The apparent molecular weights of these components are I, 42,000; II, 34,500; III, 23,000; IV, 14,000; V, 12,500; and VI, 9,500. (Although Component V actually consists of two distinct polypeptide species, it will be regarded as homogeneous in this study.)

In order to study the biosynthesis of these components, yeast cells were labeled with [3H]leucine in the presence of specific inhibitors of mitochondrial and cytoplasmic protein synthesis. Labeled cytochrome c oxidase components were then isolated from crude mitochondrial extracts by immunoprecipitation and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Labeling of the three large Components I, II, and III was insensitive to cycloheximide and sensitive to erythromycin. Labeling of the two largest Components I and II was dependent on the presence of oxygen. The three small Components IV, V, and VI were not labeled in the presence of cycloheximide but became labeled in the presence of erythromycin.

These results show that our cytochrome c oxidase preparation contains three polypeptides which are translated on mitochondrial ribosomes and three polypeptides which are translated on cytoplasmic ribosomes. Two of the mitochondrialy synthesized polypeptides are only made in the presence of oxygen.

The biosynthesis of cytochrome c oxidase shares many features with the biosynthesis of the mitochondrial inner membrane as a whole (1-4). Genetic experiments with yeast (5-7) and Neurospora cells (8, 9) have indicated that the formation of active cytochrome c oxidase is controlled by both nuclear and mitochondrial genes. This dual control was reemphasized by biochemical studies which showed that the synthesis of cytochrome c oxidase requires a cooperation between mitochondrial and cytoplasmic protein synthesis (10-12). The biosynthesis of cytochrome c oxidase is also dependent on oxygen (5) and repressed by glucose (13). A detailed knowledge of cytochrome c oxidase formation would thus be of great value for understanding the mechanism of mitochondrial biogenesis.

In the preceding publication of this series (14), we have described the isolation of a highly purified cytochrome c oxidase from bakers' yeast. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis resolved the enzyme into six polypeptide bands with the following apparent molecular weights: 42,000; 34,500; 23,000; 14,000; 12,500; and 9,500. All six components could be specifically precipitated from crude mitochondrial extracts by antisera against either the holoenzyme or the three small polypeptides only. These results opened a way for studying the biosynthesis of cytochrome c oxidase by suitable labeling techniques.

In this investigation we attempted to identify the intracellular sites at which the cytochrome c oxidase polypeptides are synthesized. We found that the three large polypeptides are synthesized in mitochondria, whereas the three small polypeptides are synthesized in the cytoplasm. These results suggest that mitochondria synthesize three subunits of cytochrome c oxidase. They also show that the interplay between mitochondrial and cytoplasmic protein synthesis can be studied at the level of a single enzyme.

**MATERIALS AND METHODS**

The *Saccharomyces cerevisiae* yeast strain D 273-10B, the isolation of mitochondria, and the procedures used for immuno-precipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis have been described earlier (14). When the yeast cells were cultured in the presence of radioactive amino acids, the medium specified in the preceding paper (14) was used. For growth in the absence of labeled amino acids, the concentration of yeast extract in the medium was raised to 3 g per liter. Unless stated otherwise, the conditions for labeling resting yeast

1 As mentioned in the preceding paper (14), the polypeptide band of apparent molecular weight 12,500 can be resolved into two components by isoelectric focusing in the presence of urea or by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in gels containing very high (>16%) concentrations of acrylamide. Our preparation of cytochrome oxidase is thus actually composed of at least seven different species of polypeptides. In the present discussion, however, we shall ignore the heterogeneity of Band V and refer to six polypeptides only.

2 Preliminary accounts of some of these experiments have been published elsewhere (15-17).
cells in the absence and presence of antibiotics were the same as in earlier studies (18).

