# Separation of Mitochondrial and Cytoplasmic Peptide Chain Elongation Factors from Yeast*

Dietmar Richter and Fritz Lipmann

ABSTRACT: Two polypeptide chain elongation factors have been isolated from yeast mitochondria, $G_{mit}$ and $T_{mit}$, which are analogous to peptidyl translocase and aminoacyl-tRNA binding factors. The mitochondrial and cytoplasmic factors differ in molecular weight and chromatographic mobility. Antiserum against the cytoplasmic factors does not react with factors from mitochondria. Mitochondrial factors and ribosomes are interchangeable with bacterial ribosomes and factors, but cytoplasmic ribosomes respond only to the binding factor, mitochondrial T. This is similar to their response to bacterial T. Thus, mitochondrial elongation factors are of prokaryote rather than eukaryote type.

One of the most surprising recent developments in cell biology is the increasing evidence that the mitochondria and chloroplasts of eukaryote cells are of prokaryote origin (Cohen, 1970). Some of the earliest pointers in this direction were observations on the presence of a system of protein synthesis in mitochondria (McLean *et al.*, 1958; Rendi, 1959) and in chloroplasts (Eisenstadt and Brawerman, 1964) of bacterial rather than eukaryote character (Eisenstadt and Brawerman, 1964; Mager, 1960; Küntzel and Noll, 1967; Smith and Marcker, 1968; Leis and Keller, 1970). The relatively minor volume of mitochondrial protein synthesis, however, causes a preponderance of cytoplasmic character in homogenates of many mammalian cells that had been mistaken for exclusiveness. Thus, quite a while ago, both Rendi and Ochoa (1962) and Nathans and Lipmann (1961) concluded from comparison between rat liver and *Escherichia coli* that there was no cross-complementation between ribosome and supernatant fractions. So and Davie (1963), however, found some interchangeability between yeast and *E. coli* supernatants, and Richter *et al.* (1968) and Albrecht *et al.* (1970) could separate from yeast supernatant complements of a bacterial as well as of a mammalian type, and Tiboni *et al.* (1970) reported restricted overlap. Although a partial overlap could be explained in part by a mitochondrial complementation of bacterial T with eukaryote ribosomes (Krisko *et al.*, 1969; Richter, 1970), it appeared likely that the present rather confusing situation might be caused by a failure to distinguish clearly between cytoplasmic, *i.e.*, truly eukaryote, and mitochondrial, presumably prokaryote, elongation factors (Lipmann, 1969). It was quite obvious that if special care was not taken, homogenates of eukaryote cells would contain a mixture of both.

We will report here on experiments with yeast homogenates where careful separation of mitochondrial and cytoplasmic fractions has yielded clear-cut separation of mitochondrial and cytoplasmic elongation factors. They prove to have quite different chromatographic characteristics, and the mitochondrial elongation factors, not unexpectedly, appear to be of prokaryote rather than eukaryote type.

## Experimental Section

The yeasts *Saccharomyces fragilis* and *Saccharomyces carlsbergensis* from the American Type Culture Collection were grown as described recently (Richter, 1970; Ohnishi *et al.*, 1966). Elongation factors and ribosomes of *E. coli* were isolated as described by Gordon (1969). Reticulocyte ribosomes and factors were kindly supplied by Dr. Maria Salas. tRNA from *E. coli* (General Biochemicals, Inc.) was charged with [$^{14}C$]phenylalanine (specific activity 325 mCi/mmole) and *E. coli* S-100, and yielded preparations with 350 $\mu\mu$moles of [$^{14}C$]phenylalanine/mg of tRNA (Richter, 1970).

*Materials.* Hydroxylapatite was obtained from Clarkson Chemical Co., and DEAE-cellulose (Cellex D) from Bio-Rad. Freund's adjuvants were from Difco. [$^{14}C$]Phenylalanine (specific activity 325 mCi/mmole) was supplied by New England Nuclear Corp. Glusulase came from Endo Laboratories, chloramphenicol from Sigma, and cycloheximide from Mann. Triton X-100 was supplied by Rohm and Haas, and diphtheria toxin was a gift from Dr. Maria Salas.

*Separation of Cytoplasmic and Mitochondrial Elongation Factors and Ribosomes.* ISOLATION OF YEAST MITOCHONDRIA. All solutions were either sterilized or passed through Millipore filters (pore size 0.22 $\mu$). According to the method of Duell *et al.* (1964), 1500 g of yeast cells (wet weight) from the mid-log phase was suspended in an equal volume of 10 mM Tris-maleate buffer (pH 5.8) containing 0.6 M sorbitol, 0.03 M 2-mercaptoethylamine, and $4 \times 10^{-4}$ M EDTA, and were incubated with 7 ml of glusulase/100 g of yeast cells at 37° for 1 hr. The slurry was cooled to 4° and diluted with an equal volume of 0.9 M sorbitol. The spheroplasts were collected by centrifugation at 1500*g* for 10 min, and washed twice with the volume of 0.9 M sorbitol used above. The washed spheroplasts (500 ml) were diluted with twice the volume of 20 mM Tris-HCl (pH 7.4), 0.25 M sucrose, and 1 mM EDTA, and were homogenized for 15 sec in a Sorvall blender (Duell *et al.*, 1964). After removing the debris, the mitochondria were isolated from the cell extract by stepwise centrifugation (Mattoon and Balcavage, 1967) and washed

