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Carbohydrate and Lipid Metabolism During Germination of Uredospores of Puccinia graminis tritici[1]

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Summary. Uredospores of *Puccinia graminis* (Pers.) tritici (Eriks. and Henn.) were uniformly labeled with ^{14}C by permitting the host (*Triticum aestivum* L.) to carry out photosynthesis in $^{14}CO_2$ during the process of spore production by the obligate parasite. The use of ^{14}C labeled spores provided advantages in a study of the utilization of endogenous substrates at frequent intervals with small amounts of spores under conditions conducive to germination.

Because of previous uncertainties about the nature of the substrates of importance to germination, a detailed study of carbohydrate and lipid components, both in the spores and in the germination medium, was made during the first 7 hours after placing the spores on aqueous media. Diethyl ether and 80 % ethanol soluble metabolites each constituted approximately 20 % of the total spore carbon. During the first hour nearly 60 % of the 80 % alcohol solubles disappeared from the spores while the total ether soluble material did not change appreciably. A significant part of the 80 % ethanol soluble materials appeared in the germination medium.

During germination and germ tube extension, there was rapid utilization of trehalose, arabitol and mannitol even though appreciable amounts of these materials were present as exogenous pools in the germination medium. Although the total amounts of ether soluble components did not change as drastically as the carbohydrate fraction, there was extensive utilization of palmitic, oleic, linolenic and 9,10-epoxyoctadecanoic acids.

The results indicate that the germination process in spores of obligate parasites is not based solely on the utilization of lipids and some possible roles of the changes in internal and external pools of soluble carbohydrates are discussed.

There are several aspects of spore germination of obligately parasitic fungi, such as the rust or powdery mildew pathogens, which deserve additional attention. Earlier observations have led to the concept that lipid components are the chief storage metabolites and are the substrates primarily used during the germination process. The idea is supported by the low R. Q. of respiration during germination (32) and the fact that lipid appears to be utilized extensively during germination (2, 8, 18, 29, 34). In addition, most workers have reported extremely low endogenous concentrations of soluble carbohydrates (0.01–0.1 % of spore weight) when compared to lipid (18–25 % of spore weight) (8, 21). It has been generally believed that exogenous acetate and fatty acids are more efficient substrates than exogenous carbohydrate (26, 27, 30, 36, 37).

Most of the data on relative carbohydrate concentrations were obtained by chemical methods based on functional reducing groups of sugars, even though some earlier reports (24, 25, 34) indicated the presence of arabitol and mannitol in spores. Daly et al. (10) showed the rapid synthesis of trehalose and sugar alcohols in tissues infected by the safflower rust fungus and suggested they were fungal metabolites. An appreciable pool of pentitol was formed by reactions associated with the marked decrease in C_6/C_1 ratios which is characteristic of diseases caused by obligate parasites (10).

Coincident with this report, Reisener et al. (28) reported mannitol, arabitol and glyceritol as comprising an appreciable fraction of the hot water soluble components of ungerminated spores of the stem rust of wheat organism. However, they did not report trehalose to be present in resting spores but did observe its synthesis from exogenous valerate, presumably during germination. Subsequently, Staples et al. (37) and Wynn et al. (47) indicated the presence of trehalose in uredospores of rust fungi. Enzymes for metabolism of sugar alcohols are present in uredo-

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spores of the wheat stem rust fungus (15), but little is known about the utilization of these compounds during germination, except for the previously neglected report by Tani and Naito (40) which showed disappearance of mannitol from spores during germination.

In addition to complications due to the analytical methods employed on resting or germinating spores of obligate parasites, other features of the germination process pose problems in the interpretation of the data previously obtained. First, germination is erratic and in many experiments the percentage of spores which germinate has been low (8, 33) or not reported. Therefore the functional role of the observed changes in various metabolic components can not always be assessed. The erratic behavior of uredospores of various rust fungi appears to be due to specific self inhibitors (1, 4) produced by the spores. In the case of stem rust of wheat uredospores, the self inhibition can be overcome by addition of certain natural products (3) or by lipid derivatives such as pelaragonaldehyde (39) which may be formed from an unusual fatty acid (9, 10-epoxyoctadecanoic acid) present in large quantities in this species. Surprisingly, 2,4-dinitrophenol also stimulates germination of this organism (3) and this fact illustrates the uncertainties surrounding the metabolic events during germination.

Second, many of the analyses have been performed at time periods long after the metabolic events important for germination are over, with the danger of bacterial contamination by organisms accompanying uredospore collection (13). The principal evidence upon which the concept of lipid utilization is based was obtained 3 to 6 days after germination was initiated (34) even though the process proceeds actively within 30 minutes of placing viable spores on a suitable medium.

