Mitochondria from dormant spores of the fungus *Botryodiplodia theobromae* did not contain a functional oligomycin- or dicyclohexylcarbodiimide-sensitive adenosine triphosphatase (ATPase); however, this enzyme activity was elaborated rapidly after 120 min of a 240-min germination sequence. The development of oligomycin-sensitive ATPase in germinating spores was abolished by cycloheximide but initially was insensitive to inhibitors of the mitochondrial genetic system, chloramphenicol or ethidium bromide. Bioynthesis of the polypeptide subunits of the enzyme was measured through use of antiserum directed against the F,,-ATPase and the chloroform-methanol-soluble proteolipid of the enzyme. The immunoprecipitated enzyme consisted of at least 12 polypeptides with molecular weights ranging from 58,000–8,000. Biosynthesis studies with ribosome-specific inhibitors showed that only one polypeptide (subunit 7) of the enzyme was synthesized on mitochondrial ribosomes and that the remainder were products of cytoplasmic protein synthesis. Subunit 12 was characterized by its binding in vitro to [14C]dicyclohexylcarbodiimide, and analysis of its amino acid composition showed that the hydrophobic peptide had a polarity index of 25%. Synthesis of all enzyme subunits began together during the first 45 min of germination when little (if any) mRNA was synthesized, and this early synthesis in vivo depended upon translation of a mRNA preserved in these spores. An examination of subunit polypeptides present in the dormant spores showed that the respiration-incompetent mitochondrion contained at least two or three of the cytoplasmic subunits of the enzyme and that these preserved subunits were not assembled into a complex. *De novo* synthesis of the absent subunit polypeptides early in germination probably is required for assembly and function of the spore mitochondrial ATPase.

Dormant spores of the fungus *Botryodiplodia theobromae* contain mitochondria whose respiratory membrane is nonfunctional and structurally incomplete. These mitochondria have been shown to be deficient in cytochrome *a* (and heme *a*) and cytochrome *c* oxidase activity (1, 2). However, germination of these spores depends on a cyanide-sensitive respiration (3), and early in germination the respiratory membrane must be rapidly reorganized and assembled to provide for the respiratory demand of these cells. Certain enzyme complexes of mitochondrial respiratory membranes are composed of multiple, distinct subunit polypeptides which are synthesized on either mitochondrial or cytoplasmic ribosome systems (for a recent review, see Ref. 4). In previous analyses of the roles of the mitochondrial and nuclear genetic systems in the synthesis and reassembly of the respiratory membrane during *Botryodiplodia* spore germination, it was established that function of the mitochondrial protein synthesis system was not required for development of respiratory competence or cytochrome *c* oxidase activity, but function of the cytoplasmic protein synthesis system was essential (2, 3).

The mitochondrial ATPase (phosphohydrolase, ATPase, EC 3.6.1.3) has been purified from three fungal sources: *Saccharomyces, Torulopsis,* and *Neurospora* (7–9). The entire complex of the oligomycin-sensitive F,-F ATPase from these organisms has a molecular weight of about 470,000–520,000; preparations from the yeasts consist of 9 (7) or 12 (8) nonidentical subunit polypeptides, and the *Neurospora* enzyme contains 11 subunits (9). Most of the enzyme subunits are synthesized on cytoplasmic ribosomes and transported into the mitochondria for assembly into the respiratory membrane. However, a small number of the ATPase subunits are products of the mitochondrial genetic and protein synthesis systems; in *Saccharomyces,* two to four of the peptides are reported to be mitochondrial ribosome products (7, 10, 11), whereas in *Neurospora* (12) and *Aspergillus* (13), two or three of the ATPase subunits, respectively, are derived from mitochondrial protein synthesis. The hydrophobic DCCD-binding "proteolipid" (subunit 9) of *Saccharomyces* is a product of a mitochondrial gene, whereas the counterpart subunit of *Neurospora* is a nuclear gene product and is synthesized on cytoplasmic ribosomes (4, 14, 15).