* From The Rockefeller University, New York, New York 10021. *Received August 7, 1970.* This study was supported by a grant from the U. S. Public Health Service (GM 13972), and by a fellowship (D. R.) from Deutsche Forschungsgemeinschaft.

twice with an equal volume of 0.5 M sucrose and 1 mM EDTA. Finally, 200 ml of the mitochondrial suspension (175 mg of protein/ml) was layered on a discontinuous sucrose gradient consisting of 100 ml of 0.75 M sucrose, 150 ml of 1.0 M sucrose, and a cushion of 100 ml of 2.4 M sucrose. All solutions contained 1 mM EDTA. Centrifugation was carried out in a zonal rotor at 30,000 rpm for 1 hr. The mitochondria were collected in the 1.0 M sucrose layer; the washed mitochondria were diluted to 400 ml, in a final concentration of 20 mM Tris-HCl (pH 7.4), 10 mM $Mg(CH_3COO)_2$, and 1 mM DTT.[1] The yield was 3.75 mg of mitochondrial protein/ml of solution.

*Separation of the Mitochondrial Elongation Factors.* The mitochondrial suspension was passed through a French press at 6,000 psi. The mitochondrial sap was centrifuged at 20,000*g* for 20 min, and at 105,000*g* for 2 hr. Mitochondrial factors were precipitated from the resulting S-100 fraction (300 ml with 1.0 mg of protein/ml of solution) with 129 g of ammonium sulfate (pH 6.9). The resulting ammonium sulfate fraction (75 mg of protein) was dialyzed against the starting buffer and applied to a hydroxylapatite column (0.8 × 1.2 cm). The column was equilibrated with 10 mM potassium phosphate buffer (pH 7.2) and 1 mM DTT; $T_{mit}$[2] was eluted from the column with 70 mM phosphate buffer and $G_{mit}$ with 30 mM phosphate buffer (pH 7.2, 1 mM DTT). Both protein fractions were concentrated by ammonium sulfate precipitation as described for the S-100 fraction. The yield was 10.5 mg of protein for $G_{mit}$ and 8.5 mg for $T_{mit}$.

*Preparation of Mitochondrial Ribosomes.* Washed mitochondria (170 ml) from 1000 g of yeast cells (see above under isolation of mitochondria) were diluted with an equal volume of 40 mM Tris-HCl buffer (pH 7.4) and 20 mM $Mg(CH_3COO)_2$, and lysed by adding one-twentieth of the volume of a 20% Triton X-100 solution (Küntzel and Noll, 1967). The ribosomes were sedimented at 105,000*g* for 2 hr. The yellowish brown ribosomal pellet was dissolved in 20 mM Tris-HCl (pH 7.4) and 10 mM $Mg(CH_3COO)_2$. The $A_{260}/A_{280}$ ratio of this mitochondrial ribosomal preparation was 1.93. The yield of mitochondrial ribosomes from 1000 g of yeast cells was 2 ml with 5 mg of ribosomal protein/ml.

*Separation of the Cytoplasmic Elongation Factors.* A S-100 fraction of gently lysed spheroplasts free of mitochondrial contaminants was concentrated, as described in the legend of Figure 1, and its Sephadex G-200 eluate used for identification of the cytoplasmic G and T components by comparison to a S-100 fraction from a French press yeast homogenate (*cf.* Figure 1A,B). Since this experiment showed that the cytoplasmic factors were easily separated from mitochondrial factors by chromatography, they were routinely isolated from the French press yeast homogenate by filtration through Sephadex G-200 as previously reported (Richter, 1970). In this method, the $T_{cyt}$ factor appeared near the void volume of the column, whereas the $G_{cyt}$ factor eluted later. Both factors were further purified by passing them through a hydroxylapatite column (1.5 × 4.5 cm); 41 mg of $T_{cyt}$ or 96 mg of $G_{cyt}$ was used for this column size. $T_{cyt}$ was eluted from the column with 10 mM potassium phosphate buffer (pH 7.2; 1 mM DTT), $G_{cyt}$ with 70 mM phosphate buffer. They were concentrated by precipitation with 43 g of ammonium sulfate/100 ml of solution (pH 6.6–6.9). The yield was 2.5 mg for $T_{cyt}$ and 17.8 mg for $G_{cyt}$.

Ribosomes were isolated as reported recently by Richter (1970). Mitochondrial and cytoplasmic factors and ribosomes were stored in liquid nitrogen.