Finally, there have been no data presented on a single uniform collection of spores which offers a complete balance sheet for changes in endogenous lipid and carbohydrates during the early stages of germination. Only gross analyses of lipids, rather than analyses of components, have been previously reported. Much of the information on metabolism has been obtained for restricted groups of compounds under quite diverse conditions and with the use of exogenous ^{14}C compounds.

This paper reports studies carried out with ^{14}C uniformly labeled uredospores. The use of ^{14}C spores has several advantages. When compared to conventional methods of analysis, there is less difficulty in obtaining and germinating sufficient quantities of spores for isotopic analysis. Small quantities of spores can be analyzed for changes in individual metabolites below the limits of conventional analysis provided they can be cleanly separated by a technique such as paper or gas chromatography. More important, the elimination of quantitative analysis by special chemical reactions permits investigation of compounds or groups of compounds which ordinarily might not be detected.

Materials and Methods

Preparation of Radioactive Spores. Little Club wheat (*Triticum aestivum* L.) was inoculated with race 56 of *Puccinia graminis* (Pers.) f. sp. tritici (Eriks. and Henn.) as described previously (46). At the stage of rust development (3–5 days after inoculation) characterized by white flecks but not spores, leaves not showing at least moderate infection were removed. The plants, growing in 4 inch pots, were placed in a plexiglass chamber. Through special ports capped by serum bottle stoppers, $^{14}CO_2$ was generated in the sealed chamber by addition of 3 N H_2SO_4 to $Ba^{14}CO_3$.

The amounts of $BaCO_3$ (50 mg) were sufficient to give a final concentration of CO_2 only slightly above normal concentrations. Total radioactivity was 200 μc. It would have been desirable to generate CO_2 each day while keeping the chamber sealed continuously until spore harvest. However, preliminary tests in a continuously sealed chamber showed that spore production was abnormal. The spores were difficult to remove from the plants, yellow rather than red in color, and gave low percentages of germination. To overcome these difficulties, the following procedure was adopted.

CO_2 was generated each day approximately 5 hours before the end of the 13 hour photoperiod. The chamber was kept sealed during the dark interval and until the fifth and sixth hour of the next photoperiod. The chamber was opened for 2 to 3 hours before the next CO_2 generation. $^{14}CO_2$ monitoring of the atmosphere by withdrawal of a sample with a gas tight syringe indicated that $^{14}CO_2$ concentration was reduced to 10 % of its initial value at the end of the light period in which it was generated, rose during the dark period, but was reduced to negligible amounts before the chamber was opened in the next photoperiod. Under this regime the spores appeared normal and germinated satisfactorily. One apparent benefit was to reduce leaf surface moisture so that spores could be collected more readily.

Generation of CO_2 was carried out for 10 to 12 consecutive days. Sporulation was evident 1 to 2 days after the initial CO_2 generation. Spores were harvested on the fourth or fifth day and at 2 to 3 day intervals thereafter with a cyclone collector or by gently tapping the plants over cellophane sheets. Although the collections were relatively free of plant debris, they were further cleaned by passing through a 100 μ copper screen or by manual removal of debris with forceps. All collections from a single group of plants were pooled and thoroughly mixed.

Spore Germination. Procedures based on the work of Allen (1) and Atkinson and Allen (3) were employed as previously described (46). Within 2 to 3 days after final spore collection, 10 mg of spores were dispersed in Petri plates on the surface of 15 ml of distilled water which previously had been poured through Whatman No. 1 filter paper. Treatment by filter paper of the water used as the germination

medium was necessary in order to obtain suitable germination with spores en masse (46). The Petri plates were placed in the dark at 22°.

At intervals after germination, the spores were separated from the germination medium by filtration through Whatman No. 1 paper or millipore filters.

Protrusion of germ tubes was evident within 30 to 60 minutes and germination was reasonably synchronous. Elongation of germ tubes was continuous during the time periods examined and appearance of the germ tube was normal. Although it has been possible to obtain as high as 90 % germination with spores germinated in this manner (46), in the present experiments it ranged from 60 to 80 % perhaps because of the environmental conditions necessary for the production of ^{14}C labeled uredospores. At least 2 replicate Petri plates were employed for each analysis during the germination process.

Radioactivity Assays. A Packard Liquid Scintillation Spectrometer was employed for counting lipid components. Counting efficiency, as determined by the channels ratio method, was approximately 62 % for most samples. A Tracerlab windowless flow counter with an efficiency of 25 % was used for other samples. Samples were dried in glass planchets at 80° with a lens paper disc for even distribution. Corrections were made for background and self absorption.

Analysis of ^{14}C Released by Spores. CO_2 production was measured with duplicate weighed samples (2–3 mg each) dispersed on a volume of water in vessels described previously (9) so as to give the same ratio of spore weight to water as in Petri plates. CO_2 was collected in planchets containing 0.1 ml of 10 % KOH and radioactivity determined at hourly intervals in the gas flow system after drying.

