Short Communication

Actinomycin D Inhibition of the Zinc-induced Formation of Cytochrome c in Ustilago

D. H. Brown, R. A. Cappellini, and C. A. Price

Plant Biology Department, Rutgers University, New Brunswick, New Jersey

Received May 18, 1966.

Summary. As reported earlier by Grimm & Allen, the addition of zinc to the sporidia of the smut fungus, Ustilago sphaerogena, evokes the formation of large amounts of cytochrome c. This occurs under conditions where the rates of increase of dry weight, RNA, and DNA remain unaffected. Actinomycin D added with zinc specifically abolishes the formation of cytochrome c. The system behaves as if cytochrome c were formed de novo.

We desired a system in which a physiological response to zinc was specific and not an indirect effect of zinc being required for growth. Sporidia of the smut fungus, Ustilago sphaerogena, (Burrill) (2), were shown by Grimm and Allen (3) to accumulate such high concentrations of cytochrome c that the cells became pink. Cytochrome c accumulation was observed with cells provided 10 μM zinc but not by cells provided 1 μM zinc or less. Candida yeast shows a similar but less dramatic response (12).

Whatever is the mechanism of action of zinc in this system, cytochrome must be formed either de novo or from a protein precursor. We sought preliminary evidence on this point by means of the antibiotic, actinomycin D, which in other systems has been shown to prevent transcription (9).

Materials and Methods

Growth and Harvesting Procedures. A culture of the sporidial stage of Ustilago sphaerogena (Burrill) was originally obtained from Dr. Paul J. Allen and maintained on 2% yeast-agar slants at 20°C.

All experiments were run using a rotary shaker (Gyrotory Shaker, Model V, New Brunswick Scientific Company), 260 rpm, 2.5 cm stroke, and maintained at 24 to 26°C. The cultures were grown from exponential inocula in 2-liter Erlenmeyer flasks containing 500 ml of a synthetic medium, modified from Grimm and Allen (3) (table I).

We took the usual precautions for the control of zinc levels (8), except that it was unnecessary to purify the components of the growth medium.

Cell densities were monitored turbidimetrically; with a Klett colorimeter and a 660 μm filter, we repeatedly found 0.39 mg dry weight/ml ± 100 Klett units for dilute cell suspensions.

Protocol for Obtaining Cell Samples. The cells

Table I. Medium A. Composition of the Synthetic Medium Used for Growing Ustilago sphaerogena

<table>
<thead>
<tr>
<th>Stock Solutions</th>
<th>Conc in Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.86 m Dibasic potassium phosphate</td>
<td>1.72 × 10^{-2} m</td>
</tr>
<tr>
<td>10.7 × 10^{-2} m Sodium citrate</td>
<td>2.14 × 10^{-3} m</td>
</tr>
<tr>
<td>1.95 m Ammonium acetate</td>
<td>3.90 × 10^{-2} m</td>
</tr>
<tr>
<td>5.0 × 10^{-6} m Manganese sulfate</td>
<td>10^{-6} m</td>
</tr>
<tr>
<td>5.0 × 10^{-4} m Copper sulfate</td>
<td>10^{-7} m</td>
</tr>
<tr>
<td>1.48 × 10^{-4} m Ferric sulfate</td>
<td>3.0 × 10^{-6} m</td>
</tr>
<tr>
<td>1.20 × 10^{-2} m Ferric sulfate</td>
<td>2.40 × 10^{-4} m</td>
</tr>
<tr>
<td>0.1625 m Magnesium sulfate</td>
<td>3.25 × 10^{-3} m</td>
</tr>
<tr>
<td>50% or 1.46 m Sucrose*</td>
<td>2% or 5.85 × 10^{-2} m</td>
</tr>
<tr>
<td>2 mg/ml Thiamine hydrochloride*</td>
<td>2 μg/ml</td>
</tr>
<tr>
<td>10^{-2} m Zinc sulfate</td>
<td>2 × 10^{-7} - 2 × 10^{-5} m</td>
</tr>
</tbody>
</table>

* Added aseptically after the remainder of the medium has been sterilized. (All solutions were sterilized by autoclaving at 15 psi for 15 minutes.)

1 From the doctoral thesis of D. H. Brown (1). Supported in part by a grant from the National Institutes of Health, AM-03267. Journal paper of the New Jersey Agricultural Experiment Station, Plant Biology Department, New Brunswick, New Jersey.

2 Present address: Biology Department, Oak Ridge National Laboratory, Oak Ridge, Tennessee.

Addendum

Addendum

Addendum

Addendum

Addendum
were harvested and samples collected according to the flow diagram of figure 1.

**Cytochrome c Analyses.** Cytochrome c was extracted by an adaptation of Neilands' (5) preparative procedure.

Packed cells containing 125 to 300 mg dry weight, were frozen in 12-ml polypropylene centrifuge tubes, resuspended in 3 ml of 0.5 M borate/potassium hydroxide buffer, pH 10.8, and agitated in a vortex shaker (Rotation — Evapo-Mix; Buchler Instrument Company) at room temperature for 5 hours. At the end of this time the extracted cells were centrifuged at approximately 6000 rpm in a SS-1 rotor in the RC-2 and the supernatant was collected and made up to 5 ml with distilled water. Further washing of the pellet did not yield measurable amounts of cytochrome c. The amounts of cytochrome c in the samples were determined by difference spectra of the oxidized and reduced pigment. \( \Delta A = A_{550} - A_{340} \) was found to have a standard error of repeatability of 1.0 mmole/ml on extracted samples, which corresponded to 0.02 mmole/ml culture.