*Assay for Polyphenylalanine Synthesis.* Polyphenylalanine synthesis was measured by determination of $^{14}C$ radioactivity incorporated in hot trichloroacetic acid insoluble protein (Conway and Lipmann, 1964). The reaction mixtures (125 μl) contained 150 μg of mitochondrial ribosomes, 75 μg of poly(U), 10 mM GTP, 50 mM Tris-HCl (pH 7.4), 10 mM $Mg(CH_3COO)_2$, 100 mM $NH_4Cl$, 60 mM KCl, 7000 cpm of [$^{14}C$]Phe-tRNA (14 μμmoles of [$^{14}C$]Phe-tRNA), and enzymes as indicated. The reaction mixtures were incubated at 37° for 10 min. Since the bacterial and mitochondrial ribosomes are interchangeable (see Results), we also assayed the mitochondrial factors with *E. coli* ribosomes (50 μg/assay). The assay for the cytoplasmic polymerization system was the same as described earlier, except that the volume was reduced to 125 μl and the GTP-regenerating system was omitted (Albrecht *et al.*, 1970).

*Immunochemical Methods.* Antisera against $T_{cyt}$ and $G_{cyt}$ were prepared with New Zealand white rabbits. Cytoplasmic T factor (1 ml; 1.8 mg/ml) and 1 ml of cytoplasmic G factor (0.7 mg/ml) were each emulsified with 1 ml of complete Freund's adjuvant and injected subcutaneously. After 4 weeks, a booster injection was given with 2.0 mg/ml of $T_{cyt}$ and 0.8 mg/ml of $G_{cyt}$, emulsified in incomplete Freund's adjuvant; trial bleedings were done 6 and 7 weeks after the first injection. Finally, the total antisera were obtained by heart puncture. The antisera were fractionated by addition of an equal amount of a freshly filtered saturated ammonium sulfate solution (pH 7.0). The precipitate was dissolved in and dialyzed against 0.155 M phosphate buffer (pH 7.0); it was stored frozen at −20°.

Protein was estimated by the method of Lowry *et al.* (1959) or Warburg and Christian (1941).

## Results

The first suggestion in our hands that we were dealing with mitochondrial factors came from the fact that different Sephadex gel filtration patterns of the S-100 fraction were obtained depending on the method of cell disruption. In earlier experiments, extra components had been observed which, in some respects, resembled the bacterial factors (Albrecht *et al.*, 1970), and there the yeast was disrupted by high shear forces in a cell homogenizer or in the Manton–Gaulin mill. Figure 1 shows a comparison of the chromatographic profiles of a Sephadex G-200 gel filtration of supernatant fractions obtained from yeast cells disrupted as before by the French press (Richter, 1970; Albrecht *et al.*, 1970), with profiles obtained from yeast spheroplasts gently lysed by osmotic shock without disruption of the mitochondria. It can be seen that an additional T component is present in the sheared preparation (Figure 1A) but is absent from the lysed preparations (Figure 1A). Likewise, there was an additional G activity in the sheared preparation

[1] The abbreviation used is: DTT, dithiothreitol.

[2] The terms used for the elongation factors from yeast cytoplasm are: $T_{cyt}$, cytoplasmic aminoacyl-tRNA binding factor (identical with the earlier term FII); $G_{cyt}$, cytoplasmic peptidyl translocase (identical with the earlier term FI). The factors from mitochondria are called $T_{mit}$ and $G_{mit}$.

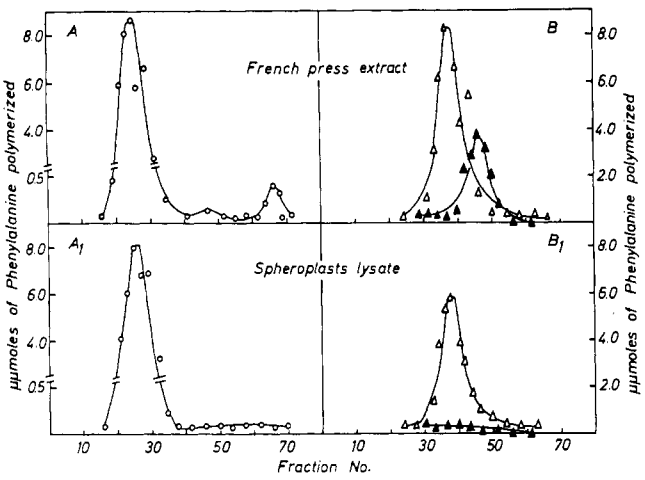


FIGURE 1: Sephadex G-200 filtrations of S-100 fractions obtained from yeast cells broken in the French press (A and B), or from lysed spheroplasts ($A_1$ and $B_1$) (*cf.* Experimental Section). The supernatant fractions were centrifuged for 2 hr at 105,000*g* and concentrated by adding 43 g of ammonium sulfate/100 ml of solution (pH 6.6–6.9); 75 mg each of the S-100 fractions was then applied to Sephadex G-200 columns (4.5 × 80 cm), and eluted with 20 mM Tris-HCl (pH 7.4) and 1 mM DTT. Fraction volume was 5.5 ml. In A and $A_1$, polyphenylalanine synthesis was measured by combining 10 μl of the eluted fractions with an excess of cytoplasmic ribosomes (50 μg of protein/assay) and cytoplasmic G factor (15 μg of protein/assay) (O—O). Similarly, in B and $B_1$, the Sephadex G-200 fractions were assayed in the presence of 50 μg of cytoplasmic ribosomes and 20 μg of cytoplasmic T (△—△). In another experiment, 10 μl of the fractions in B and $B_1$ were assayed with 50 μg of *E. coli* ribosomes and 20 μg of *E. coli* T (▲—▲).

