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Genetics, Biogenesis and Bioenergetics of Mitochondria

Editors
W. Bandlow · R. J. Schweyen · D. Y. Thomas
K. Wolf · F. Kaudewitz

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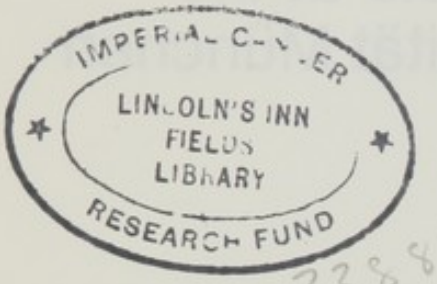
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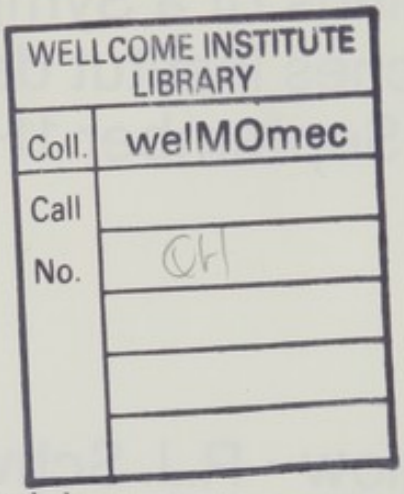
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Editors

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Preface

A biennial course on yeast molecular biology is given by members of the Genetics Institute of the University München. In August and September 1975 it was attended by students from 10 different countries and generously sponsored by EURATOM and the Deutsche Forschungsgemeinschaft. In recent years there has been an upsurge in interest in the genetics of yeast and increasing interest in its molecular biology, and it is apparent that as a model organism for cell organization *Saccharomyces cerevisiae* is destined to become the *Escherichia coli* of the eukaryotic world.

One of the main interests among the students attending the course was the use of yeast for the study of mitochondrial genetics and biogenesis, a rôle in which it has become the pre-eminent organism. This symposium was organized in order to acquaint the students attending the course with recent developments in this field, and also to bring together a wider spectrum of workers than is normally seen in Symposia on Mitochondria. The value of publishing the papers presented only became apparent during the symposium and the speakers were asked to submit their contributions for rapid publication, which with few exceptions they did. Thus we have a volume of 33 papers which presents a wide view of extrachromosomal genetics, the recent exciting developments in mitochondrial biogenesis, and the developing rôle of micro-organisms in mitochondrial bioenergetics. If yeast will be the *E. coli* of the eukaryotic world then mitochondria may well turn out to be its λ phage.

In the nature of things a volume such as this has greatest value when published rapidly and we would like to thank the contributors for their prompt submission of their papers. We would also like to thank the students for their lively participation, and our colleagues of the Genetics Institute for their enthusiastic and devoted help in the organization of the course and symposium.

The Editors

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Session 1: Genetics and Cytology of Mitochondria and Cytoplasmic Factors

Transmission, Recombination and Segregation of Mitochondrial Genes in Saccharomyces cerevisiae

B. Dujon

Within the last few years rapid progresses in the understanding of the genetic properties and functions of organelles have been made in several organisms. Among those the yeast *Saccharomyces cerevisiae* has played and continue to play an important role due particularly to the isolation and characterisation in this species of many mutations localised in its mitochondrial DNA. The mitochondrial mutations of yeast belongs to two main classes. The first one (respiratory deficient or ρ^-), discovered several years ago (1 - 3), correspond to large alterations of the base sequence of the wild type mitDNA. It is well established now that in the mitDNA of the ρ^- mutants only a small fragment(s) of the wild type mitDNA sequence is maintained and regularly repeated (4 - 10). The second class of mitochondrial mutations, described for the first time some years ago (11-13), is represented by the mutation conferring the resistance against antibiotics specifically inhibiting some of the mitochondrial functions such as mitochondrial protein synthesis (14-17) and energy conservation (18-21). Finally it should be noted that, very recently, mitochondrial mutations expressed in a deficiency of cytochrome oxydase, coenzyme $Q\text{-}H_2$ cytochrome c reductase and/or ATP synthetase have been described (22-24). In opposition to the ρ^- mutations, the other mitochondrial mutations correspond presumably to minor alterations of the wild type base sequence and can be regarded, in a first approximation, as "point" mutations although some of them might concern more than one base pair (25). The use in combination of point mutations and ρ^- deletions offers a very powerfull tool to analyse in details the mitochondrial genome of *S. cerevisiae* and, in particular, to study the mechanisms which control the transmission of the mitochondrial genetic information to the progeny. This article will be devoted to a short summary of the recent ideas on the transmission, recombination and segregation of mitochondrial genes. The reader is referred to recent review articles (26,27) for other aspects not described here.

From the analysis of the results of a great number of multifactorial crosses performed with the antibiotic-resistance mutations (11,14,17,18,25 28,29) as genetic markers, the characteristic features of the transmission and recombination of mitochondrial genes have emerged. The progeny of a mitochondrial cross is characterised by an <u>assymetry</u> in the transmission of the alleles of the two parents for a given locus and by a <u>polarity of recombination</u> (18,30). A specific locus on the mitochondrial genome (ω) closely linked to the RIB1 locus determine the polarity of recombination in the small segment of the mitochondrial genome characterized by the 3 linked loci RIB1, RIB2, RIB3 (11). Each cell has either the ω^+ or the ω^- allele (25). The segment of the mitochondrial genome closed to the ω locus (RIB1-RIB2-RIB3) is called <u>a polar</u> segment while other segments characterized by the loci OLI1, OLI2, PAR1 etc... are called <u>non polar</u>.

The major rules governing the transmission and recombination of mitochondrial genes have recently been described in a general model for recombination and segregation of mitDNA (30). This model emphasizes the idea

that the mitochondrial genetics is the genetics of a population of mitDNA molecules and makes a parallel between the genetics of mitochondria within a eucaryotic cell and the genetics of phages within a procaryotic cell (31). The main assumptions of this model are briefly summarized as follow. In every yeast cell many copies of the mitochondrial genome, genetically competent and complete are present. Such an idea is in agreement with the fact that the number of mitDNA molecules per cell is high (50 to 100) (32) and suggest that every mitDNA molecule is genetically competent. In each cell all the copies are forming a pool of mitochondrial genomes in which pairing and recombination events take place at random, the pool being therefore regarded as a panmictic population. The pool is characterized by the input fraction i.e.the relative number of copies from each parent at time zero of the cross. The input fraction varies from one cross to another under the influence of the nuclear genetic backgrounds of the two parents as well as the influence of experimental conditions. Pairing and recombination events between mitDNA molecules of the same cell occur continuously and can be regarded as non synchronous.Therefore the frequency of recombinant type found in the progeny of a cross will be the final result of multiple rounds of pairing and recombination events. Pairing and recombination events occur in all cell:haploid or diploid and ρ^+ as well as ρ^- (33). They are genetically detectable only in heteroplasmic cells i.e.in cells containing different mitDNA molecules with different genetic markers. The heteroplasmic state may be introduced either by crosses or by $\rho^+ \rightarrow \rho^-$ mutagenesis. In the first case two homoplasmic cells carrying different genetic markers fuse to form a heteroplasmic zygote. In the second case the heteroplasmic state is introduced within a homoplasmic cell by the action of a mutagene (such as ethidium bromide) which induce large alterations of the base sequence in many mitDNA molecule of the pool (10). The segregation of molecules into daughter cells (buds) occur at every cell division and produce quite rapidly homoplasmic cells from heteroplasmic ones (47). Segregation occurs at random.

The elementary acts of genetic recombination between markers of the polar region when two mitDNA molecules with different ω allele are paired (ω^+/ω^- pairing events) are non reciprocal.A mechanism of correction or gene conversion initiated dissymetrically at the ω locus itself and reaching sequentially the neighbouring markers can be envisaged and accounts for the different properties associated with the polarity of recombination (30). Each event produces as many parental as recombined molecule and produces only one recombinant type between two markers. The two reciprocal types of recombinants are produced amongst the total population either with similar frequencies (non polar regions or polar regions in homosexual crosses) or with very different frequencies (polar region in heterosexual crosses).

In such a multiple copy genetic system the frequencies of the different cell type amongst the progeny of a cross are the final results of many elementary events of pairing,recombination and segregation.

This model accounts for the main features observed in multifactorial crosses (30). Furthermore it allows us to predict the frequencies of the different recombinant and parental classes amongst the progenies of multifactorial mitochondrial crosses as functions of elementary parameters of the model (input fraction, number of mating rounds and probabilities of genetic exchanges). A satisfactory conformity between these prediction and

experimental results is found (34). The predictionsof this model, especially on the variations of frequencies of transmission of alleles and frequencies of recombinants as functions of the variations of input in polar and non polar regions of the mitochondrial genome,are in good agreement with the experimental results of multifactorial crosses obtained from our laboratory and some others (35-43).

Experiments of UV irradiation applied either to one parent prior to crossing or to newly formed zygotes have been recently reported (29). The effects of UV have been studied on the transmission and recombination of mitochondrial genetic markers in the progeny of crosses. It has been shown that the effects of UV depend on the nature of the cross (homosexual on heterosexual). In all cases UV irradiation of one of the parents diminishes the transmission of the mitochondrial alleles originated from the irradiated parent. In homosexual crosses the decrease of transmission is the same for alleles at all the loci. In heterosexual crosses, when the ω^+ parent is irradiated, there is a differential decrease of transmission depending on the distance of the resistance locus relative to the ω locus. In heterosexual crosses irradiation of the ω^+ parent increases the frequency of recombinants while irradiation of the ω^- parent slightly decreases it. In homosexual crosses the frequency of recombinants diminishes when a high UV dose is applied to one of the parents. No or only minor modifications of the polarity of recombination are observed. All these effects can be interpreted in terms of our model if one assumes that UV irradiation leads to a modification of the input fraction in favor of the non irradiated parent. As a consequence of this modification the output of alleles and the frequency of recombinants are changed. A good quantitative agreement between the predictions calculated on the basis of the model and the experimental data is found. Other experimental methods to modify the input have been recently reported such as high glucose repression (43,44),growth at non permissive temperature for temperature sensitive mutants(45,46).In all the cases effects observed on the transmission of markers and frequency of recombinants amongst the progeny are in good agreement with the predictions of the model and we believe that different experimental modifications of the input will be one of the important experimental approach used to unravel the mechanisms of transmission and recombination of mitochondrial genes in the next future.

A new allelic form of the ω locus has been found amongst some of the mutants selected at the RIB1 locus (either $C^R \rightarrow C^S$ or $C^S \rightarrow C^R$ mutations) from ω^- strains but not from ω^+ strains (25). In crosses involving ω^n mutants, recombination is possible between the different mitochondrial genetic markers but no high polarity of recombination is observed and the frequencies of recombinants are similar to those found in homosexual crosses ($\omega^+ \times \omega^+$ and $\omega^- \times \omega^-$). A striking property, exists in crosses between ρ^+ ω^n C^S strains and some ρ^- $C^R E^O$ mutants : the ρ^- $C^R E^O$ are unable to integrate by recombination their C^R allele into the ρ^+ mitDNA of ω^n C^S strains while being capable of integrating it into ω^+ C^S or ω^- C^S genomes. All these properties suggest that ω^n mutations correspond to macrolesions (probably deletions) of a segment of the mitDNA covering the ω and RIB1 loci. If ω^n is a partial deletion of the ω^- sequence the ω^+ could be an additionnal deletion of the ω^n sequence. Since the model proposes that the ω locus is the site of initiation of non reciprocal recombination events, it can be concluded that ω^+/ω^- pairing specifically initiates the

non-reciprocal act while ω^+/ω^n or ω^-/ω^n pairings do not.

Finally it must be mentionned that some recent experiments (47), based on the kinetics of formation of homoplasmic cells in the progeny of a synchronous population of zygotes, indicate that the segregation process occurs at random and that the number of segregating units entering the bud during cell division is low in comparison to the total number of mitDNA molecules present in the mother cell.

In conclusion the multiple copy model presented accounts for the different genetic properties observed in multifactorial mitochondrial crosses in yeast and permits some quantitative predictions which can be directly tested experimentally. Furthermore it proposes a series of definitions which can be used as formal basis for future developments of the studies on the mechanisms of transmission and recombination of the mitochondrial genetic information.

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On the Formation of rho⁻-Petites in Yeast: V. Mapping by rho⁻ Deletion Analysis and rho⁺ x rho⁺ Recombination Analysis of Mitochondrial Genes in Saccharomyces cerevisiae

R.J. Schweyen, B. Backhaus, S. Mathews, F. Kaudewitz

Summary

The results of different approaches of mapping four mitochondrial genes (P, T, C, O) are compared with each other:
a) analysis of recombinant frequencies in rho⁺ x rho⁺ multifactorial crosses,
b) analysis of retention or loss of mitochondrial markers by deletion in i) a population of rho⁻ clones induced by ethidium bromide, ii) a population of rho⁻ clones generated by the action of nuclear tsp mutations (temperature sensitive pet), iii) subclones derived from individual rho⁻ clones of genotype $P^R T^R C^R O^R$ isolated from populations i) or ii).

The results of all approaches allow to establish the unique gene order P-T-C-O-P, and reveal partial agreement of relative distances between markers. The analysis of populations of tsp-induced rho⁻ clones is regarded as the most useful attempt.

I) Recombination between five mitochondrial markers

Several attempts to map mitochondrial genes by analysing recombinant frequencies have been published during the last few years (1). Except in one series of experiments involving three closely linked ribosomal loci (RIB1, RIB2, RIB3) (2) this approach of determining order and distances of mitochondrial genes turned out to be unsuccessful : Recombinant frequencies found with any pair of the markers C or E (loci RIB1, RIB3) and O_I, O_{II}, P_I (loci OLI1, OLI2, PAR1) are roughly equal and approach the upper limit observed for mitochondrial gene recombination (1), i.e.

the markers appear to be unlinked.

Using additional markers (mit$^-$ mutants, (3)) slight indications were found that recombinant analysis might allow the determination of the order of the new markers and the previously known resistance markers.

With a new mitochondrial marker conferring temperature sensitivity of mitochondrial function (conditional mit$^-$, tsm$^-$, referred to as "T marker") we were able to perform the first detailed mapping analysis of five mitochondrial markers (4). In contrast to crosses involving the non-conditional mit$^-$ marker all genotypes in these crosses can be unequivocally determined. Frequencies of recombinants observed in a series of such crosses are given in Table 1. Previously published results of crosses involving three or four of the resistance markers (2, 5, 6, 7) are given for comparison.

Recombinant frequencies between the pairs of markers C-O, C-P, E-O, E-P, O-P and T-O are close to 20% and are assumed to indicate absence of linkage. Tightest linkage is found between C-E (9% recombinants). The pairs T-C, T-E and T-P exhibit intermediary recombination values (12.5 to 14%) which are interpreted as indicating weak linkage and the positioning of T between the markers C/E and P. According to these crosses the marker O is outside and unlinked to the P-T-C/E segment of the mitochondrial genome (cf. Fig. 1).

II) Deletion of four mitochondrial genes in rho$^-$ mutants

Rho$^-$ mutation results in very large deletions of mit-DNA and the arrangement of the mitDNA segments retain-

		Markers considered									
		C-E	C-O	C-P	E-O	E-P	O-P	C-T	E-T	O-T	P-T
Schweyen, Kaudewitz (1976)	Recombinants	249	794	322	1334	528	843	841	1461	2403	824
	Percentage	9.3%	17.6%	17.1%	21.9%	18.7%	19.9%	12.5%	14.0%	19.9%	13.9%
Kleese et al. (1972)	Recombinants	206	--	427	--	445	--	--	--	--	--
	Percentage	9.0%		18.6%		19.4%					
Avner et al. (1973)	Recombinants	850	1724	--	1683	--	--	--	--	--	--
	Percentage	9.5%	20.2%		19.0%						
Wolf et al. (1973)	Recombinants	302	454	873	519	989	797	--	--	--	--
	Percentage	8.2%	17.3%	18.3%	20.1%	21.0%	21.7%				
Netter et al. (1974)	Recombinants	198	149	--	--	--	--	--	--	--	--
	Percentage	9.0%	22.0%								
Total recombinants		1805	3121	1622	3536	1962	1640	841	1461	2403	824
Overall percentage		9.2%	18.8%	18.3%	20.2%	20.0%	20.7%	12.5%	14.0%	19.9%	13.9%

Table 1 : Synopsis of the frequencies of recombinants observed in mitochondrial crosses $rho^+ \times rho^+$. Frequencies of recombinants given for a single pair of markers are the sum of reciprocal recombinants (e.g. $X^RY^S + X^SY^R$) among the progeny of a population of zygotes (e.g. in a cross $X^RY^R \times X^SY^S$). The data are derived from homosexual crosses only ($\omega^+ \times \omega^+$, $\omega^- \times \omega^-$).
The markers studied correspond to the following loci (except in Kleese et al. 1973, where the corresponding loci are unknown) : C=RIB1; E=RIB3; O=OLI1; P=PAR1; T=TSM1.

ed are often arranged repetitiously. Rho$^-$ cells lack mitochondrial protein synthesis and, thus, phenotypic expression of mitochondrial genetic information. In order to determine whether mitochondrial genes are retained, rho$^-$ cells are crossed to rho$^+$ cells. As rho$^-$ mitDNA can recombine with rho$^+$ mitDNA, the mitochondrial information of rho$^-$ cells will be transferred to and expressed in a portion of the rho$^+$ progeny of the crosses. From the outcome of such crosses retention or loss of mitochondrial genes in rho$^-$ clones can be inferred.

The retention of more than one genetic marker (co-retention) on deleted mitDNA has been studied by several authors (8, 9, 10) and has been used as a means of mapping the mitochondrial genome (3, 10, 11). The basic idea behind this approach to map is that the frequency of co-retention of two genes on rho$^-$ mitDNA molecules will increase with decreasing distance between two genes on the mitochondrial genome.

We have studied co-retention of genes on rho$^-$ mitochondrial genomes which were induced by ethidium bromide (EB) in rho$^+$ mitDNA (analysis of a random population of rho$^-$ clones) or of spontaneous deletion in rho$^-$ genomes (analysis of individual, established rho$^-$ clones). By these analyses we were able to deduce the order of and relative distances between the four markers C^R, O^R, P^R, T^R (12).

In a similar analysis we have now determined the co-retention of the same four markers in mitDNA molecules deleted by the endogenous action of the nuclear conditional mutations tsp20 and tsp25 which cause high rates of spontaneous mutation to rho$^-$ (12) at restrictive temperature. This formation of rho$^-$ cells is

referred to as "tsp-induced rho$^-$ mutation".

1) Frequencies of marker retention in rho$^-$ petites

We found that the rho$^-$ cells induced by tsp mutants (primary cells) frequently segregated cell lines carrying different mitochondrial genomes which has also been observed in EB-induced rho$^-$ cells (12). This indicates that some of the primary rho$^-$ cells were heteroplasmic. Unequivocal demonstration of genotypes requires the analysis of the mitochondrial markers present in colonies derived from homoplasmic cells. The following analyses were made with rho$^-$ clones derived from single cells which had been rendered homoplasmic during 12 generations of growth by allowing segregation of mitDNA molecules (yielding secondary clones). The frequencies of genotypes occurring among such populations of secondary clones are regarded as representative of the genomes originally generated by the mutagenic process (although we are aware that recombination between different molecules present in the same heteroplasmic cell and inherent differences in the stability of certain genome configurations, may draw this assumption into question).

In a population of tsp-induced secondary rho$^-$ clones we find - in contrast to EB-induced secondary clones (Table 2A) -

i) a high and equal frequency of retention for each of the markers P^R, T^R, C^R, O^R

ii) a high frequency of co-retention of all four markers (genotype $P^R T^R C^R O^R$).

From these results we conclude that i) loss of mitochondrial genetic information from a population of cells is low during tsp-induced rho$^-$ mutation as compared to EB-induced rho$^-$ mutation where the loss of

	Clones observed and percentage			
	tsp25-induced (b)		EB-induced (a)	
	BM 1421-3C/B2		KL 14-4A/GΣ+GAΣ	
P^R	470	49%	443	2.2 %
T^R	465	49%	247	1.3 %
C^R	469	49%	237	1.2 %
O^R	460	48%	395	2.0 %
$P^RT^RC^RO^R$	384	40%	34	0.17%
$P^OT^OC^OO^O$	395	42%	18600	94.6 %
Total colonies observed	951		19668	

Table 2A : Induction of rho$^-$ cells and analysis of retention of mitochondrial markers

a) Population of secondary clones from ethidium bromide treatment of cells of strain KL 14-4A; data taken from (12)

b) This population of secondary clones was isolated as follows: (For media, growth of cells and crosses see 12, 13) Cells of strain BM 1421-3C which carries the nuclear mutation tsp25 (temperature-sensitive petite, 13) and the mitochondrial markers P^R, T^R, C^R, O^R were grown in YP-dextrose medium for 8 cell generations at non-permissive temperature (35°C), this leads to the presence of about 95% rho$^-$ cells in the culture (13). This population was transferred to permissive temperature and grown for a further 12 cell generations in order to allow segregation of deleted mitDNA molecules into single cells. (These cells are now essentially homoplasmic and rho$^-$ clones derived from such single cells are referred to as "secondary clones", cf.12). Secondary clones were analysed for retention of the markers P^R, T^R, C^R, O^R by the replica cross technique (9) : Cells of single rho$^-$ colonies were mated with a rho$^+$ tester strain (SM 50-11C, P^S, T^S, C^S, O^S). Diploid cells were replica plated onto selective media (12).

Since the rho$^-$ tester strain was $P^ST^SC^SO^S$, it was concluded that i) a rho$^-$ colony was P^R (or T^R or C^R or O^R) if the diploid progeny of a cross exhibited the respective resistant phenotype, ii) a rho$^-$ colony was assumed to be P^O (or T^O, C^O, O^O) if the respective resistant phenotype was not detected in the diploid progeny of a cross i.e. that the resistance marker had been deleted in the original rho$^-$ cell.

mitochondrial genetic information is high, ii) the mutation process gives rise to rho^- genomes which very frequently retain long sequences of the rho^+ genome (genotype $P^RT^RC^RO^R$).

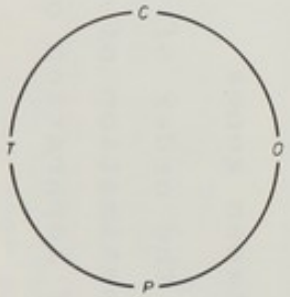
2) Determination of the gene order

As in a previous paper (12) we have analysed secondary rho^- clones induced in a strain carrying four mitochondrial markers the frequency of co-retention of two markers and the loss of the other two markers (double loss/double retention analysis, Table 2B). Of the three possible pairs of this genotype (cf. Figure in Table 2B) two pairs (I and III) can be generated by one continuous deletion event whereas the other pair (II) can be generated only by two or more deletion events, followed by subsequent joining of deleted molecules, each one carrying one of the two markers considered. This second pair is thus expected to occur with lower frequency than the other two pairs.

Table 2B shows that the pair of genotypes $P^RT^OC^RO^O$ + $P^OT^RC^OO^R$ is significantly less frequent than the other two possible pairs. This was found to be so after tsp-induced rho^- formation and after spontaneous deletion in tertiary clones from two different rho^- mutants carrying all four markers. This result is in agreement with the gene order P-T-C-O and thus confirms the gene order derived in our previous work with EB-induced rho^- clones.

3) The relative distances between genes

Besides the determination of the order of genes the deletion analysis allows an estimation of relative distances. The probability of separation of two genes



Genotype	No. of continuous deletions	Number of clones observed in experiment						
		strain BM 1421-3C/tsp25					strain KL 14-4A(a)	
		BΣ	A11	A41	Total		Total	
$P^{R}_{T}R_{C}O_{O}O$	1	10	10	22	42	52	246	275
$P^{O}_{T}O_{C}R_{O}R$		4	2	4	10		29	
$P^{R}_{T}O_{C}R_{O}O$	2	0	2	4	6	12	26	30
$P^{O}_{T}R_{C}O_{O}R$		3	1	2	6		4	
$P^{R}_{T}O_{C}O_{O}R$	1	9	5	2	16	36	412	456
$P^{O}_{T}R_{C}R_{O}O$		2	7	11	20		44	

Table 2B : Analysis of double loss/double retention of markers
BΣ: Analysis of secondary clones of tsp25-induced rho⁻ mutants (cf. Table 2A)
A11, A41 : Analyses of individual, established rho⁻ clones of high genetic stability (genotype $P^{R}_{T}R_{C}R_{O}R$). Two rho⁻ clones (A1 and A4) obtained after temperature-dependent induction of rho⁻ cells in mutant tsp25 were subcloned. Single clones (A11, A41) of genotype $P^{R}_{T}R_{C}R_{O}R$ were subcloned once more (tertiary clones) and all possible genotypes were analysed in a total of 212 (A11) and 317 (G41) subclones (not including subclones of genotype $P^{O}_{T}O_{C}O_{O}O$).
(a) : Data taken from previous analysis of rho⁻ clones of genotype $P^{R}_{T}R_{C}R_{O}R$ ((12), sum of experiments GΣ, GAΣ, HΣ, G1p, G4Σ, F3p).

by deletion (e.g. $X^{R}Y^{R}$ --→ $X^{R}Y^{O}$ and $X^{O}Y^{R}$) is assumed to increase proportionately with increasing distance between these genes. This mapping approach is analogous to the determination of distances in terms of recombination frequencies (e.g. $X^{R}Y^{R}$ x $X^{S}Y^{S}$ --→ $X^{R}Y^{S}$ and $X^{S}Y^{R}$). We will express relative distances between the genes analysed as the ratio of the observed to the expected frequencies of separation of two genes (cf. legend to Table 2C). A value of 1 indicates random pairwise association of markers X^{R}, X^{O}, Y^{R}, Y^{O} and the absence of linkage; values significantly lower than 1 indicate preferential co-retention of X^{R} and Y^{R} i.e. linkage between X and Y.

Frequencies of separation and relative distances calculated are given in Table 2C. Both in the population analysis of tsp25-induced rho^{-} mutants and in the analysis of spontaneous deletion in individual rho^{-} clones values close to 1 are consistently found with the the pair of markers O-P only. These two markers thus appear to be unlinked. All other pairs show significantly lower values and thus appear to be linked. Linkage being tightest in case of the markers C-T.

Essentially the same relative distances of separation of markers have been found in a population of rho^{-} clones induced by another tsp mutation (strain AM 1311-2D, tsp20; the relative distances are : C-T=0.13, C-O = 0.26, T-O = 0.30, P not tested).

Variation in the relative distances calculated for two markers is thus found to be low when we compare the results of the population analysis of deletion caused by tsp mutations and the analysis of spontaneous deletion in individual rho^{-} clones originating from these populations. Similar relative distances were

Separation of markers	Number of colonies and relative distance () in experiment					
	strain BM 1421-3C/tsp25				strain KL 14-4A	
	B Σ	A11	A41	Total	G Σ	G1p+G3p+G4 +F3p
$T^R C^O + T^O C^R$	27 (0.18)	30 (0.33)	45 (0.26)	102 (0.26)	345 (0.80)	369 (0.07)
$T^R O^O + T^O O^R$	103 (0.70)	65 (0.72)	129 (0.88)	295 (0.77)	551 (0.98)	970 (0.28)
$C^R O^O + C^O O^R$	86 (0.57)	45 (0.51)	116 (0.78)	247 (0.62)	507 (0.92)	1165 (0.25)
$T^R P^O + T^O P^R$	92 (0.60)	63 (0.70)	107 (0.67)	262 (0.66)	532 (0.89)	3058 (0.84)
$C^R P^O + C^O P^R$	91 (0.59)	63 (0.70)	108 (0.68)	262 (0.66)	562 (0.95)	3797 (0.87)
$O^R P^O + O^O P^R$	145 (0.95)	78 (0.90)	152 (0.96)	375 (0.94)	705 (1.02)	3492 (1.05)

Table 2C : Frequencies of separation and relative distances of markers
B Σ : Analysis of tsp-induced deletion (same population of secondary clones as in Table 2A and 2B).
A11, A41 : Analysis of spontaneous deletion in individual rho⁻ clones induced by tsp25 (same experiment as in Table 2B).
G Σ : Analysis of EB-induced deletion (secondary rho⁻ clones, data taken from (12)).
G1p, G3p, G4 , F3p : Analysis of spontaneous deletion in individual rho⁻ clones of genotype $p^R T^R C^R O^R$ induced by EB (11).
Relative distances correspond to the ratio of observed to expected frequencies of separation of two markers X and Y.
Observed frequencies : $X^R Y^O + X^O Y^R$; expected frequencies : $\frac{X^R \cdot Y^O}{N} + \frac{X^O \cdot Y^R}{N}$
X^R, X^O, Y^R, Y^O = respective frequencies of rho⁻ clones observed (not including $P^O T^O C^O O^O$)
N = number of rho⁻ clones observed (not including genotype $P^O T^O C^O O^O$)

found in the previous analysis of spontaneous deletion in individual rho$^-$ clones originating from EB-induced rho$^-$ cells. Relative distances were found to be much higher in an analysis of a population of EB-induced secondary rho$^-$ clones (cf. Table 2C).

It may be concluded from these findings that the sequence of the mitochondrial genome retained in rho$^-$ cells is much smaller after EB-induction as compared to the endogenous deletion in rho$^+$ mitDNA caused by tsp or occurring spontaneously in rho$^-$ mitDNA. A striking similarity is observed in the latter two cases. Further analyses with different tsp mutants and different rho$^-$ mutants of high genetic complexity will reveal if this similarity reflects a common mechanism in the formation of rho$^-$ petites.

III) Comparison between deletion and recombination mapping

Both approaches of mapping studied by the work presented here are found to lead to an unambiguous determination of the order of the four genes regarded. This order (P-T-C-O) can be derived from each of the experimental approaches employed, namely from i) the analysis of recombinant frequencies in rho$^+$ x rho$^+$ crosses , ii) the analysis of deletion in populations of rho$^-$ mutants induced by ethidium bromide (a) or by the action of the nuclear mutation tsp25 (b), iii) the analysis of spontaneous deletion in mitDNA of established rho$^-$ clones originating from population (a) or (b). Furthermore the relative distances of markers as derived from the results of the different approaches are consistent in that : i) Partial additivity of distances is observed. ii) T and C show close, O and P show no significant linkage to each other. iii) Both

O and P or at least P (by recombination analysis) show linkage to the T-C segment of the genome.

The homogeneity of the results obtained by all three mapping methods is remarkable for two reasons : First, it leaves no doubt that the order of these four genes and the relative distances between them is identical in different strains of S. cerevisiae and secondly, it can be excluded that deletion processes can significantly obliterate this order by frequently producing re-arrangements within the mitDNA molecule.

Although the linkage data do not necessarily permit a circular presentation of a single mitochondrial genome, circularity of the mitochondrial genome as shown in Figure 1 is suggested by electron microscopy (14), by physical mapping of mitochondrial DNA (15) and by the fact that co-retention of O and P is frequently observed (cf. Table 2B). As non-neighbours on a linear map co-retention of these two markers would be expected to be rare. Figure 1A and 1B clearly show that according to rho^- deletion analysis the neighbouring markers P-T, T-C, C-O show linkage. Thus we have indications of the relative distances between markers in the segment P-T-C-O. Since O and P appear to be unlinked, the other, as yet silent segment of the circular genome has to be assumed to equal or to exceed the length of the known region P-T-C-O.

In terms of recombination frequencies (Fig.1C) the marker O appears to be unlinked to the region of linked markers P-T-C/E. Differences were found in the relative distances of markers observed by deletion and recombination analysis (e.g. C-O, C-P) and may be due to preferential deletion of/or recombination between markers. This could be a general phenomenon or

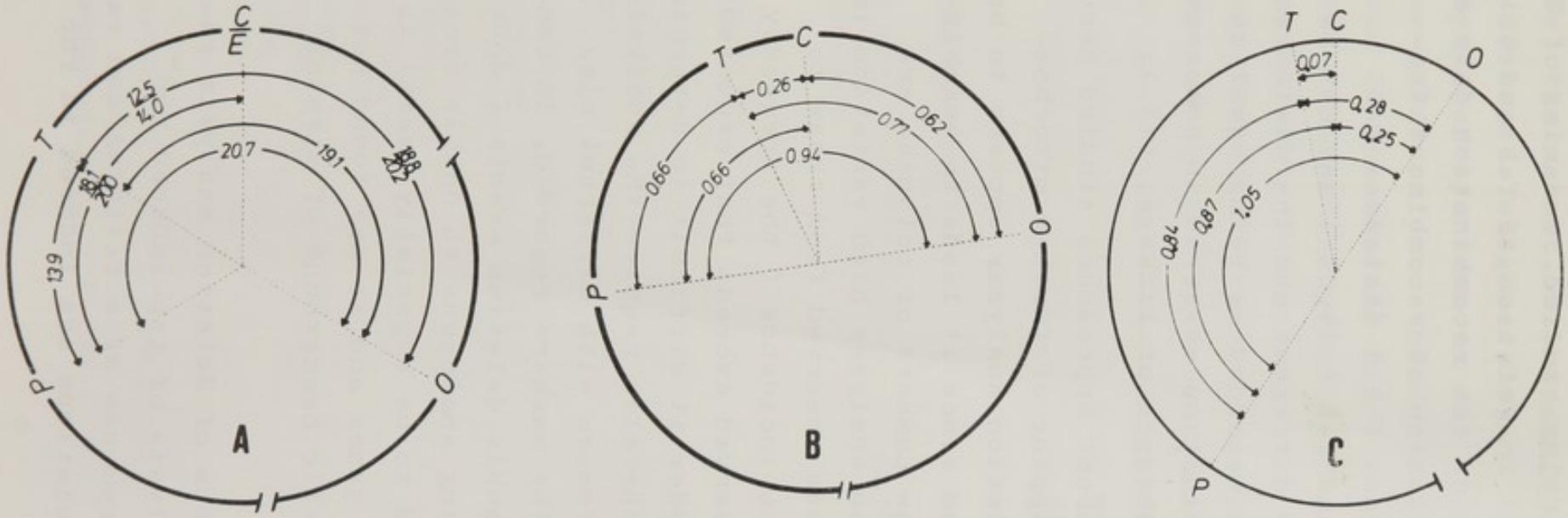


Figure 1 : Linkage maps showing distances of markers as derived from recombination and deletion analyses.
A) Data taken from Table 1 (recombination analysis)
B) Data taken from Table 2C (strain BM 1421-3C/tsp25 analysis of spontaneous deletion & tsp-induced deletion)
C) Data taken from Table 2C (strain KL 14-4A, analysis of spontaneous deletion)
—//— indicates absence of apparent linkage between markers

a result unique to the strains studied. Ordering of C and E (9% recombination) in relation to T-P and O is not possible on the basis of the recombination data available. Due to high variation of recombinant frequencies from cross to cross (4) distances T-C (12.5% recombination) and T-E (14.0%) may not be regarded as significantly different and the distances C-P, E-P, C-O, E-O may be regarded as being equal to the upper limit of recombination observed in homosexual crosses (indicating absence of linkage, (1)).

Thus any one of the different approaches studied here may be used for future mapping of further mitochondrial genes. The rho^- deletion analysis appears to be the most attractive method since it leads to unambiguous results even when low numbers of colonies are analysed. In recombination analyses high variation in frequencies of recombinants observed from cross to cross and high positive co-incidence (the frequency of double cross-overs observed exceeds that expected by 2- to 4-fold (4)) render it difficult to determine the order of genes and generally require the analysis of several independent crosses with different cis/trans-configurations of the markers regarded. In contrast, the analysis of double deletion events (double loss/double retention being analogous to double cross-over analysis) was found to be especially useful in determining the order of genes and it was found not to be influenced by the genetic background of strains used.

Among the different methods of deletion analysis reported here only the analysis of tsp-induced rho^- clones met all the requirements of a faithful and rapid mapping system : i) Deletions are induced in rho^+

cells, the result of the analysis thus reflects order and distances of genes in rho$^+$ mitDNA. In contrast, the analysis of spontaneous deletion in an individual rho$^-$ clone leads only to the determination of order and distances in one already deleted mitDNA molecule which may differ from mitDNA molecules of rho$^+$ cells and from those of other rho$^-$ cells. ii) A random population of deleted mitDNA molecules is analysed. Since re-arrangements in the mitochondrial genomes appear to be rare, unambiguous results are obtained. iii) Retention of rho$^+$ mitDNA sequences is much more frequent in these secondary rho$^-$ clones than in EB-induced clones.

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Extrachromosomal Inheritance of Drug Resistance and Respiratory Deficiency in the Fission Yeast Schizosaccharomyces pombe

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INTRODUCTION

The yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe both have well- characterized and mapped nuclear genes (1, 2). Whereas progress has been rapid in the study of extrachromosomal genetics in Saccharomyces cerevisiae (3) no extrachromosomal mutants of Schizosaccharomyces pombe have previously been described. The mitochondrial DNA of Schizosaccharomyces pombe differs from Saccharomyces cerevisiae in that it is probably of lower molecular weight (12.5×10^6 (4), compared to Saccharomyces: 50×10^6 (5)) and does not have such a high AT- content (Schizosaccharomyces: 33 % GC (6), Saccharomyces: 17 % GC (7)). In this respect Schizosaccharomyces mitochondrial DNA might be considered more representative of eukaryotes in general.

In this paper we shall show that extrachromosomal inheritance can be studied in Schizosaccharomyces pombe and that extrachromosomal mutants of this organism could provide insight in general behaviour of eukaryotic mitochondrial DNA.

MATERIALS AND METHODS are described elsewhere (8, 9)

RESULTS

1) Criteria for extrachromosomal inheritance of antimycin-resistance

In an earlier paper we have described the isolation of a series of mutants resistant to antimycin in vivo and in vitro (10).

a) Random spore- and tetrad analysis

The results of two sets of crosses of mutant [ANT^{R}8] ade 7 h$^-$ with two auxotrophic h$^+$- strains are given in Table 1. The approximate 50 : 50 segregation of resistant versus sensitive alleles might be considered to be an indication of a single Mendelian gene. However, analysis of 64 complete tetrads showed that only 38 tetrads segregated 2 R : 2 S (R = resistant, S = sensitive) characteristic for a single Mendelian gene, the other tetrads were of 3 R : 1 S, 1 R : 3 S, 4 R : 0 S, 0 R : 4 S type. The same ratio of R : S haploids both in random spore- and in tetrad analysis was found (Table 2). This demonstrates that the deviation in random spore analysis is not due to a bias of spore viability associated with the resistance allele. Another strong argument against a chromosomal gene comes from the high amount (up to 20%) of post- meiotic segregation observed in spore colonies, which is much higher than any post-meiotic segregation rate previously observed for a nuclear gene.

TABLE 1: Random spore analysis

a) of cross [ANT^{R}8] h$^-$ ade 7 x 32 leu 1 h$^+$

[ANT^{R}8] : [ANT^{S}8]	ADE 7 : ade 7	LEU 1 : leu 1	total
612 : 755	674 : 693	758 : 609	1,367
ratio 1 : 1,23			

b) of cross [ANT^{R}8] h$^-$ ade 7 x 171 ura 1 h$^+$

[ANT^{R}8] : [ANT^{S}8]	ADE 7 : ade 7	URA 1 : ura 1	total
339 : 462	407 : 395	414 : 388	802
ratio 1 : 1,37			

TABLE 2: Tetrad analysis of the cross $[ANT^{R}8]$ h^{-} ade 7 x $972h^{-}$

$[ANT^{R}8]$: $[ANT^{S}8]$	Number of complete tetrads
2 : 2	38
3 : 1	5
1 : 3	13
0 : 4	7
4 : 0	1
ratio $[ANT^{R}8]$: $[ANT^{S}8]$ =	1 : 1.25

b) Mitotic segregation of diploids

Mitotic segregation is one of the criteria for extrachromosomal inheritance in Saccharomyces cerevisiae. The mutation mei 1 (11) in the mating type locus blocks the sexual cycle of Schizosaccharomyces pombe before the first meiotic division. Using this mutation one can maintain the diploid state (except for some spontaneous haploidization). The sum of 6 different mitotic segregation experiments using mei 1 is given in Table 3. Including the sectored (R to S) colonies together with the apparently totally resistant colonies, transmission of antimycin resistance is about 20 %. This low value of transmission is characteristic for $[ANT^{R}8]$ and depends on premating growth conditions. Using glycerol instead of glucose for growing the haploids before mating, one can enhance transmission of the resistance gene up to 40 %. In another mutant $[ANT^{R}14]$, a transmission rate of about 50 % can be achieved when cells are grown in glycerol. The occurrence of mitotic segregation is a third argument in favour of extrachromosomal inheritance.

An interesting feature concerning segregation of extrachromosomal elements was revealed by the mitotic analysis. Many of the sensitive diploid colonies originating from single zygotes (selected

by their prototrophic growth and by their characteristic shape) only contained sensitive cells. The occurrence of pure sensitive diploid offspring from zygotes resulting from a cross of a resistant and a sensitive haploid cannot be explained only by the fact that resistance determinants are biassed. This behaviour is very surprising in view of the mode of cell division of this organism by fission which presumably divides the cytoplasm into equal portions. Consequently at least one (when cytoplasmic mixing is low) or both daughter cells of a zygote should contain enough determinants to express resistance if multiple copies of the genome are present. It is possible that there exists a mechanism of sorting out one or the other genome.

TABLE 3: Mitotic segregation of diploids originating from crosses [ANT^{R}_{8}] ade 7 h^{-} x mei 1 aux^{-}

	[ANT^{R}_{8}]	[ANT^{S}_{8}]	sectored [$ANT^{R/S}_{8}$]	total
colonies	183	1,562	183	1,928
%	9.5	81.0	9.5	100

c) m- fluorophenylalanine- induced mitotic haploidization

Mitotic haploidization is an alternative method of analysis to sporulation, which is not possible in diploids carrying mutation mei 1. The sequence characteristics of haploidization are:

- genes on the same chromosome always segregate together in the haploids (except in rare cases of mitotic recombination or gene conversion),
- genes on different chromosomes are distributed randomly among the haploid descendants,
- any heterozygous diploid carrying recessive or dominant chro-

mosomal mutation segregates,

- diploids selected for the presence or absence of an extrachromosomal mutation always produce haploids with the same extrachromosomal phenotypes as their diploid parents.

This technique distinguishes chromosomal and extrachromosomal genes. Table 4 shows the result of haploidization of resistant and sensitive diploids. As predicted for an extrachromosomal mutation the haploid offspring of resistant diploids was always resistant and that of sensitive diploids sensitive. (Two exceptional diploids were found, where 2 - 3 % resistant haploids appreared, presumably originating from impurity of the diploids). Controls showed normal segregation of nuclear markers. Together with tetrad analysis (including post- meiotic segregation), and mitotic segregation these results are the fourth argument in favour of an extrachromosomal, very likely a mitochondrial mutation conferring resistance to antimycin.

TABLE 4: m- fluorophenylalanine- induced haploidization of diploids with 3 genetically marked chromosomes

diploid	ade 7	URA 1	ARG 1	h^-	$[ANT^R_8]$
	ADE 7	ura 1	arg 1	mei 1	$[ANT^S_8]$

ura 1 - chromosome I, ade 7 - chromosome II, arg 1 - chromosome III

a) sensitive diploids taken from glycerol- complete- medium

	$[ANT^R_8]$	$[ANT^S_8]$
URA 1	128●	0
ura 1	308	0●
ADE 7	228	0^+
ade 7	208●	0
ARG 1	124●	0
arg 1	312	0●

●parental configurations. 4 independent haploidizations

b) resistant diploids taken from antimycin medium

	$[ANT^{R}8]$	$[ANT^{S}8]$
URA 1	0●	111
ura 1	0	138●
ADE 7	0	177●
ade 7	0●	198
ARG 1	0●	142
arg 1	0	184●

●parental configurations. 2 independent haploidizations

2) Respiratory deficiency in the wild- type and in $[ANT^{R}8]$

a) Occurrence of respiratory deficient mutants in glucose- grown cultures of $[ANT^{R}8]$

Extensive, but unsuccessful attempts have been made to isolate spontaneous or induced stable respiratory- deficient extrachromosomal mutants in Schizosaccharomyces pombe (12, 13, 14). Mutant $[ANT^{R}8]$, however, exhibited a surprising new property not shown by the wild- type. When a glucose- grown stationary-phase culture was plated on the same medium and the colonies were replicated onto glycerol- medium enriched with 0.1 % glucose, up to 0.5 % of the colonies showed a respiratory- deficient phenotype. This was stable even after several subcloning.

b) Induction of respiratory deficiency with ethidium bromide (EB) and Janus green (JG)

Both drugs are known to induce cytoplasmic petites in Saccharomyces cerevisiae (15, 16). In Schizosaccharomyces pombe the frequency of respiratory - deficient (RD) mutants can be considerably increased by growing cells in glucose- complete- medium in the presence of these drugs.

TABLE 5: Induction of respiratory deficiency by ethidium bromide (EB) and Janus green (JG) in mutant [ANT^{R}_{8}]

Concentration of EB (µg/ml)	% RD	Concentration of JG (µg/ml)	% RD
0	0.5	0	0.5
2	13.1	1	1.0
5	9.6	2	2.6
7.5	7.8	3	3.7
10.0	9.2	5	6.3
20.0	4.6	10	29.6
30.0	8.0	20	17.6

About 500 colonies tested per concentration.

c) EB- effects in the wild- type

Similar treatment with these drugs of the wild- type results in induction of an unstable cell state with a petite- like phenotype, which always reverts spontaneously to RC. Ade 6 and ade 7 mutants in Schizosaccharomyces pombe produce a red pigment when grown on adenine- limited medium. Colonies of RD- mutants carrying these mutations are brown. After EB- treatment of the wild- type brownish colonies appeared (phenotypically similar to RD- mutants) and colonies with various degrees of brownish sectoring. The number of brownish colonies and the amount of brownish sectors were dependent on the EB- concentrations used. Brownish colonies also showed a delay in growth on glycerol- medium after replica- plating. Occasionally these colonies failed to grow after replica- plating onto glycerol- complete- medium and could only recover respiratory-competence, when replica- plated on glycerol- complete- medium supplemented with 0.1 % glucose. This type of unstable respiratory deficient variant was also found among the RD colonies originating

from [ANT$^{R}_{8}$] , but most of the RD colonies proved to be stable respiratory- deficient. Treatment of wild- type and [ANT$^{R}_{8}$] for 10 generations with 20 µg/ml EB gave microcolonies in both wild-type and [ANT$^{R}_{8}$]. These colonies could recover normal colony size after cloning them onto drug- free medium. These colonies were all RC in case of the wild- type and stable RD in [ANT$^{R}_{8}$].

d) Genetic classification of RD- mutants originating from [ANT$^{R}_{8}$]

The high number of RD mutants and the inducibility by petite- inducing agents in Saccharomyces cerevisiae suggests that these mutants of Schizosaccharomyces pombe might be extrachromosomally and very likely mitochondrially inherited. Several independently isolated, EB- induced RD- mutants were checked for mitotic segregation and in mitotic haploidization. Since the results were similar, one example (mutant mit-25) is shown here. The distribution of the different phenotypes after mitotic segregation is given in Table 6.

