Biochemistry of Germination in *Peronospora tabacina* (Adam)
Conidia: Evidence for the Existence of Stable Messenger RNA

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SUMMARY

In an effort to understand the mechanisms controlling germ-tube differentiation in *Peronospora tabacina* the biochemical events occurring early in germination have been investigated. Actidione \([2 \times 10^{-8} \text{ M}]\) inhibited germination, indicating that differentiation required the synthesis of new protein. However, not all the protein synthesized prior to emergence was essential; only the protein synthesized within 30 min. of the start of germination appeared to be necessary. Inhibitors of RNA synthesis prevented the incorporation of \(^{3}H\)uridine into RNA, yet had no effect on germination, indicating that differentiation did not require the synthesis of RNA. It is concluded that the protein required for differentiation is synthesized on a stable template of messenger RNA present within the dormant conidium.

INTRODUCTION

Although a number of treatments are known to break the dormancy of fungal spores (for review see Sussman & Halvorson, 1966), at present the biochemical events responsible for the initiation of fungal spore germination are poorly understood. A knowledge of these events would provide information on the control of differentiation, and might also be of use in research aimed at controlling fungal plant pathogens.

In saprophytic species, one of the earliest events occurring in germinating spores is the synthesis of protein (Ohmori & Gottlieb, 1965; Staples, Syamananda, Kao & Block, 1962) but attempts to relate this synthesis to the appearance of certain enzymes have so far yielded only tentative results. In germinating spores of obligate parasites \(^{14}\text{C}\)-labelled amino acids are incorporated into protein indicating that some protein synthesis does occur, but the rates of incorporation are always low and an increase in total protein does not occur. Nucleic acids are also synthesized prior to germ-tube emergence in several fungi (Staples *et al.* 1962) and it is likely, as in *Aspergillus niger*, that this is primarily RNA and not DNA. Gottlieb (1966) suggested that this might be messenger RNA needed for the synthesis of early protein. However, the data of Ono, Kimura & Yanagita (1966) for *A. oryzae* indicated that RNA synthesized early in germination was ribosomal and transfer RNA and that messenger RNA was not formed until germination was well advanced. The fact that many purine and pyrimidine analogues failed to inhibit the germination of *Peronospora tabacina* conidia (Shepherd, 1962) cast doubt on the direct involvement of RNA synthesis in germination. In addition, Shaw (1967) has observed that actinomycin D \((20 \mu\text{g.}/\text{ml.})\) failed to inhibit the germination of *Puccinia helianthi* uredospores, although it inhibited the incorporation of \(^{3}H\)cytidine into RNA.
I have attempted to resolve the problem of whether the germination of *Peronospora tabacina* conidia requires the synthesis of both protein and/or RNA. The obligate parasite *P. tabacina* was chosen because conidia were readily available and, in addition, much was already known about the effects of environmental factors on germination (Shepherd 1962). The present experiments provide evidence that protein synthesis is an important early event in germination whereas RNA synthesis, as measured by the incorporation of \(^{3}H\)uridine, is not essential before germ-tube emergence. It is concluded that dormant spores contain stable messenger RNA.

**METHODS**

**Preparation of conidia.** Infected 8-week-old tobacco plants (*Nicotiana tabacum* cv. *Virginia Gold*) were induced to sporulate by placing them overnight in a chamber at 20° and 100% humidity. Conidia were washed from the fourth youngest leaves with ice-cold water. In these conidia dormancy is maintained by a germination inhibitor which, when washed from the conidia, allows germination to commence (Shepherd & Mandryk, 1962). To remove traces of this inhibitor conidia were washed three times with ice-cold water, centrifuging after each washing (3000 g for 5 min.). Conidia were then suspended in a small volume of magnesium + phosphate buffer (1·0 g. MgSO\(_4\)·7H\(_2\)O; 1·52 g. KH\(_2\)PO\(_4\); 4·608 g. Na\(_2\)HPO\(_4\); 1·000 ml. distilled water, pH 7·0) and held in ice while the concentration was determined with a haemocytometer slide. Conidia were germinated in the same buffer in 5·0 cm. diameter Petri dishes at 15°. The final volume in each Petri dish was 2·5 ml. A concentration of 5·0 x 10\(^6\) conidia per ml. was used unless otherwise stated.