Three 1.0 ml aliquots of the filtered germination medium were dried on planchets. The remaining solution was reduced to dryness at 40° under vacuum in test tubes. Using 3 ml volumes the dried film was extracted 3 times each with diethyl ether, 80 % (v/v) ethanol and with water. Aliquots were taken for total radioactivity in each fraction and the 80 % ethanol fraction was further characterized as described below.

Extraction of Soluble ^{14}C-Components of Spores. In order to inactivate enzymes rapidly, germinated and ungerminated spores were dropped into beakers with 10 to 15 ml of boiling acetone in which they were readily dispersed. After 10 to 15 minutes, the acetone was allowed to cool and contents of the beakers transferred to 20 ml Mickel disintegrator vessels and dried. The beakers were rinsed with acetone, ether, 80 % ethanol and with water in succession and each solvent also was added to the Mickel vessels and evaporated. Two grams of glass beads (Unispheres No. 608, Calaphor Corp.) and 2 to 3 ml of ether were added and the vessels oscillated for 10 to 15 minutes. This resulted in nearly complete spore breakage, which we have found to be a necessity for complete extraction of carbohydrate and lipid. Extraction of unbroken spores (36, 37) yields only 50 % of the ethanol soluble and 10 % of the lipid soluble materials. After settling, the supernatant fraction was decanted into 15 ml conical tubes and centrifuged at 2000 RCF for 10 minutes. This supernatant was transferred to a separate tube. The entire extraction procedure was repeated 3 times with ether, 4 times with 80 % ethanol and with water. In each extraction, care was taken to rinse both the Mickel vessel and the residue in the centrifuge tube thoroughly with extracting solvent. The solvents were reduced to dryness at 40° under vacuum, made to a uniform volume (usually 5 ml) and three 0.1 ml aliquots of each were assayed for radioactivity.

Analysis of the Ether Fraction. Free fatty acids were extracted with 5 % $NaHCO_3$. The aqueous fraction was acidified and re-extracted with ether. Both the ether and the aqueous phases, the latter probably containing some aromatic compounds, were assayed for radioactivity. The methyl esters of the free fatty acids in the ether were prepared by treatment with diazomethane according to the method of Schlenk and Gellerman (31) and subjected to vapor phase chromatography.

The fatty acid esters not extracted from the original ether solution were transesterified by a procedure modified from that of Tulloch and Ledingham (43). The sample was refluxed with 5.0 ml of 0.02 N NaOH in absolute methanol for 1 hour. A few drops of glacial acetic acid were added and the solvent reduced to less than 0.5 ml in a warm water bath under a stream of nitrogen. The mixture was then partitioned between 5 ml of water and 10 ml of ether. The aqueous layer was extracted twice with 5 ml of ether. The ether fractions were pooled and washed twice with 5 ml portions of water. The water fraction contained glycerol and other alcohols soluble in H_2O. The ether was reduced in volume under nitrogen and dried over sodium sulfate before application to thin layer chromatography plates.

A 250 μ layer of silica Gel G was spread on 20 × 20 cm plates which were air dried at room temperature and then activated by heating for 1 hour at 100°. Development of the plates in Ligroine (Bp. 66–75°) : diethyl ether (70:30) separated carotenes (R_F 0.74), the methyl ester of *cis*-9,10-epoxyoctadecanoic acid (R_F 0.48) : the methyl ester of 9,10-dihydroxyoctadecanoic acid (R_F 0.40). An unknown band at an R_F of 0.30 was also observed as well as some material that remained at the origin.

To determine precisely the location of the fatty acid methyl esters, the developed plates were sprayed lightly with 0.2 % 2′7′-dichlorofluorescein in 95 % ethanol and observed under UV light. The individual bands were scraped into centrifuge tubes and extracted 3 times with ether. The recovery of the fatty acid methyl esters and carotene was complete but the recovery from the bands of unknown compounds and the compounds at the origin was not determined.

The individual methyl esters of both the free fatty acids and those produced by transesterification of the

ether extract were separated by vapor phase chromatography on a 12 foot column, 0.25 inch in diameter. The column was packed with 14.3 % diethylene glycol succinate on 90/100 mesh Anakrom AB and operated at 185°. Injection temperature was 240°.

The Jarrell-Ash model 700 was equipped with a fixed splitter which allowed 1 part of the effluent vapor to pass the argon diode detector and 80 parts to flow to the heated trapping port. The splitter, collection port and detector were maintained at 210°. The carrier gas, argon, had a flow rate of 90 ml per minute. Specific activity measurements were made by trapping vapors at the collection port in 10 mm × 150 mm pyrex tubes packed loosely with defatted cotton wetted with methanol. The quantity of ester trapped was determined from the integrated area of the recorder peak and the radioactivity determined by eluting the material directly with 15 ml of liquid scintillation solution into counting vials.

