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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c Andrew Otis Jackson 1970

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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-ketoglutaric

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 glucoseamine 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.