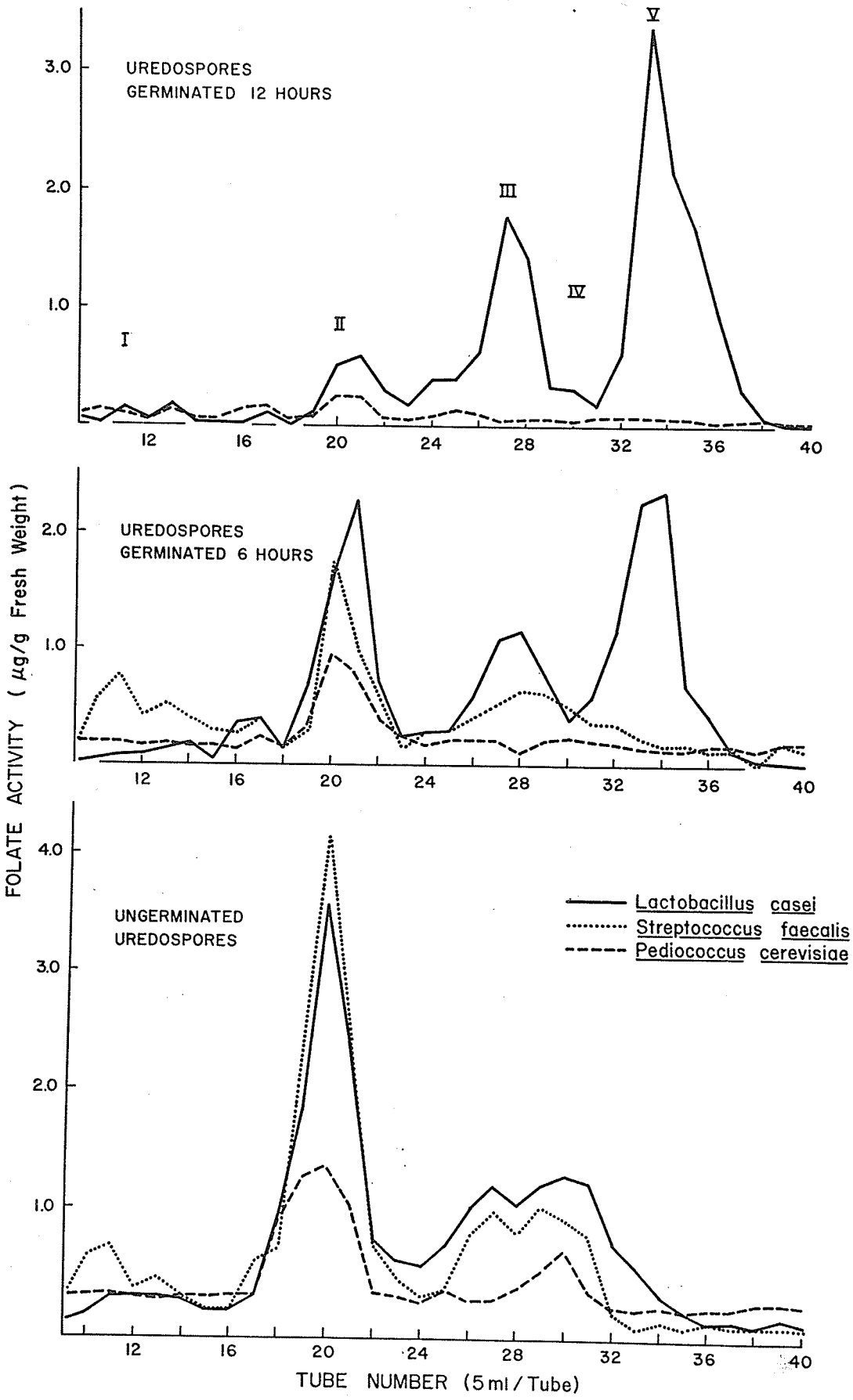

L.C.
S.F.
P.C.
100
80
60
40
20
0
6
12
RESPONSE, %
GERMINATION PERIOD (HOURS)

Fig. 2. Folate profiles of ungerminated and germinated wheat stem rust uredospores. Extracts of ungerminated uredospores, uredospores germinated 6 hours and uredospores germinated 12 hours were assayed with *L. casei* (————), *S. faecalis* (·····) and *P. cerevisiae* (– – – – – – –), after fractionation on DEAE-cellulose columns. The amount of extract applied to each column was equivalent to 8 mg fresh weight of uredospores. The eluate fractions were assayed with the test organisms after treatment with chicken pancreas conjugase. Folate content (ordinate) is expressed as μg 5-formyl-H_4PteGlu (L-leucovorin) equivalents per gm uredospores.