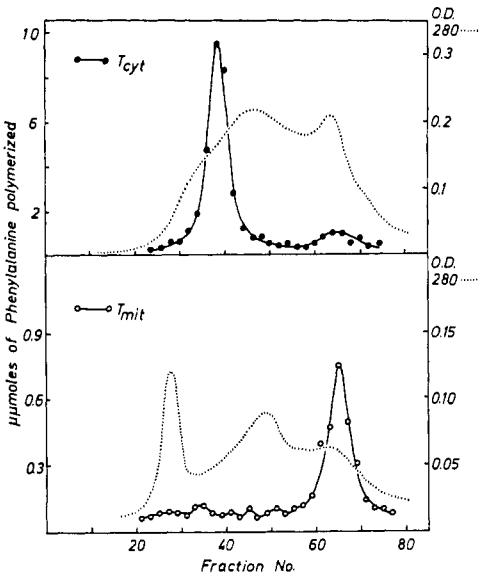


FIGURE 2: Gel filtration of the mitochondrial and cytoplasmic T factors on Sephadex G-200 columns. $T_{cyt}$ (35 mg) or $T_{mit}$ (24 mg) was passed through a Sephadex G-200 column (4.5 × 60 cm). The $T_{mit}$ factor (from 2500 g of yeast cells) was obtained from the hydroxylapatite step (see Experimental Section). Elution was carried out with 20 mM Tris-HCl (pH 7.4) and 1 mM DTT. Fraction volume was 4.5 ml; 10 μl of eluted fractions was assayed. For polyphenylalanine synthesis with an excess of homologous ribosomes and factor: 50 μg of cytoplasmic ribosomes and 12 μg of $G_{cyt}$ or 75 μg of mitochondrial ribosomes and 85 μg of $G_{mit}$ were used.

(Figure 1B), the activity of which was only revealed in the presence of bacterial ribosomes (see below).

To prove that these additional components were uniquely different proteins presumably derived from mitochondria, we proceeded to separate the mitochondria from the cytoplasm. We prepared spheroplasts by glusulase treatment of the yeast cells; these were lysed by mild shearing in a blender, and by osmotic shock. The mitochondrial fraction was isolated on a discontinuous sucrose gradient, and the mitochondria were then disrupted in the French press. The S-100 derived from mitochondria was fractionated by a stepwise gradient on hydroxylapatite columns.

For preparation of mitochondrial ribosomes, the mitochondria were lysed in the neutral detergent Triton X-100. Full details of all these procedures are given in the Experimental Section.

The cytoplasmic factors and ribosomes were prepared as before by disruption of the cells in a Manton–Gaulin mill (Richter, 1970), but with the addition of a hydroxylapatite step, also described above. Figures 2 and 3 show that the extra components seen in extracts derived from homogenization by the Manton–Gaulin mill (Figure 1A, B) were identical with those obtained from the mitochondrial fraction of lysed spheroplasts. The cytoplasmic T factor had a considerably higher mobility than the mitochondrial one on Sephadex G-200. Their molecular weights were estimated to be about 60,000 for the mitochondrial and 220,000 for the cytoplasmic (Albrecht *et al.*, 1970). The two G activities also differ (Figure 3) in that the mitochondrial G had a