The indication that cytoplasmic protein synthesis is required for elaboration of mitochondrial respiratory activity early in germination suggests (2, 3) that some protein components assembled into the developing respiratory membrane could be products of the latent nuclear mRNA which is preserved in these spores and translated during the earliest phase of germination in the absence of new nuclear mRNA synthesis (3, 5, 6). Alternatively, the cytoplasmic protein synthesis may be required only indirectly for reactivation of the respiratory membrane whose components could be preserved in the spores for assembly or activation upon initiation of spore germination. In the present study, we sought to test these hypotheses by examination of the catalytic activity of the oligomycin-sensitive ATPase in mitochondria of germinating spores and the ATPase peptide subunit structure and biogenesis during germination.

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The abbreviations used are: DCCD, dicyclohexylcarbodiimide; SDS, sodium dodecyl sulfate.
The results obtained showed that the respiration-incompetent mitochondria of dormant spores did not contain a catalytically active ATPase and that this activity was elaborated during germination through steps requiring cytoplasmic (but not mitochondrial) protein synthesis. The Botryodiplodia ATPase contained about 12 nonidentical subunits of which was a product of mitochondrial ribosomes. We found that the cytoplasmically synthesized subunits of the ATPase F₁ sector were synthesized in vivo from the mRNA preserved in the dormant spores. This suggests that translation of the mRNA of dormant spores is involved in the reassembly of at least part of the mitochondrial respiratory system.

EXPERIMENTAL PROCEDURES

Growth of Cells and Preparation of Cell Fractions—Techniques for production and germination of Botryodiplodia spores have been described in detail previously (1, 3). Neurospora crassa (74A) mycelia were grown as described elsewhere (16). Mitochondrial fractions of Botryodiplodia and Neurospora were prepared from small quantities (0.2–6 g) of radiolabeled cells which were disrupted in a mechanical homogenizer as described previously (2, 3) or from large quantities of Neurospora mycelium which were disrupted in liquid nitrogen; 0.5 g of a mycelial suspension supplemented with sodium [35S]sulfate contained no standard trace element solution, and MgCl₂ was substituted for MgSO₄. Submitochondrial particles were generated by sonic irradiation and isolated by centrifugation as described (18); the pellets were resuspended in a buffer solution containing 10 mM Tris-HCl at pH 7.5 and 500 mM (v/v) glycerol.

Isolation of ATPase Subunit 12 (DCCD-binding Protein)—The DCCD-binding protein was isolated by chloroform-methanol extraction of mitochondrial membranes (19). Coupling of DCCD to chloroform-methanol-soluble protein was performed by incubating mitochondrial suspensions with an ethanolic solution of [14C]DCCD (10 μCi/mg of mitochondrial protein, 0.1 μCi) for 4 h at 0 °C.

Immunological Techniques—Antiserum against subunit 12 was prepared by injecting rabbits subcutaneously with 1.5 mg of subunit protein (dissolved in 2% SDS (v/v)) which was mixed with 2 volumes of equal portions of Freund’s complete and incomplete adjuvants (Difco). Three consecutive weekly injections of 1.0 mg of protein each (in the incomplete adjuvant) subsequently were administered. Animals were bled via the central artery of the ear 2 weeks after the final injection; the serum was prepared by centrifugation of whole blood and was stored in small portions.

The antiserum developed against subunit 12 gave a precipitin line (in Triton X-100-glycerol-antiserum-agarose double diffusion plates with a 9-mm diffusion radius) at a 1:4 dilution ratio (in 5 μl volume) of the serum against a sample of purified subunit 12 protein (7.5 μg in 5 μl). Antiserum developed against Neurospora F₁-ATPase was donated by Dr. Walter Sebald (Gesellschaft für Biotechnologische Forschung mbH, Braunschweig-Stockheim, BHD).