RESULTS

Mitochondrial Synthesis of Cytochrome c Oxidase Polypeptides—Are any of the six polypeptides associated with yeast cytochrome c oxidase (14) synthesized on mitochondrial ribosomes? In order to answer this question, yeast cells were grown in the presence of [U-14C]leucine, harvested, and then labeled with [3H]leucine in the presence of cycloheximide. This antibiotic specifically inhibits translation on cytoplasmic ribosomes and thus allows the in vivo labeling of mitochondrial translation products (19, 20). Cytochrome c oxidase was then isolated by immunoprecipitation; the immunoprecipitate was electrophoresed on sodium dodecyl sulfate-polyacrylamide gels, the gels were sliced and each slice was counted for 3H and 14C. The distribution of 14C (reflecting total yeast protein) shows the expected peaks corresponding to the six cytochrome c oxidase polypeptides. (The two closely adjacent components of molecular weight 14,000 and 12,500 are not clearly resolved by our slicing technique.) The distribution of 3H (reflecting mitochondrionally synthesized polypeptides) shows three distinct peaks that coincide exactly with the three large (14C-labeled) cytochrome c oxidase polypeptides. Identical results were obtained if the concentration of cycloheximide was varied between 25 and 250 μg per ml or if the length of labeling was varied between 10 and 60 min. The fact that cycloheximide completely inhibited labeling of the three small polypeptides attests to the effectiveness of this antibiotic under our conditions. Since labeling of the three large polypeptides is resistant to cycloheximide and sensitive to inhibitors of mitochondrial protein synthesis (see below) it is obviously mediated by mitochondrial ribosomes.

In the experiment shown in Fig. 1, the specific radioactivities of the three large polypeptides (measured as the 3H:14C ratios) were roughly identical. However, in several labeling experiments with different batches of the same yeast strain, the 3H:14C ratios of the two largest cytochrome c oxidase polypeptides were up to 2-fold lower than the 3H:14C ratio of the 23,000-dalton component. We have no clear-cut explanation for this variability, but it may reflect variations in the pools (21) or in the turnover rates of the 42,000- and 34,500-dalton components.

Cytoplasmic Synthesis of Cytochrome c Oxidase Polypeptides—Antibacterial antibiotics such as erythromycin or chloramphenicol inhibit translation on mitochondrial ribosomes and do not materially inhibit translation on cytoplasmic ribosomes (1–4). When cytochrome c oxidase was isolated from yeast cells that had been labeled with [3H]leucine in the presence of erythromycin, only the three small polypeptides proved to be radioactive (Fig. 2). Labeling in the presence of acriflavine (another specific inhibitor of mitochondrial protein synthesis) gave a similar result (not shown). Since the three small polypeptides are labeled in the presence of erythromycin, but not in the presence of cycloheximide, they must be synthesized on cytoplasmic ribosomes.

Table I lists the relative amounts of radioactivity which are incorporated into cytochrome c oxidase under different conditions. It can be seen that, in the absence of antibiotics, 3% of the [3H]leucine incorporated into sub mitochondrial particles is associated with cytochrome c oxidase. If all polypeptides of the enzyme were equally labeled, roughly one-third of this percentage value (i.e., 1%) would reflect label associated with the cytoplasmically synthesized Components IV to VI (cf. Table IV of Reference 14). One might thus expect that, upon labeling in the presence of cycloheximide, they must be synthesized on cytoplasmic ribosomes.

FIG. 1. Cycloheximide-resistant labeling of cytochrome c oxidase. Yeast cells were grown aerobically for 8 to 10 generations to the early stationary phase in the medium described earlier (14), supplemented with 0.025 μCi of L-[U-14C]leucine per ml. The cells were washed and labeled for 60 min with [3H]leucine in the presence of cycloheximide as described under “Materials and Methods.” The isolated mitochondria (1.54 × 10⁶ cpm of 14C and 1.38 × 10⁶ cpm of 3H per mg of protein) were fractionated and subjected to immunoprecipitation with an antiserum against native yeast cytochrome c oxidase. An aliquot of the immunoprecipitate (3,150 cpm of 14C and 18,620 cpm of 3H) was analyzed by sodium dodecyl sulfate-acrylamide gel electrophoresis.