Analysis of 80 % Ethanol Fraction. Aliquots representing 20 to 40 % of total fraction were passed successively over small columns (8 × 60 mm) of 200 to 400 mesh Dowex 1 − X8 (formate form) and Bio-Rad Ag 50W-X8 (hydrogen form). In some instances, removal of ionic compounds was accomplished by mixing appropriate amounts of resin with the fraction followed by removal of the resin with centrifugation or filtration. Acidic components were eluted from the Dowex resin with 6 N formic acid and basic components from the Ag 50W resin with 2 N NH_4OH.

The acidic, basic and neutral (non-ionic) fractions were reduced to dryness at 40° in test tubes and made to standard volumes for radioassay. The neutral fraction was chromatographed on Whatman No. 1 in methyl ethyl ketone:acetic acid:boric acid-saturated water (9:1:1). All chromatograms were developed by allowing solvent to descend to the edge of the paper strips. They were removed and scanned with a Tracerlab strip counter. If appreciable radioactivity was observed at the solvent front, this area was cut from the chromatograms which were then redeveloped in the same solvent until the carbohydrates were cleanly separated. The papers were then sectioned for measurement of radioactivity on the paper with the gas flow detector. All areas representing peaks were eluted with water, made to a standard volume and recounted before chromatographing in pyridine: ethyl acetate:boric acid-saturated water (25:60:20). When freshly prepared, this solvent separates ribitol and arabitol which co-migrate in the methyl ethyl ketone developer. The procedure also served as a second check on identity of the carbohydrates.

Analysis of the Water Fraction. The water soluble fraction obtained after 80 % ethanol extraction of the cells was chromatographed in both solvents mentioned above. There were 3 components with R_F's of 0.01, 0.10 and 0.15 in the methyl ethyl ketone solvent. The material with lowest migration rate comprised 85 to 90 % of the total fraction. Re-extraction of the compound from paper was followed by hydrolysis in 1 N H_2SO_4 for 6 hours at 95 to 100°. Chromatography of the radioactive components indicated that partial hydrolysis to hexose had occurred suggesting that this fraction may have contained either a glucan or glucomannan (25). Although other experiments employing exogenous mannose-^{14}C indicated considerable incorporation into this fraction during germination, its relatively small amount and contribution to the changes observed in the present work did not justify further characterization for the present paper.

Calculation of Data. In all experiments reported, no less than 20 mg of spore material was employed for analysis at the times indicated during germination. No less than 40 mg of ungerminated spores were analyzed. The amounts employed were adjusted to the specific activity of the spores which ranged from 38,000 cpm/mg to 160,000 cpm/mg spores in the gas flow counting system. In radioactivity analysis, the quantity of radioactive materials and counting times were sufficient to give a counting error of less than 1 %.

In the analysis of components separated by paper chromatograms, the quantitative data were obtained by determining the total radioactivity of all areas (peaks and interpeaks) of the chromatogram and expressing the data for each detectable peak as a percentage. The percentages were used for determining the radioactivity of the compounds in the fractions being examined. This procedure takes into account the presence of radioactive debris and compounds of such low activity that they might escape detection in the strip scanning procedure.

In table VI, the data are given only as cpm per fraction or component. The rest of the data presented were obtained with a single high specific activity spore lot in which the specific activity of carbon was determined by measurement of the specific activity of linolenic and palmitic acids. An average of 1.02×10^3 dpm/μg carbon was obtained from 4 determinations varying from 1.00×10^3 to 1.06×10^3 dpm/μg. This specific activity corresponded to 265 cpm/μg carbon when radioactivity was measured in the gas flow counter and 612 cpm/μg carbon for liquid scintillation counting.

With the procedures employed for production of labeled spores, it is a reasonable assumption that the ^{14}C was randomly and uniformly labeled in all cellular components. In some of the separations of the various solubility fractions, it was not possible to recover all the radioactive carbon initially present in the fraction. In some instances, this may have been due to incomplete elution from adsorbents (resins, thin layer plates) employed in fractionation. In other instances, the necessity for reduction of samples by evaporation may have resulted in loss of volatile components or transformation to insoluble components. In either event, the losses were unavoidable but they are recorded. However, the recovery of the compounds of major concern (fatty acids and soluble neutral carbohydrates) have been tested quantitatively and the recoveries are above 90 %.

Results

Ether and 80 % Alcohol Soluble Components in Uredospores. Analysis of the ether soluble components by radioactivity assay rather than by conventional chemical methods was consistent with previous reports in the literature (43). The ether extractables constituted 22 % of the total carbon. In addition, the percentages of the individual fatty acids were very similar to the percentages found earlier (43).