Conditions for Triton X-100 lysis of the radiolabeled mitochondrial fraction, for the immunoprecipitation reaction (4 h), and for processing of the ATPase immunoprecipitates are detailed in Ref 17. Staphylococcus aureus protein A coupled to Sepharose CL-4B (Pharmacia) was used for precipitation of specific IgG bound to subunit 12 protein (20). Prior to the indirect immunoprecipitation of the subunit 12 protein, diethyl ether precipitates from chloroform-methanol-extracted cellular fractions were dissolved in 50 μl of 0.1% SDS (w/v). The SDS solution was then diluted 20-fold with the immunoprecipitation reaction solution. The washed precipitates were incubated in an electrophoresis buffer containing SDS and β-mercaptoethanol for 8 h at 3 °C and subjected to electrophoresis in polyacrylamide gels (12.5% or 15%) as detailed previously (17, 18).

Radiolabeling Techniques—The [3H]amino acids used had specific activities of 9.6–115 Ci/mmol, the [14C]lcucine had 270 mCi/mmol, and the sodium [35S]sulfate had 945 mCi/mmol. These labeled compounds were obtained from New England Nuclear Corp. [3H]DCCD (50 mCi/mmol) was a product of Commissariat a L’Energie Atomique and was obtained from Research Products International Corp. The germinating spores and sporulating cultures were labeled as described previously (3) in a chemically defined medium.

Miscellaneous Procedures—Amino acid analysis of the DCCD-binding subunit 12 was performed with aliquots of the hydrolyzed with 6 N HCl at 110 °C for 12, 24, and 36 h. The quantities of individual amino acids were measured with a Beckman 120 analyzer modified with a Durrum system for single column analysis of protein hydrolysates. ATPase activity of spore submitochondrial particles was determined at 34 °C and pH 7.5 with 3 mM ATP as substrate (21); concentration of oligomycin and DCCD used in these assays was 0.5 μg/ml. Measurement of the inorganic phosphorus released in the ATPase assay was performed by the method of LeBel et al. (22). Protein was determined by the method of Lowry et al. (23), with bovine serum albumin used as the standard. Other technical procedures, inhibitor concentrations, and sources of miscellaneous supplies are published (2, 3, 17, 18).

RESULTS

General Characteristics of Spore Germination—The 240-min germination sequence of Botryodiplodia spores consists of a pre-emergence phase (150 min) and an emergence phase (90 min) during which 90–95% of the spores develop elongating germ tubes. After 300 min, the dry weight of the cells begins to increase with a mass doubling time of 240 min. Germination requires oxygen and a functional electron transport system; antimycin A and cyanide completely inhibit both germination and oxygen consumption (3). Spores incubated in the presence of chloramphenicol or ethidium bromide (which have been demonstrated directly to disrupt function of the mitochondrial genetic system in Botryodiplodia) germinate and respiration rates comparable to those of untreated spores (3, 16, 18, 24); however, cycloheximide (an inhibitor of cytoplasmic ribosomes) completely inhibits germination and respiration if it is administered to the spores during the first 135 min of incubation (2). Cerulenin (a reversible inhibitor of fatty acid synthesis in these spores) severely retards spore germination and respiration (25).

Catalytic Activity of ATPase in Dormant and Germinated Spores—The specific activity of the oligomycin-sensitive ATPase in submitochondrial particles from germinated spores (240 min) was 1.51 pmol of Pi/h/mg of protein and the activity of the same fraction from dormant spores was 0. When the oligomycin was replaced with another inhibitor of this enzyme, DCCD, the inhibitor-sensitive enzyme activity of the submitochondrial particles from germinated spores was 1.72 and that of the dormant spores was 0 (0 ≤ 0.03 μmol of Pᵢ).

Using dormant spores, we were unable to detect any activity of the oligomycin-sensitive ATPase in the submitochondrial particles or in intact mitochondria when the concentration of mitochondrial protein in the assay mixture was increased to 10 times that used in assaying the ATPase activity of mitochondria in the germinated spores. To test the possibility that a soluble, endogenous inhibitor of the oligomycin-sensitive ATPase could cause the apparent absence of enzyme activity in dormant spore extracts, a series of mixing experiments was conducted in which the active enzyme fraction from germinated spores was combined in the assay mixture with fractions from the dormant spores. Neither the submitochondrial particle fraction from dormant spores, which was a counterpart of the active fraction from germinated spores, nor the postmitochondrial supernatant fluid from extracts of dormant spores had any effect upon the activity of the fraction from germinated spores.