TABLE 6: Mitotic segregation of diploids from the cross mit-25 ade 7 h^{-} [ANT$^{R}_{8}$] x mei 1 ura 1 his 2

Colonies	RC	RD	[ANT$^{R}_{8}$]	[ANT$^{S}_{8}$]
1,221	1,198	23	50	1,171
100 %	98.1 %	1.9 %	4.2 %	95.8 %

It is evident that both antimycin resistance and respiratory deficiency segregate during mitosis indicating that a non- Mendelian mutation was also responsible for respiratory deficiency. The low number of RD diploids shows that transmission of RD determinants is more strongly biassed than that of [ANT$^{R}_{8}$] determinants. The occurrence of RC [ANT$^{R}_{8}$] diploids suggests a recombinational event between [ANT$^{S}_{8}$] RC and [ANT$^{R}_{8}$] RD determinants rather than complementation, since during 20 generations of growth [ANT$^{R}_{8}$] RC

diploids never segregate into [ANT^{S}8] and RD diploids. This can be taken as good evidence that [ANT^{R}8] and mit-25 mutation are on the same genome, which is very likely the mitochondrial DNA.

Haploidization of [ANT^{S}8] RC, [ANT^{R}8] RC, and RD diploids show pure haploid descendants, being an additional proof for extrachromosomal inheritance.

In a series of crosses of 53 RD mutants of independent origin (spontaneous, EB-, and JG- induced) 40 showed no segregation of RD diploids, behaving like neutral rho$^-$- mutants or recessive nuclear petites. In this case, haploidization served as criterion for extrachromosomal inheritance. Since all the progeny haploids were RC, the cytoplasmic nature of these mutants was established. A control experiment with nuclear recessive petite mutants showed segregation into RD and RC haploids.

Some of the 53 RD- mutants transmitted antimycin resistance up to 40 %, some to the same degree as [ANT^{R}8] (20 %), some to a much lesser extent or not at all. The failure in some cases of transmission of [ANT^{R}8] could be due to a reversion to [ANT^{S}8] in the RD- mutant (perhaps by deletion of this segment) or the failure of recombination between the [ANT^{R}8] region of the RD- genome and the RC- genome with subsequent loss of the RD- genome.

Established rho^o- and rho$^-$- mutants of Saccharomyces cerevisiae do not revert, whereas some mitochondrial respiratory deficient mutants with intact mitochondrial protein synthesis (called mit$^-$) do (17). The RD- mutants of Schizosaccharomyces pombe fall in 3 main categories. Some revert very frequently (up to 1 in 10^3), some at a rate of 1 in 10^6 - 10^8, and some not at all. The 'rst and second group might be mitochondrial point mutations, hereas the third group could well represent small deletion mutants.

d) Biochemical characterization

The mitochondrial properties of mutants mit-12, mit-25, and mit-30 were analyzed. Since they proved to be rather similar only mutant mit-25 was extensively characterized.

Neither whole cells nor mitochondrial particles of mit-25 showed KCN- sensitive respiration (Table 7). Apparently this is due to the lack of cytochrome c- oxidoreductase activity. NADH: and succinate: cytochrome c - oxidoreductase show lower specific activity than in [ANT^{R}8], but about the same sensitivity to antimycin was found as in [ANT^{R}8].

Low temperature spectra reveal that the cytochromes aa_3 and cytochrome b-566 (absorbing at 561 nm at 77°K) are not present in mit-25, although they are present in [ANT^{R}8] (10). The residual cytochromes b, c, and c_1 are reducible by substrate to a smaller extent than in [ANT^{R}8].

The biochemical analysis also excludes that mit-25 is the equivalent of Saccharomyces cerevisiae rho^{-} - or rho^{o}- mutants. Except for complex IV, all respiratory enzyme complexes are still present and active in all three mit^{-} - strains. In contrast, rho^{-} - mutants in Saccharomyces cerevisiae are completely devoid of active complex III. Thus from genetical and biochemical evidence the [ANT^{R}8] marker is not only present but also expressed in mit-25.

3) Spontaneous mutations in wild- type and [ANT^{R}8] conferring resistance to chloramphenicol and erythromycin

From the data presented, it is evident that [ANT^{R}8] mutation is the key to extrachromosomal respiratory deficient mutants in Schizosaccharomyces pombe. Does the [ANT^{R}8] mutation have a similar effect upon the occurrence of other extrachromosomal mutations conferring resistance to chloramphenicol and erythro-

TABLE 7: Analysis of the electron transport activities or mutant mit-25

Section of electron transport system	% activity of [ANT^{R}8]	Special properties
Respiration of whole cells with glucose as substrate	0.3	insensitive to KCN
NADH - oxidase	0.5 - 1.0	insensitive to KCN
NADH: cytochrome c-oxidoreductase	28	
stimulated by 26 μM coenzyme Q	45	Sensitivity to antimycin similar to [ANT^{R}8]
Succinate: cytochrome c-oxidoreductase	32	
stimulated by 26 μM coenzyme Q	50	
Cytochrome c oxidase	0	

mycin? About 200 spontaneous mutants of [ANT^{R}8] and about 30 of the corresponding wild- type were isolated from plates containing 4 g/l erythromycin. Mitotic segregation analysis revealed that all [ANT^{R}8 ERY^R] mutants were of the extrachromosomal type, whereas all erythromycin resistant mutants from the wild-type were apparently recessive nuclear (ery^r) (18). 80 mutants conferring resistance to 4 g/l chloramphenicol were selected from [ANT^{R}8] and 20 from the wild- type with the same result as for erythromycin resistance (18). These results clearly demonstrate that isolation of extrachromosomal resistance mutations appears to be much easier in [ANT^{R}8] than in the wild- type. Possible

reasons for this will be discussed.

4) Effect of $[CAP^R]$ - and $[ERY^R]$- mutation on induction of extrachromosomal RD- mutants

Can extrachromosomal RD- mutants only be induced in $[ANT^R{}_8]$ or can they also be found in extrachromosomal mutants resistant to chloramphenicol and erythromycin? Tetrads from a cross $[ANT^R{}_8\ ERY^R] \times [ANT^S{}_8\ ERY^S]$ were dissected and an $[ANT^S{}_8\ ERY^R]$ spore was obtained. No spontaneous or EB- induced RD- mutants could be obtained from this strain. Similar results were found using an $[ANT^S{}_8\ CAP^R]$- spore (18). This emphasizes the unique properties of the $[ANT^R{}_8]$ mutation for the production of further extrachromosomal mutations.

DISCUSSION

We have presented evidence that antimycin resistance in Schizosaccharomyces pombe is extrachromosomally, very likely mitochondrially inherited. There are several lines of evidence:

- the occurrence of tetrads in which the spores continue to segregate genes conferring resistance and sensitivity, similar to the situation, when zygotes of Saccharomyces cerevisiae are sporulated immediately,
- the high amount of this post- meiotic segregation,
- mitotic segregation of resistance versus sensitivity in stable diploids, and
- the uniformity of the progeny for the resistant and sensitive clones after haploidization of resistant and sensitive diploids.

Transmission of antimycin resistance in diploids is rather low and can be increased by growing cells in glycerol before mating. This suggests a high susceptibility of the resistance determinants to glucose- repression. Similar effects of glucose- repression were

also reported for the transmission of mitochondrial genomes in Saccharomyces cerevisiae (19, 20, 21).

According to the mode of cell division by fission one would assume that pure clones would segregate out very late. Therefore it was surprising to find diploid colonies resulting from one zygote, consisting of sensitive cells only (18). This phenomenon is not without precedence. In Chlamydomonas, equal amounts of chloroplast DNA of both parents are transmitted to the zygote, but then the paternal DNA is destroyed leading to the expression of maternal genes only (22). A similar mechanism might also act in zygotes of Schizosaccharomyces pombe.

The absence of conditional mutants blocking meiosis lead us to the choice of haploidization for further analysis of the stable diploids. Using this method 3 chromosomes have been identified in Schizosaccharomyces pombe (23), which is in contradiction to the findings of Egel and Pentzos- Daponte (24) and Fischer et al. (25) using light microscopy (24) and electron microscopy (25). To decide whether a mutation is chromosomally or extrachromosomally inherited, however, is independent of the number of chromosomes involved, since one only has to look for heterogeneity (in the case of nuclear markers) or homogeneity (in the case of extranuclear markers) of the haploid segregants.

Biochemical and genetical data suggest that the respiratory-deficient mutants described in this paper are extrachromosomally, very likely mitochondrially inherited.

The fact that all mutants conferring resistance to chloramphenicol or erythromycin isolated in the wild- type, were nuclear recessive, whereas those originating from $[ANT^{R}_{8}]$ were cytoplasmic, indicates that $[ANT^{R}_{8}]$ has some "mutator" or selective activity for respiratory- deficient mutants and other extrachromo-

drug resistance mutations. This "mutator" activity is not shared by the other drug resistance mutations.

Results have been obtained which show that recombination occurs between $[ANT^{R}8]$, $[CAP^{R}]$, and $[ERY^{R}]$, using the mitotic as well as the meiotic systems (18). Thus we feel that way is now open for mapping the mitochondrial genome in Schizosaccharomyces pombe. A further search for mitochondrial mutants could contribute to the question of coding capacity of this short mitochondrial DNA. This might be one step towards the understanding of petite- negativity in yeast.

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Temperature-Sensitive Nuclear Petite Mutants of Saccharomyces cerevisiae

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SUMMARY: 270 temperature-sensitive Saccharomyces cerevisiae mutants have been isolated. They grow on nonfermentable carbon sources like lactate, glycerol or ethanol only at 22°C, but not at 36°C. By complementation analysis, these mutants could be attributed to 106 different complementation groups. In general, these mutants are respiratory deficient when grown at non-permissive temperature. Interestingly, however, among 66 complementation groups studied, the mutants of about 16 groups still exhibit wild type-like respiration rates even at non-permissive temperature. These mutants are considered as potentially uncoupled or phosphorylation-deficient strains. Mutants of about 20 other groups are almost completely converted into cytoplasmic petites when grown for 3-5 generations at 36°C. Most of these secondary cytoplasmic mutants still contain mitochondrial DNA as indicated by the CsCl or $Cs_2SO_4/HgCl_2$ density gradient centrifugation profiles obtained with total cellular DNA.

INTRODUCTION

The genetic investigation of respiratory deficient mutants represents one of the most promising approaches to study mitochondrial structure, function and biogenesis at the molecular level. Thereby, many of the problems encountered by the biochemist when disintegrating and analyzing the highly organized, membrane-bound enzyme systems of this organelle, may be circumvented. Available genetic as well as biochemical (1,2) evidence indicates, that mitochondrial DNA itself codes for only a very limited number of mitochondrial proteins the majority of them being of nuclear origin. Especially from yeast, nuclear respiratory deficient mutants have been isolated in many different laboratories (3,5). However, there seems to be no report on a systematic biochemical and genetic characterization of them on a large scale, so far. From such studies, representative mutants for most of the nuclear genes controlling mitochondrial structure and functions may be obtained. In yeast, the biochemical as well as genetic

characterization of nuclear petite mutants has often been hampered by their frequent and irreversible conversion into pleiotropic cytoplasmic ρ^--mutants (6). Therefore, the present study has been restricted to conditional temperature-sensitive petite mutants. With them, many operations may be performed under permissive conditions with the ρ^+- character conserved. For instance, the secondary cytoplasmic ρ^--character will not interfere with the complementation behaviour of the original nuclear mutations. On the basis of these considerations, the isolation and characterization of a large number of temperature-sensitive respiratory-deficient Saccharomyces cerevisiae mutants has been initiated in this laboratory. First results regarding the genetic and biochemical characteristics of these mutants will be presented in this study.

MATERIALS AND METHODS

Yeast strains. The mutants included in this study were derived from the two haploid Saccharomyces cerevisiae wild type strains X 2180-1A (a-mating type) and X 2180-1B (α-mating type) originally obtained from Dr. R.K. Mortimer (Berkeley). The neutral cytoplasmic petite strain D243-4A/8 (a ade 1 lys 2) was obtained from Dr. G. Michaelis (Würzburg).

Mutant isolation procedure and media. For mutagenesis, stationary phase cells were incubated with 1 % ethyl methane sulfonate for 3 hours at 22°C. The survival rate under these conditions was about 30 %. The mutagenized cells were washed with sterile water and plated, at a density of 100 viable cells per plate, onto lactate (LAC)-agar (3 % lactate pH 5.4, 0.5 % yeast extract, 0.6 % peptone, 2 % agar). After 5-7 days incubation at 22°C, the outgrown colonies were replicated onto LAC-agar plates equilibrated at 36°C. After 2-3 days incubation at 36°C the replica plates were screened for temperature-sensitive mutants. Among them, conditional respiration-deficient mutants were characterized by their ability to grow at 36°C on sugar-containing YEPD-agar (2 % sucrose, 0.7 % peptone, 0.5 % yeast extract, 1 % K_2HPO_4, 1 % KH_2PO_4, 2 % agar) but not on LAC-agar.

Complementation analysis. For genetic complementation analysis the mutants isolated were first converted into the corresponding strains of opposite mating type by meiotic segregation. Subsequently they were crossed at 22°C on YEPD-agar in all possible pairwise combinations. After 2 days incubation at 22°C, the resulting diploids were stamped onto LAC-agar plates equilibrated at 36°C. These replicas were examined for respiratory competent crosses after 5-7 days incubation at 36°C.

Respiration measurements. Respiration rates were determined polarographically with a Beckman-oxygen electrode in a 2 ml cell fitted with a water jacket and magnetic stirrer. Aliquots were taken from 30 ml cultures of each mutant after growth for 40-55 hours under rigorous agitation in YEPD-liquid medium at both temperatures, 22°C and 36°C. If necessary, the samples were diluted with fresh YEPD-medium to a cell density of 1.5×10^7 cells/ml. At the same time, the proportion of ρ^--cells in these samples

was determined by replica plating 50-150 cells onto YEPD and LAC-agar at 22°C (Table 1a). Another portion of each sample was plated onto YEPD-agar at 36°C and, after 3-4 days incubation at non-permissive temperature, stamped from there onto 22°C LAC-agar (Table 1b).

Conversion of susceptible rho^+ petite strains into cytoplasmic rho^- mutants. About 100 cells of a single rho$^+$ clone grown at 22°C on YEPD-agar were inoculated into 5x5ml liquid YEPD-medium equilibrated at 36°C. After growth to stationary phase at 36°C, the cells were inoculated into 5x1 liter of liquid YEPD-medium at 36°C. The cultures were kept on a rotary shaker at 36°C for 3 days. Then, they were harvested by centrifugation and used for subsequent DNA isolation (7). At the same time, samples of the cultures were spread on YEPD-agar at 22°C at a cell density of 100 - 150 cells per plate and incubated at 22°C for 2-3 days. Then, these plates were replicated onto LAC-agar at 22°C. Only those strains were used for DNA isolation which were essentially free of rho$^+$ cells.

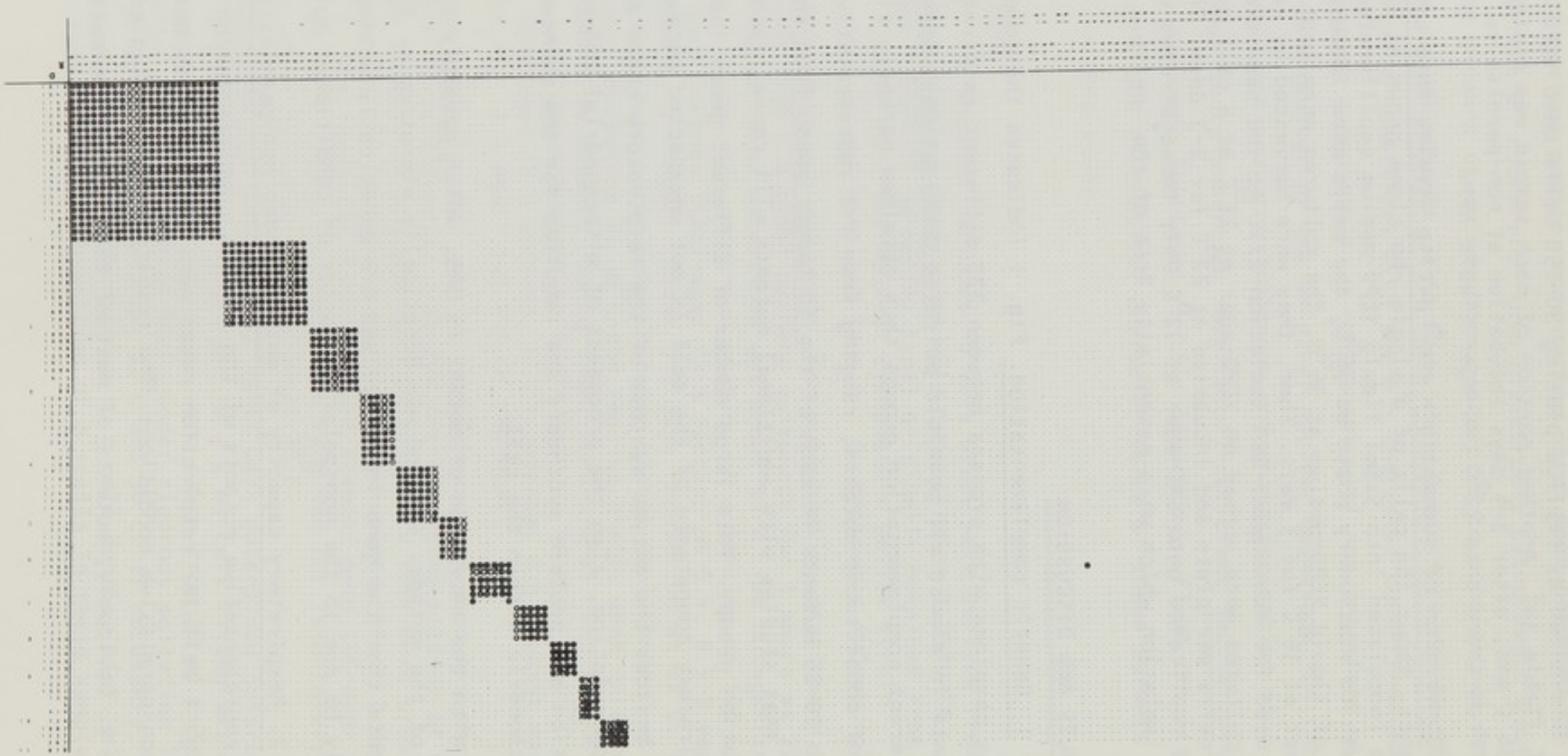
RESULTS AND DISCUSSION

1. Genetic complementation. Fig. 1 indicates the complementation patterns obtained with crosses between 270 different respiratory deficient mutants in almost all possible pairwise combinations. Apparently, the 106 different complementation groups thus obtained markedly differ in the number of constituent mutants, ranging from 1-2 representatives in most groups up to 10-25 mutants in each of the 10 larger ones. From this it seems likely that studies with additional mutants will reveal even more complementation groups. This large number of different genes involved in the respiratory competence of the cell is not unexpected because it is obvious that besides the enzyme systems of the respiratory chain and of oxidative phosphorylation, also the processes of mitochondrial DNA-replication, transcription and protein synthesis are required for the maintenance of functional mitochondria within the cell.

With the exception of mutant ts 545, which possibly is a double-mutant, none of the mutants studied was genetically pleiotropic. This may either indicate that the gene loci involved are genetically unlinked or it may simply be due to the non-polar character of conditional mutants, in general.

2. Respiratory capacity of mutants under non-permissive conditions. With representative mutants of the various complementation groups contained in Fig. 1 cellular respiration rates were determined at both, permissive and non-permissive conditions. The results of these studies are shown in Table 1. Obviously, mutants of most of the 66 complementation groups studied

Fig. 1. Complementation pattern of petite ts-mutants. Mutants of opposite mating type were crossed on YEPS at 22°C and after incubation at 22°C for 2 days, replicated onto LAC-agar at 36°C. After 5 - 7 days incubation at 36°C growth on lactate was examined. ● = no growth; o = weak growth; x = not determined; • = good growth. Mutants are indicated by both, allele and complementation group numbers.



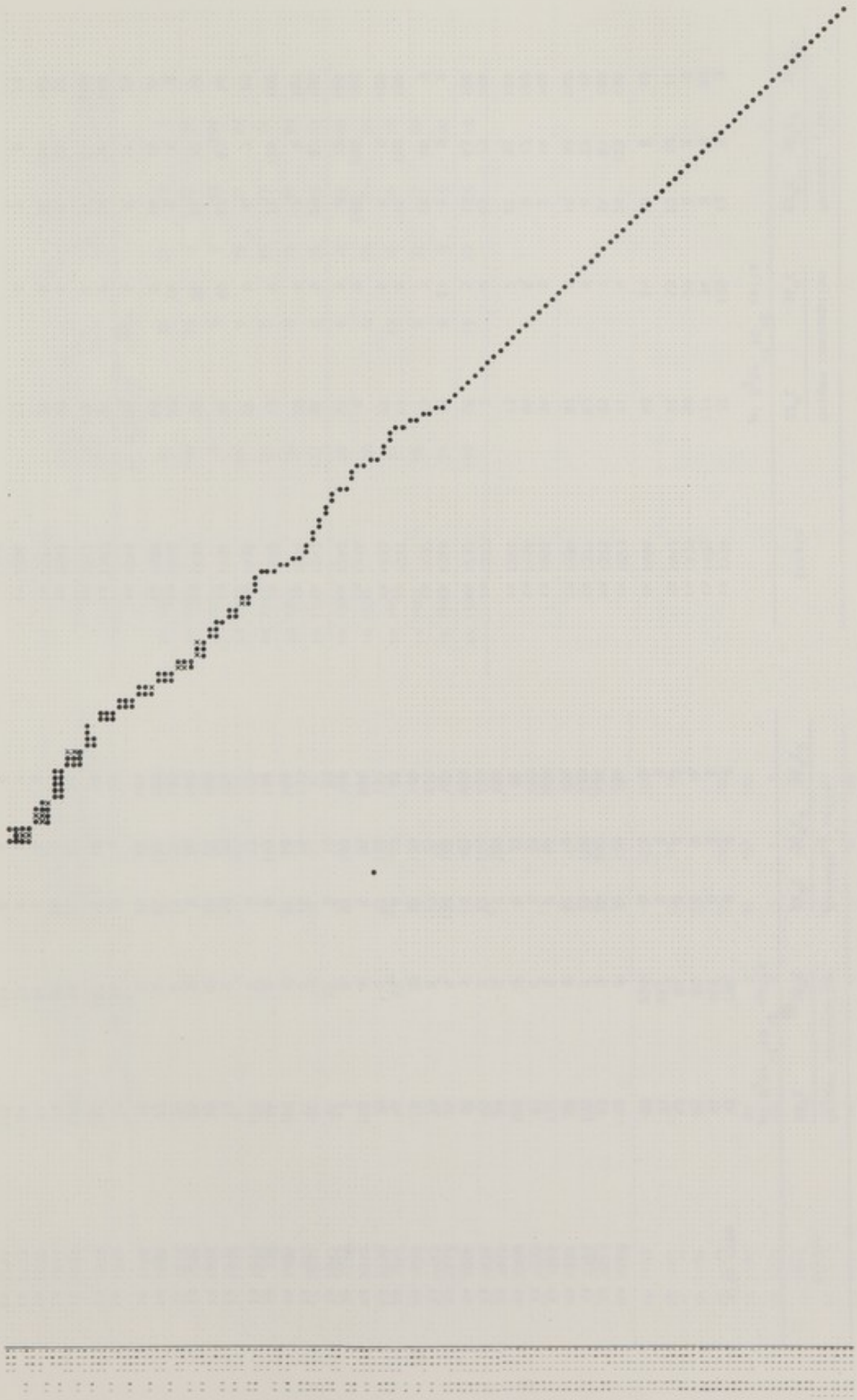


Table 1. Respiratory capacity of conditional petite mutants at permissive and non-permissive temperatures. The percentage of ρ^- cells was determined after growth at 36°C in liquid culture (36°C[a]) and after an additional cultivation on YEPD-agar at 36°C (36°C[b]). Experimental details were as described in Materials and Methods. Different complementation groups are separated by dotted lines.

Strain	Oxygen consumption		Percent ρ^- cells		
	22°C	36°C	22°C	36°C[a]	36°C[b]
	$\mu l\ O_2 hr^{-1} 10^{-8}$ cells				
Wild type	45	196	0	0	0
" "	55	163	0	0	0
" "	65	81	0	0	0
" "	40	81	0	0	0
" "	43	263	0	0	0
" "	70	230	0	0	0
ts 1528	13	0	77	100	100
ts 1513	43	0	26	100	100
ts 1286	158	0	20	100	100
ts 1201	58	0	12	0	100
ts 1266	132	0	0	46	100
ts 1335	83	0	0	58	100
ts 1363	109	0	0	70	82
ts 1420	54	0	2	100	100
ts 1425	40	0	22	100	100
ts 1016	58	3	0	33	100
ts 1418	14	0	100	100	100
ts 1147	79	0	17	25	100
ts 1547	4	0	97	100	100
ts 1047	41	15	6	10	88
ts 1199	39	0	27	100	100
ts 1311	115	0	5	85	100
ts 1526	5	0	93	100	100
ts 1047	41	15	6	10	88
ts 1583	31	0	16	16	100
ts 1185	105	0	21	10	100
ts 198	64	0	8	100	100
ts 1003	120	0	0	17	100
ts 1098	13	5	99	99	100
ts 1350	29	0	20	24	100
ts 1094	85	9	0	76	100
ts 1414	25	0	15	54	100
ts 1596	27	0	66	100	100
ts 1146	35	0	25	48	100
ts 1202	67	34	0	0	10
ts 1529	35	107	5	10	0
ts 1495	100	39	22	27	28
ts 1011	72	95	20	0	3
ts 1100	81	86	5	5	0
ts 1119	64	57	0	0	0
ts 1244	105	144	0	0	0
ts 1134	72	106	13	5	9
ts 1189	177	74	0	0	20
ts 1439	90	27	0	2	0
ts 1447	22	53	22	10	11
ts 1466	40	54	10	6	10
ts 1023	11	4	70	100	100
ts 1325	25	1	34	81	100
ts 1371	87	0	0	27	15
ts 1396	83	0	18	92	100
ts 1055	34	0	2	14	100
ts 1156	66	8	0	17	100
ts 1260	13	2	22	93	100
ts 1087	2	0	69	79	100
ts 1237	58	5	10	63	100
ts 1360	58	21	0	0	0
ts 1597	15	1	20	26	9
ts 1511	11	0	10	100	100
ts 1033	72	0	4	0	100
ts 1116	0	0	100	100	100
ts 1242	32	9	0	72	100
ts 1042	96	0	29	87	100
ts 1594	28	0	7	4	100
ts 1503	13	0	27	100	100
ts 1438	22	0	6	7	29
ts 1150	44	40	67	63	46
ts 1120	35	88	21	37	18
ts 1416	27	14	5	5	8
ts 1588	63	0	14	15	24
ts 1245	59	0	0	11	24
ts 1187	94	0	14	36	100
ts 1017	116	0	31	61	100
ts 1563	59	0	5	91	92
ts 1571	30	0	38	100	100
ts 1226	13	0	3	0	10
ts 1008	32	2	0	78	100

Table 1. (Continued)

Strain	Oxygen consumption		Percent ρ^- cells		
	22°C	36°C	22°C	36°C[a]	36°C[b]
	$\mu l\ O_2 hr^{-1} 10^{-8}$ cells				
ts 1022	10	7	41	12	20
ts 1048	60	64	10	10	7
ts 1578	22	0	21	99	100
ts 1180	47	0	n.d.	100	100
ts 1306	66	143	0	1	10
ts 1426	43	0	50	79	100
ts 1006	62	34	24	21	30
ts 1009	44	31	19	23	22
ts 1010	20	7	0	34	100
ts 1012	6	0	89	100	100
ts 1050	133	78	2	0	0
ts 1063	33	3	10	26	26
ts 1080	5	4	93	86	100
ts 1106	24	32	63	66	53
ts 1118	80	36	0	0	10
ts 1160	6	0	15	76	100
ts 1176	58	0	35	20	20
ts 1144	81	0	19	100	100
ts 1132	127	0	7	7	22
ts 1192	98	0	0	100	100
ts 1161	106	15	10	16	16
ts 1195	94	27	29	0	0
ts 1157	3	0	56	100	100
ts 1186	81	0	24	90	100
ts 1200	24	0	15	5	5
ts 1204	19	93	0	25	35
ts 1213	32	11	45	30	31
ts 1231	18	0	5	8	100
ts 1236	111	121	9	16	100

Strain	Oxygen consumption		Percent ρ^- cells		
	22°C	36°C	22°C	36°C[a]	36°C[b]
	$\mu l\ O_2 hr^{-1} 10^{-8}$ cells				
ts 1265	11	36	21	3	3
ts 1284	148	25	3	12	36
ts 1275	8	0	7	93	100
ts 1309	104	0	39	21	29
ts 1313	22	5	55	17	10
ts 1347	34	0	72	90	90
ts 1359	112	0	38	45	13
ts 1384	24	0	0	22	100
ts 1391	30	0	20	74	34
ts 1453	31	13	18	26	15
ts 1462	56	0	33	57	60
ts 1463	34	0	9	5	100
ts 1566	150	19	20	29	10

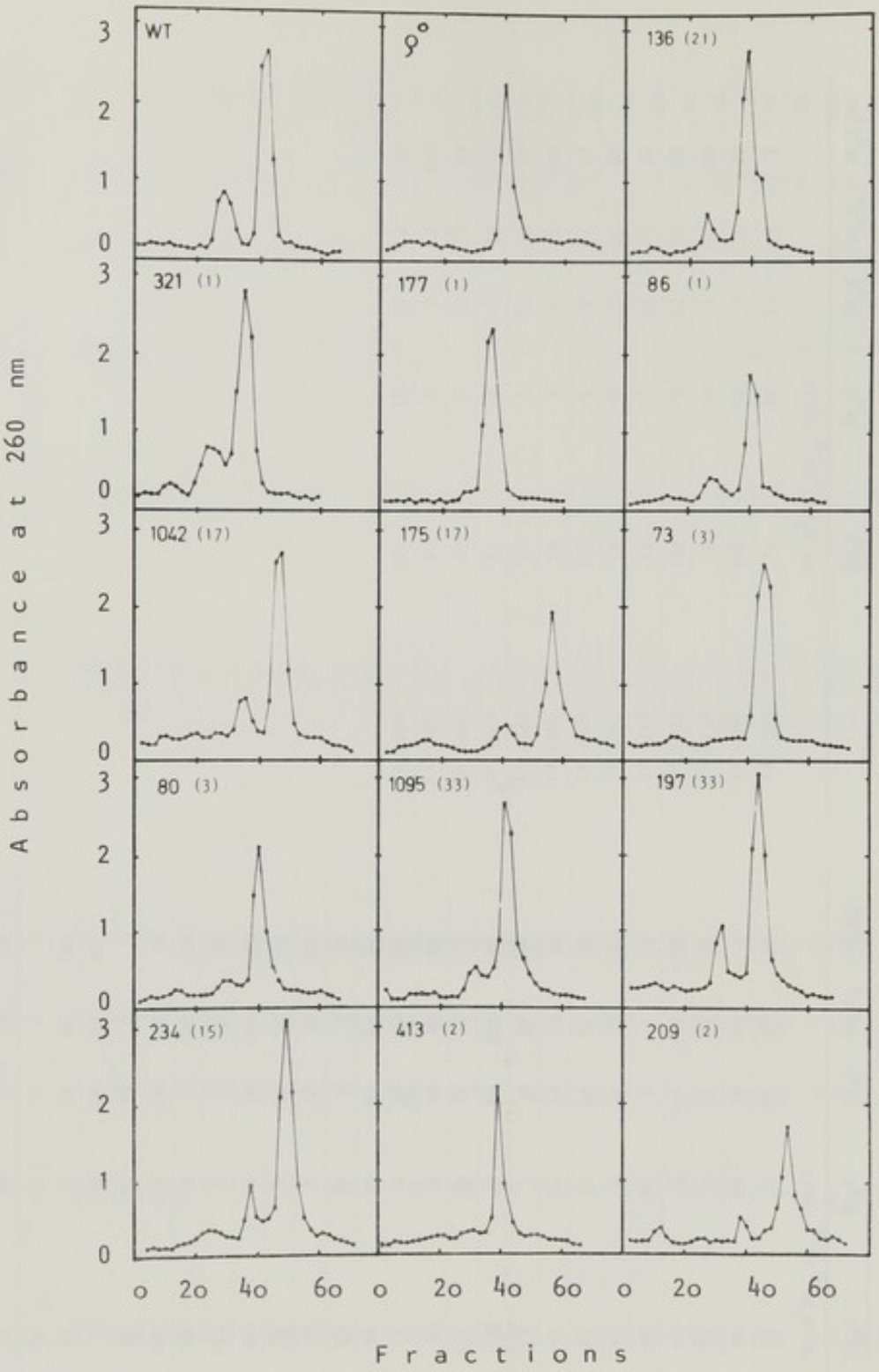


Fig. 2. Cs_2SO_4-density gradient profiles of total cellular DNA isolated from petite-ts mutants grown at non-permissive temperature. The mutants were grown at 36°C to early stationary phase in 5 liters of YEPD medium, each. DNA purification and subsequent CsCl- and $Cs_2SO_4/HgCl_2$-density gradient centrifugations were performed as described by Schweizer et al. (7) WT=Wild type. Complementation groups are indicated in brackets.

exhibit wild type-like respiration at 22°C, but fail to respire at 36°C. The few exceptions exhibiting no or weak respiration at 22°C are mostly cytoplasmic petites originated from rho^+-cells even under permissive conditions. About 16 groups, however, consist of mutants exhibiting CN^--sensitive respiration even under non-permissive conditions. Nevertheless, these latter mutants are unable to grow on non-fermentable substrates like lactate or glycerol. These characteristics might suggest them to represent uncoupled or phosphorylation-deficient mutants. ATPase and P/O ratio determination studies are in progress to confirm this idea further. As it also evident from Table 1, the characteristics studied are generally consistent within each group.

3. Conversion of conditional respiratory-deficient mutants into rho^--cells. Many of the conditional respiration-deficient mutants isolated undergo rapid spontaneous transition into cytoplasmic rho^--mutants when allowed to divide for several generations at non-permissive temperature. This new character manifests itself by the loss of the ability to grow on lactate at 22°C. The proportion of rho^+ to rho^--cells becomes less than 5 percent with the mutants of about 15 of the 106 complementation groups characterized in this study (G.Burkl, unpublished results). In Fig. 2, it is shown that apparently most of these "secondary" rho^--mutants still contain mitochondrial DNA. The relative amount of it, however, is clearly reduced compared to wild type cells. Thus, the original nuclear mutation apparently introduced a defect into the mitochondrial DNA rather than it lead to its complete disappearence. The molecular nature of this defect remains to be clarified.

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Recombination of Yeast Mitochondrial DNA Segments Conferring Resistance either to Paromomycin or to Chloramphenicol

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In a previous paper we have used cytoplasmic petite mutants of Saccharomyces cerevisiae which have retained either the mitochondrial chloramphenicol resistance C^R or the closely linked erythromycin resistance E^R (Michaelis et al., 1973). These petites were crossed with each other and stable C^RE^R recombinants have been found in two of seven crosses. Such a fusion of DNA segments might be restricted to closely linked regions only or as an alternative it might be possible to fuse quite a variety of mitochondrial DNA segments.

In order to understand more about this fusion process we have crossed 6 petites carrying the mitochondrial paromomycin resistance marker only (P^RC^O; isolated from strain KL14-4A) with 9 petites (isolated from strain IL8-8C) carrying the chloramphenicol resistance marker (C^RE^O) or the chloramphenicol and erythromycin resistance markers (C^RE^R). The mitochondrial C^R and E^R loci are closely linked whereas the P^R and C^R loci are known to be unlinked as demonstrated in two and three factor crosses (Kleese et

al., 1972; Wolf et al., 1973). The random diploid petites obtained from the 54 crosses mentioned above have been analyzed for their mitochondrial genotype by a marker-rescue in triploid crosses.

Each triploid colony results from the fusion of one diploid ϱ^- and one haploid ϱ^+ tester cell, which is sensitive to the antibiotics. Since the haploid cell is $P^S C^S E^S$, the diploid ϱ^- parent contributes entirely the resistance markers :

- if this cell is $P^O C^O$ the entire ϱ^+ descendance will be $P^S C^S$ and the colony will be scored as sensitive.
- if this cell is $P^R C^O$ (or $P^O C^R$) some ϱ^+ triploid cells will be $P^R C^S$ (or $P^S C^R$) and the colony will be scored as $P^R C^S$ (or $P^S C^R$).
- if recombination between P^R and C^R segments has occurred in the diploid parent, the triploid colony will contain $P^R C^R$ cells and be scored as $P^R C^R$ growing on the mixture of both antibiotics. Theoretically such $P^R C^R$ triploid cells might also originate from nonsegregated petite cells, containing a mixture of the two parental segments without recombination. Such heteroplasmic cells in yeast have been shown to be extremely unstable and to segregate into the two homoplasmic cell lines. Our conditions of crossing petites include many cell divisions of the 2n petites and therefore heteroplasmic cells should occur with so low frequencies not interfering with the test.

In table 1 we have listed for each cross the number of triploid colonies tested, the number of triploid ϱ^+ colonies and the percentage of triploid ϱ^+ colonies. Only the triploid

			$C^{R}E^{O}$				$C^{R}E^{R}$				
			C 42	D 41	D 61	F 91	D 21	F 11	F 21	R 53	F 41
$P^{R}C^{O}$	E 131	N	1172	1375	942	1159	1155	1074	1261	1128	1008
		$♀^{+}$	324	389	258	265	300	283	276	293	213
		$\%♀^{+}$	28	28	27	23	26	26	22	26	21
	E 121	N	1729	1375	1803	2281	555	1434	1600	564	502
		$♀^{+}$	446	257	449	403	96	277	337	135	103
		$\%♀^{+}$	26	19	25	18	17	19	21	24	21
	A 311	N	752	617	761	382	401	1332	1403	389	661
		$♀^{+}$	579	443	646	261	253	311	323	259	198
		$\%♀^{+}$	77	72	85	68	63	23	23	67	30
	A 211	N	1031	1307	695	1324	1173	1524	1567	1314	4290
		$♀^{+}$	372	370	286	387	225	279	346	303	526
		$\%♀^{+}$	36	28	41	29	19	18	22	23	12
	E 112	N	1123	1463	1042	1299	1272	1185	1005	1375	1169
		$♀^{+}$	389	342	454	471	362	273	223	443	281
		$\%♀^{+}$	35	23	44	36	28	23	22	32	24
	F 343	N	3369	1822	2314	1697	1626	1448	1822	1008	1527
		$♀^{+}$	1746	739	994	845	689	623	848	470	578
		$\%♀^{+}$	52	41	43	50	42	43	47	47	38

Table 1. Number of triploid colonies tested. The total number of 3n colonies (N), the number of 3n $♀^{+}$ colonies ($♀^{+}$) and the percentage of 3n $♀^{+}$ colonies are given for each cross.

φ^+ colonies can be used for analysis of the mitochondrial genotype.

The fusion of a P^R and a C^R DNA segment by recombination is visualized by the occurrence of recombinant triploid P^RC^R colonies. The number (R) and percentage of these P^RC^R colonies, found (f) in each cross, is given in table 2. It is shown that recombination occurs in many crosses and up to 85% of the triploid φ^+ colonies can be recombinants. As in general about 200 triploid φ^+ colonies were analyzed, only recombination values higher than about 1% are detected in our experiments. Assuming that the P^R and C^R DNA segments recombine frequently and recombination is not a rate limiting step at all, the transmission of the P^R and C^R markers into the diploid petite progeny might determine the maximum number of recombinants. This value R_c (maximum number of P^RC^R colonies calculated), which is the product of % colonies growing on paramomycin and % colonies growing on chloramphenicol, is included in table 2 for comparison. Recombination is frequent in all the cases in which R_f and R_c are similar, and the number of recombinants is roughly determined by the transmission frequencies of the two parental markers. In one cross (A311 x F91) the C^R marker is not transmitted at all. No recombinants can be expected and none are found in such a case, which might be due to an unstable C^R petite mutant. No conclusions can be drawn from this cross. Recombination does definitively not occur between two petite DNA segments, if both parental markers are transmitted to the progeny, a large number of recombinants can be calculated from the transmission values but no colonies are found (see for example cross A211 x D41). Our recombination results are summarized in table 3. A plus (+) indicates that recombination occurs

			$C^R E^O$								$C^R E^R$							
			C 42		D 41		D 61		F 91		D 21		F 11		F 21		R 53		F 41	
			R	%	R	%	R	%	R	%	R	%	R	%	R	%	R	%	R	%
$P^R C^O$	E 131	f	0	0	0	0	3	1	0	0	3	1	15	5	14	5	6	2	3	1
		c	46	14	8	2	25	10	4	1	40	13	39	14	38	14	26	9	22	10
	E 121	f	28	6	3	1	37	8	6	1	13	14	42	15	76	23	23	17	14	14
		c	32	7	5	2	37	8	6	2	15	15	47	17	82	24	25	19	16	15
	A 311	f	286	49	117	26	549	85	0	0	112	44	220	71	203	63	165	64	112	57
		c	271	47	124	28	556	86	0	0	133	53	249	80	204	63	177	68	112	57
	A 211	f	3	1	0	0	2	1	0	0	8	4	3	1	2	1	22	7	3	1
		c	76	20	58	16	50	18	35	9	50	22	61	22	30	9	58	19	82	16
	E 112	f	139	36	46	13	315	69	46	10	98	27	128	47	116	52	199	45	127	45
		c	118	30	34	10	291	64	57	12	97	27	137	50	118	53	186	42	140	50
	F 343	f	274	16	34	5	584	59	12	1	28	4	223	36	217	26	179	38	253	44
		c	361	21	39	5	572	58	38	4	37	5	254	41	270	32	201	43	312	54

Table 2. Number and percentage of $P^R C^R$ recombinants. For each cross the number (R) and percentage of recombinants experimentally found (f) are compared with the number of recombinants calculated (c) from the transmission frequencies of the P^R and C^R markers.

and at least 3 recombinant colonies have been found in this cross.

The interpretation of our results is based on two assumptions. 1. Cytoplasmic petite mutants contain a small amplified segment of wild type mitochondrial DNA which does not carry additional deletions. 2. Sequence homology between such segments of mitochondrial DNA is a prerequisite for mitochondrial recombination in petites. Each type of DNA segment was represented by several petite mutants in order to reduce the possibility of a bias caused by secondary deletions.

We have shown in this work that P^RC^R recombinants are frequently found in $\varsigma^- P^R \times \varsigma^- C^R$ crosses. Furthermore the P^RC^O segments can be fused equally well with segments carrying the C^R marker or the two closely linked C^R and E^R markers. This point is emphasized by the fact that we have found 2484 P^RC^R colonies within the 24 $P^RC^O \times C^RE^O$ crosses and have calculated a maximum of 2843 colonies. The ratio of recombinants found (R_f) to recombinants calculated (R_c) is $2484/2843 = 0{,}87$. A very similar value is obtained for the 30 $P^RC^O \times C^RE^R$ crosses: $R_f = 2627$; $R_c = 3258$; $R_f/R_c = 0{,}81$. Such a result is compatible with an arrangement of the P^R segment to the left and not to the right of the C^RE^R region in wild type mitochondrial DNA : $P^RC^RE^R$.

Currently the petite x petite crosses are extended to segments of mitochondrial DNA conferring resistance to erythromycin only and to oligomycin. These studies will demonstrate whether recombination between series of cytoplasmic petite mutants can be used for deletion mapping of mitochondrial markers in Saccharomyces cerevisiae.

		$C^{R}E^{O}$				$C^{R}E^{R}$				
		C 42	D 41	D 61	F 91	D 21	F 11	F 21	R 53	F 41
$P^{R}C^{O}$	E 131			+		+	+	+	+	+
	E 121	+	+	+	+	+	+	+	+	+
	A 311	+	+	+		+	+	+	+	+
	A 211	+				+	+		+	+
	E 112	+	+	+	+	+	+	+	+	+
	F 343	+	+	+	+	+	+	+	+	+

Table 3. Occurrence of recombination. A plus (+) indicates that recombination occurs and at least three $P^{R}C^{R}$ recombinant colonies have been found in the respective cross.

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Mapping of Transfer and Ribosomal RNA Genes of Yeast Mitochondria

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Gene mapping is presently an urgent task in the elucidation of functional organization of the yeast mitochondrial genome. Only a limited number of mitochondrial mutations have been used until now in the genetic study of yeast mitochondria. Most of the mutations used as genetic markers are widely scattered over the mitochondrial genome and their positions could not be ordered by recombination frequency analysis.

Our previous studies (Faye et al., 1973, 1974; Casey et al., 1974a; Fukuhara et al., 1974; Michel et al., 1974; Lazowska et al., 1974; Cohen et al., 1972) on several established petite clones suggested that large continuous segments are lost in all rho$^-$ mtDNA, though the analysis was not detailed enough to establish if there are multiple deletions of relatively small size in addition to the large ones. A precise knowledge of the deletion required more genetic markers and also, ideally, physically quantifiable gene sequences.

The deletions in petites can be a good tool for the mapping of mitochondrial genes. The approach is simple in theory if the deletion corresponds to the loss of a single continuous segment of the mtDNA. A pair of closely located genes should be retained or lost together more frequently than a pair of distant genes during the deletional events.

In several studies of deletions in petites (Faye et al., 1975 a, b; Fukuhara et al., 1975; Morimoto et al., 1975), we have examined the association of five drug resistance markers, eleven transfer RNA (tRNA) genes and 23S

and 16S ribosomal RNA (rRNA) genes in a large series of stable petite clones. From those studies, we chose three related topics to discuss: (i) mapping of mitochondrial genes by petite deletions and its limitation, (ii) presence of mitochondrial isoaccepting tRNAs with distinct genes and (iii) modified genetic behavior of genes in petites.

Mitochondrial tRNA and rRNA genes in petites of various genotypes.

From the grande strains labeled with four genetic markers (C^R, E^R, P^R, and O_I^R or O_{II}^R)*, several series of petite clones were isolated which represented various combinations of the markers. All of the clones have been checked for clonal purity (cf. Fukuhara et al., 1974) before extraction of their mtDNA. Although these clones have been selected for their genetic stability (spontaneous loss of markers was between 0.1 and 1% per cell generation), it was occasionally necessary to repurify the stocks for the designated genotype. Since the preparation of mitochondrial nucleic acids required a large number of cell divisions, a small fraction of the nucleic acids may come from the cells which have spontaneously lost some genes. Therefore, the possibility of minor heterogeneity must be considered in the interpretation of results.