The results for carbohydrate components were at variance with some previous reports. Although fructose, glucose and sucrose are present in infected tissues (10) we could not identify them with certainty in spore extracts. Some very low levels of activity were associated with the chromatogram areas corresponding to glucose and fructose, but there was never enough to permit precise identification. We did not observe any of the other reducing sugars reported (38) to occur in rust spores.

Contrary to the report of Reisener et al. (28), trehalose was present in ungerminated spores in agreement with the recent work of Wynn et al. (47). However, we have never been able to detect, in a number of isolations from spores or infected tissues, the presence of a water or ethanol soluble radioactive component that corresponded to glycerol which has been reported to comprise 2 % of the fresh weight of stem rust uredospores (28). With other extraction procedures (28), it is possible that lipase activity might account for glycerol formation. Mannitol was approximately equal in amount with trehalose. Arabitol was the pentitol found in the experiments to be discussed but we have observed the presence of significant amounts of ribitol in some preparations of bean and wheat rust uredospores.

In some but not all preparations, radioactive material was detected at the solvent front (table III) but it did not appear to be a carbohydrate by several criteria and tests applied to it.

Loss of Carbon to the Environment. Carbon dioxide production was linear when measured at hourly intervals during germination but only the cumulative loss at 1, 3 and 7 hours is given in table I. At the end of 24 hours, the total loss via respiration was 39.6 μg carbon per mg of spores or 6.5 % of the initial spore carbon.

Loss of carbon by this route was small during the early stages of germination when compared to the amounts found in the germination medium (table I). In this experiment 7 % of the total spore carbon was found in the germination medium, however, the value has ranged from 4 to 9 % in other experiments. Most of the carbon in the medium was extractable in 80 % ethanol. At the end of the third hour the carbohydrates external to the spore constituted a larger pool of material than the carbohydrates within the spore (compare with table III). Since the external amounts decreased at the end of 7 hours, they were probably taken up for subsequent metabolism.

Table I. *Loss of Carbon into the Environment from Germinating* Puccinia graminis *f. sp.* tritici *Uredospores*

The initial spore carbon was 614 μg c/mg fresh weight of resting spores.

Fraction	Germination time 1 hr	3 hr	7 hr
	μg c/mg spores		
Carbon dioxide	2.0	5.6	12.1
Total in water medium	42.8	47.9	46.5
Ether soluble	2.0	2.4	2.6
80 % Ethanol soluble	31.4	34.4	27.6
Trehalose	3.2	4.1	2.9
Mannitol	6.8	8.4	5.1
Pentitol	5.7	6.4	3.8
X_1*	1.4	1.4	1.3
Acidic	6.0	7.0	6.9
Basic	6.1	6.9	7.2
H_2O soluble	1.9	1.7	1.6
Not accounted	7.4	9.5	14.6

* Unknown compound with R_F slightly higher than pentitols.

Table II. *Amounts of Carbon in Various Solubility Fractions of Germinating* Puccinia graminis *f. sp.* tritici *Uredospores*

The initial spore carbon was 614 μg c/mg fresh weight of resting spores.

Fraction	Germination time 0 hr	1 hr	3 hr	7 hr
	μg c/mg spores			
Ether	135.7	129.7	118.7	102.2
80 % Ethanol (total)	132.0	50.4	26.1	28.2
Neutral	76.6	30.2	10.2	15.6
Acidic	12.9	10.1	8.9	5.3
Basic	8.2	6.5	5.6	3.1
Not accounted	44.3	3.6	1.1	4.2
Water	29.2	31.0	17.3	18.9

In other experiments, they have been completely removed at the end of 24 hours. The pools of acidic and basic metabolites external to the spore did not change appreciably and were approximately equal to the pools in the spores at all stages.

A significant amount of carbon could not be extracted from the dried residue of the germination medium with the 3 solvents used. Since exoenzymes have been reported to be released from spores of fungi (41) and during germination (45), it is possible that this carbon was present in protein initially soluble in water but which was denatured during the reduction of the germination medium prior to extraction. Losses on resins or by volatilization of components is also possible.

Changes in Soluble Metabolites of Spores During Germination. The total amounts of carbon in the ether and ethanol fractions of ungerminated spores were approximately equal, each comprising about 22 % of the total spore carbon. During the 7 hour germination time there was a slow loss of carbon from the

ether fraction, but a pronounced drop in the ethanol fraction occurred during the first hour (table II). Of the amount which disappeared (81.6 μg c/mg spores), some could be accounted for in the germination medium (31.5 μg c/mg spores, table I). However, there was a further drop (24.3 μg c/mg spores) during the next 2 hours with a net increase of only 2.9 μg c/mg spores in the carbohydrates of the germination medium. There was a small increase measured at the end of an additional 4 hours which could be 1 result of carbohydrate uptake from the external medium.