**Assessment of germination.** A high inoculum concentration (> 3·0 x 10\(^6\)) and a low temperature kept conidia dormant. The germination process was initiated by raising the temperature to 15° and by lowering the conidial concentration, and was considered complete when the first sign of a germ tube was noted. The first germ tubes appeared 60 min. after the start of incubation, but poor synchronization meant that some conidia only germinated after 4 hr. Therefore, in experiments where percentage germination was recorded, conidia were killed with formalin after 4 hr and an assessment made on a sample of 300 conidia.

**Amino acid and nucleotide pool determinations.** Both the amino acid and nucleotide pools were measured at intervals during the germination process. 1·25 x 10\(^6\) conidia were extracted twice with hot ethanol, followed by two further extractions with cold 70% ethanol. Extracts were combined, evaporated to dryness, and purified by elution from Dowex 50 (H\(^+\)) with 1·5 N-ammonia. After removing the ammonia, amino acids were estimated by the method of Cocking & Yemm (1954). For nucleotide estimations, 7·5 x 10\(^6\) conidia were extracted twice with cold 10% perchloric acid. These extracts were combined and the nucleotides adsorbed on to 10 mg. of acid washed charcoal by shaking at room temperature for 30 min. The charcoal was separated by centrifugation and the nucleotides eluted at 37° using 50% (v/v) ethanol + 1% (v/v) ammonia. The E\(_{270}\) of this eluate was taken as a measure of the nucleotides.

**Radioisotopes.** Reconstituted [\(^{14}\)C]yeast protein hydrolysate was purchased from Schwarz Biochemicals Inc., Orangeburg, N.Y., U.S.A. [\(^{3}H\)]uridine (13 c/mmole) and [\(^{3}H\)]cytidine (30·2 c/mmole) were purchased from the Radiochemical Centre, Ams- sham, U.K.
Germination of Peronospora conidia

RESULTS

Protein synthesis and germination

Figure 1 shows the rate of incorporation of $^{14}$C-labelled amino acids into protein during the first 90 min. of germination. The initial rate of incorporation declined after the first 15 min. period and the lower rate was maintained during the rest of the germination process. This decline was not due to changes in the amino acid pool, as this remained constant throughout germination. Thus, protein synthesis occurred before germ-tube emergence, although no significant increase in total protein could be detected (Fig. 1). Evidence obtained by using the specific protein-synthesis inhibitor, actidione (cycloheximide), showed that this protein synthesis was required for germ-tube formation. Actidione ($2 \times 10^{-6}$ M) inhibited both $^{14}$C-labelled amino acid incorporation into protein (Fig. 1) and germination. Table 1 shows that actidione only inhibited germination when added within 30 min. of the start of germination, indicating that the protein synthesis required for germ-tube formation occurred early in the germination process. However, in additional experiments germination was not inhibited by puromycin (160 $\mu$g./ml.) or amino acid

![Graph showing the rate of incorporation of $^{14}$C-labelled amino acids into protein during the early stages of germination in Peronospora tabacina.](image)