UREDOSPORES
GERMINATED 12 HOURS
I
II
III
IV
V
UREDOSPORES
GERMINATED 6 HOURS
UNGERMINATED
UREDOSPORES
Lactobacillus casei
Streptococcus faecalis
Pediococcus cerevisiae
FOLATE ACTIVITY (µg/g Fresh Weight)
TUBE NUMBER (5 ml / Tube)

DISCUSSION

One of the objectives of the present study was to obtain information on the metabolism of aromatic compounds by rust fungi and to assess the contribution of the parasite to the aromatic metabolism of rust infected wheat. Poor uptake and low turnover rates of the precursors limited the usefulness of this study. Perhaps more useful information could be obtained by studying changes that occur during germination with spores uniformly labelled with ^{14}C.

The aromatic amino acids, phenylalanine and tyrosine, appear to be synthesized via the shikimate pathway by rusts. This is undoubtedly the major pathway for biosynthesis of monocyclic aromatic compounds but other pathways using acetate as a precursor are involved in synthesis of more complex aromatic compounds such as flavonoids, isoflavones and isocoumarins (18). Compounds with complex aromatic ring structures have been implicated in disease resistance (5,16) but nothing is known of the metabolism of such compounds in the wheat-rust host-parasite complex or their prevalence in the rusts. However, in future studies experimental procedure should be given special consideration since defence reactions may result in the formation of highly insoluble compounds. Hyodo *et al* (14) have recently shown by cytochemical methods that the browning reaction of cells is correlated with resistance of sweet potato to *Ceratocistis fimbriata*. This reaction proceeds independently

of the formation of compounds previously thought to be phytoalexins and suggests that insoluble compounds formed during the browning reaction may present physical and chemical barriers to fungal invasion.

Uredospores of leaf rust and stem rust were able to convert tyrosine but not phenylalanine to cinnamic acids and tyrosine ammonia-lyase but not phenylalanine ammonia-lyase was extracted from uredospores. The fact that these rusts are parasitic on the Graminiae is of particular interest since plants belonging to other families either do not possess tyrosine ammonia-lyase or have much lower concentrations of this enzyme than the Graminiae (19, 30). The presence of tyrosine ammonia-lyase in cereal rusts may be of phylogenic significance and a survey of the distribution of this enzyme in rusts parasitizing plants containing varying amounts of tyrosine ammonia-lyase might be of taxonomic and evolutionary interest.

Indirect evidence indicated that rusts infecting plant tissue are able to synthesize aromatic compounds to a greater extent than observed in this study. The cell walls of uredospores and teliospores contain dark colored pigments which appear to be melanins. The melanins isolated from higher plants and fungi, unlike those of animals, are largely composed of a catechol based pigment (1, 21, 22). Melanins are probably not synthesized by germinating uredospores but the dark color of spores and the fungal stroma surrounding teliospores suggests that melanins are synthesized at a later stage of

development.

The rusts may be capable of synthesis of a variety of aromatic compounds but during the initial stages of germination the genes for these synthetic processes may be repressed and remain so until a particular stage of development. Evidence was presented some years ago indicating that synthesis of extra cellular enzymes could be induced in germinating uredospores (27). Indeed, induction and repression of enzymes involved in biosynthesis of aromatic compounds is well known in fungi. Induction of 3-deoxy-D-arabino-heptulosonic acid 7-phosphate synthase has been demonstrated by the use of auxotrophic mutants of Neurospora crassia (11). Repression of the tryptophan operon by tryptophan and induction of prephenate dehydrogenase by phenylalanine has also been reported for Neurospora (11). Another enzyme, dehydroshikimate dehydratase, is induced by the accumulation of dehydroshikimate resulting from a malfunction in the dehydroshikimate reductase of N. crassa. Dehydroshikimate dehydratase subsequently catalyzes the conversion of dehydroshikimate to protocatechuate which is excreted into the growth medium (13).