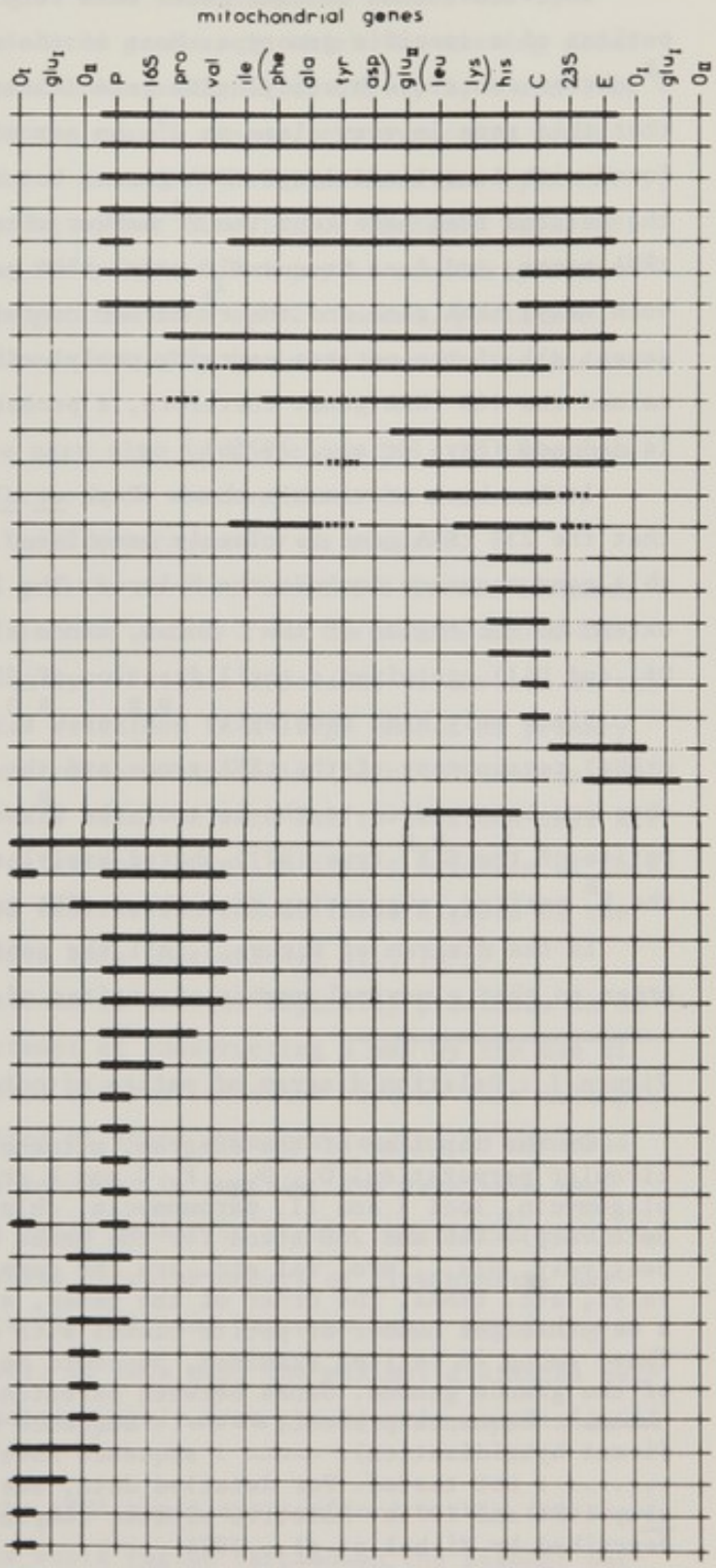
Mitochondrial DNA was isolated from each of the mutants and hybridized with eleven individual ^{3}H-aminoacyl tRNA and the two ^{3}H-rRNA, 23S and 16S, isolated from the purified grande mitochondria. The absence of significant hybridization was interpreted as a physical loss of the sequence complementary to these RNA. The results of the hybridization experiments are summarized in figure 1 (detailed data and experimental procedures are to be found in Fukuhara et al., 1976, Faye et al., 1974 and Faye et al., 1975a, b).

It is apparent that, in general, the petites carrying the C^R or P^R marker often retained some tRNA genes and rRNA genes, while the petites carrying only the O_I^R or O_{II}^R or both rarely retained these genes except the glutamyl $tRNA_I$ gene (see next section). Four petites which have kept C^R, E^R and P^R markers together contained all the eleven tRNA genes and both of the two rRNA genes.

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* C^R, E^R, etc. stand for the antibiotic resistance form of the locus, and C^S, E^S, etc. for the sensitivity form; C^O, E^O, etc. mean deletion (see Fukuhara et al., 1974).

Petite clone	Short name	Relative kinetic complexity $e^+=1$
MH41-7B/R12	CEP 1	0.50
MH41 7B/AB83	CEP 2	0.54
TR3-15A/F857	CEP 6	0.57
TR3-15A/F5262	CEP 7	0.50
MH32-12D/J4762	CEP 4	0.36
MH41-7B/AC219	CEP 3	(0.26)a
TR3-15A /F218	CEP 5	(0.21)a
IL8-8C/D21	D21	0.36 b
IL8-8C/D21/H141	C 1	0.11
IL8-8C/D41	D41	0.13 b
IL8-8C/F11	F 11	0.24 b
IL8-8C/R53	R53	0.13 b
IL8-8C/C42	C42	0.06 b
IL8-8C/D61	D61	0.06 b
IL8-8C/F11H421	C 2	0.05
IL8-8C/F21B13	C 3	0.06
TR3-15A/A115H112	C 4	0.06
MH41-7B/G42	C 6	0.05
IL8-8C/F21A15	C 5	0.03
MH41-7B/AA12	C 7	0.05
IL8-8C/F12	F 12	0.05 b
MH41-7B/AB25	EO_I 1	–
IL8-8C/F13	F 13	0.002 b
MH41-7B/L721	O_IP 2	0.67
MH41-7B/L661	O_IP 1	– a
MH41-7B/P31	P 3	(0.14)a
MH41-7B/P11	P 1	0.12
TR3-15A/F857P27	P 27	–
MH41-7B/L721Q1171	Q1171	–
MH41-7B/R12P22	P22	–
MH41-7B/P21	P 2	0.09
MH41-7B/AB83P25	P25	–
MH32-12D/L434	P 5	0.06
MH32-12D/H383	P 6	–
MH32-12D/C25	P 7	0.04
MH32-12D/H241	P 4	0.06
MH32-12D/C144	$O_{II}P$ 1	0.12
MH32-12D/T917	$O_{II}P$ 2	0.16
MH41-7B/L721Q3122	Q3122	–
MH32-12D/K416	O_{II} 1	0.07
MH32-12D/Y41	O_{II} 2	0.11
MH32-12D/K385	O_{II} 3	0.02
MH41-7B/C516	O_IO_{II} 1	–
MH41-7B/B82	O_I 3	0.14
MH41-7B/M92	O_I 1	0.06
MH41-7B/E91	O_I 2	0.07



Individual tRNA or rRNA genes were very frequently associated to the petites of a specific genotype. Most of the petites that have retained the C^R marker contained histidyl tRNA gene (sixteen out of twenty), suggesting that this gene is very close to C^R. An association of C^R marker was also found with leucyl and lysyl tRNA genes, but to a lesser extent. In contrast, the petites that have kept the P^R marker often contained 16S rRNA and prolyl tRNA genes, and less frequently valyl tRNA gene. All of the petites carrying both valyl tRNA gene and the P^R marker contained 16S rRNA and prolyl tRNA genes; all of the petites carrying prolyl tRNA genes and the P^R marker contained the 16S rRNA gene. Therefore, a probable order, P^R - 16S - pro - val is deduced (Faye et al., 1975b).

It has been previously shown (Faye et al., 1974; Nagley et al., 1974) that the 23S rRNA gene is closely associated to the E^R marker, and probably this gene sequence includesthe E locus. The 23S rRNA gene sequence appears to extend to the region of the C locus, since all C^RE^O clones examined (C42, D61 and D41) contained a small fraction of 23S rRNA gene sequence.

Since only high complexity C^RE^R or C^RE^O petites (reassociation rates of mtDNA) retain many of the tRNA genes and the petite EO_I-1 lacked most of them, they are best placed to the left of the C^R marker. Also, a high complexity petite of the C^RE^R type (D21) shared prolyl and valyl tRNA genes with some of the P^R petites, suggesting that these tRNA genes are between C^R and P^R.

In the diagram of Figure 1, all the genes were arranged in a circular order so that a maximal number of petites of any genotype represent a mtDNA

Figure 1 : Deletion diagram of petite mitochondrial DNAs.

On the top line of the diagram, mitochondrial genes are indicated in a circular permutation. O_I, O_{II}, P, C and E are genetic markers (resistance to oligomycin, loci I and II, paromomycin, chloramphenicol and erythromycin, respectively). 16S and 23S stand for the genes coding for 16S and 23S rRNA respectively. Glu_{II}, pro, val etc. are the genes coding for glutamyl, prolyl, valyl, etc. tRNAs. The order of the genes, as indicated, was chosen in such a way that the number of petite clones with double deletions is minimized. Thus, among 46 petites examined, 38 could be represented by a single deletion of the grande genome. Genes between parentheses may be ordered differently. ▬▬ : sequence present; ▬ ▬ ▬ : sequence probably present (low but significant hybridization); ——— : sequence absent (negligible hybridization); ..... : not tested. For detailed data, see Fukuhara et al., 1976, Faye et al., 1974 and 1975b. Relative kinetic complexity of mtRNA was determined as described by Michel et al., 1974

a. Wetmur-Davidson plots of DNA association showed a marked skewness.

b. From Michel et al., 1974

segment without internal interruptions. From this diagram, clones CEP-3, 4 and 5 appear to contain internally deleted genomes in which the missing genes form a continuous segment. In the gene order assigned to CEP-1, 2, 6 and 7 is assumed to reflect the gene order in the grande genome, the structure found in CEP-3, 4 and 5 may be generated by a secondary deletion from the type CEP-1, 2, 6 and 7 whose DNA are presumed to be either circular or tandemly repeated. The interrupted sequence assigned to the petite O_IP-1 may be similarly derived by a secondary deletion from the petites like o_IP-2. Indeed, petitesof the former type ($O_I^R P^R O_{II}^O C^O E^O$) have been actually isolated from the petites of the latter type ($O_I^R P^R O_{II}^S C^O E^O$)

An internal interruption can be seen also in the clone D61. In this case, with only one marker, it is difficult to know whether there is a secondary deletion or, as is more probable, clonal heterogeneity is present.

The circular order of the five genetic markers indicated in the diagram, i.e, $O_I - O_{II} - P - C - E - O_I$, was derived solely from the petite mtDNA-RNA. hybridization experiments. Independently of this approach, we arrived at the same scheme by the analysis of marker retention in a large number of primary petite clones generated by ethidium bromide mutagenesis (Bolotin-Fukuhara and Fukuhara, in preparation). In that case also, we were led to conclude that all petites cannot arise from a single deletional event. A direct evidence of this has been obtained by the analysis of restriction endonuclease digests of the mtDNA from some of the petites studied here (Morimoto et al., 1975).

The presence of secondary deletions in petites logically precludes an unequivocal ordering of genes. Our attempt at constructing a map by the use of petites is thus limited. The presented results indicated however that multiple deletions and/or extensive reshuffling of short segments leading to numerous combinations of different genes are uncommon in petites. The data show that the combinations of markers and RNA genes appear in highly restricted patterns in petite clones. Among twelve possible circular orders of five genetic markers, that proposed in Figure 1 fits the RNA gene patterns best, requiring a minimal number of secondary deletions. In this map, the maximal distances between some of the genes may be estimated roughly from the kinetic complexity of petite mtDNAs.

In the proposed map, most of the tRNA genes are confined within the segment between the two rRNA genes. The whole region carried on the segment between C^R and P^R markers represents at most 50% of the total grande genome of

50 million daltons. The distant separation of the two rRNA genes is a unique situation as compared to other known chromosomes in which the two rRNA genes are located in tandem. In Hela mtDNA, the two rRNA genes are also located close to each other, but are separated by just one 4S RNA gene (Wu et al.,1972).

Because of the limitations discussed above in the mapping procedure using petites, it is obvious that other independent approaches are necessary to construct a detailed unequivocal map of the yeast mitochondrial genome. A promising approach is the use of the numerous "mit minus" mutations (Tzagoloff et al., 1975 a, b) for producing a more refined map.

Mitochondrial isoaccepting tRNA species for glutamic acid.

In the diagram of Figure 1, we placed two glutamyl tRNA genes at two distinct loci. This was first based on the observation that the glutamyl tRNA isolated from the purified rho^+ mitochondria hybridized to two very different classes of petite mtDNA: one of $C^R E^R P^R$ type, another of O_I^R type. Since the O_I locus is completely unlinked by recombination to C, E and P loci (Avner et al., 1973), there are two major alternatives: (i) there are two separate genes for glutamyl tRNA or (ii) there is only one but this gene (or the region carrying it) may attach to other regions through rho^- mutation. If the second possibility occurs frequently, the use of petite mutants in mapping would be hopeless. Fortunately the first hypothesis was shown to be correct in the following way. The glutamyl tRNA which hybridized to, for example, CEP-2 mtDNA was eluted off the hybrids and re-hybridized to O_I-3 mtDNA. No significant hybridization was obtained, while a homologous rehybridization formed hybrids. A reciprocal experiment showed similarly the absence of cross-hybridization. Thus it was predicted that the rho^+ mtDNA codes for two isoaccepting glutamyl tRNA of distinct sequences, originating from two separately located genes. A direct demonstration came from the finding that the grande mitochondria contained two major species of glutamyl tRNA which could be separated by chromatography (Figure 2). Both of them hybridized to the grande mtDNA. The species eluted first hybridized only to the mtDNA of O_I-3 clone and not to the mtDNA of CEP-2. The second species hybridized exclusively to the mtDNA of CEP-2 (Figure 3). Competition and additivity experiments confirmed these results.

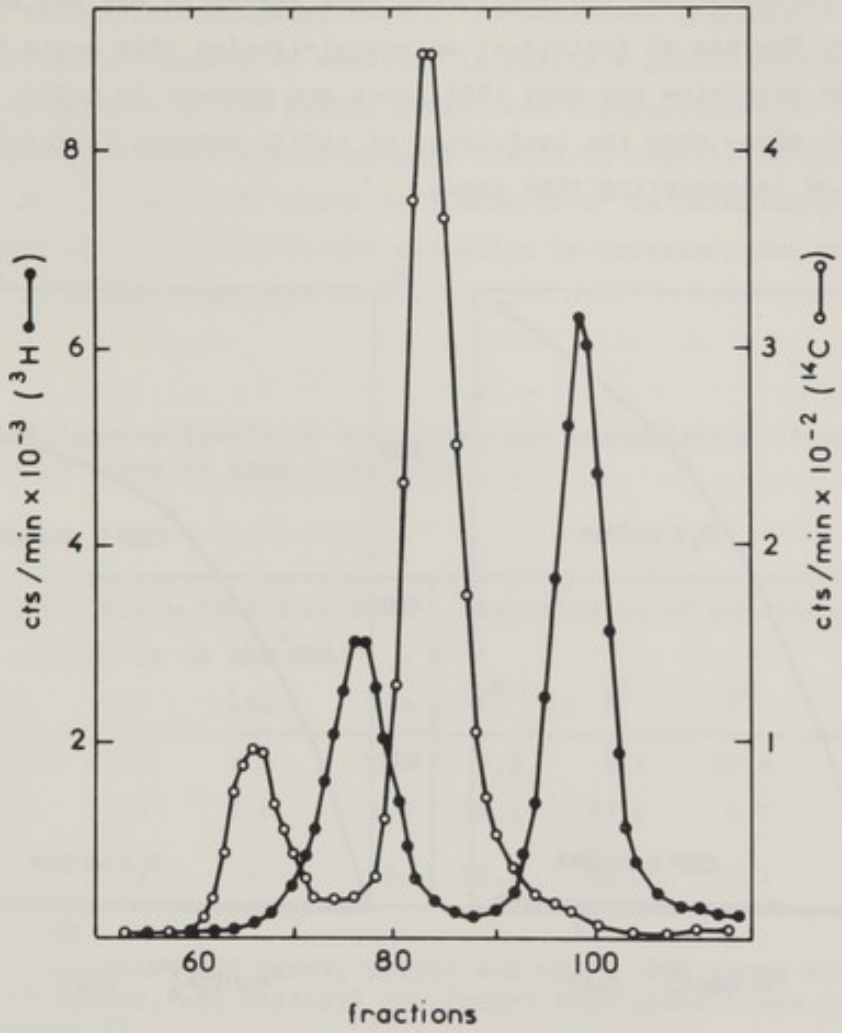


Figure 2 : Two glutamyl tRNA species from the grande mitochondria.

Mitochondrial ^{3}H-glutamyl tRNA from purified grande mitochondria (●—●) was co-chromatographed on a RPC-5 column with cytoplasmic ^{14}C-glutamyl tRNA (o—o) isolated from the supernatant fraction of a rho^{o} mutant. The mitochondrial tRNA was acylated by mitochondrial enzyme and the cytoplasmic tRNA by cytoplasmic enzyme. Details in Martin et al., 1976.

These experiments provided the first straightforward evidence for mitochondrially coded, heterogeneric, isoaccepting tRNAs (Martin et al., 1976). It may be mentioned that multiple genes appear to exist for some of the other tRNA as well (Martin and Rabinowitz, unpublished). Some of the anomalies in the deletion diagram may be due to multiple tRNA genes.

Although in yeast (S. carlsbergensis and S. cerevisiae) there appear to be a sufficient number of genes to code for at least one tRNA for each amino acid (20-25 genes; Reijnders and Borst, 1972; Schneller et al., 1975), there do not seem to be enough genes to provide for the recognition of all synonym codons. It is however possible that the estimates of the number of tRNA genes on mtDNA are too low. When ^{32}P-labeled bulk mitochondrial 4S RNA preparations

are used for hybridization, the total tRNA gene sequences may not be titrated quantitatively. The use of individual aminoacyl-labeled tRNA would be a more reliable way to determine how many tRNA genes are present in mtDNA. The example of the glutamyl tRNAs show the usefulness of petite mutants in detecting separate location of isoaccepting tRNA genes.

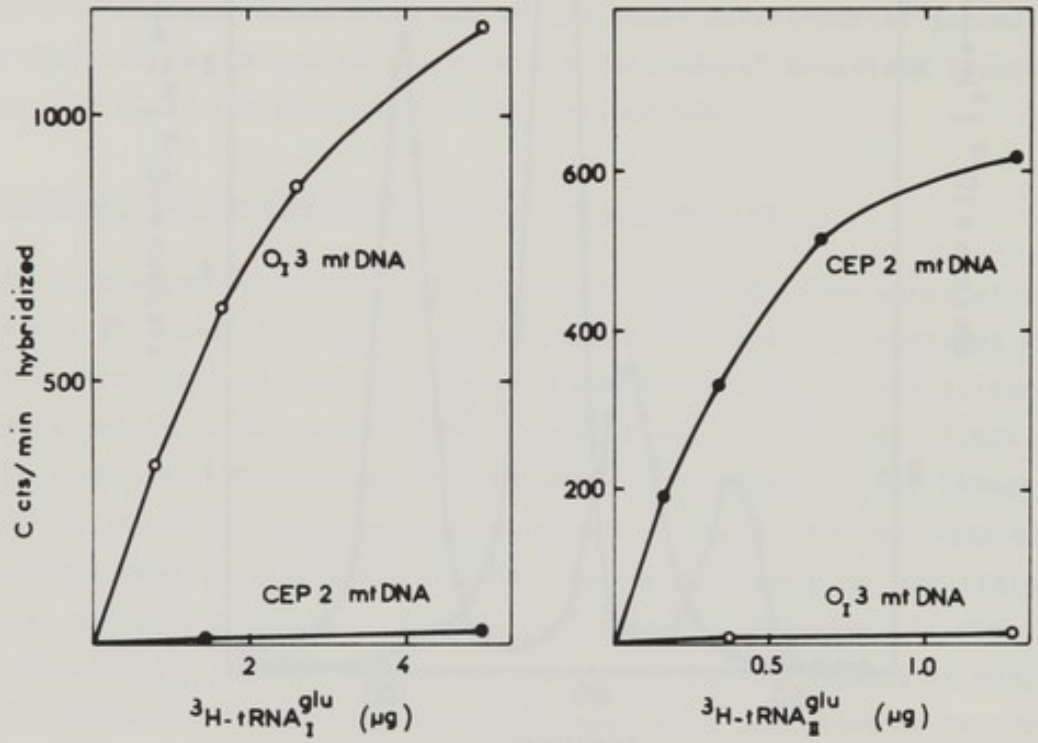


Figure 3 : Hybridization of mitochondrial $tRNA_I^{glu}$ and $tRNA_{II}^{glu}$ to petite mtDNAs.

Increasing amounts of mitochondrial ^{3}H-glutamyl $tRNA_I^{glu}$ (spec. act. 901 cts/min/µg) or ^{3}H-glutamyl $tRNA_I^{glu}$ (spec. act. 8800 cts/min/µg) were hybridized with filters charged with 20µg of O_I-3 (○—○) or CEP-2 (●—●) mtDNAs. See details in Martin et al., 1976.

Modified behavior of genes in petites and the problem of sequence repetitions.

The genetic markers retained in petites have a number of genetic properties which are different from those in grandes.

In grande x grande crosses (non-polar, or homosexual crosses, cf. Bolotin et al., 1971), all genetic markers of the parents are transmitted with similar

frequency to the diploid progeny. Such a "co-ordinated transmission" (Dujon et al., 1974) was not generally observed in petite x grande crosses: the different genetic markers of the petite parent are transmitted with different frequencies to the diploid grande progeny (Table 1, right). In CEP-1, the transmission of C^R (and of E^R which is linked to C^R in recombination) is much lower than that of P^R. In CEP-2, the situation is inversed, the transmission of C^R and E^R is higher than that of P^R.

Table 1: Hybridization levels of some tRNAs and transmission of markers located close to them in petites.

Petite clones or mtDNA	Hybridization levels of tRNA (relative to rho$^+$DNA)*				Transmission of genetic markers (%)**			
	pro	val	leu	his	C^R	E^R	P^R	colonies counted
CEP-1	1.3	1.2	1.2	1.1	7.2	8.7	27.9	319
CEP-2	1.8	1.7	1.6	1.5	14.1	23.5	4.7	192
MH41-7B (rho$^+$)	1	1	1	1	55.7	62.3	56.1	271

* With respect to other tRNA genes, prolyl and valyl tRNA genes are located close to the P^R marker, and histidyl and leucyl tRNA genes close to the C^R marker (see Figure 1).
** Petites were crossed to a grande drug sensitive tester strain (DP1-1B, ω^+) by the standard random cross technique (Coen et al., 1970). Diploids obtained were plated and the frequency of each marker was determined among the grande progeny. MH41-7B is the grande strain from which the petites were isolated.

One of the possible explanations of the uncoordinated transmission of genes in petites is the hypothesis of selective repetitions of some sequences in petite mtDNA. Some of the petites which contain many tRNA genes offer a possibility of examining whether such local repetitions exist.

When we determine the saturation hybridization levels of a tRNA on the petite mtDNAs that have retained the corresponding gene, the hybridization levels (per unit weight of mtDNA) are, in most cases, higher than the level observed with the rho$^+$ mtDNA. This can be explained at least in part by the decreased sequence complexity of petite mtDNA, due to deletion. An additional

possibility is that some specific sequences may be more repeated than others in petite mtDNAs, as we have suggested earlier (Faye et al., 1973; Fukuhara et al.,1974; Casey et al., 1974b; Cohen et al., 1972). If a tRNA gene is much more repeated than others within the same petite, the hybridization level, relative to that with rho$^+$ mtDNA, of that tRNA should be higher than the levels of other tRNAs. Table 1, left part, shows the hybridization levels of two petites, CEP-1 and 2. Clearly, the difference of hybridization levels is rather small between tRNAs, suggesting that these mtDNAs do not contain, measurably amplified regions.

According to Figure 1, histidyl and leucyl tRNA genes are close to the C^R marker, and prolyl and valyl close to the P^R marker. Since none of these tRNA regions appear to be selectively amplified, as mentioned above, it is difficult to explain the differential transmission by a difference of sequence repetition.

Table 2 : Differential loss of genetic markers from petites.

Petite clone	Genotype of subclones obtained after mutagenesis								
	$C^RE^RP^R$	$C^RE^RP^O$	$C^OE^OP^R$	$C^OE^OP^O$	O^RP^R	O^RP^O	O^OP^R	O^OP^O	colonies counted
CEP-1 ($C^RE^RP^R$)	20.4	0	72.1	7.5	-	-	-	-	226
CEP-2 ($C^RE^RP^R$)	79.4	0	15.5	5.2	-	-	-	-	271
O_IP-2 ($O_I^RP^R$)	-	-	-	-	4.3	0.9	71.3	23.5	115

The three petite strains were treated by ethidium bromide (20 µg/ml) for 4 hours at the exponential phase of growth in a glucose yeast extract complete medium. Cells were plated and individual clones were tested for the presence of each genetic marker by replica cross technique. Values represent the number (% of total) of clones for each indicated genotype. Very similar patterns were obtained when the petites were analyzed for spontaneous loss of markers in old stocks.

The petite mutants show also modified rates of loss of genetic markers. When a grande is mutagenized by ethidium bromide, various genetic markers are rescued in petite clones with approximately the same frequencies (Bolotin-Fukuhara and Fukuhara, in preparation). In contrast, in petites, some markers are much more frequently lost than others as shown in the Table 2. Such a phenomenon may also be ascribed to a differential repetition within the mtDNA sequence (cf. Faye et al., 1973), since the genes in many copies would have a better chance of survival than the genes with fewer copies. But again this hypothesis appears unsatisfactory: the P^R marker shows a strikingly high survival compared to other markers in these examples, despite the similar hybridization levels of the tRNA genes associated to these markers.

The hypothesis of local repetition of specific sequences within a petite mtDNA molecule remains to be demonstrated. More detailed molecular analyses are necessary to understand the anomalous genetic properties of genes in petites.

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Molecular Genetics of Yeast Mitochondria

G. Bernardi

I would like to report at this Symposium recent results on a number of problems concerning the mitochondrial genome of yeast, which have been studied using as major tools two restriction enzymes. A detailed presentation of these data will be given elsewhere (Prunell et al., 1976 ; Prunell and Bernardi,1976 ; Fonty et al., 1976).

As an introduction to the problems to be discussed here, it is appropriate to mention our earlier results on the physical organization of wild-type S.cerevisiae mDNA. The investigations we did along this line (Bernardi et al., 1970 ; Bernardi and Timasheff, 1970) immediately revealed that yeast mitochondrial DNA is characterized by two particular features.

The first one is that a number of properties, like buoyant density, T_m, $[\alpha]_{290}$, elution molarity from hydroxyapatite, silver binding, terminal nucleotides released by DNases are "anomalous" compared to what could be expected for a bacterial DNA having the same base composition. The origin of such "anomalies" does not reside in chemically modified nucleotides, which we had shown not to exist in yeast mitochondrial DNA (at least to a level high enough to account for the "anomalies"), but in the deviation of nucleotide sequences from randomness. In fact, all the properties under consideration are sequence-dependent. In bacterial DNAs, where short nucleotide sequences are essentially random (Josse et al., 1961), the sequence-dependence is not apparent because of the averaging out of the contributions from a very large number of random sequences ; for this reason the properties we are examining now have been usually considered as composition-dependent. In DNAs containing short repetitive sequences (both synthetic and natural), in contrast, the sequence-dependent is quite evident, as we have shown for mammalian satellite DNAs (Corneo et al., 1968). In other words the "anomalies" of yeast mitochondrial DNA point to the presence of short repetitive sequences in it.

* This paper was also presented at the Xth FEBS Meeting,Paris, July 1975.

The second characteristic feature is a striking compositional heterogeneity. In sharp contrast with repetitive DNAs which melt in an extremely cooperative way because of their high compositional homogeneity, yeast mitochondrial DNA exhibits a most complex melting pattern. The differential melting curve (fig. 1) is characterized by : 1) a gaussian component which represents about half of the DNA ; this must be extremely rich in A+T,

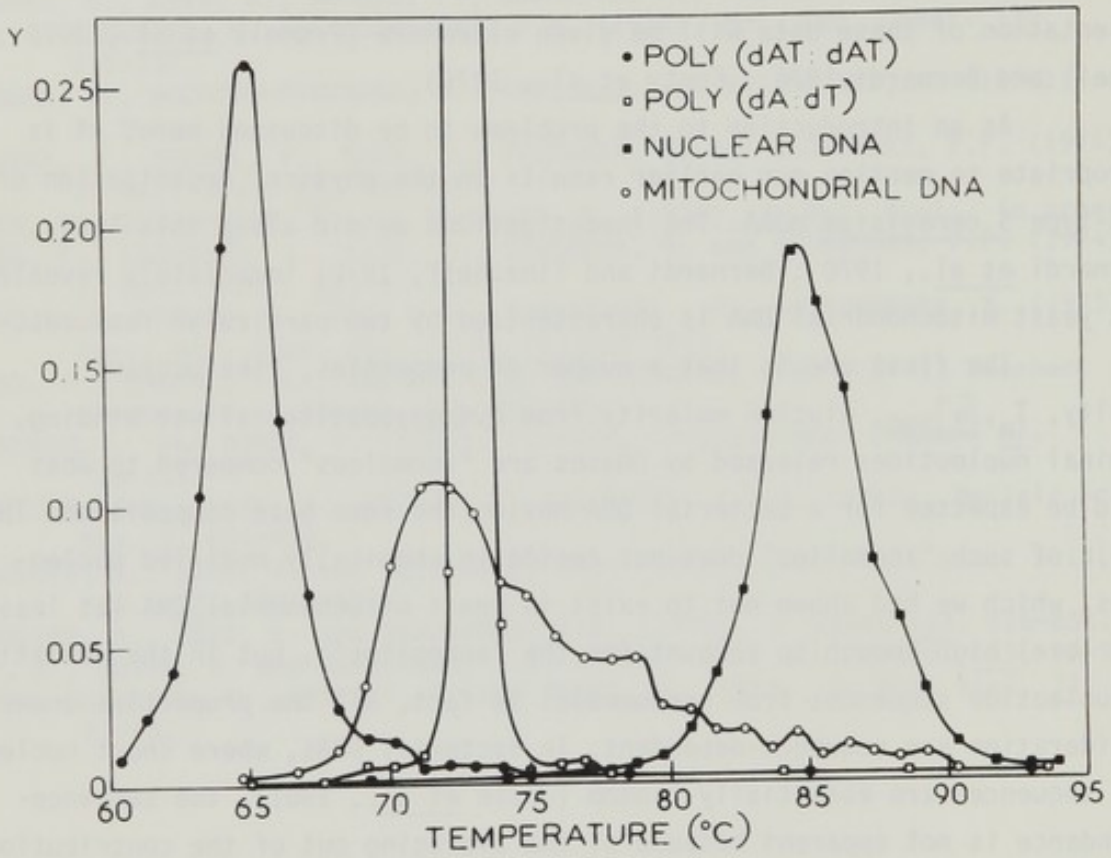


Fig. 1 Differential melting curves obtained with poly (dAT:dAT) (•), poly (dA:dT) (○), mitochondrial DNA from strain B (○), and nuclear DNA from strain B (•). The ordinate indicates the increment in relative absorbance per degree : $Y = \frac{At_1 - At_2}{A_{100} - A_{25}} / (t_1 - t_2)$, where At_1, At_2, A_{100}, A_{25} are absorbance measured at temperature t_1, t_2, 100°C and 25°C, respectively. The abscissa values are equal to $t_1 + t_2 / 2$. Y_{max} of poly (dA:dT) had a value of 0.71 (Bernardi et al., 1970).

because of its very low T_m, and must contain both alternating and non-alternating AT sequences, because of its circular dichroism spectrum; and 2) by a number of discrete melting components covering an extremely wide range of G+C levels. These two sorts of components are intimately interspersed with each other, as shown by the fact that mitochondrial DNA having a molecular wright as low as $1.2.10^6$ (versus the 50.10^6 size of the intact genome, see below) still show a unimodal, symmetrical band in CsCl density gradients (Bernardi et al., 1970).

At this stage of the game, it is inescapable to think of the mitochondrial genome of yeast in terms of a system in which very A+T-rich stretches are interspersed with stretches having higher levels of G+C (fig.2). Subsequent investigations in our laboratory aimed at checking the validity of the interspersion model and at better defining the organization of the mitochondrial genome of yeast. Three approaches were used : 1) degradation

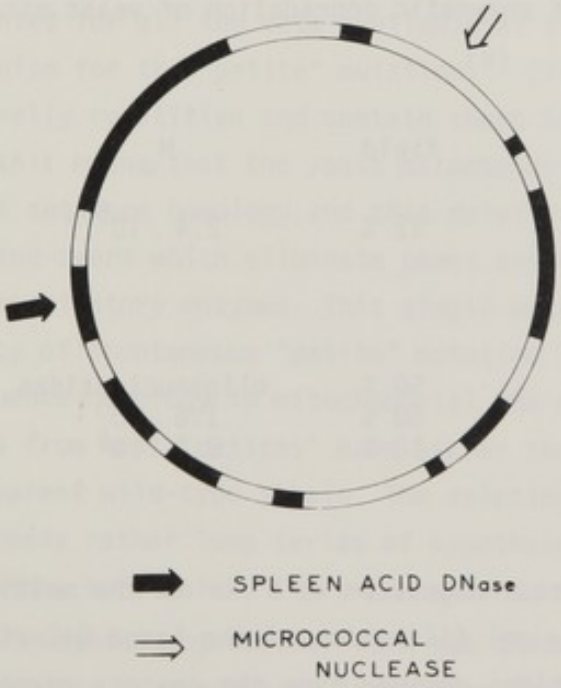


Fig. 2 A scheme of our working model for the organization of yeast mitochondrial DNA and of the experimental approaches used to test it. Black stretches correspond to the G+C-rich regions, white stretches to the A+T-rich region. Spleen acid DNase splits mitochondrial DNA with a slight preference for the G+C-rich regions ; micrococcal nuclease splits mitochondrial DNA with a very high specificity for the A+T-rich regions (Prunell and Bernardi, 1974).

of mitochondrial DNA with spleen acid DNase, an enzyme which preferentially breaks G+C-rich sequences ; 2) degradation with micrococcal nuclease, an enzyme which breaks A+T-rich sequences with a high selectivity, and 3) pyrimidine tract analysis.

Spleen acid DNase degradation combined with chromatography of the fragments on hydroxyapatite led to the preparation of short fragments as rich as 27 % in G+C (versus the 18 % of the total DNA) and particularly of fragments having a molecular weight of $0.24.10^6$ and a G+C content of only 10 % ; these were recovered in a yield of 12 % and shown to melt in a very narrow temperature range and to be responsible for the "anomalous" properties. These results (Bernardi et al., 1972 ; Piperno et al., 1972) provided the first direct evidence for the interspersion model (Table I).

Table I

Results of enzymatic degradation of yeast mitochondrial DNA

Spleen acid DNase	Yield	M_w	G + C
A+T-rich fragments isolated on HAP	12 %	$2.4 . 10^5$	10.4 %
Micrococcal nuclease			
Material derived from :			
A+T-rich segments	50 %	oligonucleotides	5 %
G+C-rich segments	50 %	$1.6 . 10^5$	32 %
	(10 %	$0.4 . 10^5$	65 %)

Micrococcal nuclease digestion at 6° or at the melting temperature combined with separation by gel filtration of the large G+C-rich fragments form the short oligonucleotides derived from the spacers revealed that spacers on one hand and genes with their regulatory elements on the other were present in equal amounts in mitochondrial DNA ; the G+C-level of the former was lower than 5 %, whereas the average G+C level of the latter was about 32 %. Very interestingly, fragments of 40,000 daltons as high as 65 % in G+C could be prepared in a yield of 10 % by this method. These data (Prunell and Bernardi, 1974) gave a quantitative estimation of the relative amounts of genes and spacers in the mitochondrial genomes and of their G+C levels (Table I).

The pyrimidine isostich analysis (Ehrlich et al., 1972) confirmed our previous conclusion (Bernardi and Timasheff, 1970) that the A+T-rich spacers are basically formed by intermingled short alternating and non-alternating AT sequences. Some isostich components, T_1, T_2 and T_3 are very frequent, since they appear in every 3, 8 and 25 base pairs, respectively. This suggests that short runs of 10 to 30 nucleotides having the same or a very similar base sequence may often exist in the A+T-rich spacers.

To summarize our results on the organization of mitochondrial genome of yeast the major conclusions arrived at were 1) the demonstration that this genome is an interspersed system of genes, with their regulatory elements, and spacers ; 2) the estimation of the amount (50 % each), the average length (over $1.5.10^6$) and the average G+C level (32 % for the genes, 5 % for the spacers) of these two elements ; 3) the indication that the A+T-rich spacers are formed by short repetitive sequences.

The interest of this interspersed gene and spacer model, is that it not only accounted for all the data available at that time, but also suggested a mechanism for the "petite" mutation[(a)] (fig. 3). In fact, if the spacers are internally repetitive and contain short identical or quasi-identical sequences, this means that the yeast mitochondrial genome has an extremely high level of sequence homology and that deletions can easily take place by internal crossing-overs which eliminate genes essential for the synthesis of mitochondrial respiratory enzymes. This simple model accounts for the very high frequency of spontaneous "petite" mutation in terms of the very high level of sequence homology in mitochondrial DNA and for the fact that mitochondrial DNAs from most "petites" have either the same or a lower G+C content than the parent wild-type strain. The deletion model was another addition to an already rather long series of hypotheses put forward to explain the "petite" mutation, but, unlike its predecessors, it was there to stay. The only modification brought in by further work was that deletions are accompanied by amplifications, a phenomenon indicating that the recombitional events underlying the "mutation" may involve the spacers of different genome units (see below).

(a) This model was first presented in a lecture at the Karolinska Institute, Stockholm, in October 1969. It was subsequently presented at a number of Meetings and mentioned by Piperno et al. (1972)and by Prunell and Bernardi (1974). A very similar model has been developed by Clark-Walker and Miklos (1974).

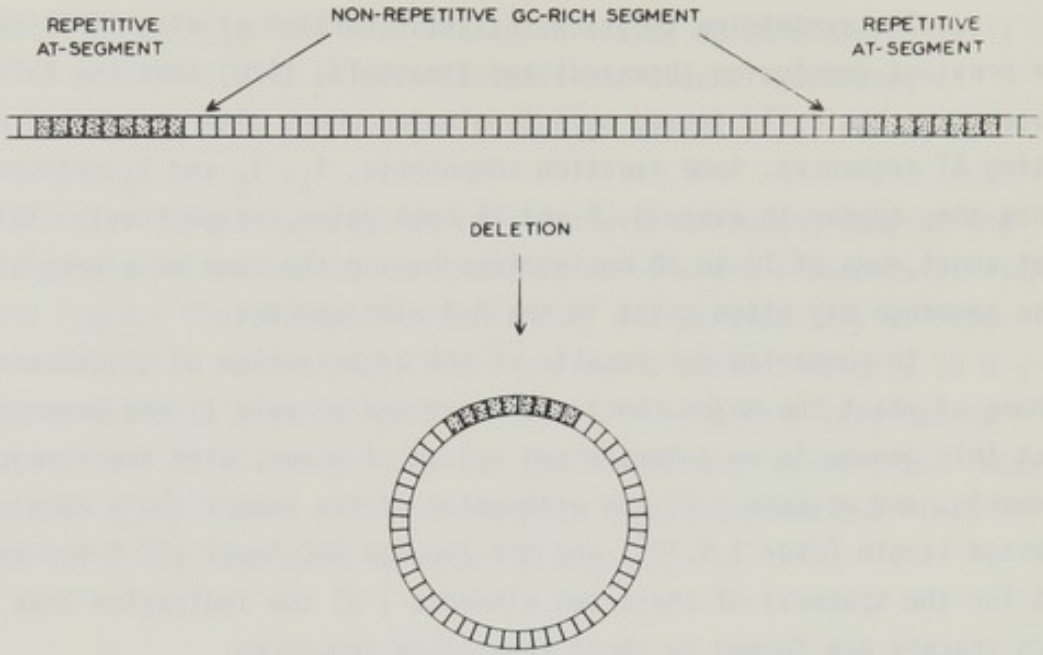


Fig. 3 The deletion model.

Our more recent investigations dealt with a number of problems such as the unit size, the homogeneity and the evolution of the mitochondrial genome of wild-type yeast. All these problems were essentially investigated by studying in detail the fragment patterns obtained with Hae III and Hpa II, two restriction enzymes, from the mitochondrial DNAs of one S.carlsbergensis (C) and three S.cerevisiae (A,B,D) strains. The sequences split by these enzymes are GGCC and CCGG, respectively.

a) Unit size and homogeneity of the genome

The number of fragments varied from 71 to 116 according to the DNA and the enzyme used. The molecular weight of fragments ranged from 4.10^6 to 10.10^3. A satisfactory separation of the fragments could only be achieved on a series of long slabs of 2 to 8 % polyacrylamide plus 0.5 % agarose. Plate I is given as an example of the fragment patterns obtained. The scheme corresponding to the 2 % gel is shown in fig. 4.

Since five gels of different porosity were used to resolve most of the fragments, the first problem was to identify corresponding bands, as

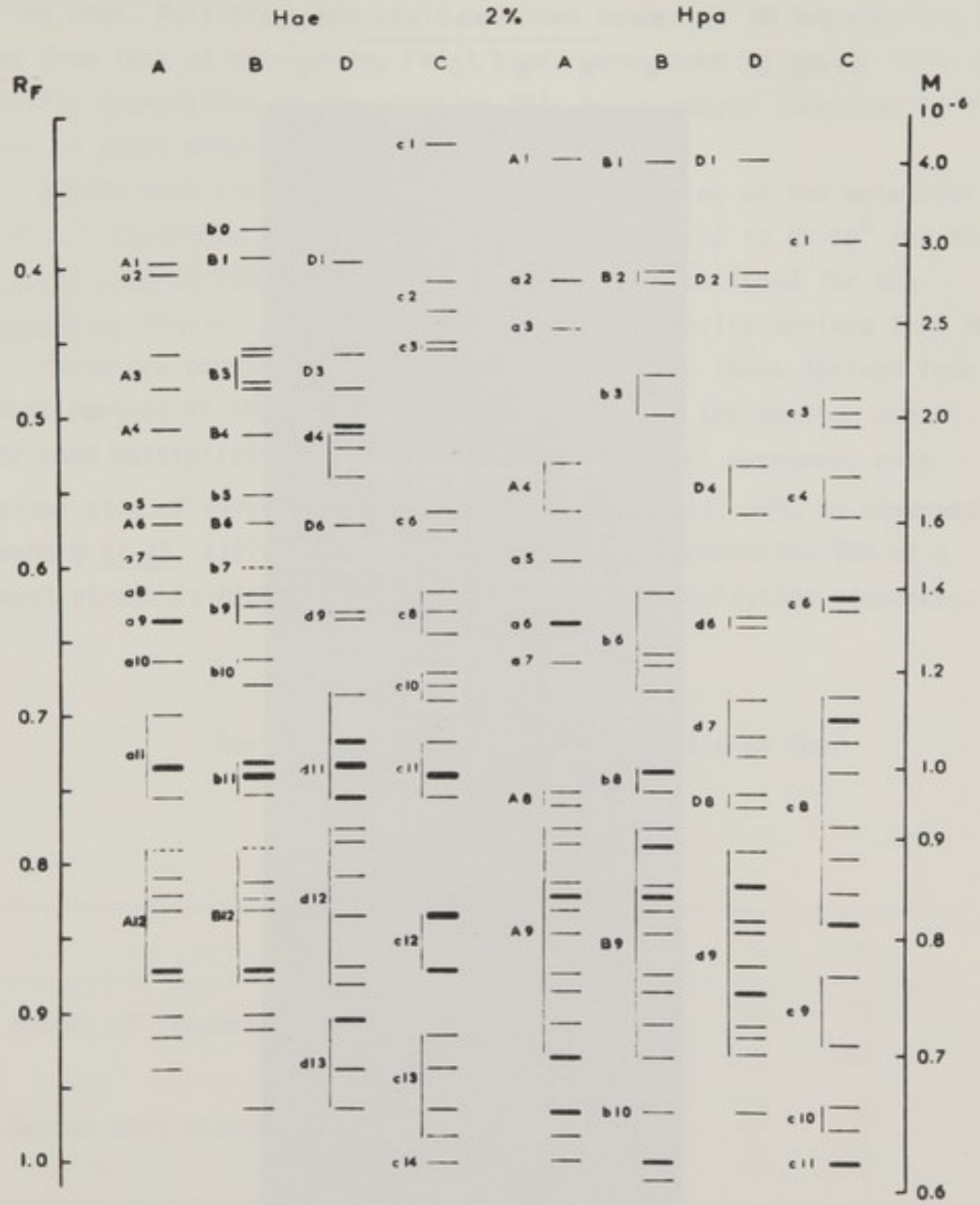


Fig. 4 Scheme of the band patterns obtained on the 2 % gel. The relative mobilities and molecular weights of the fragments are indicated. Bands containing one fragment, two fragments, and more than two (3 or 4) fragments are indicated by a different thickness; faint bands are indicated by dashed lines. Corresponding bands, or band clusters, as seen on different gels, are indicated with the same letter-number combination. Capital letters indicate bands exhibiting interstrain homology, low-case letters all other bands. The homology between B_2,$D2_2$ and a_2 of the Hpa pattern and between A_4,B_4 and d_{4_2} of the Hae pattern were not indicated to avoid crowding of the figure.

observed on different gels, in order to establish the necessary overlaps. This could be easily done on the basis of the molecular weights of the fragments and with the help of characteristic features in the band patterns, such as fragment clusters and multiple bands.

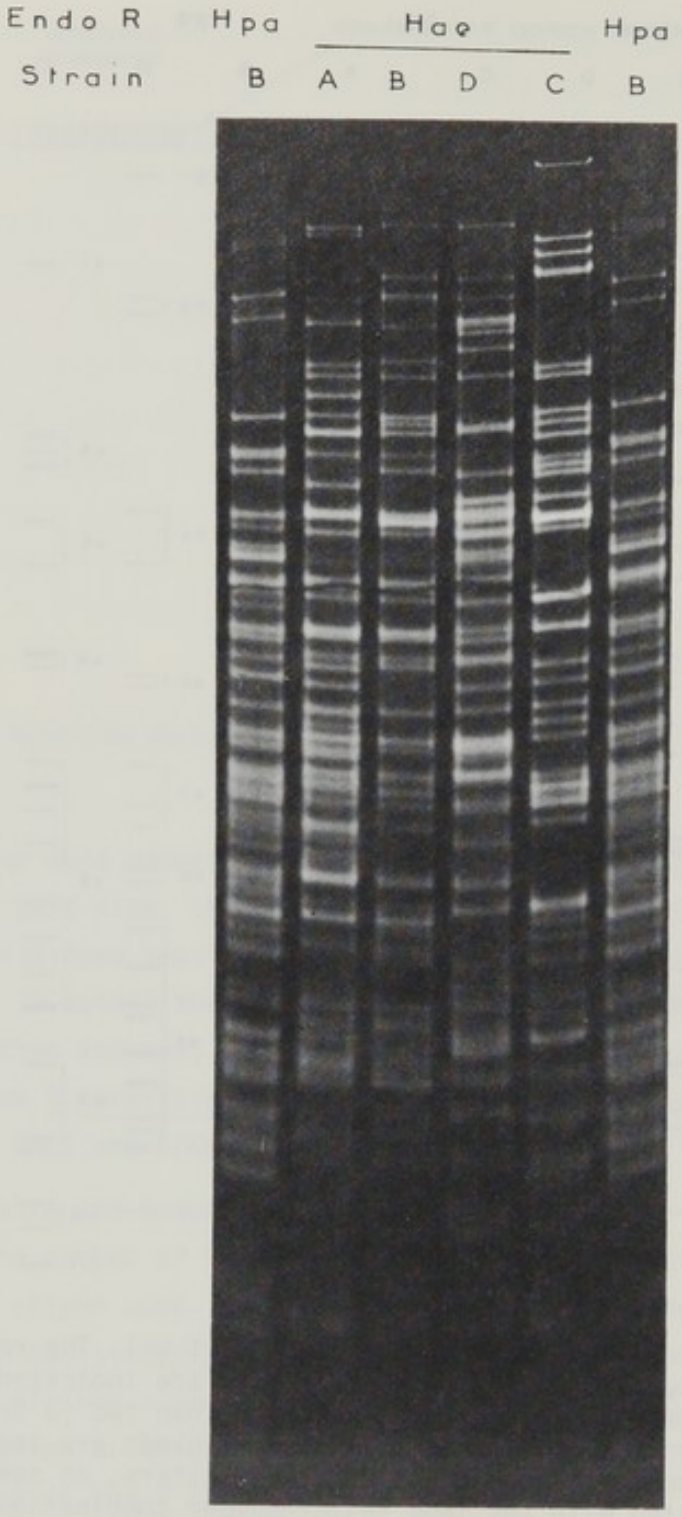


Plate I Electrophoresis patterns obtained on a 2 % polyacrylamide - 0.5 % agarose gel. The enzyme used and the DNA source are indicated.

neighboring ones. Multiple bands could be shown to derive in the majority of the cases from lack of resolution. Faint bands were shown to result from a very specific degradation of the starting DNAs by an enzyme localized in all likeliness in yeast mitochondria.