The neutral carbohydrate fraction showed the greatest change during the first hour (46.4 μg c/mg spores), only partially accountable in the germination medium (17.12 μg c/mg spores). There was also a large proportion of the ethanol fraction which could not be accounted for in ungerminated spores, but this was not observed in spores during germination. These results were obtained in 2 separate analyses of the 80 % ethanol fraction at zero time.

The water soluble fraction obtained after alcohol extraction showed little change during the first hour but decreased during the next 2 hours. Subsequently there was a slight increase which occurred concurrently with increases in the carbohydrates of the ethanol fraction of spores and decrease in the external pool of carbohydrate (table I).

Metabolism of Soluble Carbohydrates. Data are presented in table III showing the amounts of carbon present (P) within the spore or metabolized (M) to other compounds. The quantity metabolized was obtained from the total amount lost from both the spore and the medium. The content of trehalose, mannitol and the pentitols represented only slightly less than 10 % of the total spore carbon. Within 1 hour, nearly 30 % of the carbohydrates had been metabolized, and at the end of 7 hours nearly half of it had disappeared (3.4 % of initial spore carbon). Mannitol was utilized most extensively, but utilization of the pentitol pool also was significant.

Metabolism of Lipids. Although the total amount of carbon in the ether soluble fraction did not change greatly with time (table II), individual groups of compounds showed marked losses, even within 1 hour (table IV). The major loss was in the esterified fatty acids, amounting to 42.7 μg c/mg spores. This represented about 7 % of the initial spore carbon. Subsequent losses were not as marked. The greatest percentage change in the fatty acids was in the *cis*-9,10-epoxyoctadecanoic acid but considerable loss was noted for palmitic, oleic and linolenic acids (table V). The 9,10-dihydroxyoctadecanoic acid, shown previously to be an enzymatic hydration product of the epoxy acid molecule (42), increased in amount with time.

The water soluble components of the lipid fraction, including the free fatty acids soluble in 5 % $NaHCO_3$ (table IV), were not analyzed separately for ungerminated spores. However, other experiments have indicated that the amount of carbon in these fractions is similar to the values obtained for germinating spores at 1, 3 and 7 hours. This fraction contained small amounts (10^{-3} μg c/mg spores) of fatty acids including the epoxy acid.

Table IV. *Quantities of Lipid Components in the Uredospores of* Puccinia graminis *f. sp.* tritici *after Various Periods of Germination*

The initial spore carbon was 614 μg c/mg fresh weight of resting spores.

Components	Germination time 0 hr	1 hr	3 hr	7 hr
		μg c/mg spores		
Water soluble	...	3.8	3.6	2.2
5 % $NaHCO_3$ soluble	...	1.8	1.1	...
TLC Fractions				
Alcohols of fatty acid esters	8.0	6.9	6.9	6.3
Esterified fatty acid	106.6	63.9	51.9	49.9
Carotene and hydrocarbons	5.0	0.6	0.2	0.1
Unknown	...	8.5	9.5	8.7
Origin	9.7	10.1	14.0	9.0
Not accounted	8.4	34.1	31.4	26.0

Table III. *The Quantity of Carbohydrates and Other Components of the Neutral Fraction Present (P) or Metabolized (M) During Germination of* Puccinia graminis *f. sp.* tritici *Uredospores*

The initial spore carbon was 614 μg c/mg fresh weight of resting spores. The quantity metabolized is corrected for losses to the germination medium.

Component of chromatogram	Germination time 0 hr P*	1 hr P	1 hr M	3 hr P	3 hr M	7 hr P	7 hr M
			μg c/mg spores				
Origin	1.2	0.6	0.6	0.1	1.2	0.3	0.9
Trehalose	10.6	3.2	4.2	1.7	4.8	2.4	5.3
Glucose-fructose**	0.8	0.5	0.3	0.1	0.7	0.5	0.3
Mannitol	27.7	14.0	6.9	3.0	16.3	4.7	17.9
Arabitol	18.4	7.6	5.1	2.0	8.0	2.8	11.8
X_1	2.5	1.0	0.0	0.4	0.7	0.8	0.4
Solvent front	13.0	2.5	11.5	2.4	11.6	3.6	9.4

* P represents the amounts present in spores; M represents the amount metabolized from the onset of germination.
** Represents combined areas where glucose and fructose would migrate but positive identification not made.

Table V. *Quantities of Individual Fatty Acids in the Uredospores of* Puccinia graminis *f. sp.* tritici *after Various Germination Periods*

Initial spore carbon was 614 μg c/mg fresh weight of resting spores.