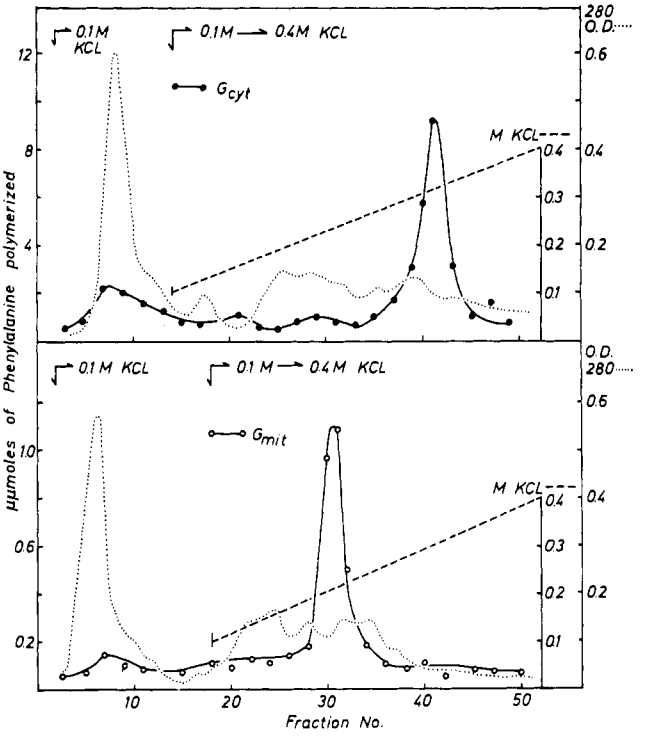


FIGURE 3: Chromatography of the mitochondrial (46 mg) and cytoplasmic (75 mg) G factor on DEAE-cellulose columns (size 1.2 × 15 cm). To elute the factors, a linear gradient (----) was used from 0.1 M (50 ml) to 0.4 M KCl (50 ml) containing 20 mM Tris-HCl (pH 7.4) and 1 mM DTT. Fraction volume was 3.4 ml. Polyphenylalanine synthesis was measured as described in Experimental Section in the presence of 20 μg $T_{cyt}$ or 165 μg of $T_{mit}$.

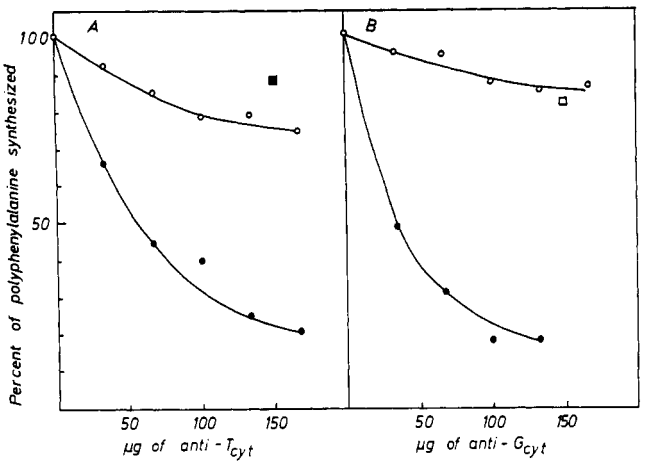


FIGURE 4: The effect of antisera against cytoplasmic factors on mitochondrial and cytoplasmic polypeptide synthesis. Antisera against $T_{cyt}$ and $G_{cyt}$ were prepared as described in Experimental Section and assayed with the mitochondrial (O—O) and cytoplasmic (●—●) systems. The experimental conditions in the absence of antisera were such that incorporation in the control (= 100%) was in A proportional to the amount of T factor and in B to that of G factor. The assay with 150 μg of a control serum from rabbits had only slight effects with the mitochondrial (□) or with the cytoplasmic (■) systems.

lower affinity for DEAE-cellulose than the cytoplasmic G; the mitochondrial G was recovered from the column at about 0.2 M KCl, whereas the cytoplasmic G eluted at 0.3 M KCl. Similar patterns for the two G activities were obtained when the S-100 fraction from cells disrupted by high shear forces was chromatographed on DEAE-cellulose in the same way as described in Figure 3 (not shown).

The fact that fractions with comparable chromatographic properties were isolated either directly from mitochondria

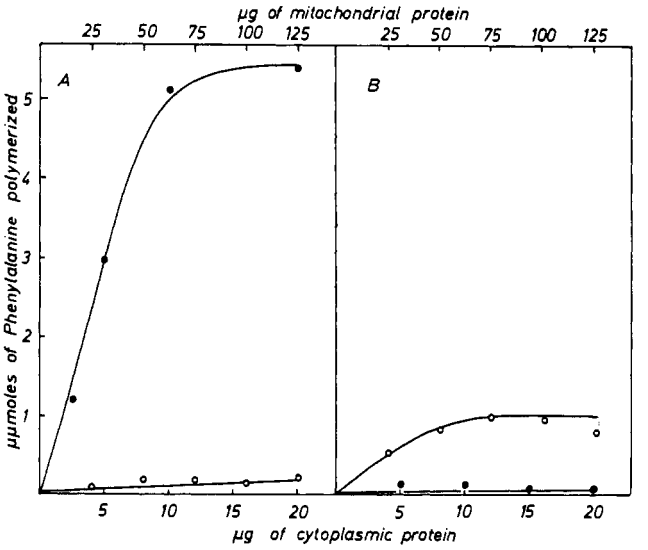


FIGURE 5: Polyphenylalanine synthesis was assayed in the presence of cytoplasmic in A and in B of mitochondrial ribosomes, using either the homologous or heterologous enzymes. Assays were carried out with saturating amounts of mitochondrial or cytoplasmic G and variable amounts of $T_{mit}$ (O—O) or $T_{cyt}$ (●--●).