Genome unit sizes could be calculated by adding up the molecular weights of all fragments (Table II). These ranged from 52 to 55.10^6 for the three S.cerevisiae strains,whereas a value of 50.10^6 was found for the S.carlsbergensis strain. The quoted values refer to results derived from Hae digests ; these are considered to be more reliable than those derived from Hpa digests because of the smaller number of bands and the smaller contribution of band multiplicity. These values are in general agreement with the physical size of circular twisted yeast mitochondrial DNA, as observed by Hollenberg et al. (1970) and indicate that the mitochondrial DNA of a given yeast strain is highly homogeneous in terms of nucleotide sequences.

Table II

Restriction fragments and unit size of four yeast mitochondrial DNA

	Enzyme	A	B	D	C
Number of fragments	Hae	84	81	83	71
	Hpa	116	107	113	107
Genome unit size($\Sigma M_i.10^{-6}$)	Hae	52	55	52	50
	Hpa	55	52	52	49

b) Homology and divergence of yeast mitochondrial genomes

i. Sequence homology. Qualitative indications of sequence homology in the mitochondrial DNAs from all strains investigated come from the following findings concerning the fragments released by the two restriction enzymes used : 1) The approximately equal number of fragments and therefore of restriction sites (Table II) ; 2) the similar size distribution of the restriction fragments (fig. 5); 3) the similar base composition of fragments

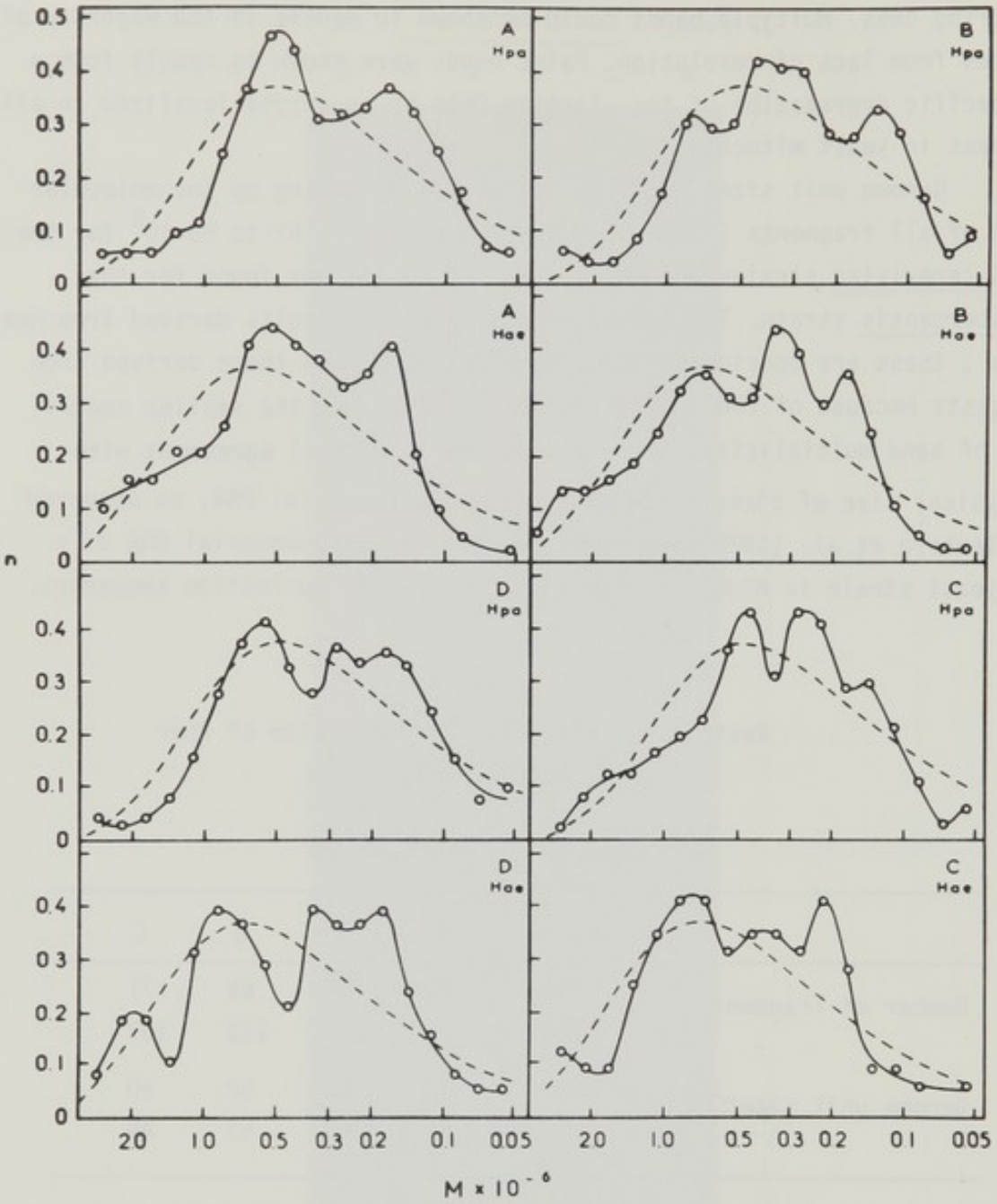


Fig. 5 Semilogarithmic plot of n versus M for the restriction enzyme fragments obtained from mitochondrial DNAs.

$$n = \frac{1}{N_0} \cdot \frac{\Delta n}{\Delta \log M},$$

in DNAs from strains A and C (Prunell and Bernardi, 1976) ; 4) the same bias in the number of Hpa versus Hae sites the former being largely predominant (Table II ; see further comments in Prunell and Bernardi, 1976). It should be noted that homology among the mitochondrial DNAs from S.cerevisiae strains is larger than between them and S.carlsbergensis as judged by all possible criteria : number of sites, fragment homology, M_w/M_n ratio of fragments.

All these findings point to a remarkable conservation of the genes and their regulatory elements in all the strains under consideration, if account is taken of the distribution and clustering of restriction sites (Prunell and Bernardi, 1976).

ii. Origin of differences in fragment patterns in the mitochondrial DNAs from different strains. Two, not mutually exclusive, possibilities exist : 1) mutations (point mutations, insertions or deletions) and/or modifications (methylation) at the sequences split by the restriction enzymes ; and/or 2) deletions and/or additions between such sites. It is of obvious interest to judge the relative importance of these mechanisms.

Since the number and the size distribution of fragments is very similar in different strains, the disappearance of "old" restriction sites should be accompanied by the formation (by point mutations, deletions, insertions, demethylation) of an approximately equal number of "new" restriction sites ; in addition, these should have a very similar distribution on the genome. This explanation clearly is so unlikely that it can definitely be ruled out as a mechanism counting for more than a very small percentage of the changes observed. Two additional problems with a point mutation mechanism arise from : 1) the clustering of restriction sites (Prunell and Bernardi 1976), which requires point mutations at several neighboring sites to cause changes in the patterns ; and 2) the inacceptably high divergence time mutation rate pf 10^{-7} per generation) between strains like S.carlsbergensis and S.cerevisiae, which show 100 % homology in their nuclear and mitochondrial genomes by DNA-DNA hybridization (Groot et al., 1975).

It is evident, therefore, that the predominant mechanism underlying the changes in restriction site distributions is one involving deletions and/or additions between such sites. This allows the number of sites to be conserved and the distribution of the restriction fragments to be affected much less than if the site number greatly changed. The deletion/addition mechanism is supported by two findings : 1) the differences in the mitochondrial genome unit sizes observed among S.cerevisiae strains and, more so, between S.cerevisiae and S.carlsbergensis (Table II); such differences are quite remarkable in view of the internal compensations between additions and deletions ; 2) deletions and additions very clearly underlie the cytoplasmic "petite" mutation (Bernardi et al., 1976 ; and paper in preparation) ; this suggests that the same mechanism is responsible for the "petite" mutation and for mutations in which the wild-type phenotype is preserved because gene functions essential for respiratory competence are not affected (Plate II).

iii. Localization of deletions and additions. Deletions and additions certainly affect essentially the A+T-rich spacers, which make up to 50 % of mitochondrial DNA (Prunell and Bernardi, 1974) and do not contain

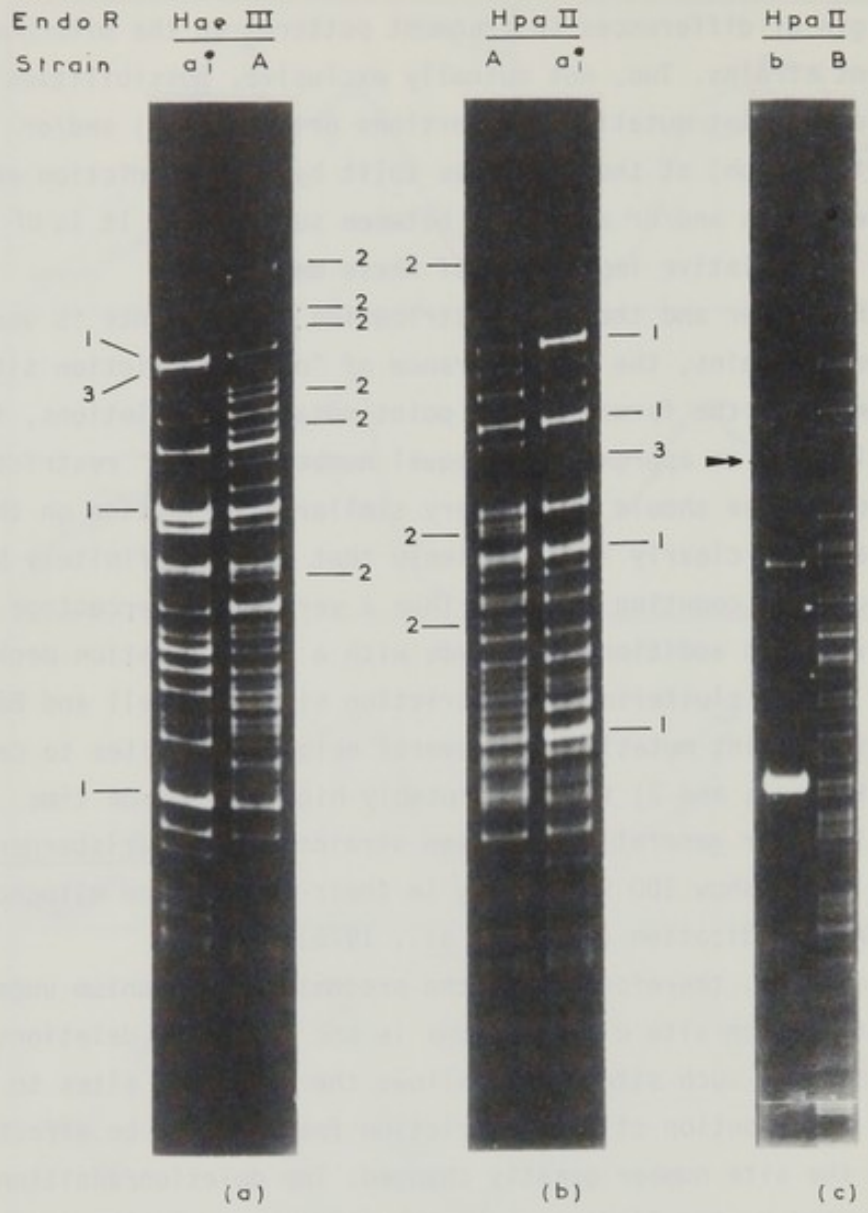


Plate II Electrophoretic patterns obtained on a 2 % polyacrylamide - 0.5 % agarose gel. The enzyme used and the DNA source are indicated. (a) and (b) : 1,2,3 indicate bands which are reinforced, missing or novel, respectively, compared to the parent wild-type. (c) : the arrow indicates a very faint band.

any restriction sites (Prunell and Bernardi, 1976). This explanation is the only one resolving the apparent conflict between : 1) the 100 % homology found by DNA-DNA hybridization of mitochondrial DNAs from S.cerevisiae and S.carlsbergensis, which is associated with an absence of mismatch in the hybrids as judged by their melting curves (Groot et al., 1975) and 2) the

almost complete absence of homologous restriction fragments found in the present work. In fact, base pairing in the DNA-DNA hybrids can be expected to take place first in the genes and their regulatory elements, because of their higher G+C level ; differences in length of the spacers due to deletions and/or additions are not very important since longer spacers could undergo looping out ; moreover some extent of base pairing by folback could take place in the loops, if palindromic sequences exist in the spacers. An additional observation pointing in the same direction is that fragment homology between S.cerevisiae and S.carlsbergensis was seen in the smallest fragments, which do not contain spacers (Prunell and Bernardi, 1976). Finally, it is interesting to note that the mitochondrial DNAs of four yeasts which in all likeliness do not contain A+T-rich spacers, since their physical genome size is comprised between 1/2 and 1/4 of that of Saccharomyces and their G+C content is in the 32-42 % versus the 17-18 % of Saccharomyces are "petite negative" (O'Connor et al., 1975).

iv. Mechanism underlying deletions and additions. The most likely basis for the deletion/addition mechanism is given by unequal crossing-overs events taking place in the spacers of different genome units. Such events can obviously lead to unequal exchanges of genome segments and therefore to changes in the location of restriction sites on the genome units which will eventually segregate. It is clear that such mechanism can lead to the formation of defective genomes and give rise to "petite" mutants as well. In other terms, we consider here recombination as the basic phenomenon underlying both mitochondrial genome evolution and the "petite" mutation, which is a frequent accident in this process. This proposal is simply an extension of the deletion model by internal recombination at the spacers previously proposed. It should be mentioned that a very strong evidence in favor of this mechanism is given by the changes in restriction fragment patterns which we have demonstrated in diploids issued from zygotes of strains A and B (Fonty et al., 1976). The Hpa band patterns (fig. 6) of 6 such diploids (1.1, 2.1, 4.1, 5.1, 8.1, 9.1) were characterized by bands identical to those of both parents and by a number of new bands, and by genome unit sizes differing by only a few percent from those of the parents. An important feature of the recombination events so observed for the first time is their very high rate, since the pattern changes were observed after as few as 20 generations. Very interestingly, three (3.1, 6.1, 7.1) diploids showed banding patterns identical to that of

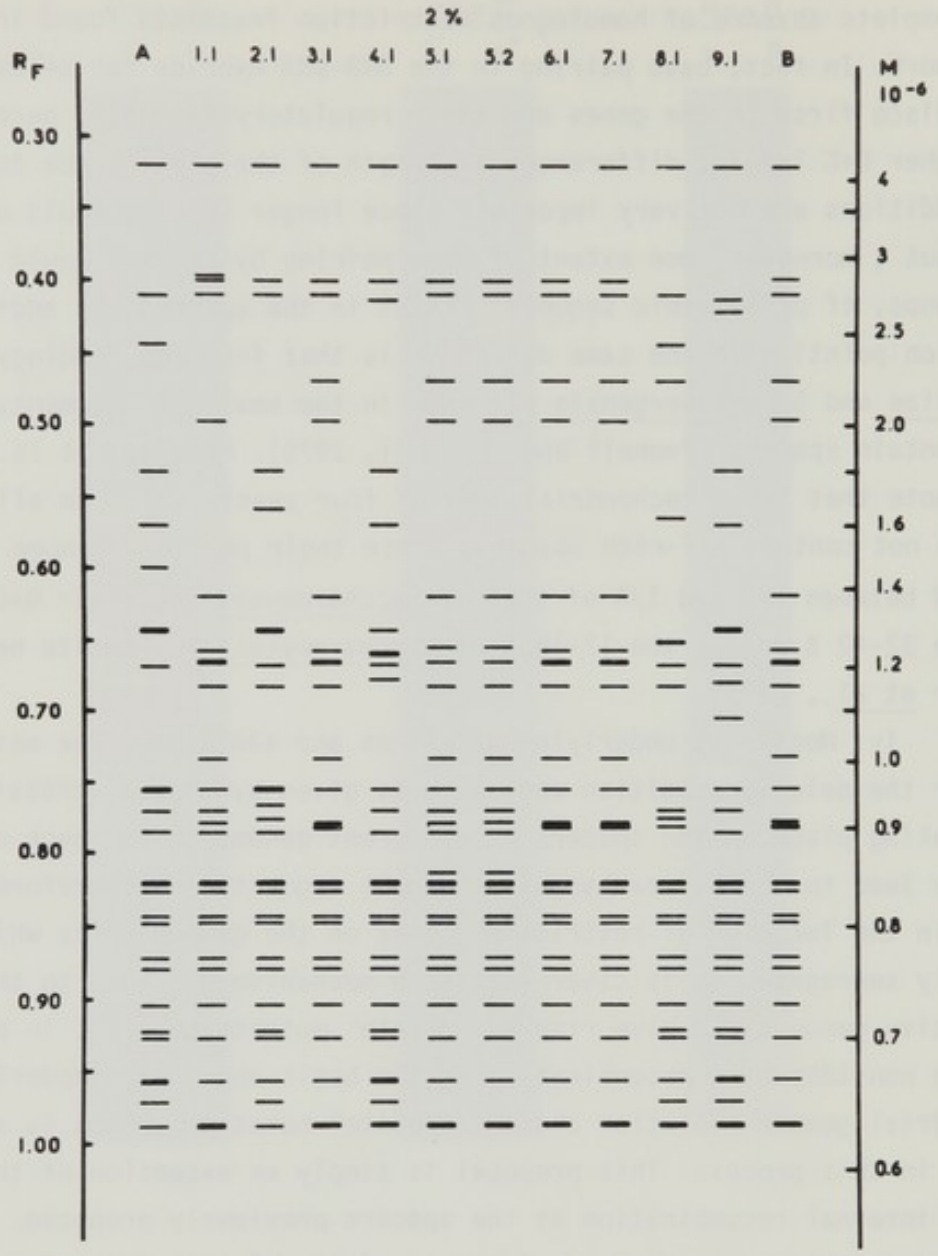


Fig. 6 Scheme of the band patterns obtained with mitochondrial DNAs from strains A, B and a number of diploids from zygotes issued from crosses of these strains.

one and the same parental strain, B. This may mean either that recombinational events were not detectable because involving homologous genome fragments or, more likely, that mitochondrial DNA segregation in the buds took place before recombination. In one particular case, DNA from two subclones of clone 5 were also examined and found to be identical in banding pattern.

v. General implications. In summary, what has been shown in the present work is that in an interspersed system of genes and internally repetitive spacers, evolution goes about essentially by recombination, this process being several orders of magnitude faster than point mutation. This conclusion is very interesting because it may apply to the genome of eukaryotes which is also made up of genes and interspersed repetitive sequences. It is conceivable that recombination processes taking place at a high rate at such sequences have played a very important role in the evolution of eukaryotes.

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Genes on Saccharomyces Mitochondrial DNA

P. Borst, J.P.M. Sanders, C. Heyting

Size, structure and genetic complexity of yeast mtDNA

The first useful information on the size and structure of yeast mtDNA was obtained in 1969 when Hollenberg et al. [1] in Amsterdam discovered the presence of 25-μm circles in electron micrographs of lysed yeast mitochondria. Although only a small fraction of the total mtDNA was present as twisted circles, Hollenberg et al. concluded from a variety of arguments that the 25-μm circles represented the true intact mtDNA of yeast and that its sequence complexity was about equivalent to its size [2]. Although this conclusion was contested at that time, the evidence for it is now quite impressive, as shown in Table I.

TABLE I: Molecular weight and complexity of Saccharomyces carlsbergensis

Method used		Molecular weight or complexity ($\times 10^{-6}$)	Ref.
Electron microscopy		50	[2]
DNA-DNA renaturation analysis		63	[2]
		49	[20]
Restriction endonuclease analysis:			
EcoRI	(9 fragments)	44	[14]
HindII+III	(11 fragments)	46	
HpaI	(6 fragments)	45	
EcoRI + HindII+III	(20 fragments)	44	
EcoRI + HpaI	(12 fragments)	44	
HpaII	(~100 fragments)	47	[16]

The complexity of yeast mtDNA estimated from renaturation experiments is in good agreement with the molecular weight calculated from the circle contour length and with the complexity estimated by cutting up this DNA by restriction endonucleases and adding up the molecular weights of the fragments obtained. None of the approaches used has as yet yielded any evidence for large-scale gene amplification, gene repetition or heterogeneity and Hollenberg's conclusions [2], therefore, now rest on solid ground.

Identification of genes on yeast mtDNA by biochemical methods

Genes for structural RNAs on yeast mtDNA have been analysed by standard hybridization techniques and the results obtained are summarized in Table II. The 0.6 rRNA gene per mtDNA molecule found by Reijnders et al. [3] is a somewhat awkward value and, therefore, Nusse and Groot (unpublished results) have repeated the hybridization experiments under a variety of conditions selected to exclude artefacts. Again they arrive at a plateau of 2.5%. If we accept that the hybridization plateau is correct, there are only three explanations left for the 0.6 copies of rRNA per mtDNA found:

i) Part of the DNA molecules have no rRNA cistrons; this is highly unlikely because the restriction enzyme analysis would have picked up such heterogeneity.

ii) We have grossly under-estimated the molecular weight of yeast mtDNA. This is clearly also very unlikely from the results in Table I.

iii) We have over-estimated the size of the rRNA, although this estimate was based on four independent and respectable methods [4]. We shall return to this point below.

In addition to one cistron for each of the rRNAs, yeast mtDNA contains genes for about twenty 4S RNAs (Table II), at least 14 of which have been shown by Casey et al. [5] to be tRNA genes. Of course, everybody believes that yeast mtDNA does not only code for structural RNAs, but that it should

TABLE II: The number of genes for rRNA and 4S RNA on Saccharomyces carlsbergensis mtDNA

RNA species	Hybridization plateau (% of DNA in hybrid)	Molecular weight (x 10^{-6})		Genes per mtDNA molecule	Reference
		RNA	DNA		
rRNA	2.4	0.7 + 1.3	50	0.6	[3,4]
	2.5	0.7 + 1.3	50	0.6	Nusse, R. and Groot, G.S.P., unpublished
4S RNA	0.9	0.025	50	18	[21]
aa*-tRNA				14 +	[25]

* The hybridization experiments with aa-tRNA were carried out with aa-tRNA labelled in the amino acid moiety; the number of 14 cistrons is a minimal estimate and will probably go up as more aa-tRNAs are tested, as indicated by the +.

also contain regulatory genes and structural genes for proteins, for instance the proteins made on mitochondrial ribosomes. Our efforts to identify these proteins in Amsterdam are discussed by Moorman et al. [6] in this volume. From Table III it is clear, however, that there is still a lot of genetic information unaccounted for, even if all proteins made on mitochondrial ribosomes are coded for by mtDNA. The question is, what this remaining 80 odd percent of the mtDNA is doing.

TABLE III: Genes on yeast mtDNA

Gene product	% of DNA accounted for*	Ref.
20 4S RNAs	1.8	[21]
2 rRNAs	4.8	[3]
3 Subunits cytochrome oxidase	3.6	[22]
1 Subunit cytochrome bc_1 complex	1.2	[23]
4 Subunits ATPase	3.1	[24]
1 "Ribosomal" protein	1.2	[25]
	15.7	

* Molecular weight DNA, 50×10^6.

Relevant to this question is the work by Bernardi and coworkers (see [7]) in the last few years on structural aspects of yeast mtDNA. They first showed by physical methods that yeast mtDNA contains many stretches that are very AT-rich and recently they succeeded in isolating these by controlled nuclease degradation. These so-called "spacers" are interspersed with the so-called "genes", because larger fragments of this

DNA have a uni-modal distribution in CsCl and because the denaturation map of this mtDNA - made by the Christiansens [8] - shows that the AT-rich segments are scattered all over the circular map.

Precise information on one of these AT-rich segments comes from an analysis of the ρ^- petite mutant RD1A isolated by Hollenberg et al. [9]. As shown in Table IV the mtDNA of this mutant has only 3 mole percent GC and it consists of a perfect repetition of a 69-nucleotide sequence. The repeat unit of this DNA has been largely sequenced by Van Kreijl and contains 67 consecutive AT base pairs, flanked by one GC doublet. A melting analysis of the heteroduplex of RD1A mtDNA and wild-type mtDNA has shown that the petite DNA is a faithful copy of part of the wild-type sequence (Van Kreijl, C.F. and Mol, J.N.M., unpublished results). This shows that even in

TABLE IV: Main properties of mtDNA from the yeast petite mutant RD1A

From [9], [26-28] and Van Kreijl, C.F. and Mol, J.N.M., unpublished results.

1. Base composition	
	Duplex DNA: 3% GC (wild-type 18%)
	Heavy strand: 3% G; <0.1% C
	Light strand: <0.1% G; 3% C
2. Sequence	
	>95% consists of perfectly repeated sequence of 69 nucleotides linked head to tail.
3. Origin	
	RD1A mtDNA forms a perfect hybrid with wild-type yeast mtDNA. There are 1-3 copies of the repeating unit per wild-type genome.

such highly reiterated petites the excision and amplification of wild-type DNA is largely error free*. It is of interest that the hybridization experiments show that there is one, at most 3 copies of this sequence present on the wild-type DNA. This proves that there is a high degree of sequence specificity in these AT-rich segments, in agreement with Hollenberg's DNA-DNA renaturation experiments, which gave no evidence for repetitive sequences in yeast mtDNA.

Nevertheless, one can ask with Bernardi whether such AT-rich segments could mainly serve a spacer function or regulatory function rather than code for part of some essential mitochondrial protein. Table V contains data that tend to support this suspicion. As was first shown by Sanders et al. [11], the mtDNA of Kluyveromyces lactis is only half the size of the Saccharomyces mtDNA and it also has only half the genetic information content of Saccharomyces by all criteria applied, renaturation, restriction enzyme analysis and rRNA hybridization plateau. More recently, Clark-Walker and co-workers [12] reported that a large particle fraction of Schizosaccharomyces pombe contains 6-μm circles and they tentatively concluded that this represents the mtDNA of this organism. If we accept for the sake of the argument that this conclusion is correct and that also in this case the complexity of the DNA is equivalent to its size, this yeast mtDNA would have hardly more genetic information than the mtDNA of animal mitochondria [13]. On this basis one could speculate that all mitochondria contain the basic information for rRNA, tRNAs and a set of essential proteins and that 5 μm of DNA is sufficient to code for this. Larger mtDNAs would only be larger than 5 μm, because they contain more regulatory sequences, involved in recombination, control of expression of mitochondrial genes or what not.

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* Before we realized that the repeat sequence of RD1A mtDNA was only 69 nucleotides long, this melting analysis was erroneously interpreted [10] to indicate that the petite DNA was an imperfect copy of the corresponding section of the wild-type mtDNA.

TABLE V: Size of mitochondrial DNAs from various yeasts

	Contour length (μm)	Molecular weight (x 10^{-6}) from			% of DNA complementary to rRNA	Ref.
		EM	Renaturation	Restriction endonucleases		
S. carlsbergensis	26	51	50	43-46	2.4	Table I
K. lactis	11	22	20	18	4.5	[11]
S. pombe	6	12.5	?	?	?	[12]

Of course, having a lot of regulatory sequences of unknown function makes yeast mtDNA only more interesting, not less interesting. DNA sequences specifying proteins are rather dull, because the sequence usually gives us only an amino acid sequence that we know already. It is the AT-rich spacer sequence that still holds secrets worth uncovering.

Location of genes on yeast mtDNA

To be able to locate genes on DNA by biochemical methods, one requires a physical map and this can now be constructed with restriction endonucleases. In the case of yeast mtDNA there are two major problems, however: First, this DNA is quite large, larger than any other DNA mapped with restriction enzymes before. Second, the DNA cannot be isolated intact, but only as large fragments and this is an obvious and formidable obstacle to the construction of a map. Nevertheless, we have managed to make a map of Saccharomyces carlsbergensis mtDNA, containing 11 HindII+III fragments and 9 EcoRI fragments [14]. Together this map has 20 fragments, which gives sufficient resolution to start ordering genes.

The first genes located are the genes for mitochondrial rRNA, because this can be done by simple hybridization. The interesting result, shown in Fig. 1, is that the two RNAs are at opposite ends of the map. This explains why we have been unable to find a typical rRNA precursor in yeast mitochondria containing both rRNAs [15]. Although the large rRNA only hybridizes with Hin_6, our best size estimates predict that this RNA should be about 50% larger than Hin_6 and, therefore, hybridize with the adjacent fragments, as indicated. Further experiments are required to resolve this discrepancy. It is possible, however, that our best estimate of the size of this RNA was not good enough and that it may not be much larger than Hin_6. If this is so, this will also raise the number of rRNA genes per mtDNA molecule, calculated from our hybridization experiments (see Table II), to 1 and this is a more satisfactory value than 0.6.

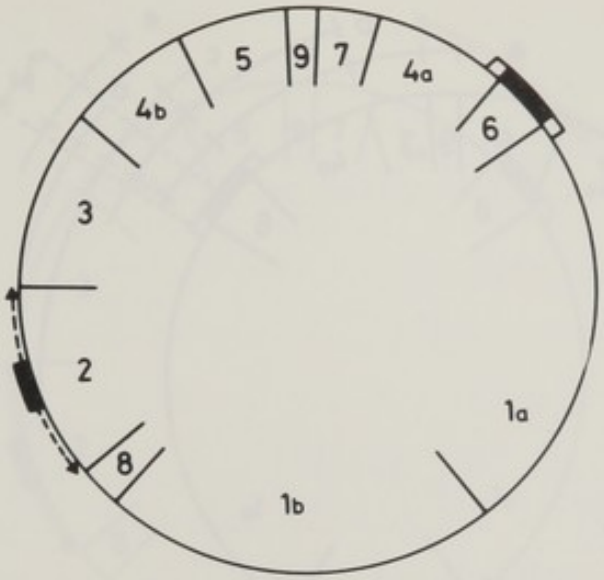


FIG. 1 - Location of the genes for rRNA on the map of Hind-II+III fragments of S. carlsbergensis mtDNA (from [29]). Note that the large rRNA only hybridizes with fragment 6, though its published size [4] is greater than the estimated size of fragment 6 [14]. This discrepancy is indicated by the open blocks.

It is clear that eventually this map can be filled with other transcription products and by using a coupled transcription-translation system it should also be possible to locate the genes specifying proteins, if any.

Location of genetic markers on yeast mtDNA

I would like to end by briefly discussing our attempts to couple the physical map to the genetic map by a procedure which is unique to yeast and which employs genetically-marked petite mutants. Fig. 2 illustrates the principle of the approach. Let us assume that petite mutants arise by simple deletions and that the remaining DNA is a faithful copy of wild-type DNA without scrambling. Then, if we compare the mtDNA of a series of petites carrying the marker M, this should be on the segment that these DNAs have in common. There are two methods to map the petites on the wild-type map: the simplest one is to make complementary RNA on the petite DNA and hybridize this to wild-type DNA. This method has obvious pitfalls

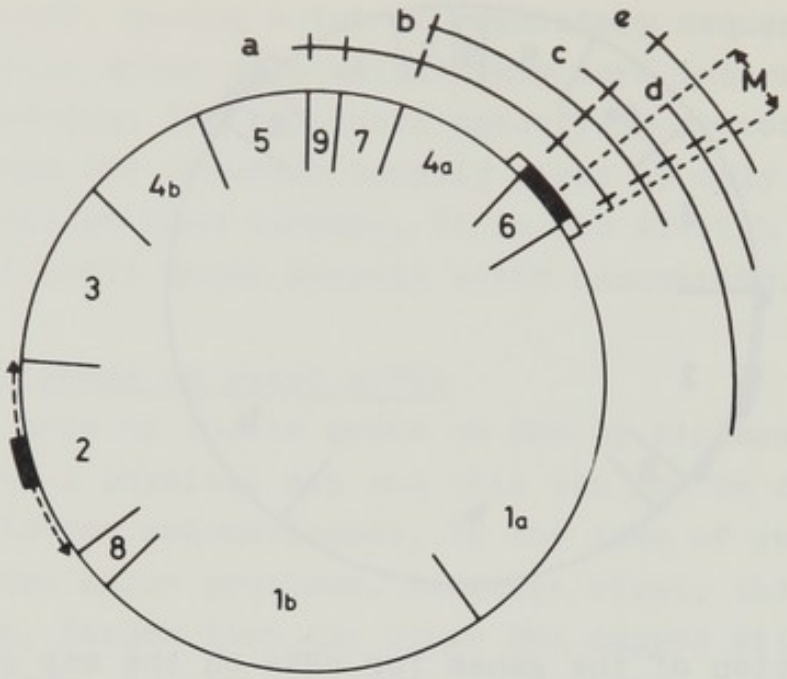


FIG. 2 - Schematic drawing illustrating the procedure used to locate the genetic marker M on the fragment map of yeast mtDNA, using petite mtDNAs carrying M.

and must, therefore, be complemented with a fragment analysis of the petite mtDNA. The endonucleases HindII+III and EcoRI, unfortunately, do not give sufficient cuts in the less complex petite DNAs. For instance, the petites c and e, shown in Fig. 2, will only give Hin fragment 6, whereas petite d will not give a recognizable Hin fragment at all. We are, therefore, forced to supplement the map shown here with additional fragmentation patterns with endonuclease HapII, which cuts the wild-type DNA in about 80 fragments [14,16].

The results accumulated over the last year with this laborious approach are encouraging: We have shown that petite mtDNAs usually contain consecutive segments of the wild-type map and that the basic pre-requisite for this approach is, therefore, fulfilled. Fig. 3 shows the preliminary location of the genetic markers E_{514} and C_{321} by this approach. The result confirms the linkage of E and C to the large rRNA cistron, al-

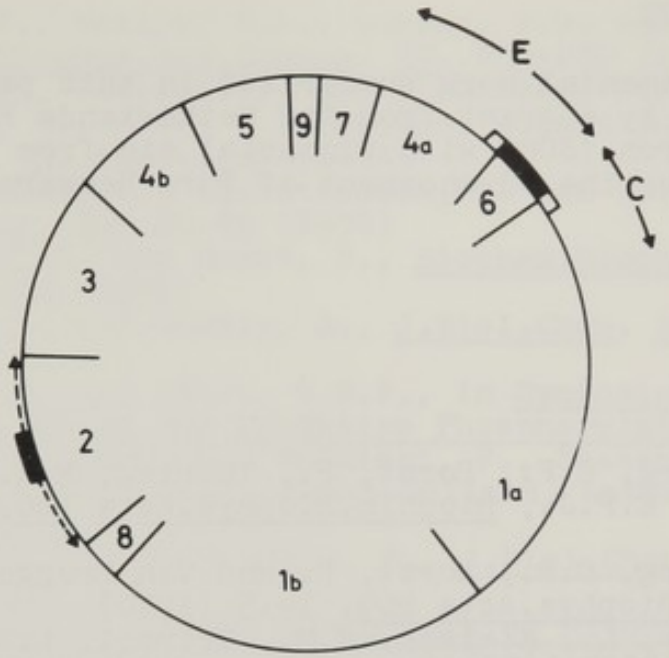


FIG. 3 - Preliminary localization of a marker for resistance against erythromycin (E) and a marker for resistance against chloramphenicol (C) on the fragment map of mtDNA. Petite mutants carrying either the marker E_{514} or C_{321} or both were used.

ready reported on the basis of petite analysis by the Gif group [17] and Linnane and co-workers [18]. The analysis of small HapII fragments now in progress will soon show whether the C marker is just inside or outside the rRNA cistron and provide sequence information about the nature of ω, which is genetically closely linked to C [19]. With Ann DiFranco and Piotr Slonimski we have started to look at the location of the markers for oligomycin and paromomycin resistance and since this kind of mapping becomes easier as more sequence information is accumulated, we expect that all known markers will be localised on the map within the next year.

In summary then, we are now in a position to couple the genetic map to the physical map. With patience and hard work it should be possible before long to identify and order all genes on this mtDNA and identify the sequences involved in the control of replication, transcription, translation and recombination. We trust that the results will be interesting.

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Experimental Variation of the Number of Mitochondrial Genomes Participating in Mitochondrial Genetics

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Linkage between the genes on mitochondrial DNA (mitDNA) has been difficult to demonstrate by conventional genetic analysis. In Saccharomyces cerevisiae haploid cells contain many copies of mitDNA, and in crosses many if not all of these molecules can participate in recombinational and segregational events. Suggestions have been made that yeast mitDNA is particularly recombinogenic : accounting for its apparently dispersive mode of replication (2) and the failure of many workers, including ourselves, to isolate significant quantities of intact genomes. Various explanations have been put forward for this behaviour - the unusually high A+T (83%) content (3) and distribution of pyrimidines (4) in yeast mitDNA, the lack of an excision repair system (5) possibly leaving just a recombinational repair system in yeast mitochondria.

Mitochondrial genetics has obvious parallels with other multigenome genetic systems found in prokaryotes and in eukaryotes. The genetics of multiple-copy bacterial plasmids has advanced chiefly through the use of deletion and physical mapping methods (6). The genetics of viruses has the obvious ad-

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vantage of being able to isolate single infective particles and to easily vary the number of genomes participating in a cross which greatly aids recombinational analysis. By analogy with other multigenome genetic systems the genomes existing within one cell (in this case the zygote or its immediate heteroplasmic progeny, 7) are capable of undergoing repetitive acts of multiple recombination with like and unlike genomes, the progeny genomes being segregated (in this case) by cell division, recombination being detectable until the cells become homoplasmic. The final frequency of mitochondrial genotypes is easily measured as effectively all cells in diploid culture have become homoplasmic after the 20 or so intervening cell divisions usually allowed in this type of analysis. Inferences may be drawn by analysis of the frequencies of mitochondrial genotypes among the progeny as to the overall type and average frequency of recombinational events. But further interpretations of mitochondrial genetics are hampered in that no obvious mechanisms exist to vary the number of genomes entering into a cross.

A number of agents which inhibit the initiation of nuclear DNA synthesis have been shown to increase the relative amount of mitDNA (cycloheximide 7, α-hormone 8). The information on conditions which decrease the amount of mitDNA is rather more conflicting. However, we have found in agreement with some others that high levels of glucose reduce the average amount of mitDNA in a culture. We show here that in crosses between cells differing in mitDNA content by growing in a high level of glucose or in glycerol the transmission of mitochondrial genes varies in a predictable manner.

EXPERIMENTAL

The relevant genotypes of the strains used in this analysis :

S 2/6B-1 $\underline{a}$ met^- ω^+ C^S E^R_{76} O^R_1

S 2/6B-2	a	met^-		$[\omega^+ \; C^S \; E^S \; O_1^R]$	
SM 1211-1B	a	ilv_5^-	trp_1^-	$[\omega^+ \; C^R_{321} \; E^R_{221} \; O^S \; T^S_8]$	
IL 16-10B	α	his^-		$[\omega^- \; C^R_{321} \; E^S \; O^S]$	
IL 126-3A	α	his^-		$[\omega^- \; C^R_{321} \; E^R_{221} \; O^S]$	
IL 187-7A	α	his^-		$[\omega^+ \; C^R_{321} \; E^S \; O^S]$	
IL 458-1A	α	his^-		$[\omega^+ \; C^R_{321} \; E^R_{221} \; O^S]$	
IL 166-7A	α	ura_1^-		$[\omega^+ \; C^S \; E^S \; O_1^R \; T^R]$	

S 2/6B-1 and S 2/6B-2 are members of a series of 6 nuclear isogenic mitochondrial recombinants (D.Y. Thomas, in preparation). O_1^R (S 2/6B) is the original oligomycin resistant mutant isolated by K. Stuart (10). The strains with the prefix IL are a series of testers from Centre Génétique Moléculaire, Gif-sur-Yvette, France. The T^S mutation confers inability for growth at 35°C on YEPG (11).

Media and Methods :

YEP is (final concentrations) yeast extract (Difco) 10g/l, bactopeptone 20g/l. Carbon sources were autoclaved separately as 50% (w/v) solutions and added to YEP stock (x4) to a final concentration of either 2% glucose YEP2D, 10% glucose YEP10D or 4% glycerol YEPG. MMD used for the selection of diploid colonies was yeast nitrogen base (Difco) 6,7g/l, 20g/l glucose. Agar was added to a final concentration of 2% for solid media.

Inhibitors were stored as 100mg/ml solutions in ethanol at -20°C. They were added to YEPG media cooled to 50°C at the following final concentrations : chloramphenicol (C) 2mg/ml, erythromycin (E) 1mg/ml and oligomycin (O) 2µg/ml. The T marker (temperature-sensitivity/resistance) was detected by non-growth on YEPG at 35°C, for crosses involving this marker prior growth was at 23°C.

Crosses were made between cells grown under the conditions shown for the number of cell generations stated. Crosses were made by depositing cells on sterile Millipore membrane filters (0.45µ pore size, 2.5cm diameter). Generally 2×10^6 cells of each mating type were mixed and filtered. The filters were placed on the surface of YEP2D plates (at 30°C). Examination by microscopy showed that zygote formation commenced in about one hour using this method. Mating pairs (not necessarily yet zygotes, as some are still separable by mild sonication) formed within 100-120 minutes of mixing the cells, were isolated

by washing the filter with 10mM NaCl and plating aliquots on MMD plates. After 48 hours growth for each cross 10^3 zygote colonies (c. $2x10^6$ cells each colony) were collected, diluted in 10mM NaCl and plated at 80-100 cells/plate of YEP2D. The colonies (after 48 hours) were replica-plated onto a series of antibiotic media.

DNA estimation

Cells from the immediate pre-crossing conditions were rapidly frozen at -20°C and were subsequently treated substantially as described (12) except that 3% Sarkosyl was used to release the DNA from the cell pellet. Aliquots were analysed in a Spinco Model E ultracentrifuge using the 4 cell ANF rotor at 44,310 rpm 20°C, equilibrium at 20 hours. 1μg M.lysodeikticus (1·731g/cc) was used as a reference for density and for nucleic acid concentration. Photographs were scanned with a Joyce-Loebel microdensitometer. Areas under the nucleic acid peaks were measured by planimetry.

Mitochondrial protein synthesis

At various times 3 aliquots of cells were removed from the glucose-treated culture. ^{3}H-Leucine was added to aliquot a), containing no antibiotics, to aliquot b), containing cycloheximide (100μg/ml) and to aliquot c), containing cycloheximide (100μg/ml) plus erythromycin (2mg/ml). Samples were periodically taken during an incubation period of 30 minutes, treated by boiling and extensively washing in 5% TCA, washed finally with ethanol, resuspended in Instagel and counted at 20% efficiency in an Isocap liquid scintillation counter.

The true rate of mitochondrial protein synthesis (essentially linear for the 30 minutes incubation period) was obtained by determining the erythromycin-sensitive, cycloheximide-resistant incorporation (a minus b), and expressing this as a percentage of the total cellular rate of protein synthesis (c).

Whole cell O_2 uptake

This was measured with a Beckman oxygen electrode (Beckman Physiological Analyser Model 160) in a 2ml cell with a thermostatted (23°C) water jacket and magnetic stirrer. Aliquots were taken from the same culture in which mitochondrial protein synthesis was determined.

A The amount of mitDNA in glucose-grown cells

Although there has been some uncertainty (13) as to whether the amount of mitDNA in a cell is actually influenced by glucose, it now appears certain that careful measurements reveal that the levels of mitDNA are influenced by growth conditions. Growth on glucose appears to decrease the amount of mitDNA per culture.

In this study a method of cell breakage and DNA extraction has been used (12) which has been shown to be remarkably efficient for a wide range of cell types and growth conditions. Table 1 shows the percentage of mitDNA recovered in aliquots of the cell samples used for the crossing experiments. It is clear that the glucose-grown cells contain considerably less mitDNA (between 20% and 50%) than those grown on glycerol, the lower amount cannot be accounted for by the small number of petites present in the cultures. Different strains growing on glycerol have different amounts of mitDNA, although the nuclear isogenic strains S 2/6B-1 and S 2/6B-2 have identical amounts of mitDNA. It is interesting that these strains show significantly different amounts of mitDNA in glucose-grown cells, since they differ only in their mitDNA (genetic) complement some control function of the level of mitDNA by the mitochondrion seems to be indicated.

Using the value of 5×10^7 daltons as the molecular weight for mitDNA (14) it is possible to arrive at an approximate figure for a number of whole mitDNA molecules present on average in each cell. This figure can only be approximate as the mode of replication and rate of initiation of mitDNA is unknown, genes close to the origin(s) of replication in growing cultures would obviously be present at a higher frequency than those more remote from the origin.

Strain		% mitDNA	% petites in culture	mitDNA genomes/cell
S 2/6B-1	D	7.1	1.1	15
	G	21.9	< 0.01	48
S 2/6B-2	D	4.6	1.2	10
	G	21.9	< 0.01	48
IL 16-10B	D	7.4	0.6	16
	G	17.1	< 0.01	38
IL 187-7A	D	4.3	8.7	9
	G	16.5	0.05	35
IL 458-1A	D	3.6	1.7	8
	G	19.8	< 0.01	43
IL 126-3A	D	10.9	4.6	24
	G	21.7	0.05	48

Table I : Amount of mitDNA in mating cells.
The level of mit DNA was measured in aliquots ($2x10^8$ cells) taken from cells grown either on YEP10D or YEPG for 6 generations and prepared as described in METHODS for analytical ultracentrifugation. From the same samples cells were also taken for determination of the proportion of petites present and for crosses. The number of mitochondrial genomes per cell was calculated assuming the unit mitochondrial genome to be $5x10^7$ daltons molecular weight (14).

B The influence of glucose upon the transmission of mitochondrial genes

A characteristic of mitochondrial genetics is that the recovery of mitochondrial alleles in the diploid progeny - which we shall term the transmission frequency - is variable from cross to cross.

The transmission frequencies of the markers C, E and O in the four crosses examined using the 4 possible permutations of pre-crossing growth conditions are given in Table II.