Similar assays were conducted with submitochondrial particles prepared from freshly harvested, dormant conidiospores of N. crassa (74A) in order to learn whether these spores also were deficient in this ATPase activity and to test whether the preparation and assay conditions would allow detection of oligomycin-sensitive ATPase in submitochondrial particles from these dormant spores. Instead, we found that these spores contained relatively high amounts of this oligomycin-sensitive activity (1.96 μmol of Pᵢ/h/mg of protein).

Elaboration of ATPase Activity during Spore Germination—Submitochondrial particles were prepared from samples of spores collected after each 30-min interval of the 240-min
germination sequence. Assays of the oligomycin-sensitive ATPase activity in these fractions provided a pattern of development of this enzyme activity in the germinating spores (Fig. 1). These results show that the spore mitochondria did not elaborate a detectable oligomycin-sensitive ATPase until after 120 min of germination, at which time development of the enzyme specific activity began to accelerate rapidly; by 240 min, the specific activity was about 75% of that present in the counterpart fraction from midlog (16-h) mycelial cells. It is likely that at least a small amount of ATPase activity was present in the germinating spore mitochondria before 120 min of germination, but it may have been undetectable because of possible instability of the enzyme or because the assay of the enzyme was insufficiently sensitive. This pattern of elaboration, however, is very similar to that observed previously for cytochrome c oxidase activity during germination of these spores (2, 16).

Chloramphenicol and ethidium bromide have been shown to inhibit mitochondrial protein synthesis (3, 16, 18) and DNA synthesis (24) in germinating spores of Botryodiplodia. We found that incubation of these spores in the presence of either of these inhibitors did not block the development of oligomycin-sensitive ATPase activity (Fig. 1), although the specific activities of the preparations from spores treated for 240 min were about 50% lower than in the control. In contrast, treatment of the spores with cycloheximide throughout incubation prevented development of any enzymatic activity (Fig. 1). If cycloheximide was added to the incubation medium at any time before 130 min, the elaboration of ATPase activity was blocked; if the drug was added to the spores at 135, 140, or 150 min, increasingly greater amounts of enzyme activity were developed (data not shown).

*Cellular Biogenesis of the ATPase*—Previous work established that an antiserum to the F1–Fo ATPase of Neurospora accurately and efficiently recognized the entire Fl–Fo ATPase of Botryodiplodia.* Precipitation of the entire F1–Fo ATPase complex with an antiserum directed against the Fl–Fo ATPase presumably is accomplished because of the stable association (in the detergent extracts of the mitochondria) of the immunoactive F1 sector with the Fo sector and other subunit(s) of the enzyme subunit complex. When an electrophoretic comparison was made of the subunit patterns of the Fl–Fo ATPase immunoprecipitates obtained from mitochondria of Botryodiplodia and Neurospora with antiserum raised against the Fl–Fo ATPase of Neurospora, the subunit compositions of the enzyme from the 2 organisms were nearly identical (Fig. 2). The Botryodiplodia enzyme consisted of 12 distinct subunit polypeptides and the apparent M, (as measured in both 12.5 and 15% polyacrylamide gels) ranged from 58,000–8,000. In the particular experiment represented in Fig. 2, the subunit pairs 1 and 2, as well as 5 and 6, were not resolved; however, in other analyses, the components of these pairs often were resolved into distinct bands.