Fig. 1. Effect of actidione on the rate of incorporation of $^{14}$C-labelled amino acids into protein during the early stages of germination in Peronospora tabacina. Conidia were labelled with 15 min. pulses of $^{14}$C-labelled amino acids (0.4 $\mu$C/ml.) given at 15 min. intervals after the start of germination. Incorporation was stopped by the addition of 0.5 ml. cold 30% (w/v) trichloroacetic acid [TCA] and conidia were transferred to centrifuge tubes washing with cold 5% (w/v) TCA + 0.1% casamino acids. After centrifugation [3000 g for 5 min.] the supernatant was discarded and the conidia resuspended in 5% TCA solution, heated to 90$^\circ$ for 10 min., collected on membrane filters [Oxoid], and dried beneath an infrared lamp. Radioactivity was counted in a micromil thin window counter [Nuclear Chicago C. 210]. Each histogram represents the radioactivity incorporated during the preceding 15 min. time interval. $1 \times 10^8$ conidia were extracted with either hot 70% (v/v) ethanol or cold 10% (v/v) perchloric acid, and the residues heated in 2 N-NaOH for 10 min. at 100$^\circ$. Protein was estimated by the modified Folin–Ciocalteu method suggested by Layne (1957). $\square$, Control; $\bigcirc$, actidione ($2 \times 10^{-6}$ M); $\bullet$, protein.
analogue (p-fluorophenylalanine, canavanine, and ethionine, all 200 \( \mu g./ml. \)), even when added at the start of germination perhaps because they failed to penetrate the conidia.

Table I. The effect of actidione on the germination of Peronospora tabacina conidia

<table>
<thead>
<tr>
<th>Time (min.) of adding actidione after the start of incubation</th>
<th>% germination at time of adding actidione</th>
<th>% germination after 4 hr</th>
<th>% conidia which germinate in the presence of actidione</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>110</td>
<td>110</td>
</tr>
<tr>
<td>30</td>
<td>0.5</td>
<td>50.5</td>
<td>50.5</td>
</tr>
<tr>
<td>40</td>
<td>0.5</td>
<td>90.0</td>
<td>89.5</td>
</tr>
<tr>
<td>50</td>
<td>0.5</td>
<td>94.0</td>
<td>93.5</td>
</tr>
<tr>
<td>60</td>
<td>3.25</td>
<td>100.0</td>
<td>97.75</td>
</tr>
</tbody>
</table>

RNA synthesis and germination

When Peronospora tabacina conidia were incubated in the presence of \(^{3}H\)uridine radioactivity was incorporated into RNA (Fig. 2). The rate of incorporation was highest during the first 15 min. period and then declined to a negligible rate after 30 min. This reduction was unlikely to result from a change in pool size because, although the uridine pool was not determined separately, only a small increase in the total nucleotide pool was detected prior to germ-tube emergence.

Actinomycin D (80 \( \mu g./ml. \)) failed to inhibit both germination and the incorporation of \(^{3}H\)uridine into RNA suggesting that conidia were impermeable to this inhibitor. No conclusions concerning the involvement of RNA synthesis in germination could, therefore, be drawn from this experiment.

Proflavine and ethidium bromide form complexes with DNA by intercalation between adjacent base pairs of the DNA double helix thus preventing the transcription of DNA. At low concentrations both inhibitors had no effect on germination (Fig. 3), although both did inhibit the incorporation of \(^{3}H\)uridine into RNA (Fig. 2). Thus germ-tube emergence would seem to be independent of RNA synthesis. At higher concentrations proflavine and ethidium bromide inhibited germination but, as Waring (1966) has pointed out, at these concentrations protein synthesis is also inhibited.

Heidelberger (1965) suggested that 5-fluorouracil mainly inhibits DNA synthesis, although a few examples are known where 5-fluorouracil, especially at high levels, directly inhibits RNA synthesis (Cohen et al. 1958). No incorporation of \(^{3}H\)cytidine into alkali-resistant, acid-insoluble material occurred during germination, indicating that \(^{3}H\)cytidine was not incorporated into DNA. In the absence of any such incorporation, it was possible to show that 5-fluorouracil had a substantial effect on the incorporation of \(^{3}H\)cytidine into RNA (Table 2) although, at concentrations below 240 \( \mu g./ml. \), it had no effect on germination. This finding demonstrated further that much of the RNA synthesized before germ-tube emergence was inessential for germ-tube differentiation.
The type of RNA being synthesized during germination was examined by extracting RNA from conidia germinated for 60 min. in the presence of $[^3H]$uridine and fractionating it on a sucrose density gradient. The radioactive profile shown in