Our prediction was that under conditions where the contribution of mitDNA from the parents were equal the transmission frequency of mitochondrial genes should be 50%. Where the parental contribution of mitDNA molecules is unequal, then mitochondrial genes from the parent contributing the most mitDNA molecules to the zygote should be in excess. For crosses II and IV ($\omega^+ x \omega^+$) this appears to be so for all 3 markers analysed. In cross IV (glycerol x glycerol) where the parental mitDNA contributions are almost equal the transmission frequency is almost 50% for all 3 markers. In cross II (glucose x glucose) where the transmission frequency is again 50% the contribution of mitDNA from the two parents was not equal (7.1% mitDNA from S 2/6B-1 and 4.3% from IL 187-7A). Again in cross II (glucose x glucose) where the contribution of mitDNA was from S 2/6B-1 21.9% and from IL 187-7A 16.5% a very high proportion of alleles from S 2/6B-1 were recovered. In situations for crosses II and IV where glycerol-grown cells were crossed with glucose-grown cells the alleles from the glycerol-grown parent are always recovered in excess in the progeny. It is evident that although there is a distinct effect of pre-growth conditions on the transmission of mitochondrial genes in crosses II and IV and on the levels of mitDNA there is an-

Cross					C^S	E^R	O^R	Ratio of mitDNA P_1/P_2
P_1	S 2/6B-1	D	x	D	92.5	68	53.5	0.96
	x							
P_2	IL 16-10B	D	x	G	97.8	79	57.9	0.42
		G	x	D	97.1	87.9	87.4	2.96
	I	G	x	G	93.3	82.9	66.5	1.28
P_1	S 2/6B-1	D	x	D	51.9	50.6	50.6	1.65
	x							
P_2	IL 187-7A	D	x	G	32.5	34.5	36.4	0.43
		G	x	D	69.9	69.9	67.7	5.1
	II	G	x	G	70.5	72.1	73.1	1.33
					C^S	E^S	O^R	
P_1	S 2/6B-2	D	x	D	77.5	51.1	41	0.42
	x							
P_2	IL 126-3A	D	x	G	85.5	53.8	39.9	0.21
		G	x	D	94.4	72.7	66.9	2.01
	III	G	x	G	96.5	66.9	48	1.01
P_1	S 2/6B-2	D	x	D	38.5	39.4	37.1	1.27
	x							
P_2	IL 458-1C	D	x	G	34.2	36.4	35.8	0.23
		G	x	D	60.5	62.5	55.8	6.08
	IV	G	x	G	52.8	54.6	50.5	1.11

TABLE II : Transmission frequency of single markers.

D signifies pregrowth on YEP10D and G on YEPG. The ratio of the contribution of mitDNA from the two parents is given. The alleles referred to are from the first mentioned parent in the cross. More than 2×10^3 colonies were analysed in each cross.

other superimposed effect not wholly accounted for by these measured levels of mitDNA. Whether this reflects the physical state of mitDNA under different growth conditions (some of the transmission frequencies appear to be slightly polar, for crosses II and IV the polarity of C and E being greater than O) or some other type of inter- or intracellular selection remains to be determined.

In the crosses I and III ($\omega^+ \times \omega^-$) the effect of glucose is best observed in the mitochondrial genes E and O. The values of C transmission for cross I are so high and similar that no distinction can be drawn for the different conditions. In both these crosses the values of transmission obtained in the glucose x glucose and glucose (parent 1) x glycerol (parent 2) crosses do not fully agree with those that would be predicted from the measured relative contributions of mitDNA from the two parents. It should again be considered that other factors than their relative concentrations of mitDNA may be important in determining the transmission of mitochondrial genes.

There remains some uncertainty about whether the mitDNA levels we have measured are in fact those existing in cells at the time of zygote formation. Using the fluorescent dye staining method for measuring the intracellular distribution of mitDNA this is being determined.

C The kinetics of the loss of mitochondrial functions after the addition of glucose

Cells for crosses described in the preceding section were always grown for six generations in the presence of glucose. We have determined the kinetics of the change in transmission of the mitochondrial genes O and T during 9 generations of growth in the presence of high levels of glucose. The repress-

ed cells were crossed with cells grown to mid-log phase on glycerol. In conjunction with this we have examined the effect of these growth conditions on several other mitochondrial functions. One parameter which can be measured with considerable accuracy is the rate of mitochondrial protein synthesis in whole cells treated with cycloheximide to inhibit cytoplasmic (80S) protein synthesis. It can be seen from the curve in Figure 1 that this function shows glucose repression in that the rate of mitochondrial protein synthesis is greatly reduced (by a factor of 10) within one cell generation in response to the added glucose (10%). The wild-type (not carrying the T_8^S mutation) showed identical kinetics for the glucose repression of mitochondrial protein synthesis at 23°C.

Respiratory function as measured by the rate of oxygen uptake showed somewhat different kinetics in that the rate did not substantially alter during the first cell generation after the addition of glucose. But it did substantially decrease. Apparently the concentration of the mitochondrial proteins supplied by the mitochondrion's own protein synthesizing system are not immediately critical in maintaining the cell's total respiratory function.

The data for transmission of the genes O and T (located in the non-polar region of the mitochondrial genome) are presented as a percentage of the initial value found for their transmission before addition of glucose to the culture. After 9 generations the transmission of these genes drops to 50% of this value. The largest drop (approximately 25%) being within the first cell generation after the addition of glucose.

Whether the level of mitDNA is sensitive to glucose repression (which could be defined as the loss of mitDNA within one cell generation) or a dilution of existing copies of

mitDNA (i.e. repression of the rate of mitDNA synthesis) is shown by the measurements of mitDNA synthesis at various times after the addition of glucose to a culture (see Fig. 1). For this experiment cells were prelabelled during growth in glycerol with ^{14}C-uracil, and the rate of DNA synthesis measured both before and at various times after the addition of glucose by labelling the culture for 40 minutes with ^{3}H-adenine. Separation of mitDNA and nuclear DNA was achieved using NaI density equilibrium centrifugation (20). The ratios of ^{14}C and ^{3}H for the mitDNA peak and the nuclear DNA peak were determined. The pre-label in mitDNA was conserved during growth on glucose indicating that there was no extensive breakdown of mitDNA. The pulse label showed that after 1 generation the rate of mitDNA synthesis had dropped by 50%. Thus the mechanism of glucose repression appears to be a slowing or a cessation of the normal (glycerol-growth) rate of mitDNA synthesis; presumably the existing copies of mitDNA are segregated to daughter cells, and the level of mitDNA is then maintained at the characteristic glucose growth level for that strain.

A simple interpretation of the results shown in Fig. 1 would be that mitochondrial protein synthesis effectively ceases upon addition of glucose to the culture and the total rate of mitDNA slows; the mitDNA molecules being distributed equally to daughter cells thus being diluted in each cell generation. The kinetics of the transmission frequency of mitochondrial genes closely following that of the number of mitDNA copies. At the repressed level of mitDNA presumably the rate of initiation is now sufficient to maintain this level of synthesis of the mitDNA present in the cell. Probably the control is exerted at the level of initiation of mitDNA synthesis rather than the rate of mitDNA elongation which in analogy with other systems tends to be rather constant under different

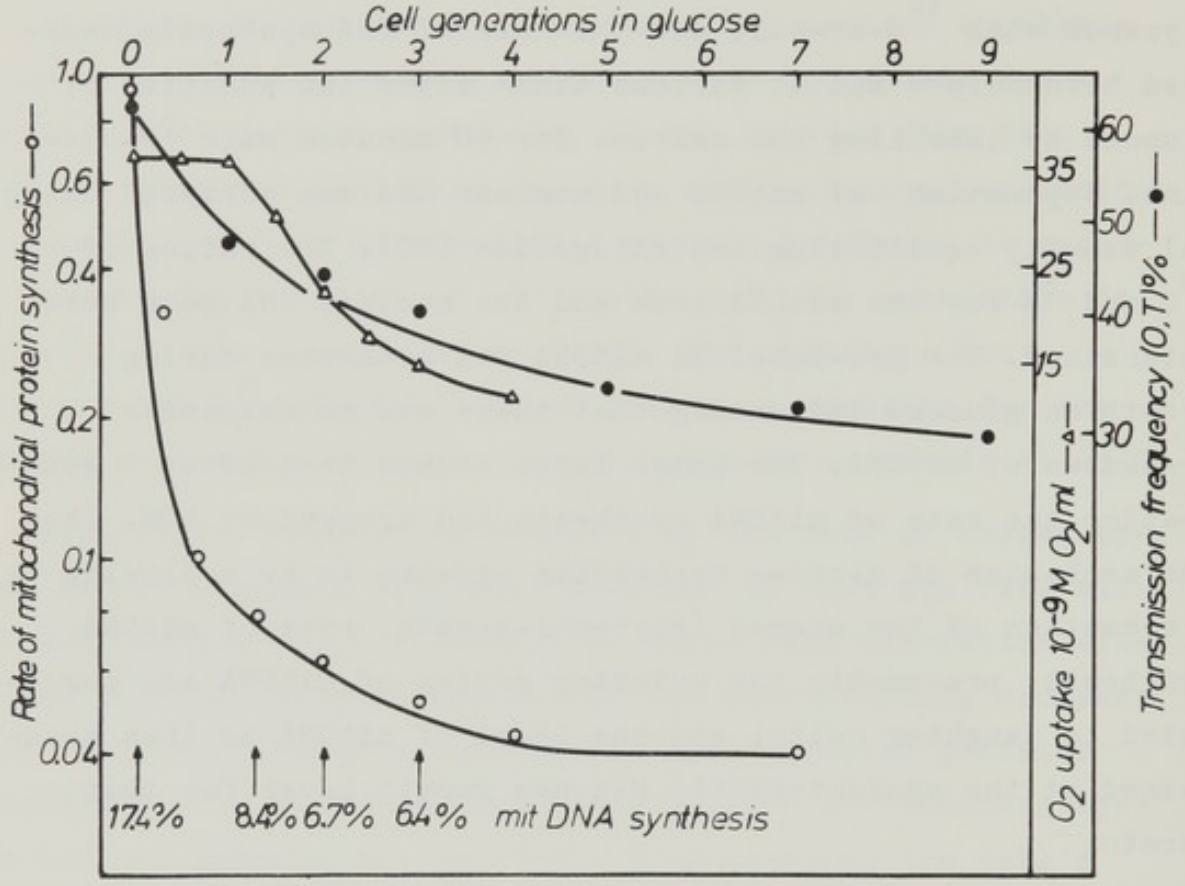


Fig. 1 Strain SM 1211-1B was grown to a cell density of 8 x 10^6/ml in YEP glycerol at 23°C and transferred to glucose at generation 0. Samples were taken for determination of mitochondrial functions before the switch and at various times after the switch. Measurements were made as described in METHODS.

growth conditions. It seems that this is another indication of a mitochondrial function controlling the cellular level of mitDNA.

DISCUSSION

Interpretation of the extensive results of mitochondrial genetics now published (see refs. 17, 18, 21 for review) awaits information on some of the basic physiological parameters of mitDNA in cells, such as the relative number of genomes present in crosses and their potentiality for recombination, replication and transmission.

Data on the mode of segregation of mitDNA to daughter cells comes from pedigree studies of the progeny of single zygotes. Several important facts were established from a study of pedigrees involving 3 and 4 mitochondrial markers (15). It was observed that the first few buds issuing from zygotes were often of pure mitochondrial phenotype (homoplasmic) in that they gave rise to pure colonies, but subsequent buds from the same zygote could be of a different mitochondrial genotype, or mixed (heteroplasmic). Thus it appears that only a few molecules of mitDNA are passed into the daughter bud or that some mechanism exists for directed segregation of mitDNA molecules (the equivalent of a nuclear spindle). Using a sensitive technique for the detection of intracellular mitDNA it appears that mitDNA may exist in cells as a few aggregates of several molecules (19, 23). Similarly data from zygotes where the position of the emerging bud was noted (16, polar or median) indicate that buds from the poles of the zygote are of a single parental type indicating that the mitochondrial genomes are not completely mixed and a pool of mitDNA molecules all participating in recombination does not occur. Data on the rate of

mitDNA initiation and replication are as yet unavailable, and data on the timing of mitDNA replication in the cell cycle is conflicting. It is with these reservations in mind that interpretations and models of mitochondrial genetics should be viewed (18).

The results presented here (see also refs. 22, 24, 25) indicate that the transmission of mitochondrial genes can be considerably modified by the previous growth history of the participating haploids. Concomitantly an alteration in the number of mitochondrial genomes by the same growth conditions is observed. The two observations appear to be causally related in that where there is a reduction in the relative number of genomes donated to the zygote by one of the parents then the mitochondrial genes transmitted to the diploid progeny by that parent are reduced. Also the kinetics and quantitative changes in transmission frequency and mitDNA synthesis are rather similar, but as we have shown other mitochondrial functions are also immediately altered upon addition of glucose. Other factors appear to be important in determining the transmission of mitochondrial genes.

Where there is an equal frequency of transmission of genes from each parent the measured amounts of mitDNA from each parent were not always equal, and conversely where the amounts of mitDNA contributed by each parent were found to be nearly equal (in crosses I and III) only the frequency of the marker in the non-polar region (O) was found to be 50%.

The results from crosses type II and IV and the kinetic experiments ($\omega^+ x \omega^+$) are easier to interpret on the basis of the relative number of mitochondrial genomes contributed by the parents. The correlation between the measured number of mitochondrial genomes from a parent and the transmission of the mitochondrial genes from that parent is clear in that from a

parent contributing fewer mitochondrial genomes fewer mitochondrial genes are recovered. The mitochondrial genes in these crosses show covariant transmission in that considering each cross the frequency of transmission of mitochondrial genes from the same parent are very similar ($\omega^+ \times \omega^+$). Also the frequency of transmission of mitochondrial genes in crosses of glycerol grown x glucose grown cells follows that expected from the relative measured frequency of mitDNA molecules.

In cross I not much effect of glucose is seen on the C marker, it appears always to be transmitted with high frequency from the ω^+ parent. But the transmission frequency of the markers E and O are altered in the same predictable manner as for crosses II and IV. Whether this means that the marker C acts independently of mitochondrial genome number or the values of transmission frequency are so high and similar that an effect cannot be detected is uncertain from the data presented for cross I. That the second interpretation is probably correct is seen by the data of cross III. Although even here the glucose x glucose cross in which there are fewer molecules participating gives a lower transmission of the C^S gene than in the glycerol x glycerol cross where there are more C^R genes and a lower frequency of C^S alleles would be expected to be recovered. It is possible that other factors are important in determining the frequency of a particular allele amongst the progeny (the total number of genomes or their physical state).

The uses of this simple method for varying the input of mitochondrial genomes for crosses to be analysed by analogy with a viral model of multigenome genetics seem to be quite clear. It would also be important to determine by pedigree analysis of these types of crosses whether in fact all mitochondrial genomes are participating in genetic recombination. Results on the effect of varying the input of mitDNA on mitDNA recombination and the polarity of mitochondrial gene recombination

will be presented elsewhere (19).

We are grateful to Peter Moore for his skilful running of the analytical ultracentrifuge, and Don. Williamson and Ilias Doxiadis for helpful discussions, Drs. Dujon and Slonimski for providing the IL strains.

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Packaging and Recombination of Mitochondrial DNA in Vegetatively Growing Yeast Cells

D.H. Williamson

The Altmann-Ris hypothesis, which supposes that mitochondria arose in evolution as bacteria that were trapped or ingested by a primordial ancestor of the eukaryotes, has attracted a great deal of interest and has much in its favour. However, its value as a stimulus to research is perhaps slightly offset by its tendency to prejudice our thinking towards a rather static view of the mitochondrion. On this view, mitochondrial organelles are seen very much as uniformly sized bacterium-like entities, regularly reproducing by equal binary fission and, like bacteria, maintaining genomes which are normally packaged independently and relatively rarely undergo recombination. On this model, the progeny of a dividing cell receive equal numbers of randomly assorted mitochondrial genomes, simply by equal partitioning of the parental cytoplasm.

In reality, this conventional picture has never stood up too well to detailed scrutiny, but it is now being increasingly eroded, particularly in the case of the yeast Saccharomyces cerevisiae. As discussed elsewhere in this volume, genetic analysis of crosses has long since established that in the zygote produced by mating two yeast cells, mitochondrial genomes normally undergo extensive recombination, and this implies that the organelles either fuse or interact in some other way that permits recombination to occur. It now looks as though fusion may not be restricted to zygotes, but could be a common feature of vegetative growth. Electron microscopic observation of serial sections has shown that yeast mitochondria are by no means uniform in size and shape but are very variable in both these characters (Hoffmann and Avers, 1973; Grimes et al., 1974; Stevens, 1974 and personal communication). In particular the work of the last mentioned author has shown that, depending on physiological conditions, individuals of the same strain may exhibit anywhere between 1 and 40-50 mitochondria. The lowest numbers are found in growing cells, and usually in such cells one organelle is much bigger and more complex than the few others which cohabit the same

cytoplasm. While we certainly need to know more about this situation, it is not unreasonable to suppose that mitochondrial reproduction occurs by some sort of unequal fragmentation rather than by equal binary fission, and also that large mitochondria may be generated by coalescence and fusion of small ones. At the very least one may surmise that individual mitDNA molecules are far more accessible to each other than would be the case on the conventional 'multi-package' concept of the mitochondrial system.

This is important because it has also emerged recently that unlike bacterial genomes in a liquid culture, mitDNA duplexes in vegetative yeast cells undergo continuous recombinational interchanges with each other (Williamson and Fennell, 1974). This phenomenon results in multiple exchanges of regions of homologous single strands between duplexes with a frequency such that old and new single strands of mitDNA rapidly become indistinguishable. The function of this activity is not understood, and it may well be that the observed exchange of single strands is merely the end result of a more complex process. It is not clear how free any given duplex is to recombine with any other in the same cell and it may well be that recombination only takes place between true sister duplexes. However, the occurrence of giant mitochondria, which must contain several mitDNA molecules, and the possibility of multiple fusions between smaller ones, makes widespread interaction between geographically distant duplexes much more feasible than would have been the case on the older multi-package model. Thus the possibility arises that all the mitDNA molecules present in a given cell cycle form a common recombining pool, which could have important genetic consequences. In any case, even if such interactions are limited to near neighbours within mitochondria, we must now seriously question the notion, inherent in the multi-package model, that mitochondrial genomes behave independently of each other, and this at least should be appreciated in attempting to interpret their genetic behaviour.

This new 'fluid' concept of the mitochondrial system may suggest solutions to some outstanding problems in this area, notably the high frequency of occurrence of cytoplasmic "petite" mutants and the apparent rapidity with which other mitochondrial mutants segregate out during

vegetative growth. However, we still have a long way to go before real progress in understanding these problems can be made. In particular we need to know more about the intramitochondrial distribution of mitDNA molecules during cellular growth and about the way in which mitochondrial genetic information is transmitted to offspring.

In order to explore these problems, we have recently developed the use of a fluorescent stain that is specific for DNA and which in yeast permits microscopic observation of both nuclear and mitochondrial DNA molecules (Williamson and Fennell, 1975). The stain in question is 4',6-diamidino-2-phenylindole (DAPI), first synthesised for use as a trypanocide (Dann et al., 1971). The technique for its use as a DNA stain has been presented elsewhere, together with evidence for its specificity towards DNA (Williamson and Fennell, 1975). Unpublished experiments with bacterophage show that it is sensitive enough to permit visualization of a single mitDNA molecule, provided it be packaged in a condensed form. The essential result of applying it to yeast cells is the finding that respiratory competent (grande) cells contain small extranuclear fluorescent stained bodies which are sensitive to DNAse and are in fact aggregates of mitDNA molecules. Similar aggregates are also present in cytoplasmic petite (ρ^-) mutants of the kind that contain mitochondrial DNA, but they are completely absent from ρ_0 mutants which for various reasons are known to lack mitDNA (Fig. 1). In fact DAPI provides a

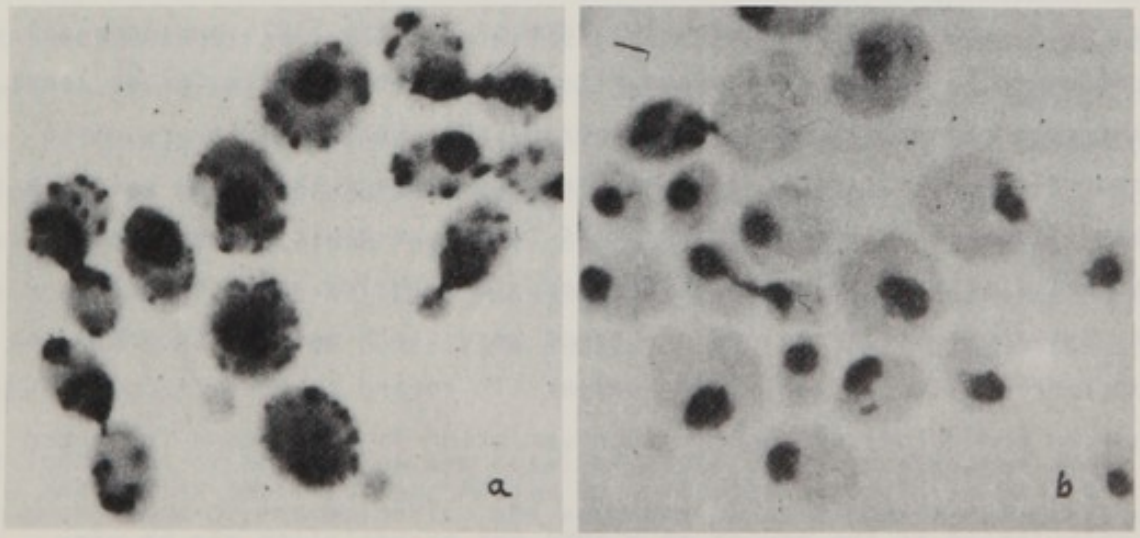


Fig. 1. Negative prints of fluorescence photomicrographs of cells stained with DAPI. (a) Grande parent, (b) ρ_0 Cytoplasmic petite mutant. Aggregates of mitochondrial DNA (chondriolites) are only present in the grande.

convenient way of diagnosing ρ_0 mutants at the cellular level and this has been discussed elsewhere (Williamson and Fennell, 1975).

The purpose of the present article is to present preliminary data concerning the occurrence and size of these aggregates of mitDNA in various situations. Detailed observations will be reported elsewhere. To avoid repetitious use of non-specific terms such as 'aggregate', it is proposed to refer to these bodies as 'chondriolites', a name which has both linguistic and operational connotations.

In randomly dividing batch cultures, the average number of chondriolites per cell varies markedly with the culture phase, stationary phase cells nearly always having more than log phase ones. The average number of chondriolites in a stationary phase cell often approaches the supposed number of mitDNA molecules (realistic present estimates suggest around 30 in a haploid), which implies that in this situation most chondriolites comprise little more, on average, than a single mitDNA molecule. In log phase on the other hand, fewer chondriolites are found; in glucose media, for example, the average number may be about half the stationary phase value, and the level is maintained fairly steadily throughout the growth period. Calculations based on estimates on the mitDNA content of such cells indicate that under these conditions each chondriolite contains an average of 2-8 mitDNA molecules. We have no direct evidence as to the numbers of chondriolites per mitochondrion in either log or stationary phase cells, but comparison with the data of Stevens (1974 and personal communication) suggests that average log phase mitochondria (or at least the larger ones) contain several chondriolites, while stationary phase mitochondria mostly contain only one (and on average therefore only one mitDNA molecule). Glycerol grown cells tend to exhibit higher numbers of chondriolites than glucose grown ones, in both log and stationary phase, but the differences are often not very striking. There seems to be considerable strain variation however, in regard to the effect of glucose on chondriolite number; such variation is also found in regard to the extent of glucose repression of mitDNA per cell, and this means that calculation of the amount of mitDNA per chondriolite can be very inaccurate unless based on direct analysis of the amount of mitDNA per cell.

In a general sense our observations on chondriolite numbers correlate very well with those of Stevens (1974) on changes in the number of mitochondria per cell in batch cultures, which are referred to above. A striking feature of our results is the rapidity with which the high stationary phase level drops to that characteristic of log phase following inoculation of resting cells into fresh growth medium. In more than one case this drop has been seen to occur within the first cell cycle, so that it could not have been caused by dilution of chondriolites amongst dividing cells. There is no net loss of mitDNA during this period (cf. Williamson and Moustacchi, 1971) and the most likely explanation for this rapid decrease in chondriolite number therefore is that these bodies coalesce or aggregate in some way early in the first cell cycle. In view of the data on mitochondrial numbers referred to above, we infer that this chondriolite aggregation also involves fusion. However, chondriolite fusion has not so far been observed in the cell cycle of established log phase cells. Exploring the idea discussed above that all the mitDNA molecules in a cell may form a common recombining pool, we have followed changes in chondriolite number during the cell cycle by examining individuals from randomly dividing cultures and measuring bud size to determine their cell cycle position. These experiments have shown that there is no single stage of the cell cycle, common to all individuals, at which all the chondriolites aggregate in one mass. However, the data are very scattered and this observation therefore does not preclude multiple small scale fusions of chondriolites at different times in different cells. Such multiple fusions could, in the course of one cell cycle, effectively result in interactions between all the mitDNA molecules in the cell and we cannot therefore define the upper limit to the size of any postulated pool of recombining molecules of mitDNA. On the other hand, it is at least reasonable to infer that all the molecules in a given chondriolite constitute such a pool, i.e. recombine with each other.

Our ignorance of so many of the details in this area, particularly of the mechanisms of recombination and segregation of mitDNA in vegetative growth, is such that the genetic consequences of the organisation of mitDNA into chondriolites must at present remain the subject of

speculation. However, if the recombination process in some way results in a unification of the genetic content of the molecules participating in a given recombining pool, the chondriolite emerges as an important heritable unit of the system.

Whether or not this is so, the newly evolving 'fluid' concept of the mitochondrial system is likely to be fruitful in interpreting genetic observations. As an example, Dujon et al.(1976) recently suggested that the appearance of mitochondrial mutants resistant to erythromycin involves a period of intra-cellular selection of the mutations prior to exposure to the antibiotic. The 'fluid' nature of the mitochondrial system discussed here may well suggest a reasonable solution to problems of this type.

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Mitochondrial Involvement in the Control of Cell Function in Saccharomyces cerevisiae

I.H. Evans, V. Egilsson, D. Wilkie

INTRODUCTION

Cytoplasmic respiratory deficient mutants (petite, p^-) of S.cerevisiae show various degrees of genetic lesion in mitochondrial DNA with an apparent total loss of this molecule in some cases (p^o). The latter show a concomitant alteration in cellular tolerance to the membrane-reacting drug chlorimipramine (CI) compared with parental (p^+) strains while p^- petites may or may not show this change (Linstead, Evans and Wilkie, 1974). From these studies with CI, the tentative conclusion was drawn that transcription products of mitochondrial DNA had a controlling influence on cell membrane development. This conclusion was substantiated by the finding that cells grown in the presence of low concentrations of acriflavine, a selective inhibitor of mitochondrial transcription, showed reversible alteration not only in CI tolerance but also in the ability to utilize galactose as a carbon source (Evans and Wilkie, 1975 & unpublished results).

A series of experiments have now been carried out extending these studies of the effects of mitochondrial change on cellular function. Mitochondrial change was both at the genetic level in petite mutants and by treatment with a wide variety of mitochondrial inhibitors.

MATERIALS AND METHODS

Culture medium contained yeast extract (1%) supplemented with one or other of the carbon sources dextrose (D,2%), galactose (GA,2%), α-methyl-D-glucoside (MG,2%), maltose (MA,2%) and glycerol (G,4%). Drugs were added to autoclaved agar medium in concentrations as indicated in the text. A multiple drop-inoculum technique was used in tests of growth capabilities of the yeast strains (for further details see Wilkie, 1972). Adriamycin was kindly donated by Farmaceutici Italia, Milan.

RESULTS

Utilization of sugars by petite mutants.

All parental strains (p^+) were able to utilize GA, MG and MA readily. Petite mutants were obtained either spontaneously or by induction with 20μg/ml acriflavine and, after purification, were tested on sugar media (for previous reports of tests of petite growth on GA and MA see Puglisi and Algeri, 1974, Schamhart et al, 1975). An average of about 30 petites were tested from each strain and it was found that nearly all petites of a particular strain showed the same pattern of ability v. inability to utilize the 3 sugars. The pattern itself was strain dependent and about 1 out of 30 petites in some strains differed from the others. In these tests, growth was recorded after 2-3 days (Table 1) but it was found that a few petites were able to utilize sugars and give colonies after prolonged incubation. Most petites gave no indication of growth after 2-3 weeks incubation. These findings applied equally to spontaneous and induced petites and the data are an extension of those reported in Evans and Wilkie(1976).

When petites from different strains with different sugar deficiencies were crossed, recombination occurred and segregation was seen among the petite diploid progeny (Evans and

Wilkie, 1976). In most of these crosses, the most frequent class among the segregants was the recombinant able to utilize all 3 sugars. It was deduced from these results that transcription products of certain segments of mitochondrial DNA were involved in control of expression of nuclear genes active in sugar metabolism. If any of these postulated segments were deleted in the petite, inability to use the sugars would result. Thus the deletion pattern in the petite would be a characteristic of the parent strain and it could be suggested that in crosses there was a tendency to reconstitute the petite genome by recombining intact segments into one genome.

A petite of strain D4 (which was able to use GA) was grown in the presence of 100µg/ml acriflavine in D medium in the expectation that the treatment might further degrade the mitochondrial DNA. 70% of treated petite cells were subsequently found to have lost the ability to utilize GA, which agreed with the prediction. In strain D18 on the other hand, further treatment of petites with acriflavine had no significant effect on their ability to use MG. In crosses between p^+ strains, meiotic tetrads were microdissected. In some of these crosses, petites derived from half of the ascospore cultures were able to use a particular sugar while petites from the remaining half were unable to do so. That is, the sugar-utilization pattern of derived petites segregated in a 2:2 ratio indicating that allelic differences in the nucleus could bring about insensitivity to mitochondrial lesion. In other words, the postulated regulation by mitochondria could be ineffective following appropriate mutational change in nuclear factors.

Petites of several strains were screened for alteration in cellular tolerance to cycloheximide (CHI) compared with the parent strain. Up to 5-fold increases in tolerance were seen but only in the petites of one strain (D26). An increase in

CI tolerance accompanied the CHI resistance in some cases, indicating change in cell membrane permeability to CHI as a likely mechanism of resistance to this drug. These findings seemed to confirm the earlier conclusions of Linstead et al (1974) that mitochondria have a role in plasma membrane biogenesis perhaps by influencing expression of nuclear factors as deduced from the results on sugar utilization.

Inhibitors of mitochondrial transcription.

Acriflavine (ACF), adriamycin (14-hydroxy daunomycin, ADR) and 5-fluorouracil (5-FU) selectively affect mitochondrial RNA synthesis and arrest growth of yeast cells in glycerol medium (Fukuhara and Kujawa, 1970; Evans et al, 1973; Oliver and Williamson, 1974). Concentrations of these drugs which had no detectable effect on growth in glucose medium, were used in GA, MG and MA containing medium. The expectation that depression of mitochondrial RNA synthesis by ACF and ADR, and aberration of transcription products by 5FU, would, in large measure, preclude the utilization of these sugars, was fully realized (Table 1). These inhibitions were reversible (except in some petites which resulted from treatment with ACF and 5FU: ADR is inefficient as a petite inducer). Furthermore, these inhibitors blocked the ability of petite strains to utilize the sugars (Table 2) and this was also expected on the assumption that transcription was proceeding in petite mitochondria.

Inhibitors of respiration.

It appeared that respiratory deficiency itself was not responsible for the inability of petites to use the sugars (otherwise all petites would be similarly deficient) but to test this, cells were incubated with a number of respiratory inhibitors in GA, MG and MA medium. Unexpectedly, these inhibitors (cyanide, antimycin A and oligomycin) prevented

Table 1 The effect of mitochondrial inhibitors and the petite mutation on ability of yeast strains to utilize galactose αmethyl-D-glucoside and maltose*.

Strain	Petite	ER	CAP	ANT	OLI	CN	DAP	5-FU	ACF	ADR	TAA
188wt	-++	-++	R	-++	R	+/---	--+	+++	--+	R	-++
B21	+++	+++	+++	+++	+++	---	+-+	+++	+-+	---	+++
D22	--+	-++	-++	-++	-++	---	--+	NT	--+	---	-++
D26	--+	-++	-++	-++	-++	+-+	--+	---	--+	---	-++
D75	+-+	R	+-+	+-+	+-+	+/---	--+	+++	--+	R	+-+
D6	-+-	-+-	-+/-+	-+-	-+-	+/---	---	-+/--	---	---	-+-
A30	---	---	---	---	---	+/---	---	---	---	---	---
B41	---	---	---	---	---	+/---	---	NT	---	---	+-+
B27	-+-	-+-	-+-	-+-	-+-	+/---	-+-	NT	-+-	---	-++
6-81	---	R	---	-+-	-+-	---	---	+/---	---	---	-+-
A15	--+	--+	--+	--+	+++	+/---	--+	---	-+-	---	-++
D11	+-+	+++	R	+++	+++	+--	+++	NT	+++	R	+++
45B	+-+	+-+	R	+-+	+-+	+--	+++	NT	+-+	R	+-+
D6R3	+-+	+-+	R	+-+	+-+	+--	++/-+	NT	+-+	R	+-+
D4	+-+	+-+	R	+-+	+-+	+/---	++/-+	NT	--+	R	+-+
D18	-+-	R	---	-+-	-+-	---	-+-	NT	---	---	-+-
A285	--+	--+	--+	--+	+++	---	--+	--+/-	--+	---	--+
Ber A	+--	+--	+--	+--	++-	+--	++/-+/-	NT	+--	---	+/---
Ber B	---	---	--+	--+	--+	---	--+	NT	--+	---	+/-+/-+

*+++/--- = ability/inability to grow on GA, MG & MA respectively.
+/- = marginal growth and R indicates that the strain is resistant to the inhibitor on glycerol, NT = not tested.
The inhibitors and concentrations used are as follows: ER: erythomycin, 2mg/ml; CAP: chloramphenicol, 1.5mg/ml; ANT: antimycin, 5µg/ml; OLI: oligomycin: 4µg/ml; CN: potassium cyanide, 10mM; DAP: 2,6-diaminopurine sulphate, 0.5mg/ml; 5-FU: 5-fluorouracil, 75µg/ml; ACF: acriflavine, 10µg/ml; ADR: adriamycin, concentrations ranging from 20 to 100µg/ml; TAA: thioacetamide, 20mM.

Table 2 The effect of mitochondrial inhibitors on the ability of petite strains to utilize galactose, αmethyl-D-glucoside and maltose*.

Petite strain	Growth	ER	CAP	ANT	OLI	CN	DAP	5-FU	ACF,10	ACF,20	ADR	TAA
1	+++	+++	+++	+++	+++	+--	+++	+--	-++	---	---	+++
2	-++	-++	-++	-++	+++	---	-++	---	-++	---	---	-++
3	++-	++-	++-	++-	++-	+--	++-	+--	-+-	-+-	---	++-
4	+++	+++	+++	+++	+++	+--	+++	---	+++	++-	---	+/-++/-
5	+-+	+-+	+-+	+-+	+-+	+--	+-+	+--	--+	--+	+++	+-+
6	+++	+++	+++	+++	+++	+--	+++	+--	-+-	-+-	--+	+++
7	+-+	+-+	+-+	+-+	+-+	+--	+-+	+--	--+	---	---	+-+
8	+-+	+-+	+-+	+-+	+-+	+--	+-+	+--	--+	---	---	+-+

*The abbreviations and inhibitor concentrations are as in Table 1, except for ACF,10: acriflavine, 10µg/ml; ACF,20: acriflavine, 20µg/ml; ADR: 20µg/ml.
Petite strains 1 and 2 are diploid and the rest haploid, of different genetic backgrounds.

cells utilizing sugars (Table 1). Cyanide was particularly efficient in blocking sugar utilization not only by parental strains but also by petites (Table 2). These effects were reversible.

Inhibitors of mitochondrial protein synthesis.

Since petite mitochondria are generally unable to synthesize proteins, it was deduced that this deficiency per se was not responsible for failure to use sugars. However, in the presence of erythromycin or chloramphenicol, the sugar-utilizing pattern of the various strains approached that of their petites (Table 1). These specific inhibitors of mitochondrial protein synthesis were unable to affect the ability of petites to utilize a sugar or sugars (Table 2). Puglisi and Algeri (1974) have already shown that erythromycin can block the utilization of GA in certain strains of S.cerevisiae and that this effect is overcome only in GA-constitutive mutants. Our strain D4 and B21 were found to be inducible for galactokinase, but were able to use GA in the presence of the antibiotic.

Other anti-mitochondrial drugs.

2-6-diaminopurine (DAP) an analogue of adenine, selectively blocks mitochondrial biogenesis in all our strains (Lee and Wilkie, 1971). The carcinogen thioacetamide (TAA), likewise has potent anti-mitochondrial activity and prevents cytochrome aa_3 and b development (unpublished results). Both drugs arrest growth on glycerol medium at concentrations which have no significant effect on glucose medium. They are weak inducers of petite but reversibly block the utilization of the sugars (Table 1).

DISCUSSION

The results clearly imply that some mitochondrial function is required in sugar utilization. The possibilities include

a) a product of the energy linked processes of the inner membrane such as ion transport, ATP synthesis or trans-hydrogenation;
b) a protein(s) synthesized on mitochondrial ribosomes;
c) a mitochondrial transcript(s)
d) a product(s) of the inner membrane enzyme system not directly related to energy linked functions.

Loss of functions a, b and c result from the petite mutation and also that of d if lack of certain mitochondrial proteins causes disruption of the inner membrane system.

The effects of ER and CAP, which mimic the petite phenotype, are compatible with a, b, c and d but less readily reconciled with c unless there is coupling of a complex nature between mitochondrial translation and transcription of certain segments of mitochondrial DNA. The effects of the respiratory chain inhibitors ANT and OLI are also compatible with all possibilities: it has been shown that cytochromes of aerobically grown cells are considerably suppressed by these inhibitors presumably in bringing about some kind of feedback inhibition of mitochondrial protein synthesis (Kovac et al, 1970). Also Luzikov (1973) has demonstrated that the inner mitochondrial membrane of S.cerevisiae is degraded under aerobic conditions in the presence of respiratory inhibitors.

The data from petite crosses showing that sugar utilization abilities can be recombined, strongly implies that the required function is not a product of the well-defined energy-linked systems since all petites are alike in possessing inner membranes incapable of energisation. The mode of inheritance seen in these crosses rather suggests that the

necessary function is related to mitochondrial DNA, for example, transcription.

The transcription inhibitors ACF, 5FU and ADR differ from inhibitors of function in being more effective in suppressing sugar utilization both in the parental strains and in sugar-positive petites (Petites transcribe their mitochondrial DNA, see Borst, 1972). In view of the likelihood that mitochondrial DNA replication and transcription are dependent on an intimate relationship with the inner membrane, and since ACF for example, in view of its similarity of action to ethidium bromide, probably interacts with the inner membrane as well as with mitochondrial DNA (see Mahler and Perlman, 1972), it is possible that the action of these inhibitors in the context of sugar utilization is at a site in the inner membrane distinct from but adjacent to, mitochondrial DNA. The especially potent effect of KCN might then be interpreted as resulting from drastic disruption of inner membrane structure and function. The data from petite crosses could also fit this interpretation if the segregation and recombination were of areas of inner membrane with a degree of conformational stability and heritability (cf. Pitot's "membrons" (Pitot, 1974)) associated with the state of individual mitochondrial DNA molecules.

The functions of these inner membrane systems is presumably enzymic and can be envisaged as the provision of a molecular species with a signalling role in regulating the expression of nuclear genes concerned, in this case, with sugar utilization (but probably also those specifying plasma membrane components although the evidence for this is still circumstantial). (That we are dealing with regulation was substantiated by the finding that petites of strain A285 which were unable to utilize GA, could not be induced to form galactokinase.) The most popular candidate for such a role is cAMP and indeed,

some of the components of a cAMP system - adenylate cyclase and phosphodiesterase - have been located in animal mitochondria (Sulimovici et al, 1975 and Van den Berg, 1975).

Other possible mitochondrial enzymes with a key regulatory role are the poly (A) polymerase (Saccone et al, 1974) and ADP ribosylation systems of the kind identified in rat liver mitochondria (Kun et al, 1975).

The systems for utilizing GA, MG and MA are all catabolite repressible and our findings suggest that the inner mitochondrial membrane system may act in mediating the derepression of some catabolite repressible nuclear genes. This hypothesis may be compared with the observations made by Subik and colleagues (1972).that a non-energy linked mitochondrial function is required for continued cell growth and division as evidenced by the growth inhibitory effects of bongkrekic acid and the $\underline{op}_1$ mutation (both acting to suppress adenine-nucleotide translocation in the mitochondrial inner membrane) in petite cells.

The data reported here for the liver carcinogen thioacetamide suggest that its carcinogenic activity may result from an effect at a site in the inner mitochondrial membrane concerned with the regulation of the expression of nuclear genes. Hadler and coworkers (Hadler et al, 1971) have also provided evidence that carcinogens may interact with the mitochondrial inner membrane, and they suggest that alteration of this membrane system may be a significant step in oncogenesis.

ACKNOWLEDGEMENT

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Ultrastructural Characterization of Mitochondria from a Yeast Mutant Sensitive to „Petite" Induction (uvs ϱ 72)

B.J. Stevens, E. Moustacchi

SUMMARY

Mitochondrial structure in a yeast mutant sensitive to *petite* induction was compared with that in the parental wild type strain and in an isogenic *petite* strain. In glucose medium, mutant cells are of 3 types : 1) normal with typical short mitochondrial cristae, 2) *petite* with small vesicular mitochondria and transverse *lamellae* and, 3) those containing a "mixed" population of mitochondria. It is proposed that the transformation *grande* to *petite* occurs at the level of individual mitochondria and involves a progressive rearrangement of cristae. In glycerol medium, the only visible abnormality in the mutant is the frequent presence of an unusual mitochondrial form, named *doublet*. The high frequency of *doublets* is a characteristic of the uvsρ 72 mutant and is likely to be related to its susceptibility to *petite* transformation.

INTRODUCTION

Five mutants of *Saccharomyces cerevisiae* (uvsρ) which are highly sensitive to induction of the cytoplasmic *petite* mutation (ρ-) by UV irradiation, but which are otherwise similar to the original wild type strain N123 in sensitivity to nuclear damage have been isolated and genetically characterized (1, 2). One of them, uvsρ 72, shows this enhanced susceptibility to *petite* induction, not only by UV irradiation but also by other agents such as ethidium bromide and thermal shock (1, 3). It also demonstrates a high frequency of spontaneous production of *petites* when grown in glucose. The uvsρ character in this mutant exhibits a non-Mendelian pattern of inheritance and further, it is associated with the mitochondrial ρ factor as demonstrated by its loss in a ρ° strain derived from uvsρ 72 (unpublished data). Indications of a mitochondrial disorder at the enzymatic level in uvsρ 72 have been provided by Mahler and coworkers (4, 5) in their studies

on the series of reactions following ethidium bromide binding to mitochondrial DNA.

The mitochondrial nature of this mutation led us to compare the fine structure of the mitochondria of the mutant with that of the wild type N123 and that of a spontaneous *petite* derived from N123. Furthermore, the ease and the high frequency of spontaneous *petite* induction in uvsρ 72 provides a suitable material for visualizing mitochondrial changes during the transformation *grande* to *petite* due to a specific mutation.

MATERIALS AND METHODS

Strains, media and growth conditions. The haploid wild type (ρ^+) strain of *S. cerevisiae*, N123 (a his_1), and two derived strains, a spontaneous suppressive petite strain, ρ- 24, and the uvsρ 72 (ρ^+) mutant strain, were used in these studies. Cultures were grown aerobically at 30° on YEP (Yeast extract Difco 1 %, Bacto-peptone 2 %, glucose 2 % w/v) or on YEPG (glucose replaced by 3 % glycerol). Cells were harvested in mid-exponential phase ($3\text{-}5\cdot10^7$ cells/ml), in early stationary phase ($1\text{-}2\cdot10^8$ cells/ml) and in late stationary phase of growth ($4\text{-}5\cdot10^8$ cells/ml). The frequency of complete *petite* colonies and of sectored colonies was routinely determined by plating suitable aliquots on solidified YEP. After 4 days growth, colonies were stained by the tetrazolium overlay technique (6).

Electron microscopy. Cells were centrifuged, washed twice in 0.9 % NaCl and prepared for electron microscopy. To avoid the limitations imposed by a single technique, we have made use of several different fixation and staining procedures.

Permanganate fixation. Cells were resuspended in a 4 % aqueous solution of $KMnO_4$ for 4 hours at 4°, washed in distilled water and in 0.1 M phosphate buffer and assembled into fibrin clots (7). The cells were stained overnight in a 1 % aqueous solution of uranyl acetate, dehydrated and embedded in Epon or in Spurr resin. In some cases, a preliminary aldehyde fixation of 1 hour was used prior to permanganate : paraformaldehyde 2.5 % and glutaraldehyde 3 % (Eastman, 50 %) in 0.1 M cacodylate or phosphate buffer at pH 7.

Aldehyde-Osmium fixation. In order to assure penetration of fixatives and resin without excessive damage to cell structure, spheroplasts were prepared by a procedure milder than that recommended by Kováč *et al.* (8). Cells were resuspended in 0.5 M thioglycollic acid, buffered with 0.1 M Tris, pH 9.3 and incubated for 5 to 10 minutes at 30°. The cells were washed in medium A (0.3 M mannitol, 0.7 M sorbitol, 10 mM citrate-phosphate buffer, pH 5.8 and 1 mM EDTA) and resuspended in

medium B (same as A but lacking buffer) containing glusulase (Endo Lab.) at a concentration of 0.01 ml/ml medium. Incubation was for 5 to 10 minutes at 30°.

Spheroplasts were washed twice in medium B, resuspended in paraformaldehyde-glutaraldehyde fixative (above) and fixed 1 hour at 4° or at room temperature. Aldehyde fixed cells were washed in buffer, post-fixed for 2 hours in 2 % OsO_4 buffered by 0.1 M cacodylate or phosphate pH 7, assembled into fibrin clots, dehydrated and embedded.

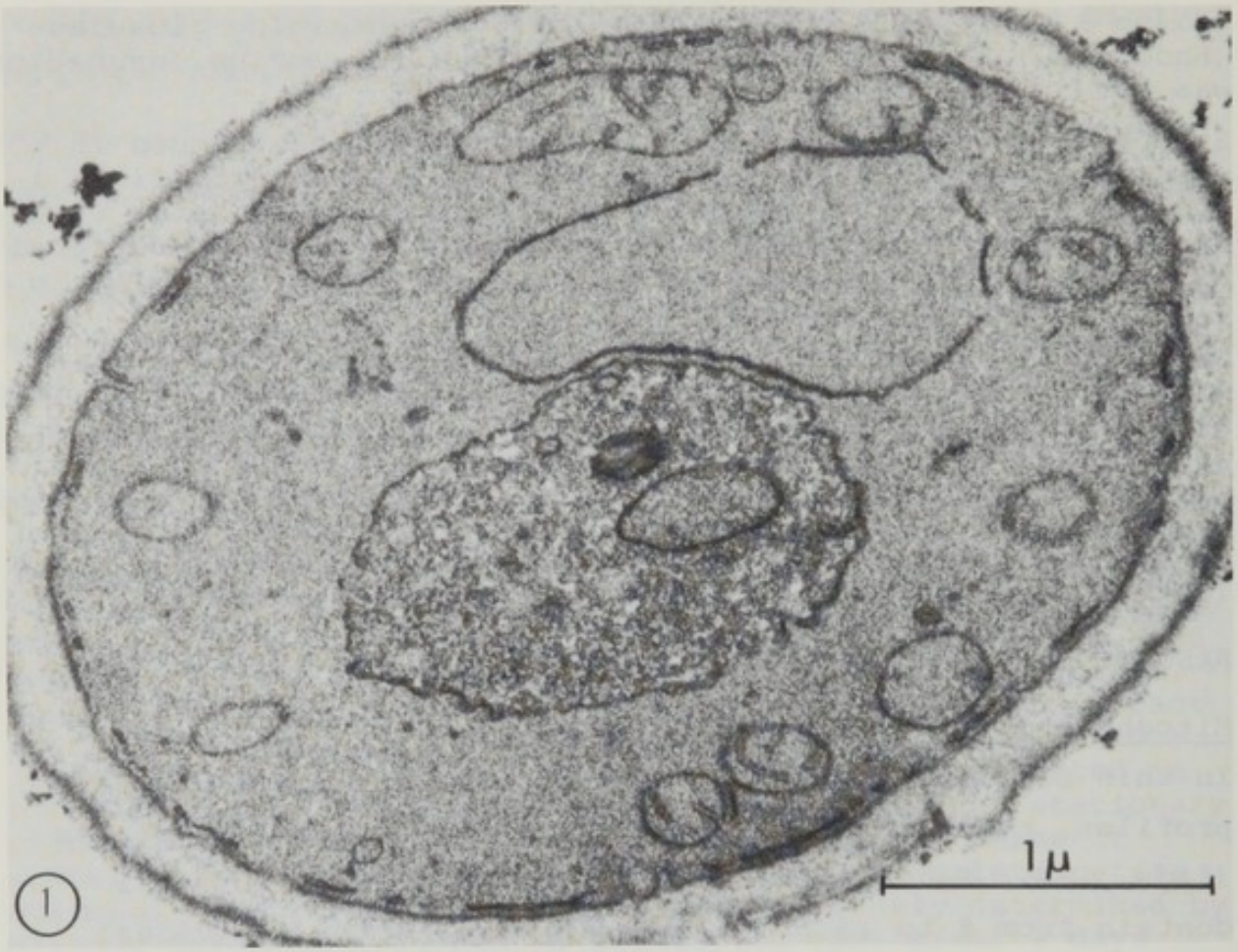
Staining. Thin sections were stained with lead citrate (permanganate fixation) or with 5 % aqueous uranyl acetate followed by lead citrate (OsO_4 fixation). Staining of polysaccharides was carried out using the periodic acid-thiosemicarbazide-silver proteinate technique (P.A.T.Ag) described by Thiéry and Rambourg (9). Demonstration of cytochrome oxidase and cytochrome c peroxidase activities by 3,3'-diaminobenzidine (DAB) (Sigma) was carried out according to Todd and Vigil (10).