Subunit 12 (M, of 8,000) of the Botryodiplodia ATPase was of particular interest to us because a protein with similar electrophoretic mobility has been reported to be the hydrophobic, DCCD-binding protonophore of the ATPase in mitochondria of yeast and of Neurospora (19), where it is a product of mitochondrial or nuclear genes, respectively. We extracted and purified the protein from Botryodiplodia with the chloroform-methanol procedure of Sebald et al. (19), which gave an electrophoretically homogeneous preparation. An experiment was performed in which [3H]leucine-labeled mitochondria from germinated spores were mixed and co-extracted with other mitochondria that had been incubated in vitro with [14C]DCCD; the chloroform-methanol-soluble proteins prepared from these mitochondria were analyzed by electrophoresis in 15% polyacrylamide-SDS gels (Fig. 3). The near congruence of the [14C]DCCD-labeled peptide with that of the [3H]leucine-labeled subunit demonstrates probable identity of the Botryodiplodia subunit 12 with the counterpart DCCD-binding subunit of the ATPases of Neurospora and yeast. The [14C]DCCD-bound protein in Fig. 3 was retarded slightly in electrophoretic mobility in relationship to the H-labeled subunit; the cause of this retardation is not known, but a similar effect has been observed with the Neurospora and yeast enzyme subunits (19). In addition, a minor peak of 14C radioactivity was present in the gel pattern (with an apparent M, of 22,000). This peptide, which was invariably extracted with chloroform-methanol from mitochondria labeled in vitro with [14C]DCCD, could be an aggregate of

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\* W. Sebald and R. Brambl, unpublished results.

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subunit 12 or another hydrophobic substance which reacts with DCCD.

The DCCD-binding subunit 12 of the Botryodiplodia ATPase was subjected to amino acid analysis for comparison to the analogous proteins from other sources. This analysis (Table I) indicated that the subunit peptide contained about 75 residues, a value which agrees with the apparent peptide had a polarity index of 25%, which agrees with the apparent molecular weight estimated by SDS-polyacrylamide gel electrophoresis. The peptide had a polarity index of 25%, as calculated by the method of Capaldi and Vanderkooi (27); three amino acids (glycine, alanine, and leucine) made up 41% of the total and their abundance probably contributes to the characteristic solubility properties of this protein. The subunit contained no histidine, and few residues of lysine and threonine were present. In all of these characteristics, the Botryodiplodia subunit 12 is similar to the subunits from Neurospora and yeast (19).

Biogenetic Origin of the Subunits of F$_{1}$-F$_{0}$ ATPase—We employed the ribosome-specific inhibitors chloramphenicol and cycloheximide to determine which of the ATPase subunits of Botryodiplodia were synthesized via the mitochondrial protein synthesis system. Previous studies with this organism (17) and with Neurospora (28, 29) showed that a direct radiolabeling of the cells in the presence of one of these inhibitors would not permit an exclusive labeling of the products of the other ribosome system. Instead, to label the mitochondrial ribosome products, it was necessary to first incubate the spores for 240 min in a medium containing chloramphenicol to allow an accumulation of the cytoplasmic ribosome products. After thoroughly washing the cells free from chloramphenicol, the spores were transferred to a fresh medium containing cycloheximide and [3H]leucine for an additional 60-min incubation during which the mitochondrial ribosomes synthesized labeled products that associated with the accumulated products from the cytoplasmic ribosomes to yield a complete enzyme complex.

In Fig. 4 are shown the results of such an experiment in which the mitochondria were prepared from germinated spores labeled in the presence of cycloheximide and treated with antiserum to the F$_{1}$-ATPase. Electrophoresis of the resulting immunoprecipitate showed that subunit 7 of the ATPase ($M_r = 20,000$) was the only component of this enzyme synthesized in the presence of cycloheximide. This same peptide has been reported to be a product of mitochondrial ribosomes in Neurospora and Aspergillus, but the ATPases of these organisms also contain, respectively, either one or two additional peptides ($M_r = 11,000$ and 14,000) synthesized in the presence of cycloheximide (12, 13).