TABLE I: Tests for Interchangeability of the Components of Cytoplasmic and Mitochondrial Peptide Synthesizing Systems.[a]

| Peptidyl Translocase G | Aminoacyl-tRNA Binding Factor T | Ribosomes: Mitochondrial Phe | Ribosomes: Cytoplasmic Polymerized[b] |
|---|---|---|---|
| Mitochondrial | + Mitochondrial | 7.1 | 0.9 |
| Cytoplasmic | + Cytoplasmic | 0.5 | 197.0 |
| Mitochondrial | + Cytoplasmic | 0.9 | 0.5 |
| Cytoplasmic | + Mitochondrial | 0.4 | 13.5 |

[a] Polyphenylalanine synthesis was carried out as described in the Experimental Section, using 50 μg of cytoplasmic or 75 μg of mitochondrial ribosomes. The enzyme concentrations were 15 μg for $T_{cyt}$, 7.5 μg for $G_{cyt}$, 125 μg for $T_{mit}$, 75 μg for $G_{mit}$. Incubations were done at 37° for 10 min. [b] μμmoles/mg of ribosomal protein.

or together with the cytoplasmic factors in cells broken by shearing (Figure 1A, B) shows that the mitochondrial factors are different proteins. This was confirmed by their lack of reactivity with antisera prepared against and strongly inhibitory to the cytoplasmic factors. In contrast, it is shown in Figure 4 that there is no significant inhibition of the activities of the mitochondrial factors by these antisera.

*Parallels between the Mitochondrial and Bacterial Factors.* In this section we describe in greater detail tests on the complementation of poly(U)-directed polyphenylalanine synthesis on cytoplasmic, mitochondrial, and bacterial ribosomes, respectively. As shown in Figure 1B, mitochondrial G factor functioned only in conjunction with bacterial ribosomes and T factor. It was shown previously that on eukaryotic ribosomes (Krisko *et al.*, 1969; Richter, 1970) bacterial and eukaryote T factors are interchangeable but not the G factors. We have now therefore tested the mitochondrial factors for resemblance to eukaryotic or bacterial factors. Figure 5 shows that the combination of $T_{mit}$ and $G_{mit}$ will not function with cytoplasmic ribosomes and the combination of $T_{cyt}$ and $G_{cyt}$ will not function with mitochondrial ribosomes. This is examined in more detail in Table I. It appears that $T_{mit}$, like bacterial T, is interchangeable with the $T_{mit}$ on cytoplasmic ribosomes when combined with $G_{cyt}$. The cytoplasmic T, however, will not function similarly on mitochondrial ribosomes, just as the bacterial T replacement, the eukaryote, is unidirectional. In Table II, mitochondrial and *E. coli* T and G factors are compared on *E. coli* ribosomes. The mitochondrial factors are both individually interchangeable with bacterial factors and ribosomes, confirming the parallels between bacteria and mitochondria.

In line with the results of others obtained with intact mitochondria (Rendi, 1959; Mager, 1960) with the isolated mitochondrial system, we find poly(U)-dependent phenylalanine polymerization inhibited by chloramphenicol but not by cycloheximide. Thirty micrograms per milliliter of chloramphenicol caused 50% inhibition of mitochondrial

TABLE II: Interchangeability of Mitochondrial and Bacterial Peptide-Synthesizing System.[a]

| Peptidyl Translocase G | Aminoacyl-tRNA Binding Factor T | Ribosomes: Mitochondrial Phe | Ribosomes: Bacterial Polymerized[b] |
|---|---|---|---|
| Mitochondrial | + Mitochondrial | 7.1 | 22.1 |
| *E. coli* | + *E. coli* | 18.1 | 421.0 |
| Cytoplasmic | + Cytoplasmic | 0.5 | 0.8 |
| Mitochondrial | + *E. coli* | 17.1 | 201.0 |
| *E. coli* | + Mitochondrial | 9.8 | 11.2 |

[a] For polyphenylalanine synthesis, see Experimental Section. The assay contained 50 $\mu$g of *E. coli* ribosomes or 75 $\mu$g of mitochondrial ribosomes; the concentrations of the mitochondrial factors were the same as in Table I. In the case of *E. coli* enzymes, 18 $\mu$g of *E. coli* T and 6 $\mu$g of *E. coli* G were used. [b] $\mu\mu$moles/mg of ribosomal protein.