Fatty acid	Germination time 0 hr	1 hr	3 hr	7 hr
	μg c/mg spores			
Shorter than palmitic	2.4	8.3	2.3	21.8
Palmitic	32.2	18.2	20.4	
Stearic	7.3	5.7	4.4	4.9
Oleic	18.7	7.6	6.2	7.4
Linoleic	7.1	3.7	3.2	2.5
Linolenic	11.6	6.7	6.1	5.4
*$C_{18\text{-Epoxy}}$	23.6	8.6	2.8	1.3
**$C_{18\text{-DiOH}}$	<0.5	2.0	3.2	6.7

* 9,10-epoxyoctadecanoic acid.
** 9,10-dihydroxyoctadecanoic acid.

There was a marked drop in the extractable carotene and hydrocarbons during the first hour of germination. One conspicuous aspect of the changes in the lipid fraction is the increase in carbon that is not accounted for in fractions extractable from the thin layer plates by ether. The polar compounds which remain at the origin are not quantitatively eluted by ether. Much of the material in the unaccounted for category probably represents polar compounds which remain at the origin of the thin layer plates. This material increases from 8.4 to 34.1 μg c/mg spores during the first hour. In addition, an unknown material comprising about 5 % the total lipid fraction is evident after the first hour and remains essentially constant during the remaining time periods.

In table VI are summarized data obtained in a separate experiment which substantiates the main observations of the previous tables although a detailed analysis of the lipid fraction was not attempted. There is disappearance of soluble carbohydrates during the germination process. Some of this loss undoubtedly was into the germination medium but not all of it. However total loss from the lipid fraction was negligible during the same interval.

Table VI. *Changes in the Radioactivity of Different Carbon Pools During Germination of Uredospores of* Puccinia graminis *f. sp.* tritici

Analysis on 20 mg spores with initial radioactivity of 54,000 cpm/mg.

Fraction	Germination time 0 hr	1.5 hr	3 hr
	cpm/mg spores		
I. External carbon			
CO_2	...	260	470
Medium	...	2240	2380
II. Internal carbon			
Ether soluble	7200	6910	6800
80 % Ethanol soluble	6500	2450	1790
Neutral	4670	1940	1350
Trehalose	1180	820	660
Mannitol	1870	550	380
Pentitol	1400	500	170
Acidic	700	350	340
Basic	690	230	200
Water soluble	1500	990	610

Discussion

The appearance of uredospore amino acids in the germination medium and their movement into host tissues has been reported (19), and several recent papers have mentioned (38, 42) possible losses to germination media, but no quantitative data have been reported. The amounts detected in the present study were surprisingly large and preliminary data indicate that nearly all are released in the first 10 minutes. The mechanisms responsible may have a considerable bearing on our understanding of metabolism during germination. Interpretation of the phenomenon is handicapped by an inability to determine whether the exogenous carbon is contributed by all spores of the population or only by a certain percentage of spores. In this study, for example, only 70 % of the spores germinated. However, we have observed as much as 5 % of the total spore carbon in the medium when 90 % of the spores germinated.

The obvious assumption that external carbon arises by diffusion through the spore wall or hyphal germ tube implies the absence of regulation of permeability but this explanation must be weighed against other alternatives. Uredospores are formed in a host lesion and the possibility exists that exudates may be carried with the spores during collection. If so, it might be expected that glucose, fructose and sucrose would be detected in proportions equivalent to those found in the parasitized host, but this was not the case. A second possibility is the release of materials through rupture of the spores or lysis of germ tubes. We have never observed significant occurrence of either process. Finally, the point at which spores are attached to the vegetative mycelium may represent a thin area in the spore wall which has not functionally been sealed. However, we know of no cytological information on such a structure or the frequency of its occurrence in a population of mature spores.

The role of external compounds in the germination process itself is unknown. It is generally thought that the failure of spores to germinate when placed under conditions of high spore density is due to local concentrations of specific chemical inhibitors produced by the spores (1). Considerable evidence has been presented to substantiate this concept, but the nature of the inhibitory materials is not known.

It has been observed, however, that concentrations of sucrose so low (0.1 M) as to be negligible osmotically will inhibit germination nearly completely (4) and Staples et al. (37) indicated that most of the metabolites they tested inhibited germination at 10 mM concentrations. In addition to germination control

by specific chemical regulators as suggested by Allen (1), it is tempting to speculate on a general mechanism, such as catabolite repression, to explain germination control under some circumstances. Under non-germinating conditions, a considerable level of metabolic activity does occur (26, 27, 33, 36). The movement of substrates from the spore may relieve a substrate-imposed metabolic block on specific pathways required for germination to ensue. At high spore densities, the concentrations of external metabolites might be sufficient to provide metabolic suppressors but at low spore densities concentrations would be reduced enough to permit germination. It is of interest that Horikoshi and Ikeda (16) have reported that mannitol inhibits the trehalose of *Aspergillus* spores.