The hydrophobic DCCD-binding subunit of the ATPase is a product of a mitochondrial gene in yeast (30) and a nuclear gene in Neurospora (14). The previous experiment in this study indicated that this subunit 12 in Botryodiplodia prob-

**TABLE I**

### Amino acid composition of the purified DCCD-binding subunit 12 from Botryodiplodia

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Percentage in mol/100 mol</th>
<th>No. of residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>6.31</td>
<td>5</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.95</td>
<td>2</td>
</tr>
<tr>
<td>Serine</td>
<td>4.97</td>
<td>4</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>6.89</td>
<td>5</td>
</tr>
<tr>
<td>Proline</td>
<td>3.33</td>
<td>3</td>
</tr>
<tr>
<td>Glycine</td>
<td>14.79</td>
<td>11</td>
</tr>
<tr>
<td>Alanine</td>
<td>15.00</td>
<td>11</td>
</tr>
<tr>
<td>Valine</td>
<td>8.17</td>
<td>6</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.33</td>
<td>2</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>7.53</td>
<td>6</td>
</tr>
<tr>
<td>Leucine</td>
<td>12.19</td>
<td>9</td>
</tr>
<tr>
<td>Tyrosine</td>
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<td>2</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>8.47</td>
<td>6</td>
</tr>
<tr>
<td>Histidine</td>
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<td>0</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.34</td>
<td>1</td>
</tr>
<tr>
<td>Arginine</td>
<td>3.24</td>
<td>2</td>
</tr>
<tr>
<td>Tryptophan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cysteine</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>99.97</td>
<td>75</td>
</tr>
<tr>
<td>Polarity index*</td>
<td>25%</td>
<td></td>
</tr>
</tbody>
</table>

* Calculated according to Capaldi and Vanderkooi (27).

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**Fig. 3.** SDS-polyacrylamide gel co-electrophoretic analysis of the ATPase subunit 12 extracted with chloroform-methanol from mitochondria of germinating spores labeled *in vitro* with [3H]leucine (○) or *in vivo* with [14C]DCCD (○).

**Fig. 4.** SDS-polyacrylamide gel electrophoretic analysis of an immunoprecipitate obtained from mitochondria of germinating spores labeled with [3H]leucine from 240–300 min in the presence of cycloheximide after a transitory incubation of the spores from 0–240 min in the presence of chloramphenicol.
ably was synthesized on cytoplasmic ribosomes; however, this conclusion is vulnerable because it depends upon incorporation of this subunit (in the presence of cycloheximide) into a complex which would be precipitated by an antiserum to the F₁-ATPase. As an alternative approach to establish the ribosomal origin of this subunit peptide, we used the chloroform-methanol extraction procedure to isolate the protein from mitochondria of spores which had been labeled with [³H]-leucine in the presence of chloramphenicol or cycloheximide. The results of an experiment in which equal quantities of drug-treated spores were extracted showed that subunit 12 (as identified by in vitro labeling with [¹⁴C]DCCD) was synthesized in the presence of chloramphenicol (Fig. 5A), but that it was not synthesized in the presence of cycloheximide (Fig. 5B). Therefore, this subunit of the ATPase, unlike that of yeasts, is a product of cytoplasmic ribosomes in Botryodiplodia and (like Neurospora) probably is a product of a nuclear gene.

Biosynthesis of the ATPase Subunit Peptides during Spore Germination—During the first 45 min of spore germination in Botryodiplodia, little (if any) mRNA is synthesized, and the rapid protein synthesis in these cells depends upon translation of a mRNA population which is accumulated during sporulation and preserved during spore dormancy (5, 31). To test the question of whether this preserved mRNA could be translated in vitro into the subunits of the ATPase, we radio-labeled the spores with [³H]-leucine during the first 45 min of germination and performed an ATPase immunoprecipitation assay of the mitochondria obtained from these spores. In addition, we labeled the spores during three later intervals of germination and performed the same assays of the spore mitochondria to establish whether there was a temporal sequence in the synthesis and assembly of the subunit polypeptides into the ATPase during spore germination.