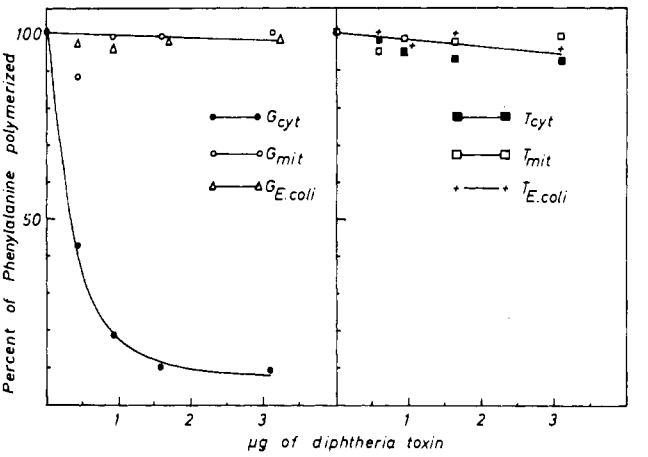


FIGURE 6: Resistance of mitochondrial G factor to inactivation by diphtheria toxin and $NAD^+$. The G and T factors from various sources were incubated with diphtheria toxin at 37° for 20 min in a total volume of 60 $\mu$l containing 10 m$\mu$moles of $NAD^+$, and either 60 $\mu$g of $G_{mit}$ or 9 $\mu$g of $G_{cyt}$, 22 $\mu$g of G (*E. coli*), 30 $\mu$g of $T_{cyt}$, 80 $\mu$g of $T_{mit}$, and 26 $\mu$g of T (*E. coli*). The reaction mixture for polyphenylalanine synthesis consisted of the homologous ribosomes, [$^{14}$C]Phe-tRNA, GTP, ions, and nontreated complementary factor (see Experimental Section), and after addition of pretreated G or T factor was incubated at 37° for 10 min. Conditions were such that incorporation in the control was proportional to the amount of G factor (on the left) and T factor (on the right). As seen on the right, the toxin did not inactivate the T factors from either source.

peptide synthesis. In contrast, the isolated cytoplasmic system was unaffected by this chloramphenicol concentration but was inhibited by 35 $\mu$g of cycloheximide to nearly 50%. Diphtheria toxin + $NAD^+$ is known to inactivate eukaryote G factor (Honjo *et al.*, 1968; Goor and Maxwell, 1970; Hameister and Richter, 1970) but not bacterial G factor (Johnson *et al.*, 1968). Figure 6 shows that, in contrast to cytoplasmic, the mitochondrial G is resistant to the toxin, extending the parallelism with bacteria. Furthermore, preliminary results seem to indicate that the mitochondrial T factor can be split into subfractions $T_s$ and $T_u$ (A. H. Morimoto, J. Scragg, J. Nekhorocheff, V. Villa, and H. O. Halvorson, manuscript in preparation; D. Richter, unpublished data).

## Conclusions

Although the yeast mitochondria carry 80S ribosomes (A. Morimoto and H. Halvorson, manuscript in preparation; Schmitt, 1969; Vignais *et al.*, 1969) of a composition somewhat in between bacterial and cytoplasmic, their protein-synthesizing system is of bacterial type. Factors and ribosomes from yeast mitochondria and bacteria are interchangeable, whereas the cytoplasmic factors cannot be interchanged with ribosomes from bacteria or mitochondria. Similar results have been reported recently by Küntzel (1969), who used mitochondrial ribosomes and a mitochondrial supernatant fraction from *Neurospora crassa*. The analogy of the mitochondrial elongation factors from yeast to those of bacteria is supported by insensitivity of mitochondrial G factor to diphhtheria toxin, by the chloramphenicol sensitivity of the mitochondrial protein-synthesizing system, and by lack of reaction with antisera against cytoplasmic factors. The mitochondrial T factor seems to be separable into $T_s$ and $T_u$ by methods similar to those used for the bacterial T factor (A. Morimoto, A. H. Scragg, J. Nekhorocheff, V. Villa, and H. O. Halvorson, manuscript in preparation). Probably these mitochondrial subfractions $T_s$ and $T_u$ are identical with those previously described as yeast $T_1$ and $T_2$ (Albrecht *et al.*, 1970; Richter *et al.*, 1968).

During the preparation of this manuscript, Halvorson and his coworkers drew our attention to their similar findings with the yeast *S. cerevisiae*.

## Acknowledgments

We thank Dr. C. Sorg of the Sloan-Kettering Institute for Cancer Research for his help in preparing the antisera. We are also grateful to Drs. B. Poole and P. Lazarow in the laboratory of Dr. de Duve for their help in carrying out the zonal centrifugation run. The criticism of Dr. Julian Gordon during the preparation of this manuscript is gratefully acknowledged.

## References

Albrecht, U., Prenzel, K., and Richter, D. (1970), *Biochemistry 9*, 316.
Cohen, S. S. (1970), *Amer. Sci. 58*, 281.
Conway, T. W., and Lipmann, F. (1964), *Proc. Nat. Acad. Sci. U. S. 52*, 1462.
Duell, E. A., Inoue, S., and Utter, M. F. (1964), *J. Bacteriol. 88*, 1762.
Eisenstadt, J., and Brawerman, G. (1964), *J. Mol. Biol. 10*, 392.
Goor, R. S., and Maxwell, E. S. (1970), *J. Biol. Chem. 245*, 616.
Gordon, J. (1969), *J. Biol. Chem. 244*, 5680.
Hameister, H., and Richter, D. (1970), *Hoppe-Seyler's Z. Physiol. Chem. 351*, 532.
Honjo, T., Nishizuka, Y., Hayaishi, O., and Kato, I. (1968), *J. Biol. Chem. 243*, 3553.