Penicillium urticae has been shown to convert 6-methylsalicyclate to gentisyl derivatives by sequential enzyme induction (10). An enzyme from N. crassa which catalyses the formation of salicyclic alcohol from salicyclic acid is induced after 50 hours in shake culture media containing salicyclic acid. After 60 hours in the presence of salicyclic acid the

fungus was also shown to produce a second enzyme catalyzing the conversion of cinnamate to cinnamylaldehyde (12).

Poor uptake of all precursors except phenylalanine and tyrosine prevented quantitative determination of intermediates involved in synthesis of aromatic compounds as well as quantitative determination of synthetic rates. Low rates of tyrosine deamination also contributed to these difficulties. Longer term studies of the metabolism of these compounds in axenic culture might help overcome these problems but uptake might not be improved even under these conditions.

The study of the folic acid components of uredospores, like most biochemical approaches of this nature, was initiated in the hope that it would provide information needed to grow the rusts in culture. Such studies have contributed extensively to the knowledge of the biochemical processes of the rust fungi but this information has not been useful in culturing the rusts.

In the past, considerable effort has been expended to grow rusts in artificial media. These attempts have been unsuccessful except for a few reports of growth into media from infected callus tissue (6, 7) but until recently, these reports were not taken seriously because they could not be repeated by other workers. However, this area of research has received increased attention since the recent reports describing growth of stem rust in culture starting with uredospores (28, 29). These reports have subsequently been confirmed with Puccinia graminis tritici (24), Melampsora lini (25) and possibly

Puccinia recondita (24). Thus it appears that the ability to grow in culture may be common among the rusts.

Growing cultures of rusts appear to be adversely affected by slight changes in the physical environment. Even routine handling of plates containing cultures appears detrimental to growth. Turel (26) has suggested that contact with agar during cultural transfers is sufficient to damage the mycelium. She also reported that axenic cultures of flax rust grew better at 16°C than at 17-17.5°C and were seriously damaged by short exposure to 24°C (26). Improved growth of the rusts obtained by suspending uredospores in gelatin solutions may also be due to improved physical conditions (4).

High concentrations of inoculum are required for maximum growth of the fungus (2, 29) but this requirement can be partially eliminated by seeding lower concentrations of stem rust uredospores in gelatin droplets (4). As pointed out by the authors (4), higher concentrations of an endogenous uredospore stimulant may be retained in the gelatin. The necessity of high concentrations of inoculum for initiation of saprophytic growth is not without parallel in other fungi. When the density of zoospore suspensions of Phytophthora infestans is low, zoospores encyst, germinate and form germ tubes but usually no further growth occurs (3). However, development of compact colonies from a single zoospore readily occurs in media amended with pectin. An observation that appears to be more similar to the situation in the rusts has

been reported for a mutant strain of Aspergillus amstelodami (9). The requirement for high inoculum concentration was overcome by seeding large numbers of conidia on agar covered with cellophane and removing the cellophane from the agar after conidial germination. Media primed in this way supported normal development of single spore cultures but the stimulant associated with the conidia was not identified. It would be interesting to repeat the latter experiment with uredospores to see if saprophytic growth of the rusts can be obtained from a single uredospore under these conditions.