The spores radiolabeled during the four intervals of spore germination were rapidly harvested at the end of each labeling interval, and the immunoprecipitates obtained from the mitochondria were subjected to electrophoresis in SDS-polyacrylamide (12.5%) gels. The results of this experiment showed that synthesis of all of the subunit polypeptides began simultaneously during the first 45-min interval of germination (Fig. 5A) and continued throughout germination (Fig. 5, B, C, and D). During the last interval (150-240 min), the quantities of the subunits synthesized (per unit spore mass) increased sharply. The results of this experiment demonstrated that synthesis of the mitochondrial and cytoplasmic subunits of the ATPase began together during the first 45 min of germination, and that the synthesis of one group of subunits did not precede others, unlike the pattern of synthesis of the subunits of cytochrome c oxidase in these spores (16).

Subunits of the ATPase in Dormant Spores—Mitochondria of the dormant spores of Botryodiplodia do not contain an oligomycin-sensitive ATPase, and the previous experiments showed that this enzyme was synthesized in the earliest phase of spore germination. Therefore, we wished to determine which (if any) of the subunits of the mitochondrial ATPase were preserved in the dormant spores, whether they were assembled into a complex and whether the absence of the catalytic activity could be due to an absence of assembled subunit polypeptides.

 Cultures of Botryodiplodia were allowed to sporulate on a low sulfur-defined medium containing sodium [³⁵S]sulfate; the dormant spores were harvested and the [³⁵S]-labeled mitochondria were immediately extracted. Immunoprecipitation assays of these mitochondria with the F₁-ATPase antiserum showed...
peptides isolated with antiserum to the F1-ATPase from mitochondria of dormant spores labeled during sporulation with sodium [35S]sulfate. Detection of only subunit polypeptides actually were absent from mitochondria in the course of spore disruption. However, this antiserum was not a reservoir for compartmentalization and preservation of some of these enzyme subunits during dormancy. Since the antiserum used in this experiment (Fig. 7) was directed against the assembled subunits of the F1-ATPase, the detection of only subunit 2 could mean that some remaining subunit polypeptides actually were absent from mitochondria of the dormant spores or that some of them were present but not assembled with the antigenically reactive subunit 2. To help decide between these alternatives, we prepared an antiserum against the purified DCCD-binding subunit of Neurospora, and we used this antiserum to determine whether the corresponding subunit 12 was present in the dormant spores of Botryodiplodia which were radiolabeled with four 3H-labeled amino acids. The mitochondria prepared from these dormant spores were treated with this antiserum and the immunoprecipitate was subjected to electrophoresis. The result (Fig. 8) showed that the mitochondrial membranes of these spores indeed contained this subunit polypeptide. A similar immunoprecipitation assay of the postribosomal supernatant fluid of the same 3H-labeled dormant spores showed that no detectable F0 sector subunit 12 was present in the extramitochondrial cytoplasmic fraction. These results indicate, therefore, that in addition to subunit 2 of the ATPase, at least one other subunit of this complex was present in mitochondria of the dormant spores, and that those subunits present were not assembled into an antigenically recognizable complex.

DISCUSSION

The ATPase of Botryodiplodia isolated in this study via immunoprecipitation contained at least 12 different subunit polypeptides. In comparison, Saccharomyces and Torulopsis have 9 or 12 subunits each (7, 8) and Neurospora and Aspergillus have 11 each (9, 13). In Botryodiplodia, only 1 of these subunits (Mr = 20,000) was synthesized on mitochondrial ribosomes, whereas the ATPases from these other fungal sources may contain from 2-4 subunit polypeptides derived from the mitochondrial genetic system. The hydrophobic, DCCD-binding subunit 12 of the Botryodiplodia enzyme proved to be a product of cytoplasmic ribosomes, as is the analogous subunit of Neurospora (15), but this is in contrast to Saccharomyces, where it is a product of mitochondrial ribosomes (4).