RESULTS

Mitochondria in wild type strain N123

In thin section, mitochondria exhibit rounded or elongate profiles, regularly distributed in the peripheral cytoplasm (figs. 1-4). Sections close to the long axis of the cell contain from 8 to 12 profiles (not necessarily individual mitochondria). The cristae in mitochondria from respiring wild type cells are short plates, irregularly positioned, which, in general, do not extend across the organelle but terminate freely about halfway into the matrix (fig. 2). The population of mitochondria in a single cell (chondriome) is homogeneous and unvaried. As already described (11) some changes in mitochondrial morphology were observed according to the growth conditions : in mid-exponential phase, mitochondrial profiles are larger and cristae are fewer (figs. 3, 4) whereas in stationary phase, profiles are smaller and more numerous (fig. 1).

Following aldehyde-osmium fixation, mitochondrial ribosomes are observed, principally along the inner membrane at the periphery and more rarely along the cristal membranes (fig. 3). DNA fibrils can often be distinguished in clear areas of the matrix. Following aldehyde-permanganate or simple permanganate fixation, only the mitochondrial membranes are preserved ; the disappearance of the opacity of the cytoplasmic background permits



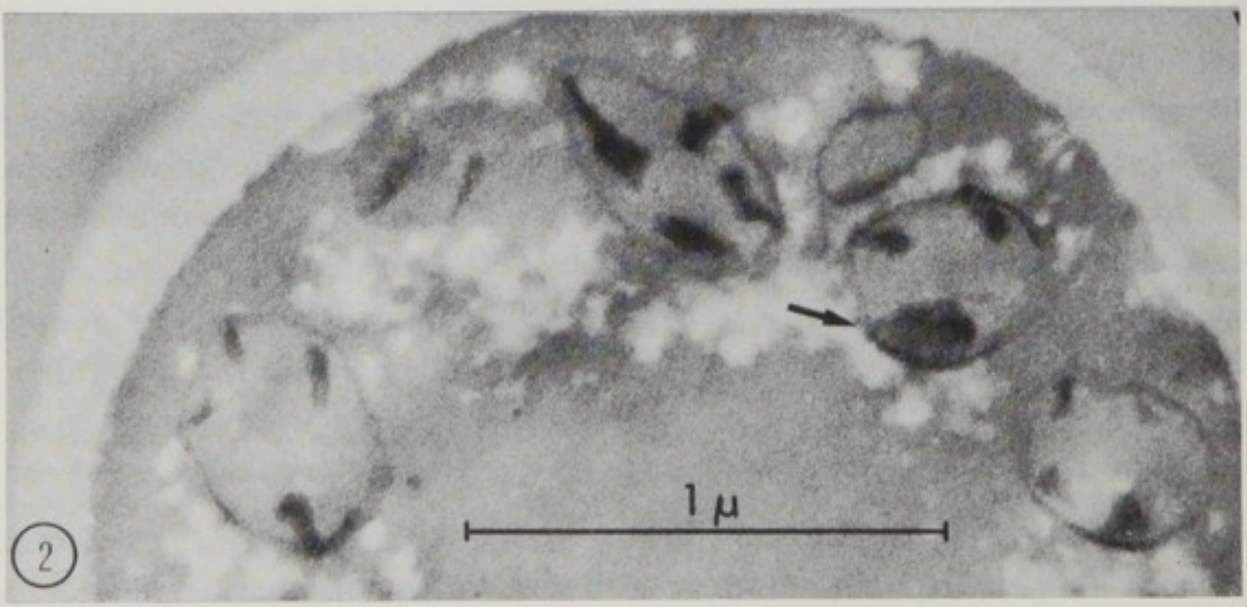
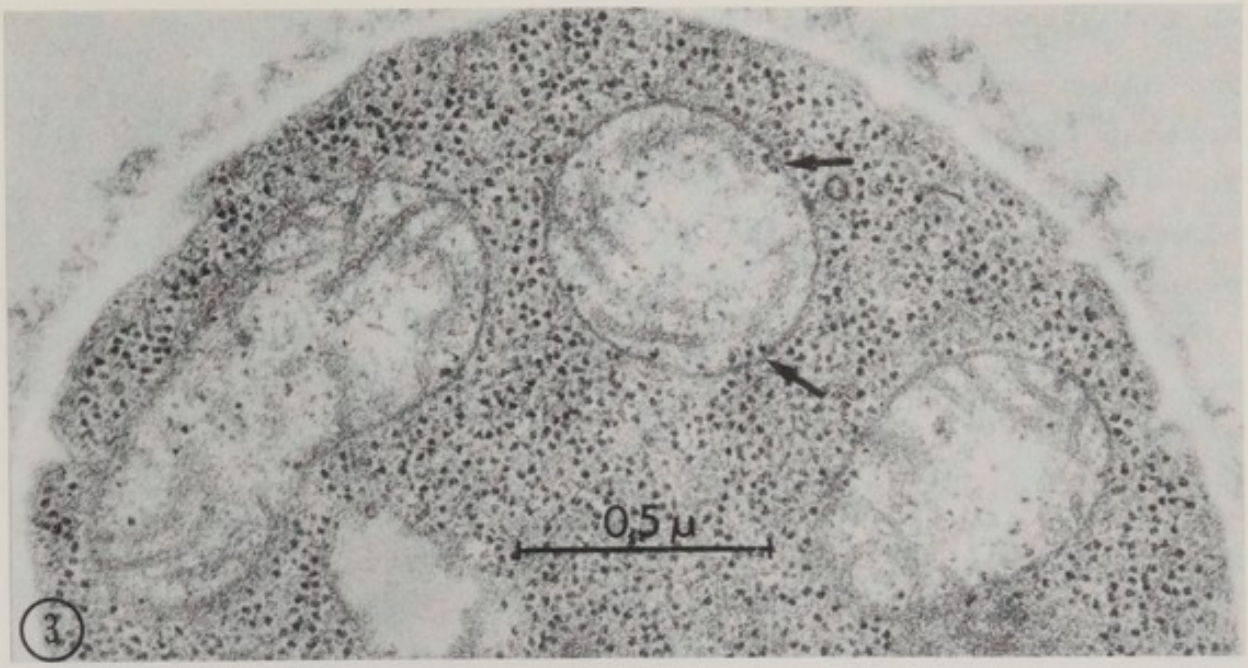


Figure 1. Wild type N123 cell from stationary phase culture in YEP. Mitochondrial profiles are evenly distributed at the periphery. Cristae are short and irregularly positioned. Aldehyde-$KMnO_4$ fixation.

Figure 2. Stationary phase N123 cell in YEP. DAB reaction. Mitochondrial cristae contain the electron-dense reaction product ; one of them is seen in face-on view (arrow).



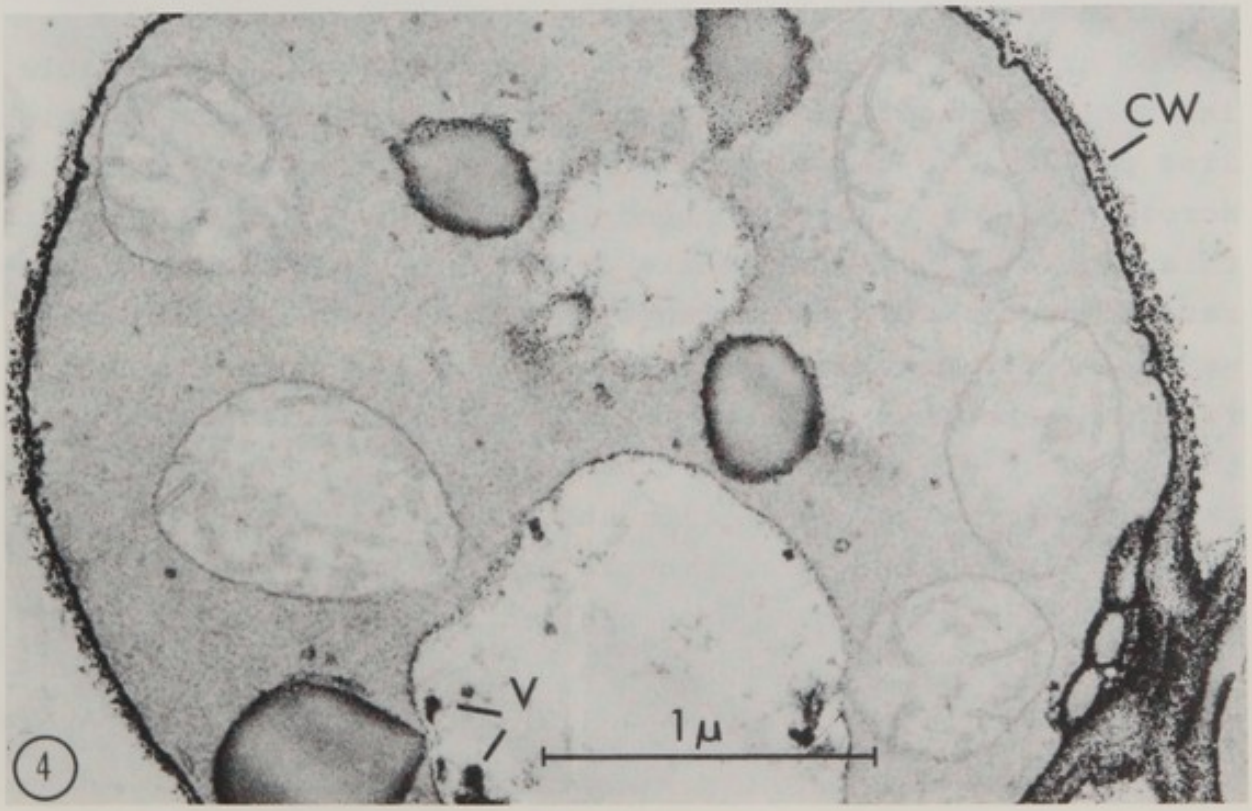


Figure 3. Mid-exponential phase N123 cell in YEPG. Aldehyde-osmium fixation, uranyl and lead staining. This technique visualizes mitoribosomes (arrows). Mitochondrial cristae are short.

Figure 4. Same as fig. 3 but stained by the P.A.T.Ag technique. Mitochondrial profiles are distinct against the pale cytoplasm. Cell wall remnants (CW), vacuolar components (V) are specifically stained for polysaccharides.

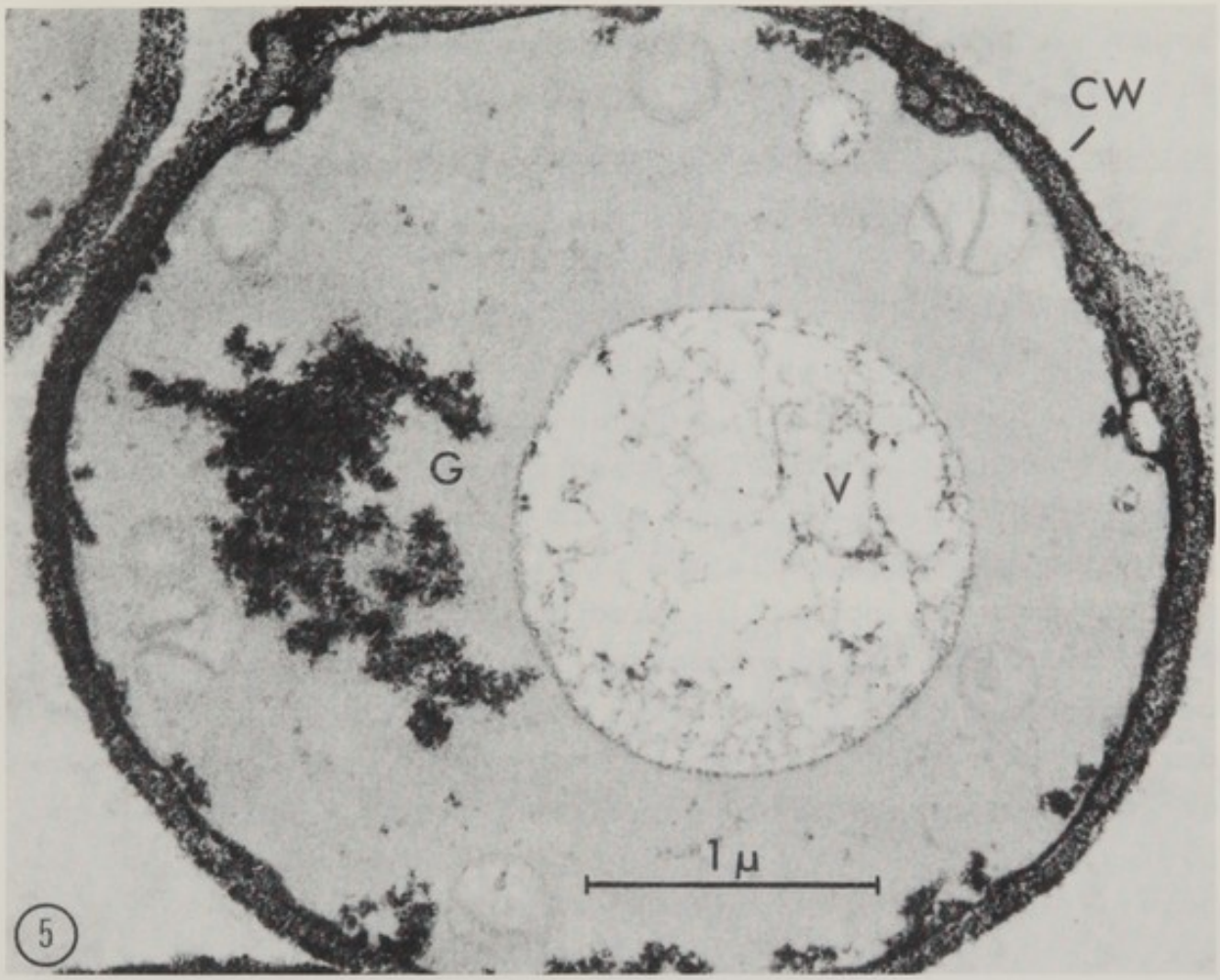
an unobstructed view of mitochondrial profiles (fig. 1).

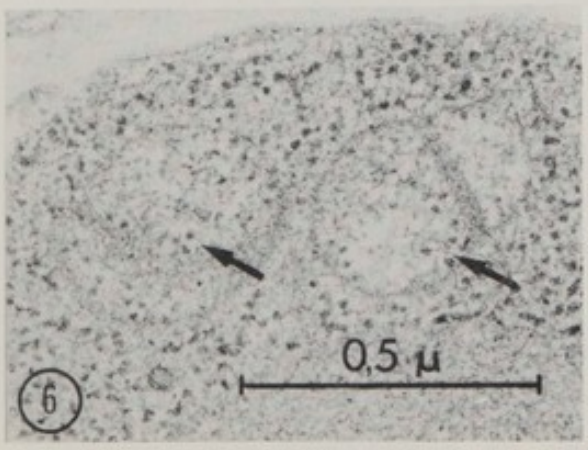
The DAB reaction clearly and densely stains mitochondrial cristae and, to a lesser extent, the outer envelope (fig. 2). The P.A.T.Ag technique was found to produce clear images of aldehyde-osmium fixed material in which only the cell wall remnants, glycogen and some cytoplasmic vacuoles are specifically stained (fig. 4). A very weak staining of mitochondrial membranes against the unstained cytoplasmic background allows a clear distinction of mitochondria, usually buried in the opaque ribosome-packed cytoplasm after uranyl-lead staining.

Mitochondria in *petite* strain, ρ-24.

The isogenic *petite* strain 24 was examined for a characterization of *petite*-type mitochondria. Most often, *petite* mitochondria are spherical or slightly elongated and they lack normal cristae. In sections, many of them have no discernible internal organization ; others contain a double membrane which lies parallel to the surface or extends as a curved partition across one end of the mitochondrion (figs. 5-7). Both ends of this double membrane terminate at the envelope, creating separate compartiments. To distinguish these from normal cristae, we will refer to them as *lamellae*. Larger and more aberrant forms of membranes such as whorls are likewise found. Owing to the presence of the *lamellae* there is little confusion possible between a normal mitochondrion and these *petite* mitochondria. Moreover, the *petite* mitochondria are not evenly distributed in the cell, but tend to accumulate in some regions and to be absent in others. Our description of the ρ- phenotype concurs with those given by Yotsuyanagi (12) and by Federman and Avers (13).

The staining properties of *petite* mitochondria are similar to those of wild type. It is noteworthy that the DAB reaction is positive and that the electron-dense reaction product is present especially in the *lamellae* (fig. 7). Due to the known absence of cytochrome oxidase in *petite* mitochondria, it is probable that DAB is reacting with cytochrome c peroxidase as





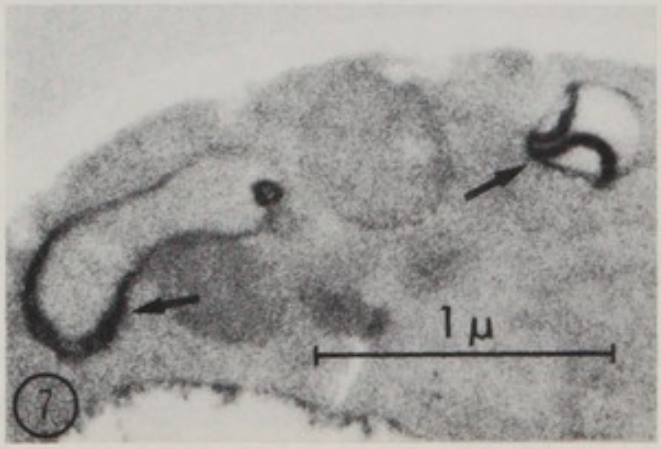


Figure 5. Stationary phase ρ⁻24 cell in YEP. Aldehyde-osmium fixation, P.A.T.Ag staining. Mitochondrial profiles are round vesicles which lack normal cristae. In some, membranous lamellae form curved partitions. Glycogen (G), cell wall (CW) and vacuolar contents (V) are specifically stained.

Figure 6. Same as fig. 5 but uranyl and lead staining. Mitochondrial profiles contain some ribosomes (arrows).

Figure 7. Stationary phase ρ⁻24 cell in YEP. DAB reaction. Mitochondrial lamellae exhibit the reaction product (arrows).

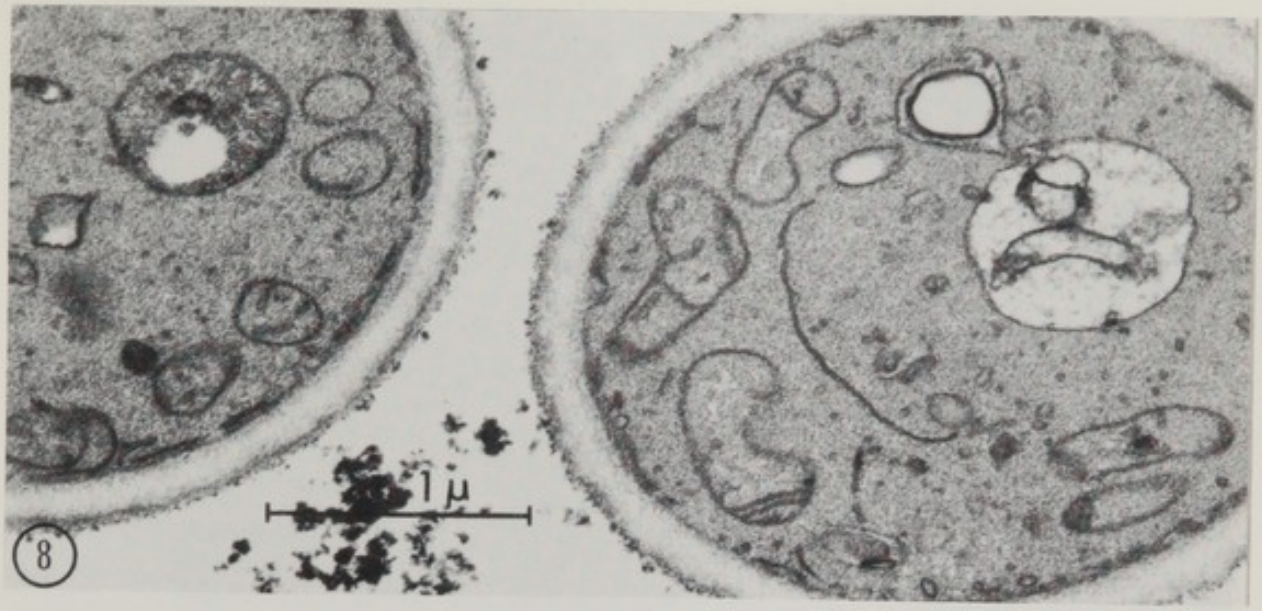
proposed by Todd and Vigil (10). Ribosome particles and DNA filaments were occasionally observed (fig. 6).

Mitochondria in the uvsρ 72 strain.

The mutant strain has a slower growth rate than N123 in glycerol medium. It regularly produces up to 10 % complete *petite* colonies and from 10 to 28 % sectored colonies when plated on glucose medium.

Glucose-grown cultures of uvsρ 72 are constituted by a heterogeneous population of cells which can be grouped into three main categories according to their mitochondrial phenotypes. The majority of the cells are similar to the wild type ; their mitochondria are normal in structure, but they may be irregularly distributed and aggregated in some regions. A second category of cells which exhibit the characteristics of *petite* cells is observed. This is expected since glucose medium allows growth of spontaneous *petite* cells. In several different experiments, estimates of their proportion in thin sections gave between 8 and 18 %. These figures are in reasonable agreement with the frequencies of *petite* colonies scored on plates. Most of these cells resemble closely the ρ-24 strain and contain only small vesicular mitochondria (fig. 10). Others, however, demonstrate more grossly altered mitochondria which are larger, swollen, sometimes distorted and possess internal *lamellae* (figs. 8,9). In *petite* cells, all mitochondria are abnormal. The third category comprises about 5 % of the population. These cells have a "mixed" chondriome, that is one or more normal mitochondria co-existing in the same cytoplasm with one or more *petite*-like mitochondria. The atypical mitochondria are generally of the vesicular type with one or sometimes two internal *lamellae* extending across one end or running parallel to the envelope (figs. 11, 12). A few "mixed" cells have one or more swollen and distorted *petite*-type mitochondria along with other normal ones. In others, the mitochondria are normal in size but most of them display atypical transverse *lamellae* (fig. 13).

When uvsρ 72 is grown on glycerol, the spontaneous *petite* cells



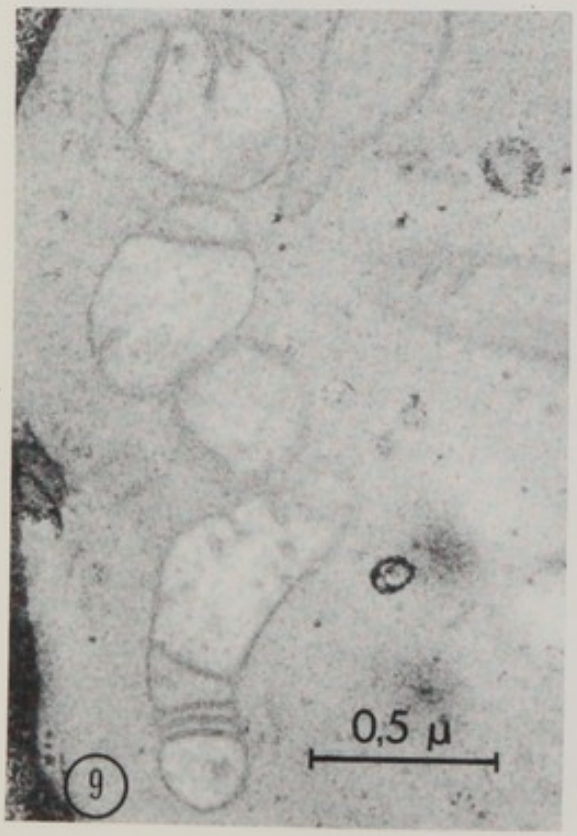
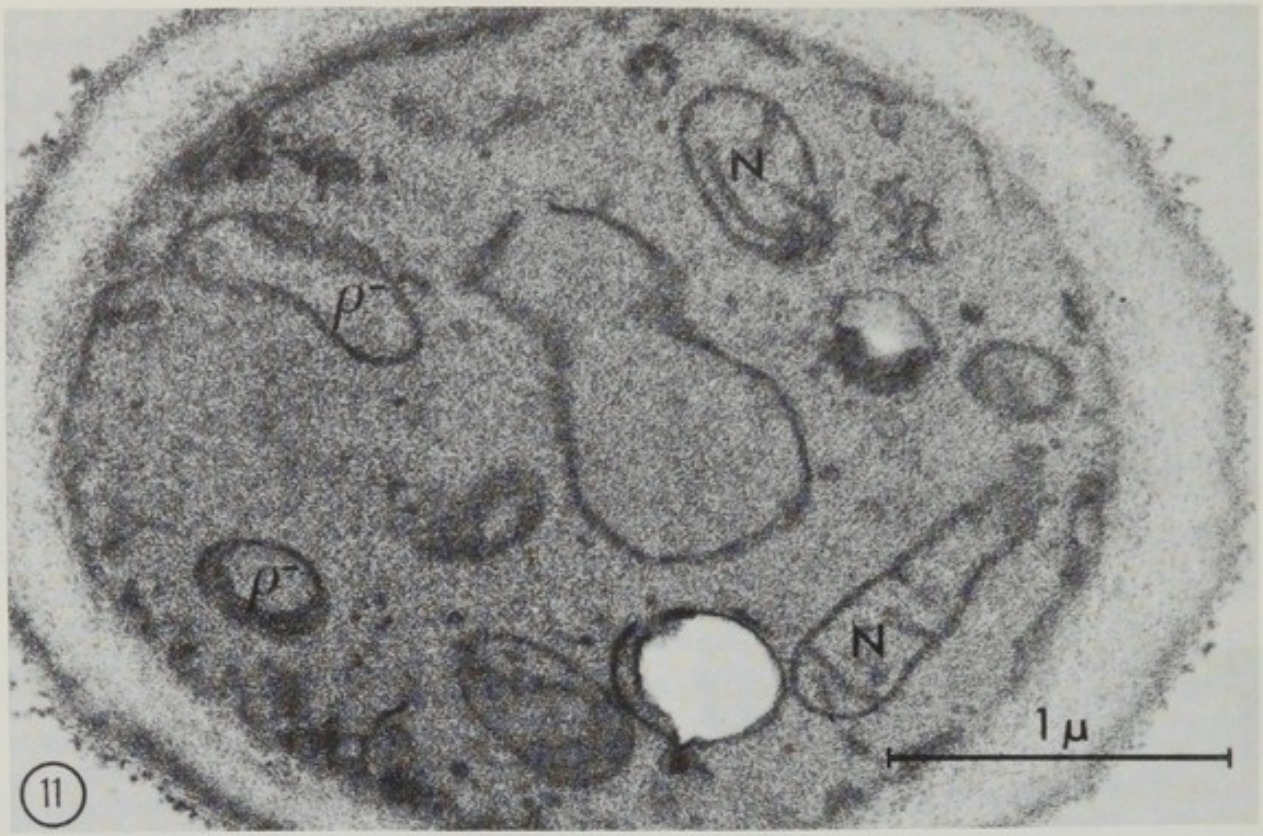


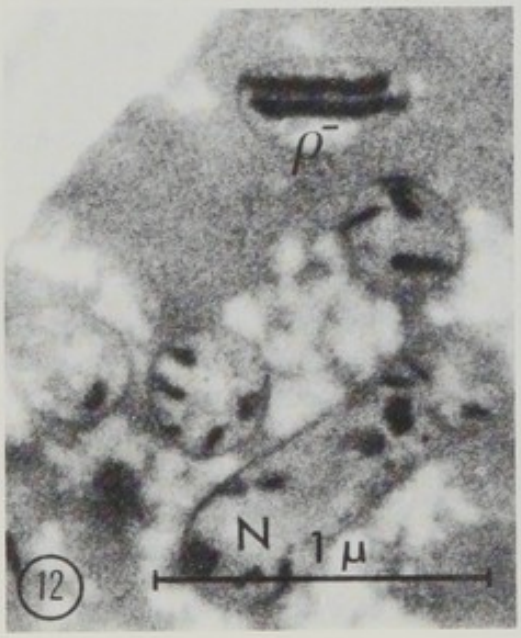


Figure 8. Stationary phase uvsρ 72 cells in YEP. Aldehyde-$KMnO_4$ fixation. Normal mitochondria appear in cell at left ; at right, a *petite*-type cell whose mitochondria are grossly altered.

Figure 9. *Petite*-type uvsρ 72 cell in YEP showing abnormal mitochondria as in fig. 8. Aldehyde-osmium fixation, P.A.T.Ag staining.

Figure 10. Typical *petite* cell in uvsρ 72 culture in YEP. Aldehyde-$KMnO_4$ fixation. All mitochondria are small and vesicular as in ρ^-24 strain.





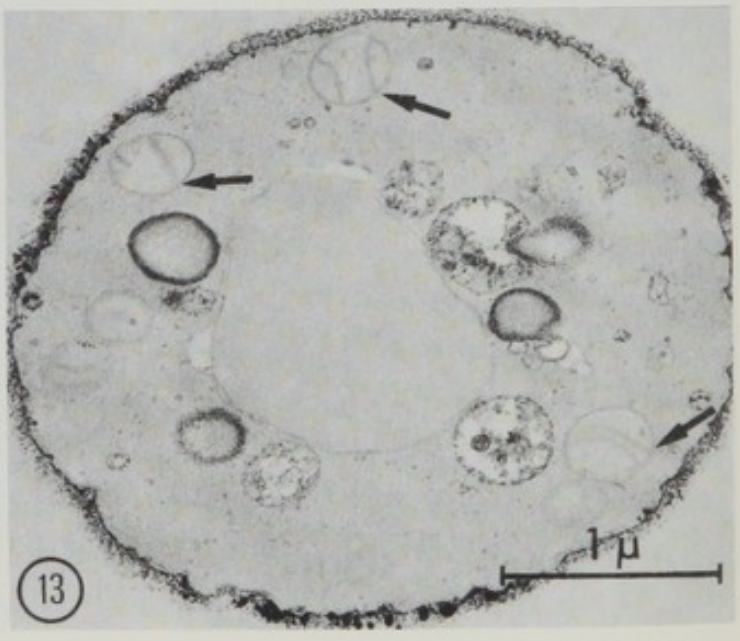


Figure 11. Stationary phase uvsρ 72 cell in YEP containing "mixed" chondriome. Normal cristate mitochondria (N), *petite*-type mitochondria (ρ^-).

Figure 12. Stationary phase uvsρ 72 cell in YEP with "mixed" chondriome. DAB reaction. Parallel *lamellae* indicate *petite*-type mitochondrion (ρ^-), others are normal (N).

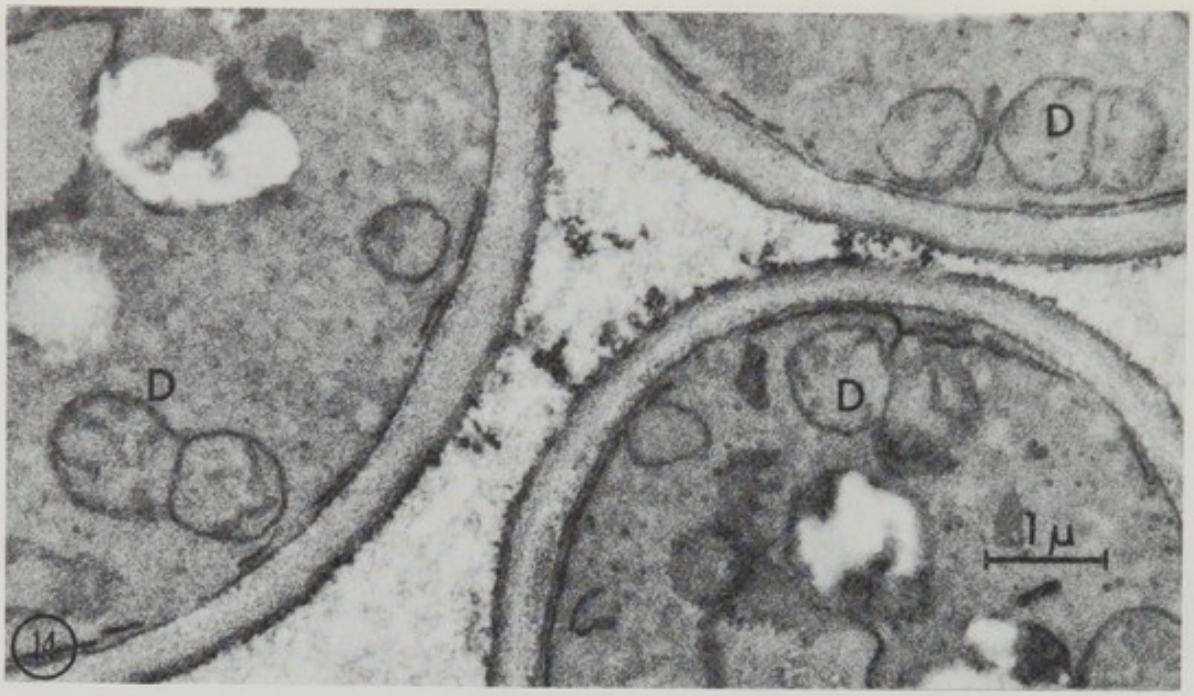
Figure 13. Stationary phase uvsρ 72 cell with "mixed" chondriome, P.A.T.Ag staining. Most mitochondria contain abnormal transverse *lamellae* (arrows).

are eliminated from the population. No cells with *petite* or "mixed" mitochondrial phenotypes are observed. However, a high proportion of cells contain one or more mitochondria which are "double". *Doublet* mitochondria are generally elongate and they have a septum parallel to the short axis of the mitochondrion and running roughly across the middle (figs. 14-16). In most cases, the mitochondrial outline is idented at the septum, forming a flattened figure eight. The outer membrane can sometimes be seen outside the septum while the septum itself appears to be formed by two apposed membranes.

Doublet mitochondria were observed in all cultures of glycerol-grown uvsρ 72 cells and after all the fixation and staining procedures used. The proportion of cells having one or more *doublets* in a section varied from about 30 to 70 %. Some glucose-grown cells likewise contained this unusual form, but usually less than 10 %. *Doublets* were also detected in N123 cells but their frequency in most cases was less than 10 % and was always lower than that in the mutant strain under the same experimental conditions. In uvsρ 72, the growth phase had little effect on *doublet* frequency, but the highest frequencies were found after incomplete spheroplast formation and aldehyde-osmium fixation. Since this procedure produces some cytoplasmic swelling, it is conceivable that some *doublets* were artifactually formed. Nevertheless, even if this is so, the constantly lower frequencies of *doublets* in N123 after identical treatment indicates a differential response and points to this mitochondrial phenotype as a character of uvsρ 72. From serial sections of one $KMnO_4$-fixed uvsρ 72 cell in late stationary phase on YEPG, of 44 individual mitochondria, 13 were *doublets* or ever tri- or quadripartite.

DISCUSSION

The uvsρ 72 mutant is unable to repair UV-induced damage to its mitochondrial DNA, while it conserves the ability to repair nuclear damage (1). In addition to or perhaps in connection with defects in the repair system, there is also evidence for a



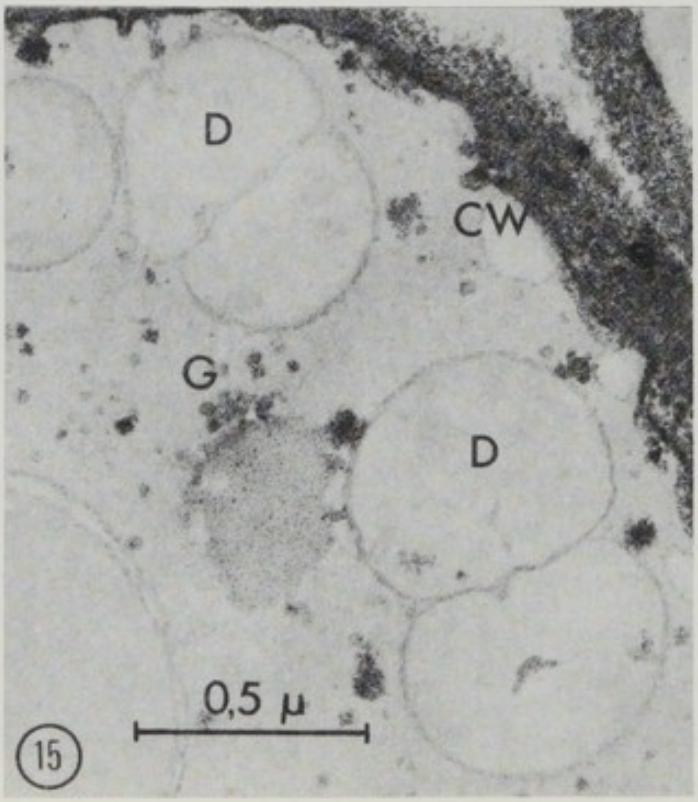


Figure 14. Stationary phase culture of uvsρ 72 in YEPG. Three cells each contain a *doublet* mitochondrion (D). $KMnO_4$ fixation.

Figure 15. Mid-exponential phase uvsρ 72 cell in YEPG. Two *doublet* mitochondria (D) are present ; each possesses a membranous, median septum. Glycogen (G) and cell wall (CW).

Figure 16. Stationary phase uvsρ 72 cell in YEPG. DAB reaction. A *doublet* mitochondrion contains electron-dense reaction product in septum and in cristae.

disorder at the level of production of mitochondrial ATPase (4, 5) as well as for other alterations in the respiratory chain (P. Perlman, A. Goffeau, personnal communications). In view of these biochemical changes, it is of particular interest that we have found some specific morphological changes in the mutant mitochondria.

When uvsρ 72 is grown on glucose, the *petite* cells which are spontaneously produced during culturing continue to grow and can be recognized by their typical *petite* mitochondrial phenotype. Those which contain large, distorted mitochondria are intriguing. This mitochondrial phenotype may represent a further intermediate form, possibly resulting from a fusion of several individual mitochondria. It is also possible that this particular *petite* cell type is specific to the uvsρ 72 strain. Examination of *petite* colonies derived from the mutant may resolve this point.

More significant, however, are the cells which demonstrate a *partial* transformation of their chondriome ; these contain only one or several abnormal mitochondria while conserving at least a few others with a normal structure. It is tempting to correlate the presence of these "mixed" cells with the high frequency of sectored colonies seen on plating. The "mixed" population of mitochondria in these cells suggests that the transformation *grande* cell to *petite* cell is a stepwise process in which each mitochondrion undergoes separately its own transformation, not necessarily in synchrony with the others in the same cell. Our observations suggest that one step in this process is the change in the internal architecture of the mitochondrion : the alteration from the normal, short cristae to the transverse lamellar type. We have been able to observe only the form and the distribution of the membranes ; other structural or biochemical changes are likely to accompany the process as well.

A "mixed" population of mitochondrial phenotypes within single yeast cells has been reported in zygotes from a $\rho^+ \times \rho^-$ cross (13) and in certain respiratory-competant mutants, on the basis

of a histochemical diversity (14). Unlike these examples in which two populations were independently perpetuated during many cell generations, the "mixed" population in some uvsρ 72 cells suggests an unstable chondriome, capable of complete transformation to *petite* phenotype in glucose medium. When grown in non-fermentable medium, only a slight rearrangement of cristae occurs, as evidenced by the formation of *doublets*.

This unusual mitochondrial form, the *doublet*, exists with a high frequency in the mutant strain, particularly in glycerol-grown cells. Indeed, serial sections from one uvsρ 72 cell, in which we found that at least a third of the mitochondria were *doublets*, indicate that most mutant cells contain one or more *doublets*. Although *doublets* were observed in N123, their frequency was always significantly much lower than in uvsρ 72. One interpretation of the *doublet* structure is that the septum results from the partitioning of the mitochondrion by a transverse *lamella*. In this case, the slight swelling which occurs during spheroplast formation serves to emphasize the phenomenon and to render these partially transformed mitochondria more evident. However, since 1) *doublets* are observed after fixation of intact cells, 2) many of these show a symmetrical figure eight form, and 3) their frequency is particularly high in uvsρ 72, a mutant known for its disposition to ρ^- formation, a more likely interpretation is that *doublets* represent a step in mitochondrial division. Figures of partitioned mitochondria in other organisms where the mitochondrial population was rapidly expanding have been linked to mitochondrial division (14). In either case, the accumulation of *doublet* forms in the uvsρ 72 mutant points to a disturbance or a defect in its mitochondrial biogenesis. This feature is likely to be related to the other known characteristics of the mutant.

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The Killer Character in Yeast: Preliminary Studies of Virus-Like Particle Replication.

E.A. Bevan, A.J. Herring

SUMMARY

The genetic determinant of the cytoplasmically inherited killer character in yeast has been shown to be the dsRNA genomes of virus-like particles. Novel RNA species which are believed to be the replicative intermediates of dsRNA replication have been isolated from particles prepared from actively growing cells. The particles have been found to possess a capsid associated RNA polymerase activity. This enzyme incorporates nucleotide triphosphates into RNA species with the same characteristics as the putative replicative intermediates and, thus, appears to be a replicase. These results are discussed in relation to those obtained from other dsRNA mycophage.

INTRODUCTION

The cytoplasmically inherited killer character of the yeast Saccharomyces cerevisiae (1,2) has been equated with the presence of dsRNA genomes of VLPs similar to the dsRNA 'mycophage' found in a variety of other fungi (3,4,5). The main features of the system follow. Two species of dsRNA (of molecular weights 2.5×10^6 and 1.4×10^6 daltons) are found in killer cells. We shall term these dsRNA-250 and dsRNA-140. Sensitive strains possess dsRNA but have either dsRNA-250 together with a second species of lower molecular weight than dsRNA-140 or dsRNA-250 alone. Some sensitive strains do not possess any dsRNA at all. In situations where the killer character is segregating, either meiotically or mitotically, the dsRNA-140 is

dsRNA = double stranded RNA. VLPs = virus-like particles.

co-inherited with toxin production. All segregants normally have dsRNA-250. However, sensitive segregants fail to maintain dsRNA-140 and in some exceptional crosses lose both dsRNA species (4,6). The dsRNAs are contained in isometric VLPs and are separately encapsidated in particles which appear to be identical in protein composition (7,8).

As yet no infective cycle has been demonstrated for any of the dsRNA mycophages(5). It appears that the particles replicate in step with the cell in a well controlled manner (9). This integration of the replicative cycle of the VLPs with host cell metabolism is, perhaps, one of the more interesting features of this group of 'viruses'. It is, therefore, of interest to elucidate the mechanism of replication so that control points can be identified. Capsid associated RNA polymerase activity has been reported for Penicillium stoloniferum viruses S and F (10,11,12), Penicillium chrysogenum virus (13) and for Aspergillus foetidus viruses (14). In this communication we report that yeast VLPs have a similar capsid associated activity which may be related to putative replicative intermediates present in particles isolated from actively growing yeast cells in which VLP replication is occurring.

MATERIALS AND METHODS

Chemicals. (^{3}H)-UTP was from the Radiochemical Centre, Amersham, Bucks.,U.K.; unlabelled nucleoside triphosphates were from Boehringer-Mannheim, Lewes, Sussex, U.K.; ribonuclease A, crystalline grade was from Sigma, London, U.K.

Strain. The sensitive strain 3/A1 which possesses only dsRNA-250 was used throughout.

VLP preparation. Cell growth and VLP purification were essentially as reported in (7) with the exception that cell breakage was achieved by agitating a paste of cells and glass beads with a Chemap 'Vibromixer',and the low pH precipitation was omitted from the purification procedure. Instead, the homogenate was processed by three cycles of high/low centrifugation. Crude

VLP preparations were then loaded onto 10-40% sucrose and spun at 27,000 rpm in the SW-27 rotor of a Beckman L265B ultracentrifuge for 3 hours. The gradients were then analysed with an ISCO 640 fractionator.

RNA polymerase assay. The assay conditions described by Chater and Morgan (11) were used with the omission of bovine serum albumin from the assay buffer. (^{3}H)-UTP specific activity was adjusted to 67 mCi/mmole. Incubation time in the experiment described was for 4 hours at 30°C, the reaction was terminated by the addition of the reagents for the phenol extraction which was carried out as described in (4). tRNA was added to the samples to act as a carrier in the ethanol precipitations at a concentration of 200 µg/ml.

RNA analysis. Polyacrylamide gel electrophoresis (15) both in aqueous and formamide gels (15) was performed as is described in (4). The gels were sliced into 5mm slices for counting which was performed in Nuclear Chicago NE-250 scintillation fluid after prior dissolution of the gels in 0.5mls of hydrogen peroxide at 60°C overnight. Ribonuclease digestion was by 1µg/ml of enzyme in 0,15M NaCl plus 0,015M Na-citrate, pH 7.0 for 10 minutes at 16°C.

RESULTS AND DISCUSSION

The Isolation of replicative intermediates of dsRNA.

The final purification step in the isolation of VLPs is performed by sucrose density gradient centrifugation. The OD profile of such a gradient in which VLPs from logarithmic phase cells were sedimented exhibits a shoulder on the slowly sedimenting side of the VLP peak. Such a shoulder is absent when VLPs are isolated from stationary phase cells. This difference in the OD profiles of the gradients is illustrated in Fig. 1.