FIG. 2. Erythromycin-resistant labeling of cytochrome c oxidase. Resting yeast cells were labeled with [3H]leucine for 60 min as outlined in Fig. 1 except that cycloheximide was replaced by 4 mg of erythromycin per ml. In this particular experiment, cytochrome c oxidase was isolated by immunoprecipitation from an extract which had been carried through the DEAE-cellulose chromatography step (14). An aliquot of the immunoprecipitate (2,196 cpm of 3H) was analyzed by sodium dodecyl sulfate-acrylamide gel electrophoresis.
Yeast cells were labeled with L-[4,5-3H]leucine and processed as described in Figs. 1 and 2. The concentration of [3H]leucine during labeling was 40 μCi per ml in Experiment 1 and 120 μCi per ml in Experiment 2. Immunoprecipitation was carried out with antisera against the small cytochrome c oxidase components.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Labeling conditions</th>
<th>Fraction</th>
<th>cpm</th>
<th>cpm/mg</th>
<th>Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No antibiotic</td>
<td>SMP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.14 × 10^4</td>
<td>3.02 × 10^4</td>
<td>(100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SP&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>10.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Immunoprecipitate</td>
<td>1.28 × 10^4</td>
<td>n.d.</td>
<td>3.1</td>
</tr>
<tr>
<td>1</td>
<td>Erythromycin</td>
<td>SMP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.02 × 10^4</td>
<td>2.20 × 10^4</td>
<td>(100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>3.54 × 10^4</td>
<td>2.49 × 10^4</td>
<td>11.7</td>
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<td>Immunoprecipitate</td>
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<td>0.19</td>
</tr>
<tr>
<td>2</td>
<td>Cycloheximide</td>
<td>SMP&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.40 × 10^4</td>
<td>3.82 × 10^4</td>
<td>(100)</td>
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<tr>
<td></td>
<td></td>
<td>SP&lt;sub&gt;2&lt;/sub&gt;</td>
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<tr>
<td></td>
<td></td>
<td>Immunoprecipitate</td>
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<td>n.d.</td>
<td>0.9&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> SMP, submitochondrial particles.
<sup>b</sup> SP<sub>2</sub>, crude cytochrome c oxidase fraction as defined in Reference 14.
<sup>c</sup> n.d., not determined.
<sup>d</sup> This represents a minimal value; it increased to 7.3% when immunoprecipitation was carried out with a carefully titrated, optimal amount of cytochrome c oxidase antiserum.

The double labeling experiment illustrated in Fig. 3 shows clearly that the two largest cytochrome c oxidase components...
migrate slightly faster than the 42,000- and 35,000-dalton peaks of the total translation pattern. The differences are within the range of variability encountered between different electrophoretic experiments but are clearly revealed in this sensitive double label experiment. This confirms our earlier suggestion (18) that the "42,000"- and "35,000"-dalton peaks of the total translation pattern are composite and include not only the two largest cytochrome c oxidase components, but other mitochondrially synthesized polypeptides as well. In contrast, the 23,000-dalton cytochrome c oxidase polypeptide coincides exactly with one of the major peaks of the over-all translation pattern. This peak may thus contain exclusively the third largest cytochrome c oxidase component (Band III). Alternately, it may include several polypeptides which are not resolved by our electrophoretic technique.

The results of Fig. 3 agree well with the finding (Table I) that the three large cytochrome c oxidase components represent only a fraction of the total mitochondrial translation products.

Effect of Oxygen on Mitochondrial Synthesis of Cytochrome c Oxidase Components—As mentioned earlier, the biosynthesis of active cytochrome c oxidase is absolutely dependent on oxygen (5). Fig. 4 suggests that oxygen is necessary for the synthesis (or integration) of cytochrome c oxidase polypeptides. Aerobically grown yeast cells were inhibited with cycloheximide and labeled with [3H]leucine in the presence and absence of oxygen. As already documented in Fig. 2, cytochrome c oxidase from cells labeled under oxygen carried label in all three large polypeptides. In contrast, the enzyme from the cells labeled under nitrogen contained label in the 23,000-dalton polypeptide only; labeling of the two larger polypeptides (of molecular weights "42,000" and 34,500) was completely suppressed.

It might be argued that this effect of oxygen was an artifact limited to cycloheximide-poisoned cells. This possibility was excluded by repeating the labeling experiment just mentioned in the absence of any inhibitor. These conditions would also reveal any effect of oxygen on the labeling of cytoplasmically synthesized cytochrome c oxidase components. As expected, the immunoprecipitate from the aerobically labeled cells now contained radioactivity in all six bands of cytochrome c oxidase (Fig. 5). (The two closely positioned bands of molecular weight 14,000 and 12,500 are again only poorly resolved.) The anaerobically labeled material, on the other hand, specifically lacked the two radioactivity peaks corresponding to molecular weights of 42,000 and 34,500. This result fully confirms that obtained in the presence of cycloheximide. It indicates, moreover, that the cytoplasmically synthesized components of cytochrome c oxidase can be made in the absence of oxygen.