As is shown in this study, mitochondria of the dormant spores of Botryodiplodia did not contain an ATPase activity which was sensitive to oligomycin or DCCD. This catalytic activity was elaborated rapidly, however, during the course of spore germination through a series of steps which required cytoplasmic protein synthesis, but which did not require mitochondrial protein synthesis. These results imply that the structural contribution of the mitochondrial genetic system to the ATPase complex (subunit 7, Mr = 20,000) must be preserved in the mitochondria of the dormant spores and must be available for assembly into the enzyme complex upon initiation of spore germination.

The obligate role for cytoplasmic protein synthesis in the reactivation of the ATPase during spore germination could be due to an indirect requirement (not related to ATPase subunit synthesis or assembly) or to a direct synthesis of one or more subunits of the enzyme for subsequent assembly and function. The former possibility is difficult to test and to exclude completely; however, we believe that other evidence favors a direct role for cytoplasmic protein synthesis de novo in the structural assembly of the ATPase of the germinating spores.

All of the subunits of the mitochondrial F1-F0 ATPase were synthesized in vivo during the first 45 min of germination, a period during which the protein synthesis in these spores depends upon translation of a population of mRNAs which are preserved during spore dormancy (5, 31). This mRNA population of the dormant spores was isolated and translated in vitro into at least some subunits of the F1-ATPase, thereby demonstrating a probable involvement of the early (0-45 min)
translation of the preserved mRNA in the synthesis and structural assembly of the enzyme.

Immunoprecipitation assays of mitochondria from radiolabeled dormant spores showed that low amounts of at least two subunits of the F1-F0 ATPase were preserved in these mitochondria. Other subunits of the enzyme may or may not be present; they could be present in a nonimmunoreactive form or in quantities too low to be recognized. However, it is clear that the subunit polypeptides preserved in these mitochondria (subunit 2 of the F1 sector and subunit 12 of the F0 sector) were not assembled into a complex and that they were present in the mitochondria as unassociated polypeptides. Whether these residual subunits preserved during dormancy eventually become a part of a functional enzyme during spore germination remains to be established; but because they also are synthesized de novo in the earliest phase of germination, it seems possible that they do not have an essential role in the reassembly of the mitochondrial ATPase.

The indication that assembly of a functional ATPase depends upon translation of the mRNA preserved in the spores contrasts with the mechanism for reassembly of cytochrome $c$ oxidase in germinating spores of Botryodiplodia (16). Elaboration of the catalytic activity of this enzyme, as with the ATPase, is insensitive to chemical inhibitors of the mitochondrial genetic system, a fact which suggested that the enzyme subunits synthesized via mitochondrial protein synthesis are preserved in the dormant spores (2). This prediction subsequently was confirmed directly, but it was also shown that cytochrome $c$ oxidase becomes functional during spore germination not as a result of de novo synthesis of any of the enzyme cytoplasmic subunits, but through a recruitment of the preserved, compartmentalized cytoplasmic subunits from the cytoplasm of the dormant spores into the mitochondria (16). Upon initiation of germination, the preserved cytoplasmic subunits are imported into the mitochondria where they are assembled with heme $a$ and the mitochondrial subunits to yield a functional complex. Unlike those subunits of cytochrome $c$ oxidase, the cytoplasmically synthesized subunits of the ATPase were not accumulated and preserved in the extramitochondrial cytoplasm of the dormant spores. It seems likely, therefore, that within the germinating spores of this organism, there are different mechanisms involved in the reactivation of two separate complexes of the dormant spore respiratory system.

This present study provides the first direct demonstration of a role for the latent mRNA which is preserved in dormant cells of many species of animals, plants, and microorganisms (26). Among the diverse functions served by this population of mRNA in the transition from metabolic quiescence to rapid growth, our earlier studies (2, 3, 18) suggested that its translation early in germination contributed to the reactivation of the mitochondrial respiratory system of the Botryodiplodia spores. The translation of this mRNA in vivo (and in vitro) into components of the mitochondrial ATPase now demonstrates one specific role of this mRNA in the initiation of spore germination.

REFERENCES