An essential nutrient or hormone required for stimulation of vegetative growth of the rusts may be present in low concentration in uredospores. These components may leach from uredospores into the growth media and once a certain concentration is reached vegetative growth of the rusts may be induced. A variety of chemicals are known to leach from uredospores (8, 15, 23) so that stimulation could result from one or more compounds. It is possible that the rusts may be deficient for a single metabolite and that addition of this metabolite to nutrient media would enhance growth. It is also possible that special combinations of nutrients are required for maximum growth. A nutrient requirement of the latter type has been shown to be necessary for growth of Ustilago nuda haplonts of the (+) mating type (20). These haplonts were all proline deficient and required proline in the nutrient media. However, when nutrient media were supplemented with amino acids

at the same concentrations as proline the haplonts could not grow and growth occurred only when the concentration of proline, relative to that of other amino acids, was increased. Inhibition of growth was thought to be the result of inhibited proline uptake or utilization caused by the additive effect of several of the amino acids present in the culture medium. If this is true for the rusts, considerable manipulation of nutrients may be required before optimum conditions are determined.

On the other hand, the rusts may also require special "shock" stimuli for induction of saprophytic growth. The genes necessary for saprophytic growth may be repressed and certain environmental conditions may be required to derepress these genes. It is known that both chemical and physical stimuli will induce infection structure development in germinating uredospores. The effects of these stimuli have recently been investigated in detail in germinating uredospores of wheat stem rust, sunflower rust, bean rust and snapdragon rust (17). The information obtained from these studies can be used to readily induce infection structures under laboratory conditions. It is also possible that a special set of physical and chemical stimuli applied at particular stages of development as well as proper physical and chemical conditions are necessary for axenic culture of the rusts.

The rusts are biotrophic organisms and the vigor of their growth during pathogenesis is directly related to the

vigor of the host. The reasons for the restriction of the rusts to living tissue are not known but several mechanisms may be operative other than simple supply of nutrients. First, the rusts may not be able to grow on dead tissue because of the presence of inhibitory substances. In the living host, however, these substances may be compartmentalized so that the inhibitory components do not affect the rust. Second, the host may act to selectively remove or detoxify staling products resulting from rust growth. Stem rust growing in culture has been reported to produce a brown substance which is released into the agar surrounding the cultures (4). The accumulation of such substances could conceivably have a toxic effect on the growth and development of rust mycelium. A third alternative is that the living host may serve to recycle some compound necessary for growth of the rusts. In connection with the folates, it is interesting to speculate that the host plant aids in the recycling of formylated folates from methylated folates and thus provides the requirement necessary for continued growth.

Biotrophism is characteristic of many plant parasites. Many of these parasites such as rusts, powdery mildews and white rusts are also obligate parasites but a few biotrophic organisms such as Phytophthora infestans, Ustilago sp. and Claviceps purpures have been grown in axenic culture. These two classifications, obligate and biotrophic, thus are dissimilar. While biotrophism is a natural evolutionary development of host-parasite interactions, obligateness is an

artificial classification indicating that an organism has not been successfully grown in artificial culture.

Since so many varied parasites are biotrophic this mode of existance must have significant evolutionary advantages. One advantage might be more efficient production of new individuals. A cereal rust pustule for example is restricted to a small area of the total leaf surface with the result that a limited amount of mycelium is formed from which many spores are produced. A metabolic sink is created at this site so that the fungus is nourished from a large area of the plant. On the other hand, parasites that rapidly kill invaded tissue are not able to mobilize nutrients to the same extent as biotrophic parasites and must use those nutrients present in the invaded tissue for spore production. Once these nutrients are exhausted, the fungus must grow into fresh tissue to obtain additional nutrients. Thus the ratio of spores to total fungus protoplasm is probably much higher in biotrophic parasites than in other parasites and presumably production of individuals by biotrophic organisms is more efficient.

Once a biotrophic existance has become established, mutations which result in an inability to synthesize nutrients that are available from the host would not jeopardize the survival of the parasite. In this manner, metabolic lesions could accumulate and result in the obligate nature of many biotrophic organisms.

At present, mycelial growth of the rusts in culture

is slow but this is not unusual even in accepted saprophytic fungi and in time, with proper manipulation of cultural conditions, growth will probably be improved. While advances in axenic culture will probably come most rapidly from empirical studies of different cultural conditions on axenic growth, biochemical studies such as the characterization of the folates may also prove to be useful. The present study, while not providing definative information regarding the inability of the rusts to grow rapidly in culture, does suggest that a study of the effects of formylated folates on the growth of rusts in axenic culture would be desirable.

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