We conclude that oxygen controls the mitochondrial synthesis (or the integration) of two polypeptides associated with cytochrome c oxidase.

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**Fig. 4.** Effect of anaerobiosis on the labeling of cytochrome c oxidase polypeptides in the presence of cycloheximide. Yeast cells were labeled with L-[4,5-3H]leucine in the presence of cycloheximide, except that one-half of the cells was labeled aerobically and the other half was labeled anaerobically. From each aliquot, cytochrome c oxidase was isolated by immunoprecipitation and analyzed by sodium dodecyl sulfate-acrylamide gel electrophoresis.

**Fig. 5.** Effect of anaerobiosis on the synthesis of cytochrome c oxidase polypeptides in the absence of antibiotics. The experiment was identical to that described in Fig. 4, except that the cells were labeled in the absence of cycloheximide. The mitochondria were isolated and subjected to immunoprecipitation with an antiserum against the small cytochrome c oxidase components. Aliquots of the immunoprecipitates (aerobic sample, 3,500 cpm; anaerobic sample, 1,880 cpm) were analyzed by sodium dodecyl sulfate-acrylamide gel electrophoresis.
The present study shows that the six polypeptides associated with cytochrome c oxidase from bakers' yeast are synthesized on two different types of ribosomes. The three large components are synthesized on mitochondrial ribosomes, whereas the three small ones are formed on cytoplasmic ribosomes. Similar results were recently obtained by Weiss et al. (22) for cytochrome c oxidase from Neurospora crassa. Initially, Weiss et al. reported that only a single polypeptide of the Neurospora enzyme was synthesized by mitochondria. Subsequent experiments suggest, however, that three large polypeptides (of molecular weight 38,000, 30,000, and 18,000) are of mitochondrial origin (21).

The following observations indicate, however, that cycloheximide-resistant labeling of our yeast strain does, in fact, represent mitochondrial protein synthesis. (a) Upon fractionation of the labeled cells, virtually all of the incorporated radioactivity is associated with mitochondria (18, 25). (b) Cycloheximide-resistant labeling is essentially completely (up to 98%) inhibited by inhibitors of mitochondrial protein synthesis such as erythromycin or acriflavine (18). (c) No cycloheximide-resistant labeling can be detected in the cytoplasmic petite mutants which have lost a functional mitochondrial genetic system (19, 26). (d) Mitochondrial synthesis of cytochrome c oxidase polypeptides is inferred from a lack of inhibition by even extremely high concentrations of cycloheximide. This greatly reduces the chance of interference by indirect effects, or side effects, of this antibiotic.

Unlike experiments with isolated mitochondria, in vivo labeling of mitochondrial translation products results in a very efficient incorporation of radioactive amino acids and avoids artifacts which may arise during labeling experiments with isolated mitochondria. The in vivo labeling procedure might also allow newly formed mitochondrial translation products to combine with cytoplastically synthesized partner proteins which may be present in excess in the cycloheximide-poisoned cells (see below). In labeling experiments with isolated mitochondria, such a trapping of mitochondrial products is unlikely since cytoplasmically formed partners would probably be removed when the mitochondria are isolated. In the context of the present study this would mean that cytochrome c oxidase polypeptides labeled by isolated mitochondria could not be assembled into a functional holoenzyme. Since they would then probably be fractionated away during the subsequent purification of cytochrome c oxidase, one might be led to the erroneous conclusion (of 26) that mitochondria do not participate in the synthesis of cytochrome c oxidase.