Fig. 2. Effect of proflavine (25 μg./ml.) and ethidium bromide (50 μg./ml.) on the incorporation of $[^3H]$uridine into RNA during the first 60 min. of germination. Conidia were labelled for 15 min. pulses with 2.0 μC/ml. $[^3H]$uridine. Incorporation was stopped by the addition of cold 30% (w/v) TCA and conidia were transferred to membrane filters as described in Fig. 1, except that the hot TCA wash was omitted. (When heated at 90° for 10 min. all radioactivity was lost from the conidia.) Filters were dried by successive washes with 95% (v/v) ethanol, and ether before the radioactivity was counted in a Packard Tri-carb Liquid Scintillation Spectrometer using 2 to 5 diphenoxazole (6-o-g./l. toluene) as scintillant. ×---×, Proflavine; △-----△, ethidium bromide. ○-○, control.

Fig. 3. Effect of proflavine and ethidium bromide on the germination of Peronospora tabacina. Proflavine and ethidium bromide were added at the start of germination and the percentage germination was assessed after 4 hr at 15°. ○-○, Proflavine; △-----△, ethidium bromide.

Table 2. The effect of 5-fluorouracil on $[^3H]$cytidine incorporation into RNA

Conidia (2.0 x 10⁹/ml.) were incubated for 60 min. in the presence of $[^3H]$cytidine (2.0 μC/ml.). Conidia were collected and prepared for scintillation counting as described in Fig. 2. The radioactivity lost on heating conidia for 10 min. at 100° in 5% (w/v) TCA was taken as a measure of the incorporation into RNA. After 4 hr incubation in the presence of 5-fluorouracil (240 μg./ml.) the percentage germination was 97%.

<table>
<thead>
<tr>
<th>Concentration of 5-fluorouracil (μg./ml.)</th>
<th>$[^3H]$Cytidine incorporated into RNA (d.p.m.)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>24,922</td>
<td>0</td>
</tr>
<tr>
<td>80</td>
<td>16,720</td>
<td>57</td>
</tr>
<tr>
<td>120</td>
<td>10,198</td>
<td>59</td>
</tr>
<tr>
<td>160</td>
<td>7,715</td>
<td>69</td>
</tr>
<tr>
<td>200</td>
<td>7,474</td>
<td>70</td>
</tr>
<tr>
<td>240</td>
<td>5,479</td>
<td>78</td>
</tr>
</tbody>
</table>
Fig. 4 shows two peaks which are characteristic for ribosomal RNA. Some incorporation into soluble RNA was observed, but no class of RNA corresponding to messenger RNA was synthesized. This ribosomal RNA was not required for germ-tube differentiation.

Fig. 4. Sucrose density gradient fractionation of RNA from germinating *Peronospora tabacina* conidia. 1·25 x 10⁷ conidia were incubated for 60 min. with a total of 50 μCi [3H]uridine. Uptake was stopped by rapidly cooling to 0°C. Conidia were collected by centrifugation and augmented with 2·5 x 10⁷ dormant conidia to provide sufficient RNA for manipulation during the isolation procedure. Conidia were suspended in 3 ml. 0·05 M-tris/HCl, pH 8·0, containing 5 x 10⁻⁶ M-MgCl₂ and 1% (w/v) sodium dodecyl sulphate (SDS) and sonicated (Raytheon D.F. 101; setting 50) for 20 min. at 4°C. Glass sinter was added to assist in breaking the conidia, with the result that breakage always exceeded 80%. The sonicate was extracted three times with tris-saturated phenol in the presence of 0·05 M-EDTA and 0·5% (w/v) SDS. All operations were carried out at 4°C and, after adjusting the combined aqueous phases to pH 5·8 with 20 M-sodium acetate, RNA was precipitated by the addition of two volumes of cold ethanol. The RNA was purified by precipitation with 1% (w/v) cetyl trimethylammonium bromide (CTAB). This CTAB + RNA precipitate was washed with 70% (v/v) ethanol in 0·1 M-sodium acetate and the resultant Na-RNA was dissolved in 0·2 ml. glass distilled water and stored at -15°C. The RNA (250 μg) was fractionated on a 5 to 20% (w/v) sucrose density gradient (0·025 M-tris HCl pH 7·0 containing 0·1 M-NaCl) by centrifugation for 4·5 hr at 37,000 rev./min. (Spinco S.W. 39 rotor). Samples were collected dropwise from the bottom after puncturing the tube, and the fractions assayed directly for radioactivity by means of liquid scintillation spectrometry. ---, control; ----, proflavine.