Sussman and coworkers have suggested permeability changes as a mechanism of germination control of *Neurospora*. Recently (6), this group has reported 2 metabolic pools of trehalose involved in the activation of germination, one of which is water soluble. Because of the differences in experimental procedures, it is not possible to equate operationally this pool with the external pools found in the present study. It would be of interest, however, to establish whether saprophytic fungi are different from obligate parasites in this respect since the absence of an external pool in the latter might complicate comparative studies of metabolism (36, 37).

Even though external compounds may not be functionally important, the existence of a large external pool of carbon complicates studies of metabolism. In recent reviews, Allen (2) Staples and Wynn (38) and Shaw (32) have summarized the evidence indicating that utilization by rust uredospores of exogenous substrates, particularly carbohydrates, is low when compared to non-obligately parasitic fungi. Isotope dilution by external pools of carbohydrate, as well as internal dilution, imposes a restriction on the interpretation of the data. Perhaps more important is the possibility that, if uptake of exogenous substrates is by an active metabolic process, there will be competitive effects upon uptake from solution between added substrates and similar compounds normally present.

One of us (J. M. Daly) has supplied hexoses and pentoses to wheat rust uredospores in concentrations calculated to approximate the total external pool of carbohydrate. At these concentrations, 20 % of supplied mannose was metabolized to other compounds in 6 hours with approximately 3 % recovered in carbon dioxide, a conversion rate higher than expected from the data in the literature (33, 37, 44). It should be noted also that washing bean rust uredospores leads to different incorporation patterns from acetate (35, 38). It has been assumed that this represents a difference in metabolism between resting (unwashed) spores and germinating spores, but removal of external metabolites by washing also may explain the differences.

The release of endogenous metabolites to the surrounding medium also imposes some additional burdens in measuring metabolic turnover since ^{14}C data based only on that incorporated into spores may not indicate true rates of conversion of exogenous substrates. In view of excretion of enzymes in other fungal species (41) this point would be of concern in estimations of protein, and possibly nucleic acid, synthesis. To the authors knowledge, the germination medium has been ignored in such studies.

Although it has been generally held (8, 11, 18, 29, 32, 38) that during germination carbohydrate is synthesized at the expense of lipid, the data available in the literature are not conclusive. Gross analysis for total fatty acid (8) showed little change during the first 6 to 8 hours after germination. In addition, most reports of carbohydrate synthesis were based on methods which did not detect polyols. However, the presence of glyoxylate pathway enzymes in spores (7, 12) does provide a mechanism for lipid to carbohydrate conversion.

Since we have measured only net changes, not turnover, it is not possible to decide from our data whether lipid was converted into carbohydrate in the important initial stage of germination. The data for the ether soluble fraction (table II) can be reconciled with previous data showing a decrease in fatty acids simultaneously with an increase in non-saponifiable lipids (8). However, a marked decrease in certain esterified fatty acids occurred within the first hour.

Although the decrease in certain fatty acids is considerable, the fact that there is an appreciable initial pool of soluble carbohydrate which is metabolized concurrently with lipid components suggests that interconversion between lipid and carbohydrate may be limited. The extent of carbohydrate disappearance is considerable. Previous estimates (28, 40) of the extent of carbohydrate utilization must be subject to reservation since the germination medium was not examined.

It is most likely that the lipid and carbohydrate pools may have distinct metabolic significances. Some of the fatty acid components which disappeared may be accounted by increases in the non-saponifiable lipid (8) or in ether soluble components which could not be recovered from thin layer plates (table IV).

The rapid disappearance of 9,10-epoxyoctadecanoic acid during the initial stages of germination (table V) is accounted for only partially by appearance of 9,10-dihydroxyoctadecanoic acid. Tulloch (42) reported essentially stiochiometric conversion under non-germinating conditions. Although other pathways of metabolisms of the epoxy acid may occur during germination, more important perhaps is the possibility that the dihydroxy acid may be further metabolized to other compounds, such as pelargonaldehyde, which specifically stimulate germination (2).

Some lipid may be used for carbohydrate synthesis (8, 12, 18, 29) via the glyoxylate pathway, but the major significance of the glyoxylate enzymes in uredospores may be only to replenish 4 carbon acids of the tricarboxylic acid cycle used in amino acid biosynthesis. Glutamic and aspartic acids (and their

amines) are very active metabolically during germination and are synthesized rapidly from acetate (29, 37).

The most conspicuous event during germination is the rapid deposition of germ tube cell walls. Although chitin has been assumed to be a major component, the existence of pentoses (20) in cell wall hydrolyzate of uredospores suggest a more complex structure than this (5, 17, 23). The direct incorporation, via sugar nucleotides, of both internal and external pools of carbohydrates to cell wall components should be investigated for significance in the problems of germination control and organelle differentiation in uredospores.

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