At present there is no direct evidence that yeast cells contain cytoplastically formed cytochrome c oxidase components in excess over mitochondrially formed ones. However, our labeling experiments may be interpreted as suggestive evidence for this notion. Thus, if yeast cells are labeled with [3H]leucine for many generations, all six cytochrome c oxidase polypeptides are equally labeled (cf. the [3H] trace of Fig. 1 with Table IV of Reference 14). In contrast, if resting yeast cells are labeled for only 60 min, labeling of the three small cytochrome c oxidase polypeptides is much less extensive than that of the three large ones (cf. Fig. 5 of Reference 14 with the upper portion of Fig. 5 in the present paper). Additional tentative evidence for excess pools of cytoplastically synthesized cytochrome c oxidase components stems from experiments with N. crassa cells (21) and with anaerobically grown yeast cells adapting to oxygen (10). We are now attempting to isolate these cytoplastic precursors by immunoprecipitation with specific antibodies.

**Isolation of Labeled Membrane Proteins by Immunoprecipitation** —The specificity of immunoprecipitation for isolating minute amounts of radioactive proteins has been exploited in many earlier investigations (27-29). However, application of this method to rather insoluble membrane proteins entails several problems that are sometimes difficult to overcome. Since most isolated membrane proteins are not pure, they will give rise to antibodies containing antibodies. We have dealt with this problem by carrying out immunoprecipitation on radioactive cell fractions and identifying the labeled antigen in the precipitate by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In addition, we have often employed antisera against the isolated small cytochrome oxidase polypeptides; preparations of these polypeptides lack most of the contaminants present in the holoenzyme and, as a consequence, elicit pure antisera.

However, pure antisera may precipitate contaminants if these are tightly bound to the solubilized antigen. To minimize such a coprecipitation, we first separated cytochrome oxidase from most of the other cytochromes and then reacted it with antisera in the presence of 1% Triton X-100 and salt. These conditions weaken interactions between membrane proteins (30) yet do not prevent the formation of insoluble antigen-antibody complexes. More recently, we have devised a simplified procedure which no longer requires a prefractionation of the membranes.

For studying supermolecular complexes, however, coprecipitation of tightly bound polypeptides may also be a definite asset. Indeed, it represents one of the most powerful methods for identifying the polypeptides with which a particular antigen (or a group of antigens) is associated. This approach has already been successfully used to probe the subunit composition of oligomycin-sensitive ATPase (29, 31). In our experiments we have exploited the fact that all six cytochrome c oxidase bands are precipitated by an antisera which was directed only against the three small polypeptides. Since the antisera also precipitated the labeled cytochrome c oxidase polypeptides which had been made in the presence of cycloheximide, these must have been combined with preexisting small cytochrome oxidase Components IV to VI. At present we do not know whether this integration process involves individual turnover of the three large subunits within the holoenzyme or utilization of excess pools of the small components. For the reasons outlined above we favor the second alternative.

Perhaps the greatest attraction of the immunoprecipitation
technique is its potential for studying incompletely assembled cytochrome c oxidase complexes. We have already reported that the nonrespiring promitochondria of anaerobically grown yeast (32–34) retain material which cross-reacts with an antiserum against cytochrome c oxidase (15). Similar observations have been made with mitochondria from cytoplasmic petite mutants (15, 35). The present experiments should make it possible to identify the cytochrome c oxidase component which is still present in these cells. It seems reasonable to expect that promitochondria have retained Components III to VI, whereas petite mitochondria have only retained Components IV to VI. An even more challenging area of investigation is opened by the large number of different yeast mutants which specifically lack cytochrome c oxidase (6, 7, 36). A detailed study of some of these mutants will be reported in subsequent publications.

Control of Mitochondrial Protein Synthesis by Oxygen—Earlier reports from our laboratory indicated that oxygen controlled the synthesis of at least two polypeptides synthesized on mitochondrial ribosomes (18). By sodium dodecyl sulfate-acrylamide gel electrophoresis in gels containing 9.6% acrylamide, the apparent molecular weights of these two polypeptides was calculated to be about 42,000 and 35,000 (18). According to the present experiments, these oxygen-controlled proteins are identical with (or include) the two largest polypeptides of cytochrome c oxidase. However, we are not certain whether anaerobiosis prevents the synthesis of these polypeptides or merely their integration into the holoenzyme.

Although this investigation is obviously no more than a first step, it has led to the probable identification of three polypeptides synthesized by functional mitochondria. It has also provided some information on how oxygen and cytoplasmic protein synthesis regulate the formation of functional mitochondria.

REFERENCES