Particles from the shoulder may be expected to have a lower RNA content than those from the main peak since sedimentation velocity has been found to reflect RNA content both in VLPs from yeasts(7) and in those from other fungi (5). To investigate the nature of the RNA present in the shoulder, RNA was prepared from both the main peak and shoulder fractions and analysed by polyacrylamide gel electrophoresis. Main peak fractions yielded only dsRNA-250 but, as may be seen in Fig.2, the shoulder fractions gave two RNA species; dsRNA-250 which runs as a very sharp band and a broad peak of lower mobility

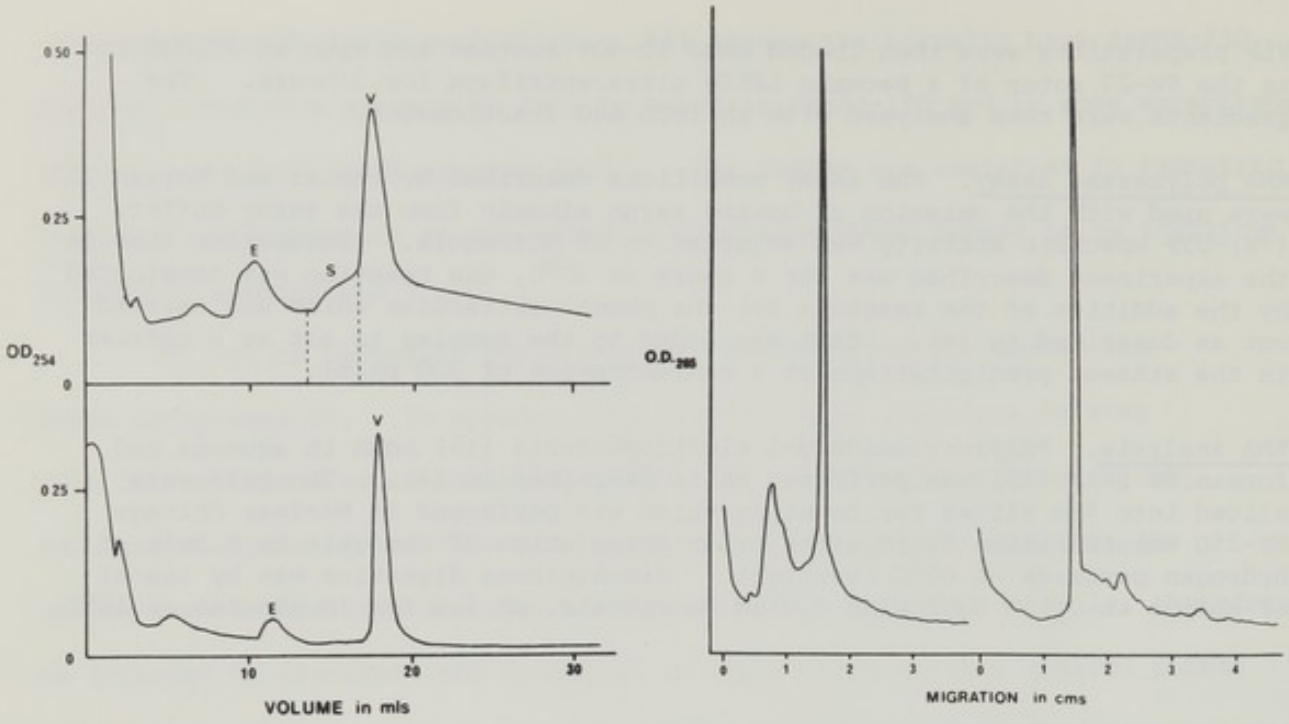


Fig. 1. OD_{254} profiles of VLP preparations analysed on 10-40% sucrose gradients. Top profile, VLPs from growing cells. Bottom profile, VLPs from stationary phase cells (24 days in culture). E indicates the peak of empty particles, V the peak of VLPs containing dsRNA and S indicates the shoulder fractions which were pooled for the preparation of the RNA the analysis of which is shown in Fig. 2.

Fig. 2. OD_{265} profiles of polyacrylamide gels loaded with RNA prepared from the shoulder fractions of the sucrose gradient shown in Fig.1. The left-hand profile is of a gel loaded with untreated RNA, the right-hand profile is of a gel loaded with the same amount of RNA after digestion with ribonuclease.

than dsRNA-250. Staining of the gels revealed that both bands stained pink with toluidine blue, indicating that they were double stranded structures (3). Staining also showed that there was a 'trailing edge' which extended down the gel from the dsRNA-250 peak and which was not clearly visible in the gel OD profile.

Digestion of this shoulder fraction RNA with ribonuclease A completely removed the slowly migrating RNA species but left the dsRNA-250 peak unaltered (as was expected under the conditions of digestion chosen). However, the 'trailing edge' of the dsRNA-250 peak became greatly enlarged and extended.

This may be clearly seen in the gel profile illustrated in Fig.2. On digestion the RNA in the slowly migrating band has thus given rise to a heterogeneous collection of dsRNA molecules ranging in size from very nearly the same as dsRNA-250 downwards.

Similar ribonuclease sensitive species of low electrophoretic mobility to that demonstrated in Fig.2 have been isolated from "H" particles of Penicillium stoloniferum S virus (15) and from Aspergillus foetidus S virus (fractions S3 and S4) (17). Both of these species have been described as aggregates; in the case of PsV-S H particle RNA, as an aggregate of ssRNA, since small fragments are produced by ribonuclease digestion, and in the case of AfV-S particle RNA, as an aggregate of dsRNA molecules with short ssRNA tails since molecules of full genome size dsRNA are produced after ribonuclease digestion. However, recently Buck (12) has shown that PsV-S H particles contain one molecule of dsRNA plus an additional molecule which ranges in size from a small ssRNA fragment to a partially replicated dsRNA molecule. Thus, in this case the actual RNA species involved in the aggregate remains in doubt.

From our results it may be concluded that the low mobility ribonuclease sensitive species is an aggregate of molecules consisting of a variable dsRNA portion with a ssRNA 'tail'. The molecules in the 'trailing edge' to the dsRNA-250 peak may also be similar in structure but unaggregated. Such molecules are the expected replicative intermediates of dsRNA replication if the process happens by an asynchronous mechanism. Aggregation may occur by interaction of the ssRNA tails as was postulated for the AfV-S RNA or, if the ssRNA species being replicated are of both senses, by the annealing of unreplicated central portions of the molecule to form a structure not dissimilar to RNA bacteriophage

PsV-S = Penicillium stoloniferum virus S.
AfV-S = Aspergillus foetidus virus S.

replicative intermediate. It is hoped that further information regarding this point may be gained by electron microscopy of the shoulder fraction RNA. An alternative explanation of the slow mobility of these molecules is simply that their mobility is anomalous; dsRNA molecules are thought to orientate with respect to the gel axis (18) and a ssRNA tail might interfere with this process.

The capsid associated RNA polymerase activity.

Incorporation of (^{3}H)-UTP into acid insoluble material was observed when yeast VLPs were tested in the RNA polymerase assay system described by Chater and Morgan (11). In the initial experiments VLP preparations consisting of both shoulder and main peak sucrose gradient fractions pooled together were used. The RNA polymerised in this reaction was extracted and analysed by aqueous polyacrylamide gel electrophoresis both before and after mild digestion with ribonuclease and by polyacrylamide gel electrophoresis in formamide (15, 4). The resulting OD and radioactivity profiles of these gels may be seen in Fig. 3 a,b and c respectively.

With the aqueous gel analysis the OD profile was identical before and after ribonuclease digestion. No low mobility peak was visible but the 'trailing edge' of the dsRNA-250 peak was observed. Without digestion counts were found at lower mobilities than the dsRNA-250, at the same level in the gel as the dsRNA-250 peak and also at slightly higher mobilities in the same region of the gel as the 'trailing edge'. After digestion the low mobility counts are not found. The counts in the dsRNA-250 peak remain unaltered but there is a considerable increase in the counts associated with the 'trailing edge' and a spreading of those counts further down the gel. The RNA species labelled in the 'in vitro' reaction thus had precisely the same mobility and response to ribonuclease digestion as the RNA isolated from the shoulder fractions.

Moreover, it is clear that the majority of the RNA product was double stranded as indicated by its resistance to degradation to small fragments by the ribonuclease digestion.

Recently we have repeated the experiment described above using particles from the shoulder fraction only. The OD profiles of the product were identical to those seen in Fig. 2. It was found to be labelled with the distribution of counts in the gels before and after digestion the same as that seen in Fig.3 a and b. Care was taken to load equal amounts of product RNA both with and without ribonuclease digestion and summation of the counts found in the two gels showed that 90% of the product was ribonuclease resistant.

To discover the molecular weight distribution of the RNA polymerised in the 'in vitro' reaction formamide gel analysis was used. When dsRNA is denatured prior to loading onto the gel it yielded a single peak of ssRNA with a mobility similar to that of 28S ribosomal RNA. This may be seen in the OD profile shown in Fig. 3 c. At the bottom of the gel there is a further peak of contaminating ribosomal RNA fragments and the tRNA which was used as a carrier. The radioactivity profile shows that much of the activity is in high molecular weight RNA with a size ranging from that of denatured dsRNA-25O down to that of 17S ribosomal RNA. In addition there was some labelled product of low molecular weight comparable in size to the 4S carrier RNA. This low molecular weight RNA would not have been detected in the aqueous gel analysis if it represents a separate labelled product and is not produced by the denaturation of dsRNA product since it would have run off the gel. It is currently being investigated further.

We interpret these results as showing that the detected capsid associated polymerase activity is a replicase which, in the 'in vitro' reaction, is extending the double stranded portion of partially replicated molecules.

Buck (12) has shown that, for PsV-S polymerase, there is no initiation 'in vitro'. We also believe that the reaction in yeast is probably restricted to particles which initiated the reaction in the cell and which were in the process of replication when isolated.

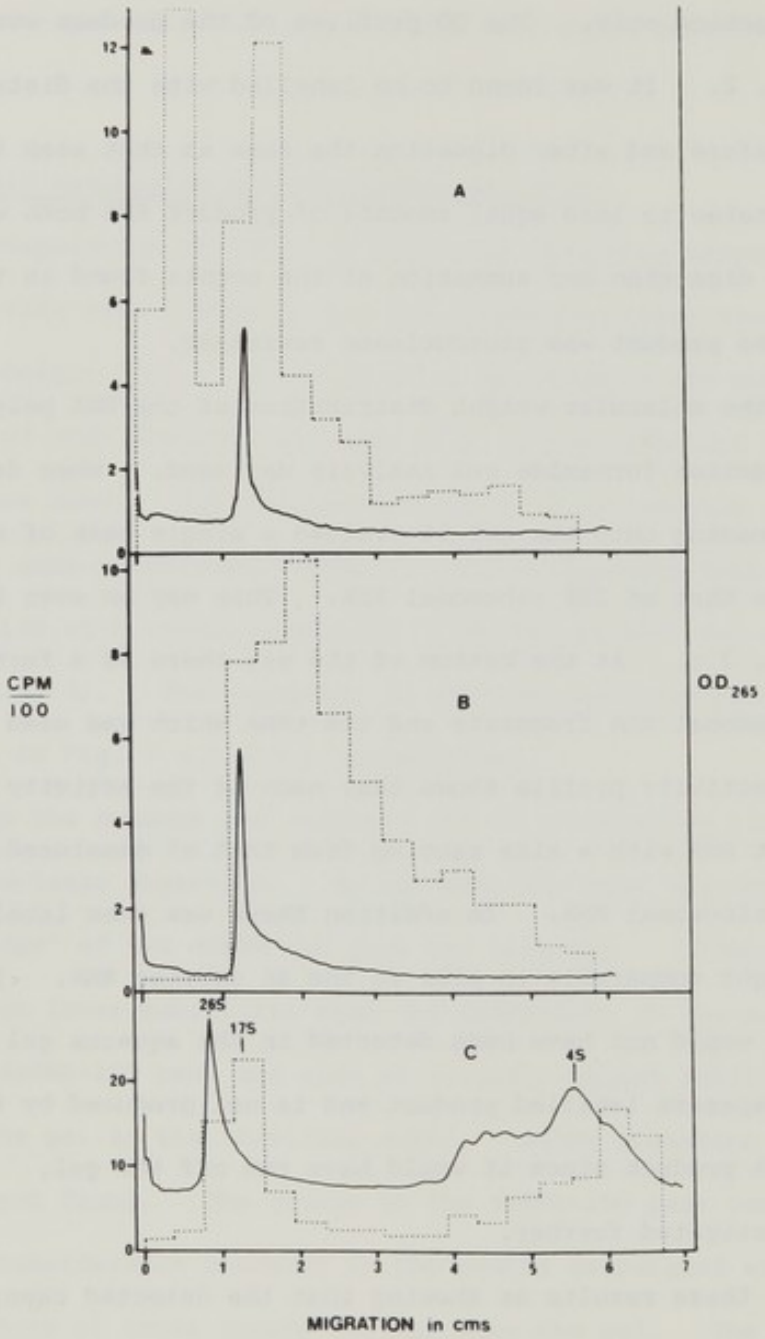


Fig. 3. OD_{265} and radioactivity profiles of gels loaded with RNA isolated from particles which had been incubated in the RNA polymerase assay mixture. a. Untreated RNA, b. RNA after ribonuclease digestion, c. Untreated RNA analysed on a formamide gel, the RNA was heated to 60°C for 3 min in buffered formamide prior to loading.

Buck's results with the PsV-S system show that the polymerase activity converts H particles into P particles which contain two molecules of dsRNA and similar particles containing two dsRNA molecules have also been described in AfV-S isolates (17). This has led Buck and Ratti to propose a model of mycovirus replication (9) in which dsRNA replication is seen to occur by a doubling mechanism by which particles with two molecules are produced and then uncoated and recoated separately to repeat the cycle. Our results are consistent with this model only if all the mature main peak yeast particles contain two molecules of dsRNA. Were this the case the particles in shoulder would contain one dsRNA and one partially replicated molecule but would still be lighter and thus more slowly sedimenting than main peak particles which would have two dsRNA molecules. If the replication of yeast VLPs resembled that of PsV-S the replicative intermediates would be expected to be found on the faster sedimenting side of the main peak as are the PsV-S H particles. Comparison of the biophysical properties of yeast particles with those of other mycoviruses and the observation that some of the preparations of RNA from the shoulder fractions appear to contain more replicative intermediate RNA than dsRNA-250 makes this possession of two dsRNA molecules unlikely. Thus, our present results suggest that the replicative cycle of yeast VLPs is similar to that of reovirus (19) in that a ssRNA precursor is encapsidated and then made up to dsRNA by the polymerase activity which we observe. Our current efforts are directed towards confirming this.

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Session 2: Biogenesis of Mitochondria

The Biogenesis of Mitochondria – a Review*

G. Schatz

Mitochondria are formed by a close interaction of two distinct genetic systems: the nucleo-cytoplasmic system and the mitochondrial system. The nucleo-cytoplasmic system synthesizes the bulk of the mitochondrial mass including about 90% of the proteins and all of the lipids and low-molecular weight components. In contrast, the contribution of the mitochondrial system is relatively restricted; it synthesizes most (and perhaps all) of the mitochondrial RNAs and roughly 10-20 hydrophobic polypeptides of the mitochondrial inner membrane (1, 2,3).

Research on mitochondrial biogenesis is currently progressing along two major fronts. On the first front, the genes localized on the mitochondrial DNA species from yeast, Neurospora and mammalian cells are being identified and mapped. Impressive advances in this area have been scored by isolating and characterizing mutants whose characteristic phenotype results from a mutation on mitochondrial DNA ("mitochondrial genetics").

Four major groups of such mutants have been described:

a) extrachromosomal petite (rho$^-$ or rho^o) mutants (4). These arise by massive deletions, rearrangements or even complete loss of mitochondrial DNA (1). While they are not overly useful for mapping studies themselves, they have proved extremely valuable for "deletion mapping" in con-

* Dr. G. Schatz was unable to attend the Symposium, we are grateful to him for providing this review article for publication.

junction with hybridization studies (5) or crosses with the more specific mutants mentioned below (6,7).

b) mutants resistant to inhibitors of oxidative phosphorylation (8,9), or of mitochondrial protein synthesis (10,11). Many of these are probably point mutants. While the altered gene product has not been identified in any of these mutants, there exists suggestive evidence that at least some of the oligomycin-resistant mutants contain an abnormal hydrophobic protein associated with the ATPase complex (12-14; cf. also 15).

c) mutants temperature-sensitive for the maintenance of normal mitochondrial function (mit^{ts} mutants). Only few mutants of this type have so far been described (16-18) even though they hold considerable promise for studying the question of how mitochondrial DNA (and mitochondria as a whole) are replicated.

d) mutants unconditionally defective in respiration and/or oxidative phosphorylation but relatively normal with respect to mitochondrial protein synthesis (mit^{-} mutants) (20,21). Although these mutants are in principle not as informative as the corresponding conditional mutants mentioned under c), they have been extremely valuable because a very large number of them has been isolated (21). These mutants (which are described in one of the preceding chapters of this book) are now being mapped (22). Although these studies are only in their early stages, they have already led to a much more detailed genetic picture of mitochondrial DNA. It is to be hoped that these mutants will lead to an identification of the cistrons coding for the mitochondrially translated subunits (2) of cytochrome _c_ oxidase, ATPase and cytochrome _b_. To achieve this, however, it will be necessary to show that a given mit^{-} mutant

carries an altered form of one of these polypeptides. A loss of one of these polypeptides could well be caused by indirect (i.e. regulatory) effects (cf. 23,24).

Genetic methods for mapping mitochondrial DNA have received a strong boost from the recent successful application of biochemical mapping studies. At present, most of the information along these lines has come from specifically cleaving mitochondrial DNA with restriction nucleases and subsequently ordering the fragments obtained (25,26). On a more limited scale, easily denatured stretches on mitochondrial DNA have been characterized and ordered by electron microscopy ("denaturation mapping", refs. 27,28). Whereas genetic methods are usually most powerful if dealing with short map distances, the biochemical and biophysical methods mentioned above are usually most reliable with respect to longer distances. It is therefore most fortunate that mit$^-$ mutants become available just when the first cleavage maps of yeast mitochondrial DNA (26) were obtained. The combination of these two approaches will almost certainly greatly expand our information on the coding function of mitochondrial DNA. Yet a third option has become available through the demonstration that isolated yeast mitochondrial DNA can be transcribed and translated in a heterologous cell-free system to yield polypeptides which can be immunochemically identified as the three large subunits of cytochrome c oxidase (29). It is conceivable that this in vitro system could be "persuaded" to accept restriction fragments as a template and thereby yield particularly clear-cut evidence on the location of the cistrons specifying mitochondrial protein products. A related approach, whose potential has not yet been realized, is the introduction of mitochondrial DNA or its fragments into a bacterial host such as E. coli via in vitro recombination with a suitable vector. The first attempts in this direction have already been made

(30). As far as can be judged at present, such experiments seem to be relatively innocuous; because of the well-documented similarity between the genetic systems of bacteria and mitochondria, they might also tell us much about the regulatory factors that govern the expression of mitochondrial DNA.

The second major approach towards studying mitochondrial biogenesis deals with the assembly of the mitochondrial membranes. This approach, which will now be reviewed in more detail, has largely focussed on a few reasonably well defined oligomeric complexes associated with the mitochondrial inner membrane. A few questions have been answered, some are just now being answered, and many continue to be an intriguing challenge.

1. Which proteins are synthesized by mitochondria?
   Studies in Saccharomyces and Neurospora suggest that mitochondria synthesize three polypeptides of cytochrome c oxidase (31-35), one (36) or two (37) polypeptides associated with cytochrome b and two (38) or four (39) polypeptides associated with the oligomycin-sensitive ATPase complex. The evidence appears to be most compelling for cytochrome c oxidase. First, all laboratories seem to be in agreement on this point (2); second, the synthesis of these subunits has also been observed with isolated mitochondria (40,41); third, the polypeptides have been isolated and at least partially characterized (42-46); and fourth, some evidence has accumulated on the function of these subunits (cf. below).

   At the most, therefore, nine polypeptide products of mitochondria have been identified. While there is reason to believe that these polypeptides constitute the major products, the recent application of highly resolving gradient (47) or two-dimensional acrylamide gel electrophoresis (48)

combined with autoradiography reveals as many as twenty labeled species. Some of these could well be artefacts (cf. below); yet it seems clear that the difficult task of tracking down minor, and thereby more elusive protein products is still ahead of us. Some of these products may be involved in the correct replication of functional mitochondrial DNA (49,50), the association of mitochondrial ribosomes with the mitochondrial membrane (cf. e.g. 51,52) or the response of the mitochondrial genetic system to oxygen and to signals from the extramitochondrial space of the cell. A detailed investigation of mitochondrial DNA-binding proteins (in conjunction with potentially interesting mutants, ref. 18) or of ribosome-membrane junctions in the mitochondrial inner membrane (52) might be most rewarding.

2. What are the properties of mitochondrially-made proteins? Most of the currently identified polypeptide products of mitochondria have been isolated in sufficient amounts to determine their gross chemical properties such as isoelectric point, molecular weight, and amino acid composition (53,42,43,37). Sequence work on the (presumably mitochondrially-made) large subunits of cytochrome c oxidase from bovine heart is currently underway in several laboratories (46,54). (At least some of these efforts, however, may have to be reevaluated since one of the large subunits, which had been considered to be a single polypeptide, has recently been shown (55,56) to be a mixture of two distinct polypeptides). The evidence available so far supports the earlier view that mitochondrially-made polypeptides are very hydrophobic and, at least in part, still carry an N-terminal N-formyl-methione residue. The primary sequence of these proteins will be indispensable for putting mito-

chondrial genetics on a solid chemical footing and for defining the interaction of these polypeptides with phospholipids and other, more hydrophilic subunits. It is to be hoped that such investigations will finally clarify the puzzling observation that some of these polypeptides can apparently be converted to smaller species by treatment with performic acid (57), alkali (58), or simply chloroform-methanol (57,58). It is possible, of course, that many of the mitochondrial polypeptide products of molecular weight $>$ 20,000 are aggregates of smaller polypeptides which are not dissociated to their monomeric state by the commonly used procedures involving dodecyl sulfate as denaturant (57,58). Conversely, however, the smaller polypeptides mentioned above could reflect artifactual fragmentation by performic acid or proteases. It is now fairly well documented that mitochondrially-made (59,60) as well as cytoplasmically-made (61) polypeptides can be quickly degraded by intracellular proteases.

3. What is the function of mitochondrially-made proteins?
Initially it was often speculated that the mitochondrially-made hydrophobic proteins were mainly concerned with connecting the more hydrophilic "partner" proteins to the hydrophobic lipid bilayer. That this concept is at least partly true was suggested by the fact that loss of mitochondrial protein synthesis weakened the membrane attachment of F_1-ATPase (62) as well as of the cytoplasmically-made cytochrome *c* oxidase subunits (24) and rendered the cytoplasmically-made cytochrome c_1 polypeptide more susceptible to proteolysis (61). It is now fairly clear, however, that some of the mitochondrially-made polypeptides also participate more directly in the enzymic function of the oligomeric complexes with which they are associated. First,

one of the mitochondrially-made subunits of the ATPase complex appears to be the inhibitory site of oligomycin and dicyclohexyl carbodiimide (cf. 63, 13 with 53). Second, the third-largest mitochondrially-made subunit of yeast cytochrome c oxidase participates in binding the substrate ferrocytochrome c (64). Third, an antibody against the second-largest mitochondrially-made subunit of yeast cytochrome c oxidase completely inhibits the activity of the purified holoenzyme (65). Fourth, the mitochondrially-made subunit(s) of cytochrome b is(are) the heme-binding site(s) (36,37).

Additional information on the essential function of these hydrophobic polypeptides of cytochrome c oxidase has come from studying their three-dimensional arrangement within the enzyme. A variety of surface probes have shown that the largest of these hydrophobic subunits is almost completely buried in purified or membrane-bound cytochrome c oxidase whereas the second-largest hydrophobic subunit is partly buried, particularly in the membrane-bound enzyme (66,67). These findings make it extremely unlikely that the large subunits are contaminants. Rather, it appears that they are necessary for the asymmetric and transmembraneous orientation of cytochrome c oxidase in the mitochondrial inner membrane (68,69) and that they may function as nuclei in the assembly of cytochrome c oxidase *in vivo*. It is likely that the ATPase complex, too, spans the membrane in an asymmetric fashion and that some of the hydrophobic, mitochondrially-made subunits function as "proton pores" which link proton transport to ATP synthesis (70). Since the enzymic activities of cytochrome c oxidase (71) and the cytochrome bc_1 complex (72) are also associated with transmembrane proton movements, it is tempting to speculate that the mitochondrially-made subunits also of these complexes

function as "proton pores". They are certainly big enough to span the membrane even if not fully extended. Clearly, more needs to be learned about the arrangement of the mitochondrially-made polypeptides relative to other membrane components. Chemical cross-linking (73), X-ray triangulation (74) and identification of protein-protein interactions by genetic methods (75) could be used to explore this problem.

4. How are mitochondrially-made polypeptides assembled with cytoplasmically-made polypeptides?

Present evidence indicates that the two genetic systems can influence each during the assembly of oligomeric membrane complexes (2). Loss of mitochondrially-made polypeptides may cause the loss of a cytoplasmically-made subunit (24); conversely, nuclear mutations may cause the loss of one or more polypeptides coded by mitochondrial DNA (24,76). It is not quite clear how these interactions work. For example, does a cessation of cytoplasmic protein synthesis lead to a slowdown of mitochondrial translation (cf. 77 for a biological precedent) or are the mitochondrially-made polypeptides simply no longer integrated into the membrane and, as a consequence, degraded by intramitochondrial proteases? The latter possibility is favored by the observation that loss of the large mitochondrially-made polypeptides in certain nuclear yeast (23) and Neurospora mutants (78) is accompanied by increased levels of mitochondrial translation products of molecular weight below 10,000. One of such small polypeptides has recently been isolated in amounts sufficient to carry out at least a limited sequence analysis (79). A comparison of this small polypeptide with some of the larger polypeptides made by wild-type mitochondria could perhaps help to shed new light on this point. Another

approach would be to carefully study initiation and elongation on mitochondrial polysomes from cycloheximide-inhibited cells.

Mutants with defective mitochondrial assembly are among the most powerful tools for unraveling the various assembly controls. Initial investigations with cytochrome c oxidase- and ATPase-less nuclearly-inherited yeast mutants have been encouraging (23,80,76) but a wider variety of mutants has to be studied. Even more importantly, some of the already available mutants must be characterized in greater detail. For example, none of these investigations (except those of cytochrome c-less yeast mutants, see ref. 81) has as yet led to the firm identification of a structural gene for a known polypeptide of the mitochondrial inner membrane. Once such a gene has been identified, structural genes for neighboring proteins may be pinpointed through second-site reversion studies involving conditional revertants (cf. 75). There are already indications that mitochondrially-inherited mutations may be suppressed by nuclear mutations (82,83) and the converse should be true as well. A very extensive characterization of a single mutant will probably prove to be more fruitful than the superficial screening of many mutants. Since assembly of supermolecular structures (84,85) as well as the movement of polypeptides across membranes (86) appear to involve proteolytic processing events, particular attention should be given to the possibility that some mutants with defective mitochondria accumulate larger precursors to some of the membrane-associated polypeptides discussed above.

Concluding remarks

This brief survey has attempted to point out some of the most notable and promising advances during the past five years;

some major questions, however, have not been raised here because no answers are as yet available. How are cytoplasmically-made proteins such as F_1-ATPase transported across both mitochondrial membranes? How can yeast cells completely lacking mitochondrial DNA regulate their content of (largely non-functional) mitochondrial structures? How are the biosynthetic events in normal mitochondria coordinated in time with events in the extramitochondrial space? These few reminders should be sufficient to justify future meetings on this topic, preferably in cities as pleasant as Munich.

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On the Formation of rho$^-$-Petites in Yeast: VI. Expression of a Mitochondrial Conditional Mutation Controlling Petite Formation in Saccharomyces cerevisiae

W. Bandlow

Introduction

Rho$^-$-mutation leads to a complete or partial loss of the genetic information of the mitochondrial genome together with a change of the physical properties of mitochondrial DNA (mitDNA) (1-3). The mechanism of rho$^-$-petite formation is unknown. It appears to be a multiple step process, the initial steps of which are reversible (4-6). Established mutants do not revert (1). They may, however, alter their mitochondrial genetic complement since elimination of genes concomitant with a decrease in genetic complexity and amplification of the retained genes resulting in tandem, inverted tandem or multiple repeats with apparent preservation of the original mitochondrial genome length occurs (7-11).

In many strains of *Saccharomyces cerevisiae* the rho$^-$-petite mutation occurs spontaneously at an extraordinarily high frequency of 1-3 x 10^{-2} per generation (compared to 10^{-7} - 10^{-8} for nuclear genes). Others like strain M12 from which the temperature-sensitive petite producing mutant tsm8 was derived, are relatively stable and show rates of petite mutation of 10^{-4}. The rate of this mutation can be increased to 100% by ethidium bromide (3,12,13). This high rate is the more surprising for a deleterious mutation mechanism since apparently about 50 copies of mitochondrial DNA are present per haploid cell (14,15).

It appears that an additional superimposed process is responsible for the rapid distribution of the mutated information among the intact mitDNA molecules in petite genesis. Various hypotheses of petite formation are currently discussed which imply the involvement of misreplication of mitDNA (16,17) or of continuously occurring mitochondrial recombination of a single mutated mitDNA molecule with the remaining wild type mitDNA genomes in the cell (7,8). Another possibility is the alteration of the attachment site for mitDNA in the mitochondrial membrane which may possess the dominant regulatory function postulated by Williamson (14). Because of the complexity of this process it is helpful to separate various stages of petite genesis. We are able to do this in a temperature-sensitive mutant giving rise to petites at restrictive temperature in glucose medium, in which the initial responses on the temperature shift are reversible (18,19). This conditional decrease in rho-factor stability under non-permissive conditions provides a starting platform for the examination of control mechanisms responsible for the fidelity of replication and continuity of mitDNA not offered by studies using intercalating dyes like ethidium bromide or acriflavin.

Results

The isolation procedure, the physiological behaviour, and a preliminary biochemical analysis of mutant tsm8 compared with the wild type, have been published previously (18-21). Figs. 1 and 2 summarize the growth rate and petite induction rates of the iso-nuclear wild type and mutant strains SM552 [TSM8] and SM551 [tsm8], respectively.

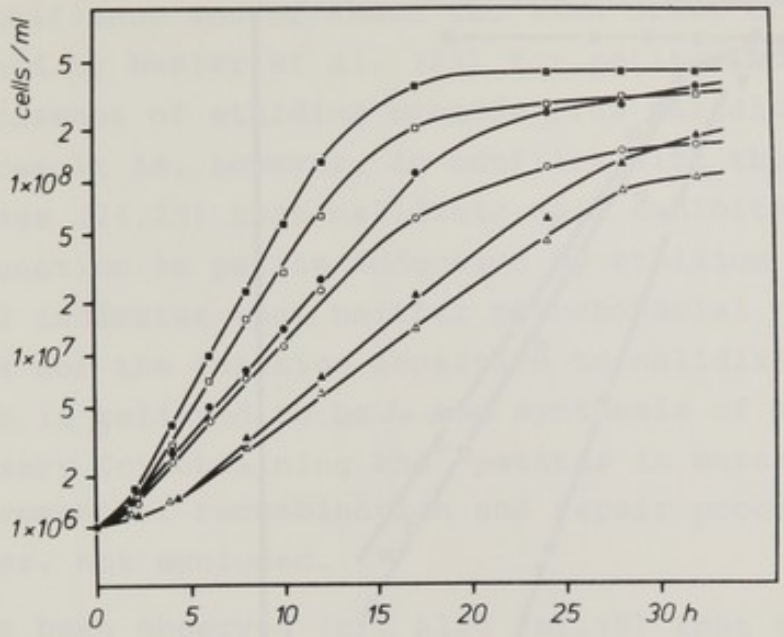


Fig. 1 Growth curves of wild type and mutant. Cells were grown in 1% yeast extract (Difco), 0.5% Bacto peptone (Difco) in test tubes with vigorous shaking. Filled symbols: wild type SM 552 [TSM8], open symbols: mutant SM 551 [tsm8]; squares: 6% glucose, 36°C; circles: 6% glucose, 23°C; triangles: 5% glycerol, 23°C.

Fig. 1 reveals that the wild type 552 does not have a significant advantage in growth over mutant tsm8, strain 551, at 23°C, both in glycerol and glucose, and at 36°C in glucose medium. However, mutant tsm8 does not grow at 36°C on glycerol (cf. Fig. 5).

Fig. 2 shows that at 23°C in glucose medium (and glycerol, and at 36°C in glycerol, not shown) petite formation rates are the same as with the nuclear isogenic wild type (1-4% within 6 generations). Petites are formed at significantly higher rates only in glucose at 36°C. Under these conditions virtually all cells are converted to petite cells within 6-8 generations, as measured by their inability to utilize glycerol as source of energy both at 23° and 36°C. These petites generated at non-permissive temperature have been shown to be of the rho$^-$-type by genetical analysis (22).

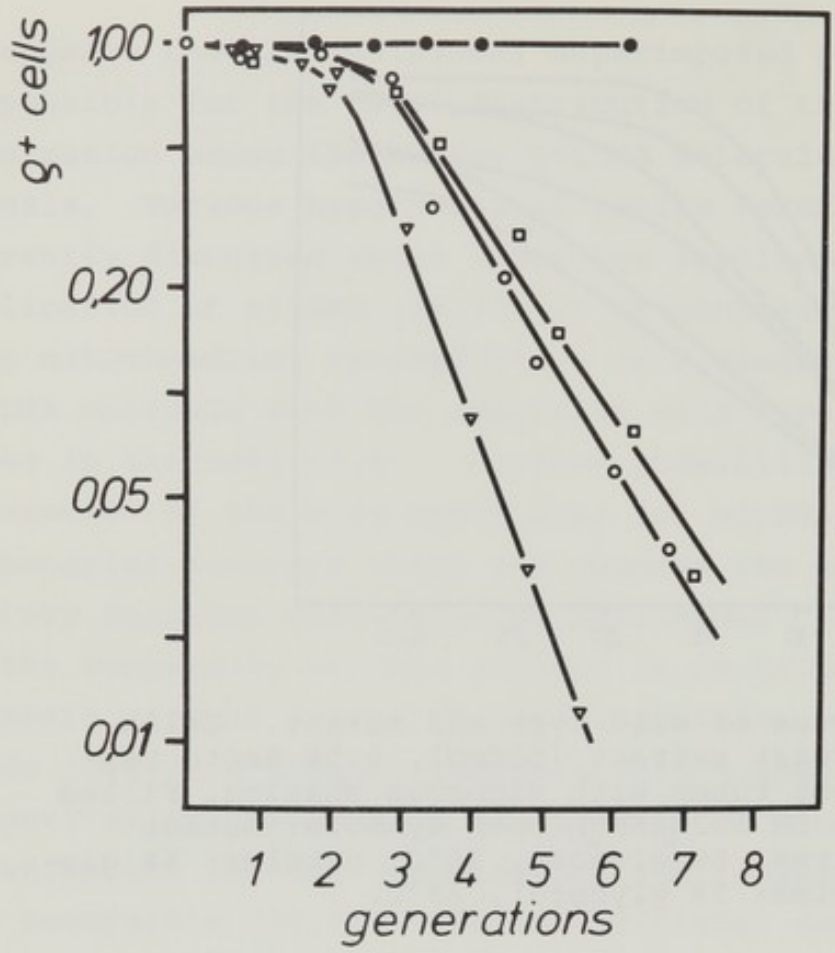


Fig. 2 Rho$^-$-petite formation rates of the mutant in the presence of various inhibitors. Cells of strain D12/61/1 were inoculated at a titer of $5x10^5$ cells per ml and grown in 6% glucose at 23°C and 36°C, respectively, as described under Fig. 1. Cells were plated on 6% glucose complete medium at times indicated and incubated at 23°C, replica-plated onto two glycerol plates (complete medium containing 3% glycerol, 0.1% glucose) and these incubated at 23°C and 36°C, respectively. Fraction of rho$^+$-cells versus generations of growth in liquid medium are given. ● control, 23°C; o 36°C; □ 4 mg/ml chloramphenicol, 36°C; ▽ 500 µg/ml nalidixic acid, pH 6.5, 36°C.

As shown in Fig. 2 the presence of inhibitors of mitochondrial protein synthesis and mitDNA synthesis does not change rho$^-$-petite formation rates significantly. The small increase in rate in the presence of nalidixic acid is reproducible with different tsm8 strains, but it may be due to the higher pH of the growth medium in the presence of this drug. The increase in rho$^-$-formation rate

is significant and of about the same order of magnitude as found by Mahler et al. (23) for petite induction in the presence of ethidium bromide plus nalidixic acid. This result is, however, in conflict with the more recent findings (24,25) that nalidixic acid exhibits a protective function in petite induction by ethidium bromide. Fig. 2 indicates that neither mitochondrial protein synthesis nor the function sensitive to nalidixic acid (which is believed to be *de novo* synthesis of mitDNA) are necessary for obtaining rho$^-$-petites in mutant tsm8. The involvement of recombination and repair processes is, however, not excluded.

It has been observed (cf. also ref.19) that tsm8 mutant cells exhibited an extremely long lag phase when replica-plated from glucose 23°C onto glycerol plates at 23°C. This phenomenon was investigated in more detail. Figs.3 and 4 reveal that mutant tsm8 appears to have a super-repressed phenotype in the presence of high glucose concentrations, even at 23°C despite the presence of oxygen, and derepression occurs considerably slower than in the wild type. The spectrum of glucose-repressed mutant cells in the presence of high glucose (Fig. 3) lacks entirely cytochrome aa_3 and other cytochromes are severely depleted.

Moreover, it is evident from the spectra that in glucose-repressed cells of both mutant and wild type, cytochrome aa_3 absorbed at 599 nm and the long wavelength peak of cytochrome *b* is at 557.7 nm. In fully derepressed cells these maxima are shifted to 602.0 and 558.6 nm, respectively, indicating that in repressed cells the long wavelength components of cytochromes *a* and *b* (i.e. a_T and b_T) are partially or totally absent. Moreover, mutant cells both repressed or during derepression contain significantly higher concentrations of cytochrome *c* (the meas-

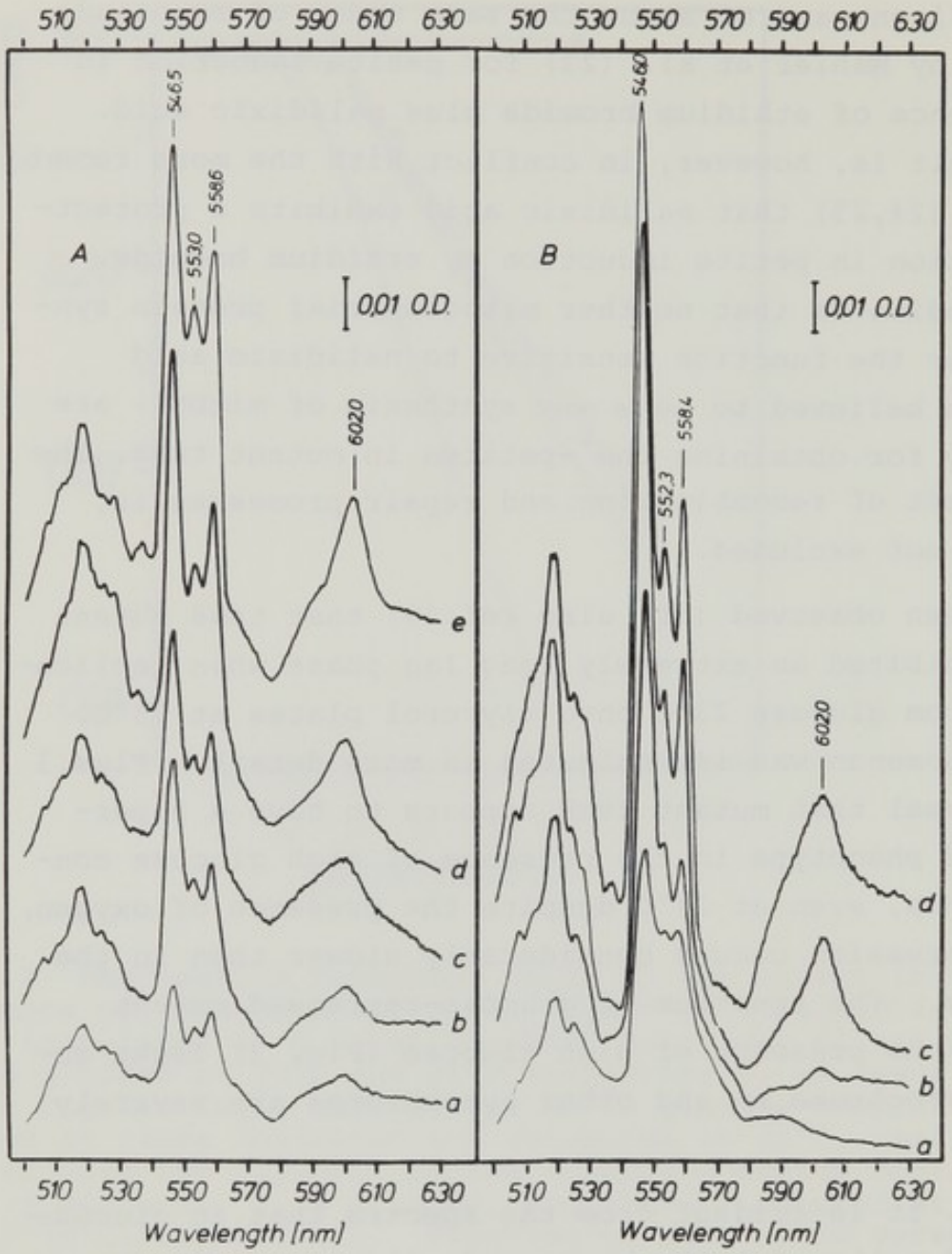


Fig. 3 Derepression kinetics of wild type and mutant illustrated by low temperature spectroscopy of whole cells. Cells of wild type SM 202 (A) and mutant SM 201 (B) were grown at 23°C under aeration in 6% glucose with the addition of another 6% glucose 2 h before harvesting until a titer of $2x10^8$ was reached. Cells were then washed and reinoculated in glycerol medium at the same titer and aerated at 23°C. At the following times samples were withdrawn, cells centrifuged and washed and spectra taken: A (wild type): spectrum a, 0 h glycerol at 23°C; b, 2 h; c, 4 h; d, 6 h; and, e, 24 h. B (mutant tsm8): spectrum a, 0 h glycerol at 23°C; b, 16.5 h; c, 41 h; d, 65 h. Cell density for the spectra was $2x10^9$ per ml in all cases.

ured cell titers being the same in all spectra). Under conditions of derepression (shift from glucose to glycerol, 23°C, high oxygen tension) cytochromes reappear only after several days in early stationary phase in the mutant, whereas they reach the normal level within 36 h in the wild type under the same conditions as documented by Fig. 4. This behaviour is parallelled by the rate of resumption of mitochondrial protein synthesis and respiration during derepression (19).

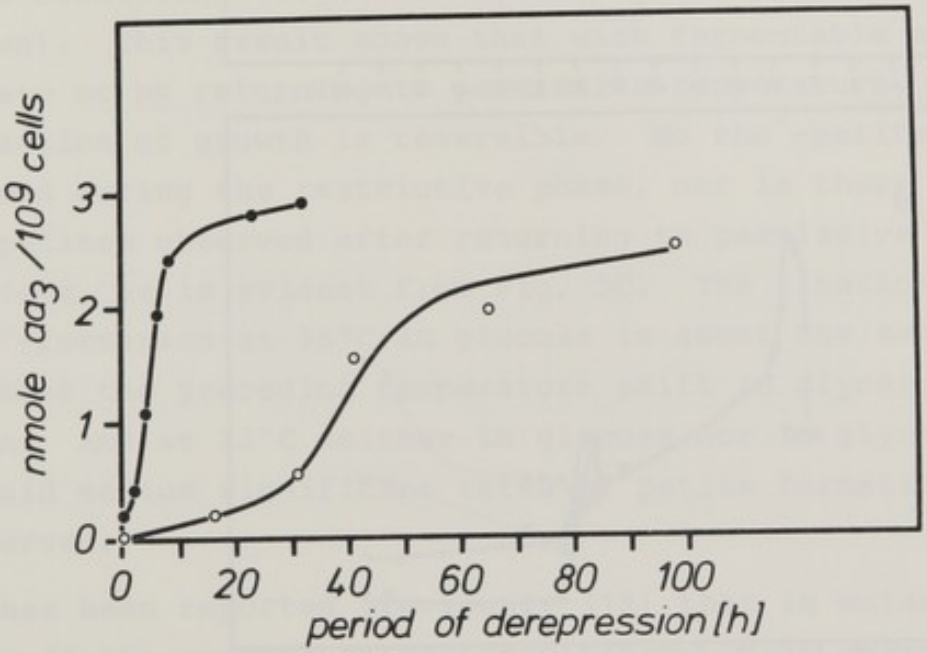


Fig. 4 Derepression kinetics of wild type and mutant cytochrome aa_3. Wild type SM 202 (●) and mutant SM 201 (o) cells were grown as described under Fig. 3. Cytochrome aa_3 content was measured at room temperature in a dual wavelength spectrophotometer using the wavelength pair 605-590 nm and an absorbance coefficient $\varepsilon_{605-590}$ = 12.4 mM^{-1} cm^{-1}

Fig. 5 illustrates the effect of the shift to restrictive temperature and of the reverse shift from restrictive to permissive temperature on growth (Fig. 5A), respiration (Fig. 5B) and rho$^-$-petite formation (Fig. 5C). In glycerol medium at 36°C, growth stops probably in the G1 phase after the completion of one cell cycle (Fig. 5A).

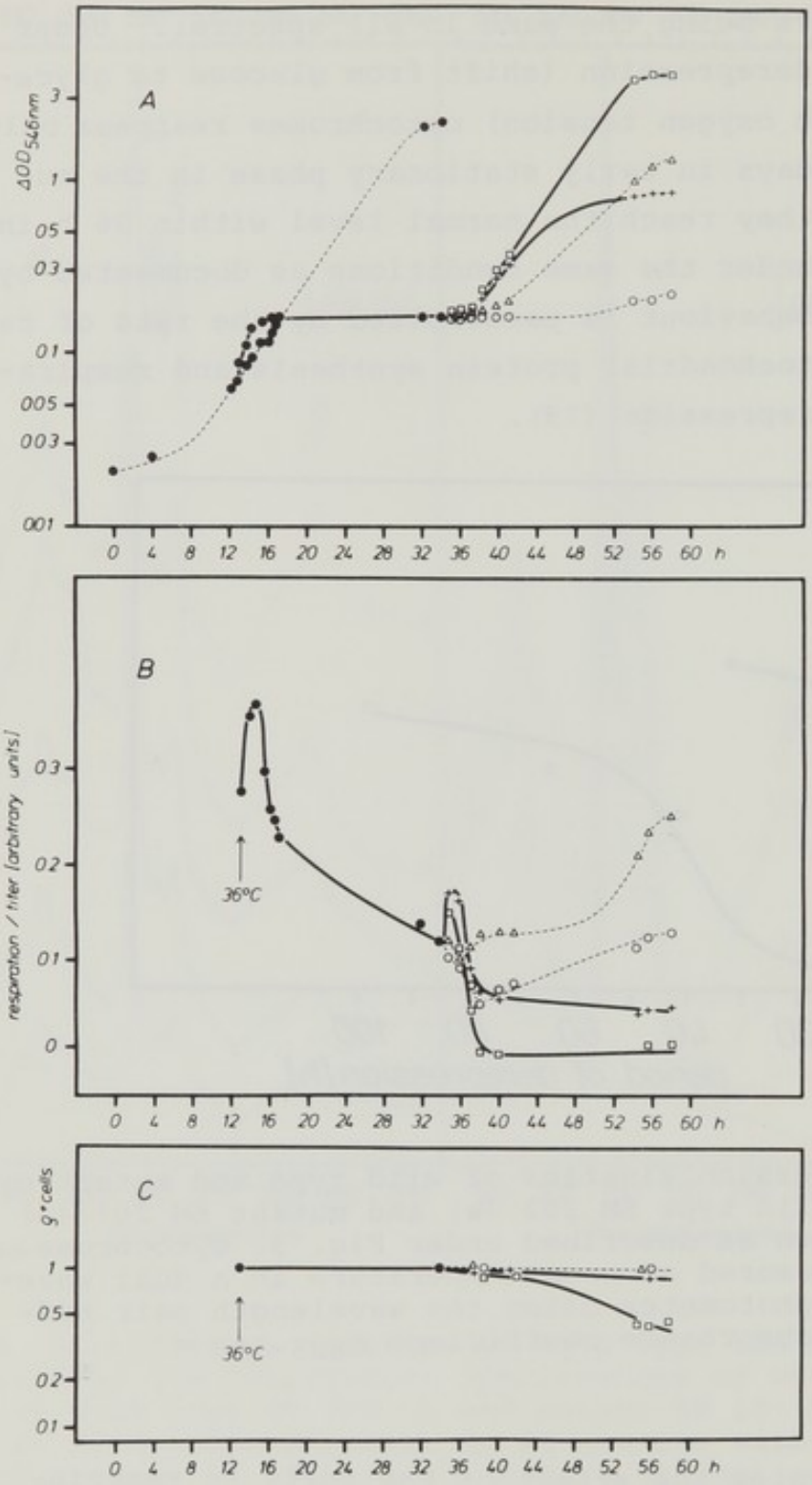


Fig. 5 Cell growth (A), whole cell respiration (B) and rho⁻-petite formation rates (C) of mutant strain D12/61/1 in glycerol liquid medium at 23°C and 36°C. Cells were grown at 23°C for 12 h (●---●) and then divided into two portions: one was further grown at 23°C (●---●) and the other shifted to 36°C (●——●). After 35 h, one fourth of the latter received 6% glucose at 36°C (□——□), one fourth received 0.8% glucose at 36°C (+——+), one fourth received 6% glucose at 23°C (△---△) and the last portion was returned to 23°C in glycerol (o---o).