Johnson, W., Kuchler, R. J., and Solotorovsky, M. (1968), *J. Bacteriol. 96*, 1089.
Krisko, I., Gordon, J., and Lipmann, F. (1969), *J. Biol. Chem. 244*, 6117.
Küntzel, H. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 4*, 140.
Küntzel, H., and Noll, H. (1967), *Nature (London) 215*, 1340.
Leis, J. P., and Keller, E. B. (1970), *Biochem. Biophys. Res. Commun. 40*, 416.
Lipmann, F. (1969), *Science 164*, 1024.
Lowry, O. H., Rosebrough, W. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Mager, O. (1960), *Biochim. Biophys. Acta 38*, 150.
Mattoon, J. R., and Balcavage, W. X. (1967), *Methods Enzymol. 10*, 135.
McLean, J. R., Cohn, G. L., Brandt, J. K., and Simpson, M. V. (1958), *J. Biol. Chem. 233*, 657.
Nathans, D., and Lipmann, F. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 497.
Ohnishi, T., Kawaguchi, K., and Hagihara, B. (1966), *J. Biol. Chem. 241*, 1797.
Rendi, R. (1959), *Exp. Cell Res. 18*, 187.
Rendi, R., and Ochoa, S. (1962), *J. Biol. Chem. 237*, 3707.
Richter, D. (1970), *Biochem. Biophys. Res. Commun. 38*, 864.
Richter, D., Hameister, H., Petersen, K. G., and Klink, F. (1968), *Biochemistry 7*, 3753.
Schmitt, A. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 4*, 234.
Smith, A. E., and Marcker, K. A. (1968), *J. Mol. Biol. 38*, 241.
So, A. G., and Davie, E. W. (1963), *Biochemistry 2*, 132.
Tiboni, O., Parisi, B., Perani, A., and Ciferri, O. (1970), *J. Mol. Biol. 47*, 467.
Vignais, P. B., Juet, G. J., and André, J., (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 3*, 177.
Warburg, O., and Christian, W. (1941), *Biochem. Z. 310*, 384.

# Effects of Guanidine Derivatives and Oligomycin on Swelling of Rat Liver Mitochondria*

C. Bhuvaneswaran and K. Dakshinamurti†

ABSTRACT: The effects of guanidine derivatives and oligomycin on energy-dependent swelling of rat liver mitochondria have been evaluated. Guanidine derivatives are known to inhibit respiration. They also inhibit succinate-linked reduction of acetoacetate with both the aerobically generated high-energy intermediates or externally added ATP as the source of energy. Guanidines do not inhibit mitochondrial swelling mediated by energy derived from either substrate oxidation or by externally added ATP. Oligomycin, on the other hand, inhibits swelling supported by externally added ATP, while having no effect on swelling mediated by substrate oxidation.

Energy changes were restricted to any one of the three phosphorylation sites using a combination of inhibitors of electron transport (cyanide, antimycin A, and rotenone), and artificial electron donors (ascorbate plus *N,N,N',N'*-tetramethyl-*p*-phenylenediamine) and acceptors (phenazine methosulfate, and ferricyanide). Under these conditions swelling still occurred in the presence of guanidines. The results indicate that the mechanism of inhibition by the guanidines is similar to that of oligomycin. However the sites of inhibition are different.

Hollunger (1955) first observed that guanidine and its derivatives induced a unique and characteristic *in vitro* inhibition of the mitochondrial process associated with high-energy phosphate-bond formation. The slow release of the guanidine inhibition by 2,4-dinitrophenol (DNP)[1] as well as the profile of oxidized-reduced state of cytochrome *b* have led to the suggestion that the guanidines form a complex with the high-energy intermediates (Chance and Hollunger, 1963; Pressman, 1963a). However, little information is available regarding the nature of this complex.

We have examined the properties of four of the site-specific phosphorylation inhibitors. Three of these are guanidine derivatives, namely, 4-methyl-3-butenylguanidine (Galegine),[2] phenylethylbiguanide (DBI), and decamethylenediguanidine (Synthalin). In addition, oligomycin was chosen since this compound blocks ATP synthesis from all the three energy-conserving sites (Lardy *et al.*, 1958; Lardy and McMurray, 1959; Slater, 1963; Ernster and Lee, 1964). We present evidence here to suggest that the guanidines exert their influence between the high-energy intermediate and formation

* From the Department of Biochemistry, University of Manitoba, Winnipeg 3, Manitoba, Canada. *Received July 13, 1970.* This investigation was supported by grants from the University of Manitoba and the Medical Research Council of Canada.

† To whom correspondence should be addressed.

[1] The abbreviations used are: DNP, 2,4-dinitrophenol; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; BSA, bovine serum albumin; PMS, phenazine methosulfate.

[2] The commercial names used are: Galegine, 4-methyl-3-butenylguanidine; DBI, phenylethylbiguanide; Synthalin, decamethylenediguanidine.