Table 3. The effect of ethidium bromide, 5-fluorouracil, and proflavine, on the incorporation of [14C]-labelled amino acids into protein

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>% germination</th>
<th>[14C]-labelled amino acids incorporated into protein (c.p.m.)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>2322</td>
<td>0</td>
</tr>
<tr>
<td>Ethidium bromide (50 μg./ml.)</td>
<td>100</td>
<td>2116</td>
<td>9</td>
</tr>
<tr>
<td>5-Fluorouracil (240 μg./ml.)</td>
<td>96</td>
<td>2252</td>
<td>3</td>
</tr>
<tr>
<td>Proflavine (25 μg./ml.)</td>
<td>98</td>
<td>1215</td>
<td>48</td>
</tr>
</tbody>
</table>
Germination of Peronospora conidia

Because, when conidia were labelled for 60 min. in the presence of proflavine (25 μg./ml.), radioactivity was not incorporated into either ribosomal peak.

At concentrations which inhibited RNA synthesis, yet had no effect on germination, ethidium bromide, and 5-fluorouracil had little effect on the incorporation of [¹⁴C] amino acids into protein (Table 3), whereas proflavine inhibited this incorporation by 50%. This experiment demonstrated not only that considerable protein synthesis occurred in the absence of RNA synthesis but also showed that not all the protein synthesized was essential for germ tube emergence.

DISCUSSION

These experiments demonstrate that protein synthesis is an essential requirement for germ-tube differentiation, but that RNA synthesis is not. Although [¹³C]uridine is incorporated into RNA prior to germ-tube emergence, this RNA synthesis is not essential and can be abolished by inhibitors which have no effect on germination. The protein which is involved in germ-tube formation appears, therefore, to be synthesized on a template of stable messenger RNA already present in dormant conidia. Alteration of this RNA in such a way that it is able to act as a template for protein synthesis may be the first step in the germination process.

Fungal spore germination involves changes in both metabolism and morphology, and as such provides a model system for studying the biochemical events controlling differentiation. Any developmental process involves the orderly and sequential expression of genetic information. Information of this nature could readily be controlled at the translation level and would require the presence of already transcribed messenger RNA within the dormant spore. Indeed, examples of translational control of differentiation are known in higher organisms. Fertilized sea-urchin eggs develop rapidly to the blastula stage in the absence of RNA synthesis, indicating the presence of stable messenger RNA within the unfertilized egg (Spirin, 1966). In the alga Acetabularia mediterranea, RNA synthesis is not required for the development of spore caps which are formed in the absence of a nucleus (Werz, 1965). Shaw (1967) reported that uredospores of Puccinia helianthi germinated when RNA synthesis was inhibited with actinomycin D, suggesting that control of germination in this fungus might also be at the translation level.

During germination, the rate of incorporation of [¹⁴C]-labelled amino acids into protein in Peronospora tabacina was exceedingly low when compared with similar rates determined for germinating conidia of saprophytic fungi (Staples et al. 1962). Low incorporation rates are found also in germinating rust uredospores and may be a property common to obligate parasites. Shaw (1967) attributed these low rates in rust uredospores to an absence of nucleoli, which appear to be the site of ribosomal synthesis (Perry, 1967). In P. tabacina ribosomal RNA is synthesized prior to germ-tube emergence, as it is in Aspergillus oryzae (Ono et al. 1966). Thus it would seem that the low rates of incorporation into protein found in P. tabacina, are not caused by a lack of ribosomal RNA synthesis.

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REFERENCES