Protein was determined by microkjeldahl on samples extracted with 5 ml of hot 10% w/v trichloroacetic acid.

Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) were determined by a modification of the method of Ougon and Rosen (6).

**RNA.** Approximately 25 mg dry weight of tissue was extracted for 18 to 20 hours with 5 ml of cold 10% w/v perchloric acid (PCA). The suspension was agitated in a vortex mixer during the period of extraction. At the end of this time the samples were centrifuged at 3000 rpm for 3 minutes. The supernatant was poured into a 100-

ml volumetric flask. The sediment was resuspended in 5 ml of cold 10% w/v PCA and the resulting supernatants added to the volumetric flask and made to volume.

**DNA.** Five ml of 5% w/v PCA was added to the sediment from which the RNA had been extracted. After heating for 20 minutes at 70° the resulting extract was filtered through Whatman No. 3 filter paper directly into a 50-ml volumetric flask. The sediment was rinsed with 5 ml of hot 5% w/v PCA and then thoroughly with distilled water. The filtrate was made to volume with distilled water.

Both RNA and DNA were determined spectrophotometrically and the results expressed in absorbancy units. RNA was taken as \( A_{260} - A_{280} \) and DNA as \( A_{260} - A_{280} \).

Actinomycin D was kindly supplied by Dr. H. D. Brown of Merck Sharp & Dohme.

**Results**

We confirmed Grimm and Allen's (3) primary observation that zinc is essential for cytochrome c synthesis in *Ustilago*. For our purpose, an equally crucial property of *Ustilago* as reported by Grimm and Allen, was that growth was independent of the zinc supply over the range affecting cytochrome c. We found that at 0.2 \( \mu \text{M} \) zinc, which is at the lower end of this range of zinc concentrations, growth, as measured by either dry weight or protein, is indeed affected and, moreover, total RNA levels are disproportionately affected (fig 2).

We then found that 0.4 \( \mu \text{M} \) zinc was insufficient for cytochrome c formation, but adequate for the synthesis of total protein. Cells were grown at this concentration until the cell density reached about 2 mg dry weight per ml (about 20 hrs) and then transferred to fresh medium containing 20 \( \mu \text{M} \) zinc or no added zinc. Dry weight, protein, and DNA increased exponentially over the succeeding 24 hours. RNA increased with more complex kinetics. The added zinc was without detectable effect on any of these components (fig 3a, b). In contrast cytochrome c formation was strongly dependent on zinc (fig 3c). The 10-fold increase in cytochrome c in 24 hours brought the level up to what it would have been if adequate zinc had been present from the beginning. Other experiments showed that the increased rate of cytochrome c formation became linear with time within 1 hour after the addition of zinc. A lag period of 30 minutes or less would not have been detected.

Our studies thus provided us with a simple test
system in which zinc would promote cytochrome c synthesis rapidly and without grossly affecting the growth of the cells as measured by 4 parameters of gross composition. The behavior of cultures made from different isolates of Ustilago have varied only in minor ways.

We expected that if the formation of cytochrome which is promoted by zinc requires the specification of new protein, actinomycin D should abolish any such increase in cytochrome c. Alternatively if zinc brought about the transformation of pre-formed protein, short term exposure to actinomycin D should not affect the rate of cytochrome formation.

We observed in fact that actinomycin D at 50 μg/ml left dry weight, protein N, and DNA unaffected for about 12 hours (fig 4a, b). The rate of RNA synthesis decreased slightly over the first 6 hours and then fell to zero. These patterns are consistent with the classical response of cells to actinomycin D. Cytochrome c synthesis however was immediately and absolutely abolished (fig 4c). Thus the promotion by zinc of cytochrome c formation is prevented under conditions where most of the proteins of the cell continue to be formed at normal rates.

Discussion

We found that after the simultaneous addition of zinc and actinomycin D, the total amounts of protein, DNA, and the bulk of the RNA continued to increase normally for at least 6 hours. If actinomycin D acts only by inhibiting the formation of mRNA (9), we should infer that most of the mRNA of the cells is long-lived. In sharp contrast to the effect of actinomycin D on total protein, the drug abolished the formation of cytochrome c. The data are thus completely consistent with the idea that the formation of cytochrome c evoked by zinc requires the release of new messenger. We take this as preliminary evidence for the view that the protein is formed de novo.

It is always hazardous to infer too much from data obtained with inhibitors, but the striking difference in the sensitivities of cytochrome c and bulk protein synthesis deserves comment. One possibility is of course that the lifetime of the cytochrome c mRNA is much shorter-lived than those of the bulk proteins. Another possibility is that the synthesis of cytochrome c occurs in a cell compartment that is especially accessible to the drug. In this connection, we note that a new class or classes of large particles are formed within Ustilago in response to zinc (7).

The net decrease in RNA in the very zinc-deficient cells is similar to that observed in Euglena (10,11), citrus leaves (4), and possibly in Mycobacterium smegmati (13).
Literature Cited