After the separation of daughter buds from the mother cells, no further budding is initiated. Growth is resumed after the addition of a fermentable carbon source (not shown) or after lowering the temperature again, the lag being dependent on the length of the period at restrictive temperature. After an initial increase whole cell respiration decreases after the temperature shift to restrictive conditions (36°C, Fig. 5B). But it increases again after lowering the temperature to permissive conditions (or after the addition of glucose, not shown). This result shows that with fermentable substrate or by returning to permissive temperature, the cessation of growth is reversible. No rho^--petites are formed during the restrictive phase, nor is there a burst of petites observed after returning to permissive conditions, as is evident from Fig. 5C. The kinetics of rho^--formation at 36°C in glucose is about the same as without the preceding temperature shift in glycerol medium. And at 23°C neither in glucose nor in glycerol liquid medium significant rates of petite formation are observed.

It has been reported previously (18) that in mutant tsm8 some of the enzymes of the respiratory chain decrease rapidly in their enzymatic activities during growth on glucose at 36°C. This difference is apparent even when correlated with the number of rho^+ cells remaining in the culture.

Fig. 6, derived from results with a single, fermenter-grown batch culture, reveals that this also holds for glycerol-grown cells at 36°C, i.e. under conditions where growth ceases and no petites are formed. However, the decrease in respiratory activities is significantly slower in glycerol than in glucose.

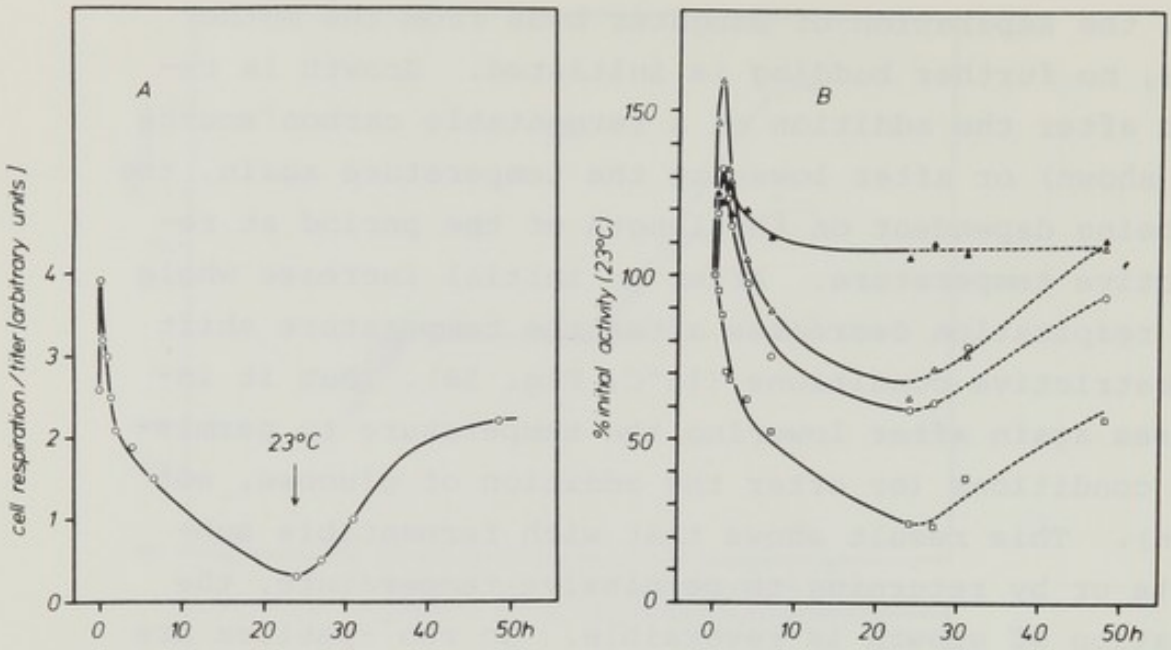


Fig. 6 Whole cell respiration and *in vitro* respiratory activities of mutant strain D12/61/1. Cells were grown on glycerol at 23°C in a fermenter until a titer of 6×10^7 cells per ml was reached. Then the temperature was raised to 36°C and samples withdrawn at the times indicated. After 24 h the temperature was lowered again to 23°C. A, cell respiration. B, respiratory activities of cytochrome *c* oxidase (□); NADH : cytochrome *c* oxidoreductase (o); succinate : cytochrome *c* oxidoreductase (△); and, succinate : duroquinone oxidoreductase (▲).

Fig. 6A shows the corresponding respiratory rates of whole cells with glucose as substrate. Fig. 6B illustrates the activities of NADH- and succinate : cytochrome *c* oxidoreductases, cytochrome *c* oxidase and succinate : duroquinone oxidoreductase of isolated mitochondria. From the comparison of the four enzyme activities measured it is evident that those which decrease in their activity at restrictive temperature, contain mitochondrially synthesized components, whereas the others probably do not. The rates of decrease are, however, slower than one might expect for an enzyme complex containing a thermolabile protein.

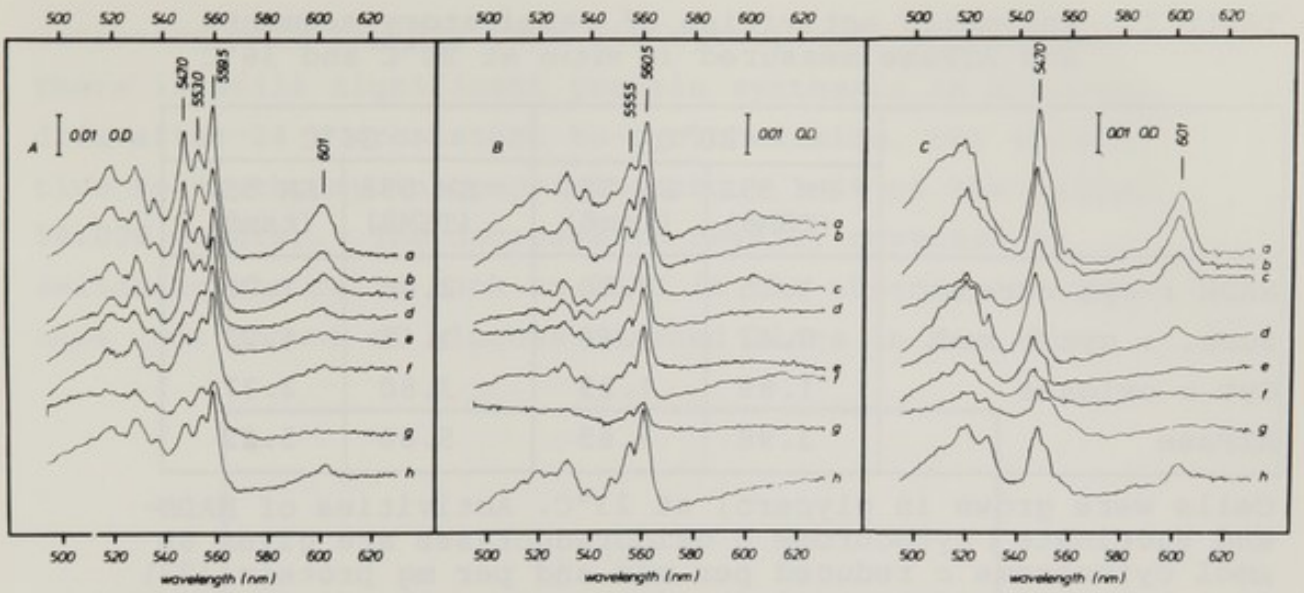


Fig. 7 Low temperature spectra of mitochondria (5 mg/ml in all cases) isolated from cells after the shift to 36°C at times indicated (same culture as in Fig. 6). A, dithionite reduced *minus* oxidized; B, NADH *plus* antimycin reduced *minus* oxidized; C, ascorbate *plus* TMPD reduced *minus* oxidized; a, 0 h; b, 1 h; c, 1.5 h; d, 2 h; e, 4 h; f, 6 h; g, 24 h after the shift to 36°C. Spectrum h was taken 24 h after returning to 23°C (glycerol). Low temperature spectroscopy and substrate concentrations were as described earlier (26).

Fig. 7 shows that in the same preparation also the content of cytochromes decreases after the shift to restrictive conditions. First, cytochrome aa_3 vanishes and returns only partly after a reverse shift to 23°C. Also cytochromes *b* and *c* are affected. The loss of cytochrome *c* may be due to the fragility of the mitochondria and a higher solubility of this cytochrome under restrictive conditions and/or to regulatory phenomena.

In order to test whether the respiratory chain or the energy conserving machinery contain a thermolabile function, cells of wild type and mutant tsm8 were grown at 23°C and the respiratory rates and ATP hydrolysis rates of the oligomycin-sensitive ATPase were compared at permissive (20°C) and restrictive (36°C) temperatures (Table I).

TABLE I. Enzymatic activities of respiratory segments and ATPase measured *in vitro* at 20°C and 36°C

	20°C		36°C	
	SM 552 (TSM8)	SM 551 (tsm8)	SM 552 (TSM8)	SM 551 (tsm8)
NADH : cyt *c* red.	1.15	0.80	2.88	1.72
Succ. : cyt *c* red.	0.42	0.46	1.15	1.23
Cyt *c* oxidase	1.89	1.29	3.80	2.37
ATPase	3.98	3.65	5.80	5.22

Cells were grown in glycerol at 23°C. Activities of NADH- and succinate : cytochrome *c* oxidoreductases are given as µmol cytochrome *c* reduced per min and per mg protein (27) Cytochrome *c* oxidase activity is given as the first order rate constant (28), oligomycin-sensitive ATPase activity is µmol H^+ released on ATP hydrolysis (29).

Table I shows that respiratory rates are enhanced about twofold when measured at 36°C as compared to 20°C, even after a pre-incubation time of 15 min at 36°C. It has been shown previously (18) that respiratory-control ratios and P/O values of both wild type and mutant are affected by the higher temperature although to a slightly lesser extent with the wild type than with the mutant. However, respiratory control is preserved in the mutant at 36°C with all substrates tested. Thus, most probably, respiratory chain and energy conservation do not contain a thermolabile function.

Fig. 8 reveals that both mitochondrial and cytoplasmic protein syntheses decrease after the shift to non-permissive conditions. Cells were labelled in glycerol medium with ^{3}H-leucine after raising the temperature to 36°C either in the presence of cycloheximide (open circles) or erythromycin (closed circles). Under both sets of conditions an effect of the higher temperature is seen but the rate of the mitochondrial protein synthesis

decreases more rapidly than its cytoplasmic counterpart. There is still significant protein synthesis in mitochondria after 24 h (resistant to cycloheximide, but sensitive to erythromycin comprising about 1-4% of the initial 54.000 counts). The decrease is readily reversed by switching the cells back to 23°C in the absence (or presence (not shown) of glucose (dashed lines in Fig. 8).

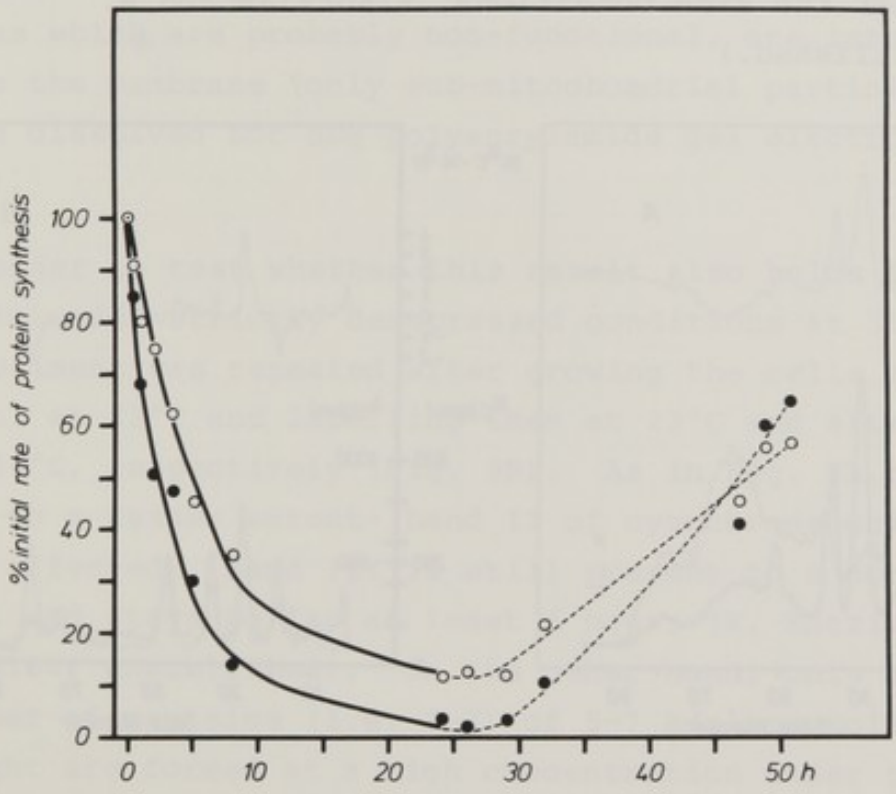


Fig. 8 Mitochondrial and cytoplasmic protein syntheses *in vivo* after the shift to 36°C with glycerol as source of energy. Cells of mutant strain D12/61/1 were grown at 23°C to a titer of 4×10^7 cells per ml, washed twice with synthetic medium, resuspended in synthetic medium and aerated for another 2 h. Then the culture was shifted to 36°C (solid lines), samples of 2 ml were withdrawn at times indicated and incubated at 36°C in the presence of erythromycin (4 mg/ml, 15 min, o) or cycloheximide (200 µg/ml, 3 min, ●), respectively. Then ^{14}C leucine (0.25 µCi/ml) was added. The invorporation was terminated by pipetting one ml of the incubation mixture into cold trichloroacetic acid and evaluated as described under Table II. After 24 h the temperature was lowered again to 23°C (broken line).

The data indicate that also mitochondrial ribosomes do not involve a thermolabile function but that mitochondrial protein synthesis is probably affected secondarily.

However, Fig. 9 shows that the tsm8 mutation causes an alteration of the labelling pattern of mitochondrial translation products synthesized at the elevated temperature. (The differences observed are not found in the wild type under the same conditions, K.Kotzias and W. Bandlow, unpublished.)

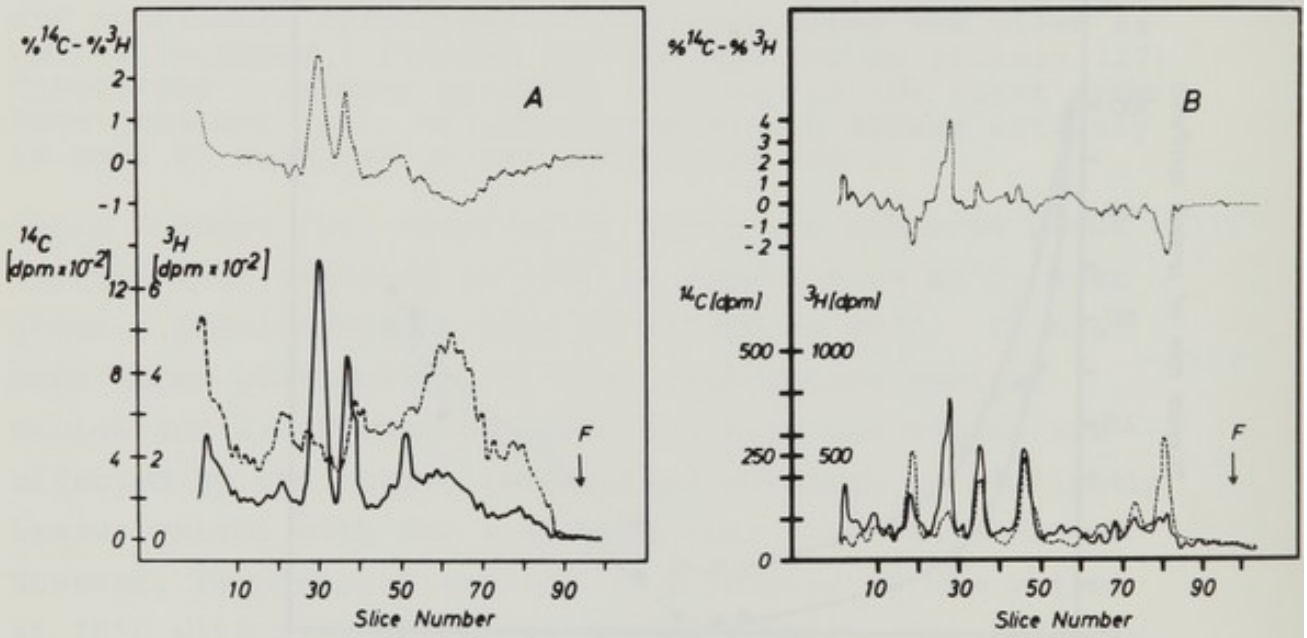


Fig. 9 SDS polyacrylamide gel electrophoretic patterns of mitochondrial proteins labelled at 23°C and 36°C, respectively, both in the presence of cycloheximide (200 µg/ml). A. Galactose. One half of the culture was labelled at 23°C with a mixture of ^{14}C leucine, isoleucine, valine and phenylalanine (solid line), the other half after 16 h at 36°C with the respective ^{3}H amino acids (broken line). B. Glycerol, at 23°C and after 90 min at 36°C, other conditions as in A. Electrophoretic and counting procedures have been described previously (18).

Fig. 9A shows the SDS-polyacrylamide gel electrophoretic pattern of mitochondrial proteins of cells grown at 23°C on galactose and labelled in the presence of cycloheximide, at 23°C and after 16 h at 36°C. It is evident

from this figure that the familiar pattern of mitochondrially synthesized proteins has altered in cells labelled at 36°C. A large number of undefined proteins of variable low molecular weight, probably not present in normal mitochondria at an appreciable concentration, is formed under restrictive conditions at the expense of the high molecular weight proteins comprising bands I, II and III of cytochrome oxidase. Apparently these proteins which are probably non-functional, are integrated into the membrane (only sub-mitochondrial particles have been dissolved for SDS polyacrylamide gel electrophoresis).

In order to test whether this result also holds for cells under strictly derepressed conditions at 36°C, the experiment was repeated after growing the cells in glycerol at 23°C and labelling them at 23°C and after 90 min at 36°C, respectively (Fig. 9B). As in Fig. 9A, band I, and -to a lesser extent- band II of cytochrome oxidase are affected. Band III is still present to a normal extent and stays so for at least 6 hours (K. Kotzias and W. Bandlow, unpublished). On the other hand, only a limited number of proteins (i.e. 2-3) of 5-7 kdalton molecular weight are formed at a high concentration under these conditions.

One possibility for emergence of low molecular weight material under non-permissive conditions may be the thermolability of a tRNA leading to premature termination of peptide chains. In order to test this hypothesis, the incorporation kinetics at 23°C and 36°C of radioactive precursors into mitochondrial proteins was measured for 35 min for formate an the 20 commonly occurring amino acids. The results are given in Table II, which shows only the cpm values of radioactivity incorporated after 35 min for these amino acids.

TABLE II Incorporation of Amino Acids (cpm / 35 min)

Amino Acid	SM 201	tsm8	SM 202	TSM8
	23°C	36°C	23°C	36°C
Formate	28,000	62,000		
Ala	380	770		
Arg	175 (15,500)	240 (24,300)	200 (1,700)	300 (22,000)
Asp	400	940		
Asn	250 (5,100)	350 (7,200)	380 (4,800)	650 (5,700)
Cys	160 (1,900)	140 (2,300)	60 (1,400)	130 (2,000)
Glu	120 (4,800)	390 (9,300	140 (4,150)	380 (9,160)
Gln	420 (13,500)	240 (13,500)	480 (8,000)	280 (14,800)
Gly	450	880		
His	570 (5,000)	790 (5,400)	950 (7,000)	2,050 (6,800)
Ile	1,950	4,950		
Leu	1,950	5,450		
Lys	640	1,190		
Met	2,250	3,400		
Phe	1,200	2,850		
Pro	4,200	7,200		
Ser	450	1,650		
Thr	850 (8,000)	1,100 (9,500)	650 (8,300)	600 (6,800)
Trp	3,100	7,400	3,800	8,400
Tyr	620	1,620		
Val	900	1,950		

Cells of SM 201 [tsm8] and SM 202 [TSM8] have been grown at 23°C in glycerol, washed, resuspended in synthetic medium and incubated at 23°C and 36°C, respectively. After 2 min, cycloheximide (200 µg/ml) and after 5 min, the label was added. The radioactivity added was 0.03 µCi/ml ^{14}C Ala, Arg, Asp, Asn, Cys, Glu, Gln, Gly, His, Ser, Thr, Tyr; 0.15 µCi/ml ^{14}C Ile, Leu, Lys, Met, Phe, Pro, Val; 0.4 µCi/ml ^{14}C Trp; and 3 µCi/ml ^{3}H formate (presence of 2 mM cold serine). The incorporation was terminated by pipetting 5×10^7 cells per sample into hot trichloracetic acid at a final concentration of 10%. After boiling for 20 min cells were given on glass fiber filters No.6 (Schleicher and Schüll, Dassel, Germany) and washed free of non-incorporated activity with 5 mM cold amino acids and finally with ethanol. After drying filters were counted in 5 ml toluene containing per liter 5 g PPO and 0.3 g dimethyl-POPOP. Only the 35 min values are given. The values in parentheses give results of incubations in the absence of cycloheximide. (The experiment was performed together with A. Klein and technically assisted by B. Rothe.)

This Table shows that none of the tRNAs can be considered as a candidate for the thermolabile function, since with most amino acids a higher incorporation rate is found at 36°C as compared to 23°C. The incorporation rates with Arg, AsN, Cys, Glu, GlN, His and Thr are poor both at 23° and 36°C and both with mutant and wild type. The incorporation rates obtained in the absence of cycloheximide indicate that whole cell incorporation is, in some cases, lowered at the restrictive temperature both in the mutant and in the wild type. This effect may be caused by lowered transport rates or by pool extension of these amino acids at the elevated temperature. A specific temperature effect in the mutant is not observed with any of the amino acids.

Discussion

Handwerker et al. (20) and Schweyen et al. (21) have shown that the tsm8 mutation, which leads to rho$^-$-petite mutation at 36°C under conditions of catabolite repression, is mitochondrially inherited and maps between P (paromomycin-resistance) and C (chloramphenicol-resistance) on the mitochondrial genome. The nature of the gene product(s) of the TSM8 gene, if any, is, however, not yet clear. From the data presented here it may be concluded that the respiratory chain and the energy conserving machinery do not contain a thermolabile protein, the non-functioning of which under non-permissive conditions in the presence of glucose may secondarily initiate rho$^-$-petite formation. It is evident that the mutation does not directly affect mitochondrial ribosomal RNAs, firstly, because the locus of the tsm8 mutation maps differently from the rRNAs (21,30-32) and, secondly, because the SDS polyacrylamide gel electrophoresis pattern of mitochondrially synthesized proteins is altered and not

generally a lower incorporation rate of radioactive precursors achieved. This alteration in the labelling pattern occurs much faster than the response of the mitochondrial translation activity to the temperature shift. All these functions including the cytoplasmatic ribosomal activity are influenced by the shift to restrictive conditions. These changes may be for secondary reasons such as exhaustion of mitochondrially synthesized protein precursor pools and lack of energy during longer periods at restrictive temperature.

The formation of low molecular weight proteins and their integration into the membrane may be closely related to the thermolabile function and to the initiation of the petite formation process. The nature of these peptides is presently not known and it cannot yet be decided whether they are breakdown products of normal functional mitochondrial proteins or low molecular weight precursors of these.

Four explanations may be considered as possible reasons for the disappearance of mitochondrially synthesized high molecular weight proteins and the emergence of low molecular weight peptides under restrictive conditions.

First, the shift to restrictive temperature may induce the action of a protease in the mutant, specifically cleaving mitochondrially synthesized proteins (but not cytoplasmic, unpublished observation). This appears to be rather unlikely because in the presence of glycerol at 36°C, band III of cytochrome oxidase persists and only subunits I and -to a lesser extent- II are diminished. Moreover, when cells are double-labelled in the presence of cycloheximide in a single culture first at 23°C and then at 36°C, the proteins labelled at 23°C remain intact, whereas those labelled at 36°C have a lower molecular weight (K. Kotzias and W. Bandlow, unpublished). Phenyl-

methylsulphonylfluoride (as an inhibitor of serine-specific proteases) does not prevent the emergence of low molecular weight material.

Secondly, it might be assumed that the peptides are translation products from shortened messengers. The mutation would then concern mitochondrial RNA polymerase, or ribonucleases, or mtDNA.

A third mechanism of the formation of these peptides might be a consequence of the action of a thermolabile tRNA occasionally leading to premature termination of peptide chains. The coding of a variety of tRNAs on the mitochondrial genome has been documented (33-36). But although it has been reported by Fukuhara (31) that a variety of tRNA markers maps in the region adjacent to the C-marker, which is the same region where the tsm8 marker maps, this explanation appears not to be very likely because temperature sensitivity of the incorporation of radioactive precursors into mitochondrial protein could not be found with any amino acid (Table II). Moreover, it is difficult to understand why in the presence of glycerol only subunits I and II vanish (Figs. 9A and 9B).

A possible fourth explanation is that these low molecular weight peptides are natural precursors of the mitochondrial proteins which are integrated into the membrane and their pool size extended because the oligomerization or their assembly into the functional complexes has become rate limiting rather than their formation which might be rate limiting under normal conditions. The pool of these peptides must then be so low under normal conditions that they are not detected by SDS polyacrylamide gel electrophoresis. If this were so, the mutation would effect a kind of organizer or assembler protein.

Which of the alternatives is correct cannot yet be decided. There is, however, no genetic evidence available for the existence of mitochondrially coded proteases, subunits of

mitochondrial RNA polymerase or RNases and also a mitochondrial "assemblase" has not yet been reported.

The crucial point is that at restrictive temperature rho$^-$-petite mutants are formed only under growing conditions with fermentable carbon sources (also with poorly repressing substrates like galactose, raffinose or mellibiose, 19). No petites are detectable in the presence of glucose under non-growing conditions (19)- Rho$^-$-petites are absent when cells are kept in glycerol at restrictive temperature and then plated on glucose medium at permissive temperature and a burst of petites is not found after returning from glycerol at 36°C to 23°C with simultaneous addition of glucose (19). This behaviour indicates that besides the restrictive temperature cell growth under catabolite repression is a prerequisite for the petite initiation in mutant tsm8. Continuation of mitochondrial protein synthesis and of the process sensitive to nalidixic acid are, obviously, not necessary, since also in the presence of chloramphenicol or nalidixic acid petites are formed with about the same frequency as in the absence of the inhibitors (Fig. 2).

On the other hand, mitochondrial DNA synthesis continues under non-permissive conditions, both in the presence of glucose and glycerol but mitochondrial DNA molecules are obviously altered in glucose-grown cells (I. Doxiadis and R.J. Schweyen, unpublished). It appears that the elevated temperature leads to an artifactual replication of mitochondrial DNA. These DNA molecules may be dimers or multimers of altered replication products. In glucose under growing conditions these may subsequently undergo recombination under the formation of inversions and,after cleavage, yield incomplete DNA molecules which may now contain tandem repeats and losses of other genes (7,8). These recombination and cleavage processes should not oc-

cur under non-growing conditions, i.e. in glycerol. The situation in mutant tsm8 may, thus, parallel that in nascent normal rho$^-$-petites, which do not replicate their DNA properly and continuously undergo rearrangements of mitDNA and losses of genes in combination with the repetition of retained sequences so that the original genome size is preserved (7,37).

One might speculate that the defective protein is a mitochondrially coded membrane factor exhibiting a control function both on the termination of DNA replication and, directly or indirectly, on the assembly of respiratory chain complexes. Its activity should, in addition, be controlled either directly by a glucose catabolite or, more likely, indirectly via a catabolite repression controlled nuclear gene product. This could explain the negative control of the assembly of respiratory components by catabolite repression and also the long lag in derepression of mutant tsm8, even under permissive conditions. How these multiple functions could be exerted by a single mitochondrially coded protein, present in mitochondria in only very low concentrations, remains to be elucidated.

Acknowledgements

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Disaggregation of Mitochondrial Translation Products to Small Polypeptides by Performic Acid Oxidation

D.E. Leister, P.J. Rogers, H. Küntzel

Proteins synthesized on Neurospora crassa and Saccharomyces cerevisiae mitochondrial (mt) ribosomes have apparent molecular weights (AMW) in SDS-containing buffers of between 40K and 10K (for review see 1). About 80 % of these proteins are extracted with acidified chloroform/methanol (C/M) (2:1) (2). In the course of isolating purified mt-products, we observed that some proteins had a pronounced tendency to aggregate in SDS-containing buffers. Complete oxidation of sulfur-containing amino acids with performic acid not only lead to an elimination of these large aggregates, but also caused an almost quantitative reduction of their AMW to about 8 - 10K (3). In this paper we demonstrate that all stable mt-translation products of AMW greater than 12K are dissociated to small polypeptides (8 - 10K) by performic acid oxidation under conditions where peptide bonds are not hydrolyzed.

Neurospora crassa (wild type Em 5256) and Saccharomyces cerevisiae (haploid strain S 288C, α) were grown and double-labeled as previously described (2). First, total cellular proteins were uniformly labeled with [^{14}C]-phenylalanine, then mt-translation products were selectively labeled with [^{3}H]-phenylalanine in the presence of cycloheximide. Mitochondria isolated from broken cells by differential centrifugation were suspended in distilled water and extracted with 20 volumes of C/M (2:1) containing 35 mM acetic acid. The extract was filtered through paper and concentrated about 5-fold. Proteins were recovered by precipitation with diethyl ether at -20°C. The precipitate was suspended in 5 mM Tris-HCl, 0.5 % SDS,

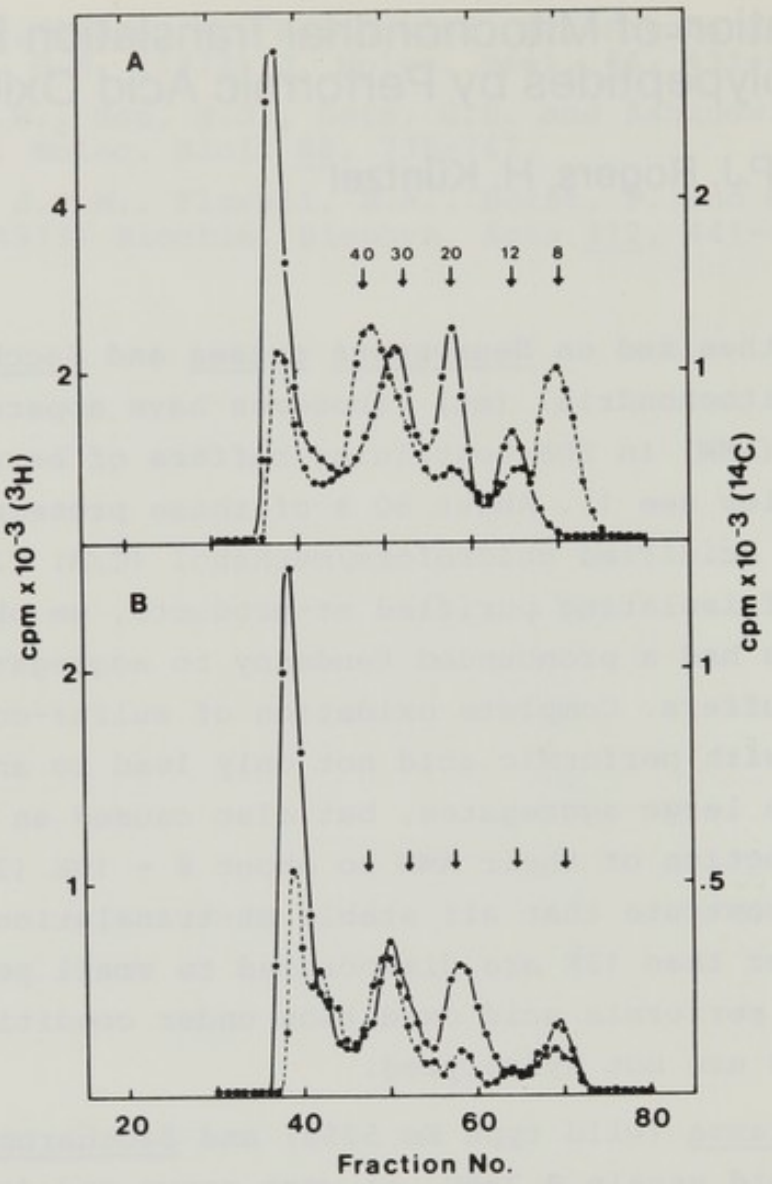


Fig. 1: SDS-Sephadex G-200 filtration of C/M-extracted proteins from Neurospora crassa (A) or Saccharomyces cerevisiae (B). The proteins were uniformly labeled in vivo with [^{14}C]-phenylalanine (o---o) and subsequently pulse-labeled in the presence of cycloheximide (0.1 mg/ml) with [^{3}H]-phenylalanine (o——o). The AMW (x 10^{-3}) indicated by the arrows are estimated from the elution positions of bovine serum albumin, ß-lactoglobulin and cytochrome c.

1 mM dithiothreitol, pH 7.5 (column buffer) with the aid of a syringe; heated at 100°C for 1 min; then applied to a Sephadex G-200 column equilibrated with the same buffer.

The extracted proteins were separated into four major peaks; eluting in the excluded volume and in regions of AMW 40K, 20K, and 12 - 8K (Fig. 1). The excluded fraction and the 20K peak contain mainly mt-translation products, whereas the 40K and the 12 - 8K peaks contain proteins synthesized on both mt- and cytoplasmic ribosomes (2).

The protein fractions in Fig. 1B were analyzed by SDS-PAGE in 10 % polyacrylamide gels as described by Weber and Osborn (4). The stain patterns (Fig. 2) revealed more protein species than seen by chromatography due to the greater resolving power of PAGE, however the AMWs of the protein bands generally corresponded to the MW region of the column from which samples were derived.

Figure 3 shows the results of re-chromatography of C/M-extracted yeast mt-products derived from a single column analysis (top row). The middle row of chromatograms were obtained by precipitating proteins in the exclusion peak, 40 - 30K, and 20K regions with 90 % acetone; solubilizing the precipitates by boiling in column buffer and re-chromatographing. Exclusion peak and 40 - 30K proteins re-chromatographed in their original positions, whereas part of the 20K proteins aggregated and eluted in the exclusion region. Aliquots of the same column fraction pools were dialyzed against distilled water to remove buffer salts and then lyophilized. The lyophilate was dissolved in 98 % formic acid and cooled on ice. Ice-cold performic acid (0.5 ml of 30 % H_2O_2 and 9.5 ml of 98 % formic acid were mixed and allowed to stand for 2 hr at room temperature in a sealed vessel; 5) was added to the protein solution and the mixture incubated at 4°C for 8 - 12 hr. 5 volumes of distilled water were added and the solutions were lyophilized. The samples were dissolved in water and re-lyophilized to thoroughly remove formic acid. Oxidized proteins were dissolved in column

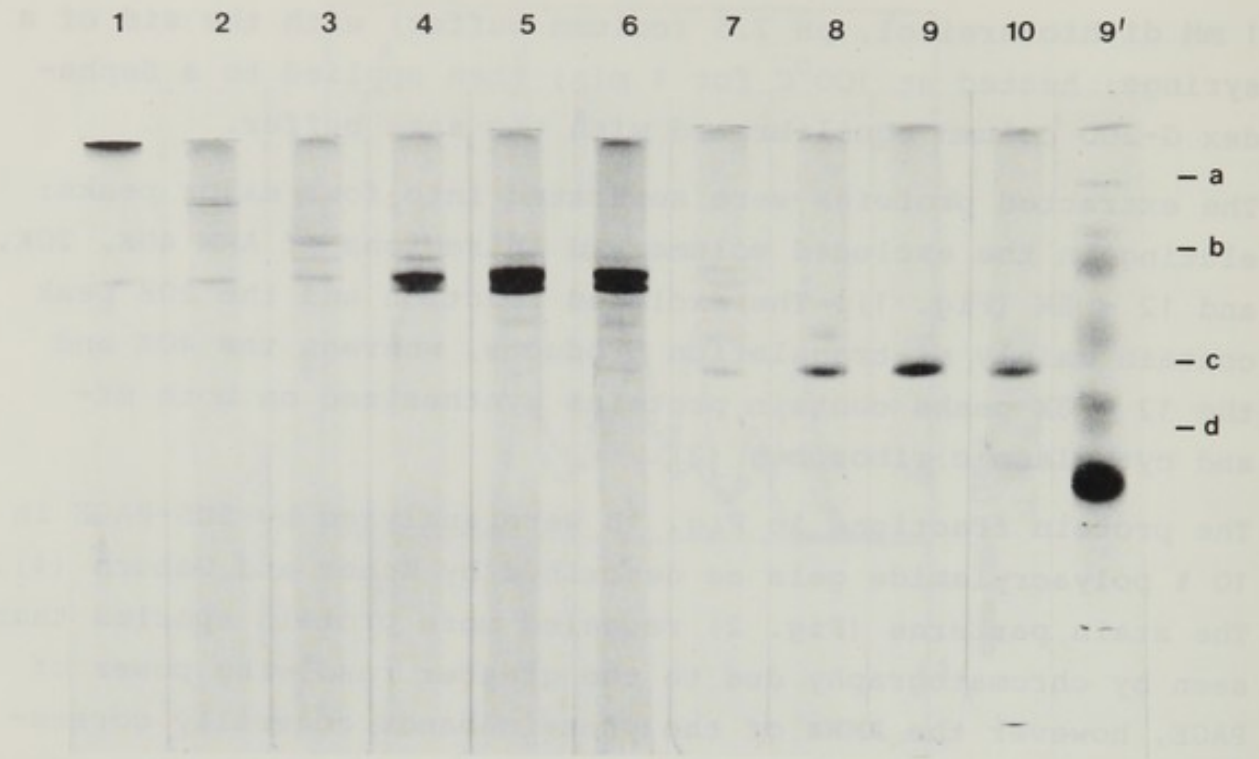


Fig. 2: SDS-polyacrylamide gel electrophoresis of C/M-extracted proteins from yeast mitochondria. The following fractions of the Sephadex eluate (Fig. 1B) were used: gel 1, fr. 37-40; gel 2, 41-42; gel 3, 43-44; gel 4, 45-47; gel 5, 48-50; gel 6, 51-53; gel 7, 54-55; gel 8, 56-57; gel 9, 58-59; gel 10, 60-62. An aliquot of fractions 58-59 (gel 9) was dialyzed against water, lyophilized, dissolved in 98 % formic acid to a final concentration of 1 mg protein/ml, and treated with 100 µl performic acid per mg protein for 12 hrs at 4°C. The mixture was diluted 10-fold with water and lyophilized; the residue was dissolved in SDS buffer and electrophoresed (gel 9'). a) bovine serum albumin (MW 69K); b) egg albumin (43K); c) ß-lactoglobulin (19K); d) cytochrome c (12K).

buffer and chromatographed (bottom row). Proteins in all MW areas of the initial chromatograms were nearly quantitatively disaggregated to about 8 - 10K AMW polypeptides.

Samples of unoxidized and oxidized 20K yeast proteins were also submitted to SDS-PAGE (Fig. 2, gels 9 and 9'). Although 6 times more oxidized than unoxidized protein was electrophoresed, the 20K band was not detected in gel 9' and a new major band appeared at about 8 - 10K. The intensely stained proteins seen above the 20K protein in 9' were detected in the unoxidized sample but were less intense because less protein was electrophoresed.

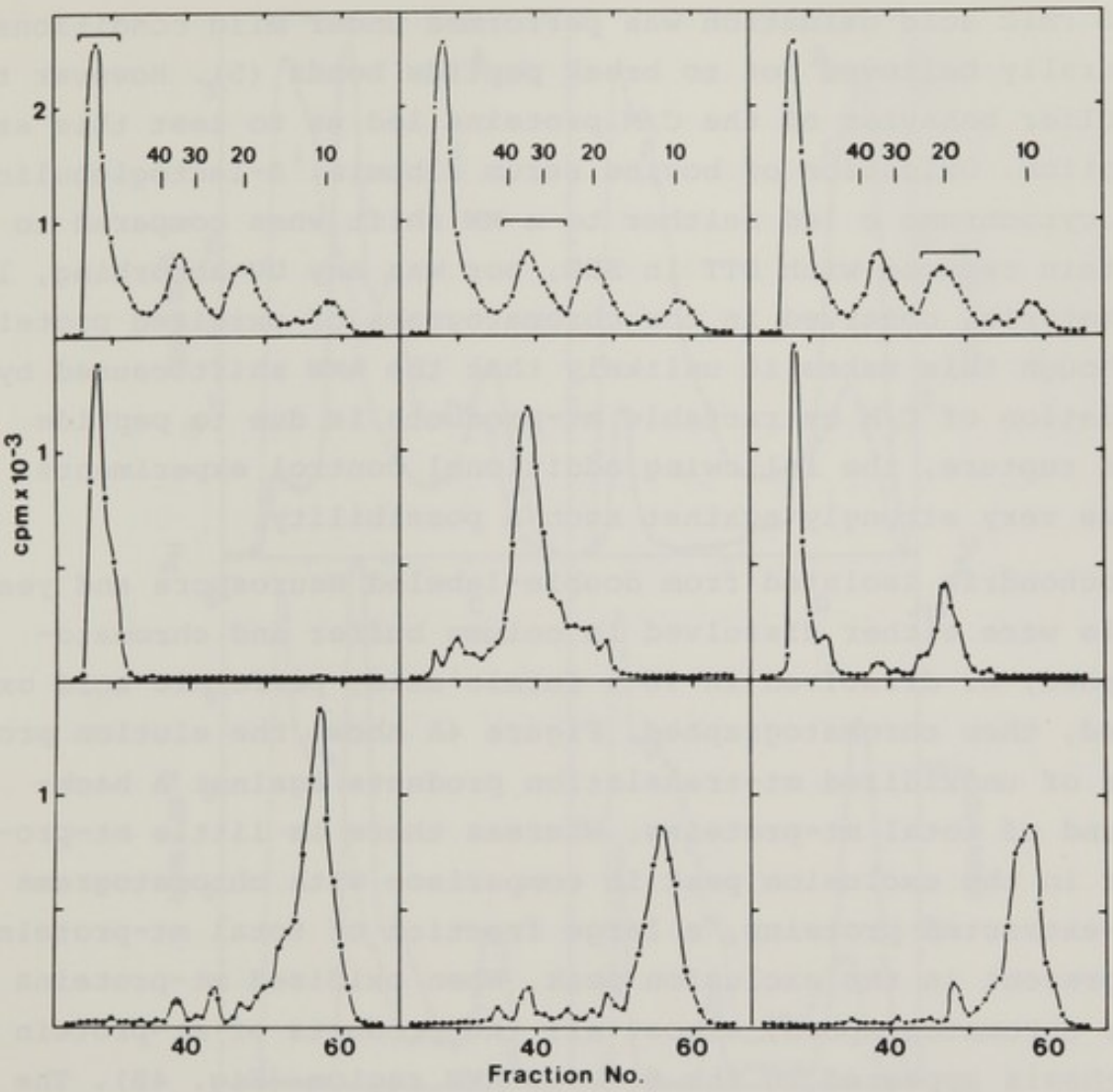


Fig. 3: SDS-Sephadex G-200 filtration of extractable mitochondrial proteins from yeast pulse-labeled with [^{3}H]-phenylalanine. The peak fractions indicated in the top row were pooled and proteins were precipitated with 90 % acetone. One aliquot of the protein was immediately re-analyzed by SDS gel filtration (middle row); another aliquot treated with performic acid (see legend to Fig. 2) before re-chromatography (bottom row).

We stress that all the AMW shifts caused by performic acid oxidation observed in the course of this work resulted from oxidation. Treatment with formic acid alone had little effect on AMWs.

Performic acid oxidation was performed under mild conditions generally believed not to break peptide bonds (5). However the peculiar behavior of the C/M proteins led us to test this assumption. Oxidation of bovine serum albumin, ß-lactoglobulin and cytochrome c led neither to a MW shift when compared to protein reduced with DTT in SDS, nor was any UV-absorbing, low MW material observed in the chromatograms of oxidized proteins. Although this makes it unlikely that the AMW shift caused by oxidation of C/M extractable mt-products is due to peptide bond rupture, the following additional control experiments argue very strongly against such a possibility.

Mitochondria isolated from double-labeled Neurospora and yeast cells were either dissolved in column buffer and chromatographed, or dissolved in 98 % formic acid, performic acid oxidized, then chromatographed. Figure 4A shows the elution profile of unoxidized mt-translation products against a background of total mt-proteins. Whereas there is little mt-product in the exclusion peak in comparison with chromatograms of C/M-extracted proteins, a large fraction of total mt-proteins is present in the exclusion peak. When oxidized mt-proteins were chromatographed, almost all the products of mt-protein synthesis appeared in the 8 - 10K AMW region (Fig. 4B). The small increase in the amount of total mt-proteins eluting in the 8 - 10K AMW region after oxidation reflects the fact that although most, if not all, C/M-extractable proteins are shifted to low AMW by oxidation, these proteins comprise only about 10 - 15 % of the total mt-proteins (1). Most importantly, the lack of any further, substantial change in the elution profile of the bulk of the total mt-proteins strongly argues that oxidation does not rupture peptide bonds.

Thus mt-products, not only alone, but also in the presence of total mt-proteins, undergo a dramatic reduction in AMW upon performic acid oxidation. This shift is a property peculiar to these proteins, shared apparently only by products of cytoplasmic protein synthesis which are extracted from mitochondria

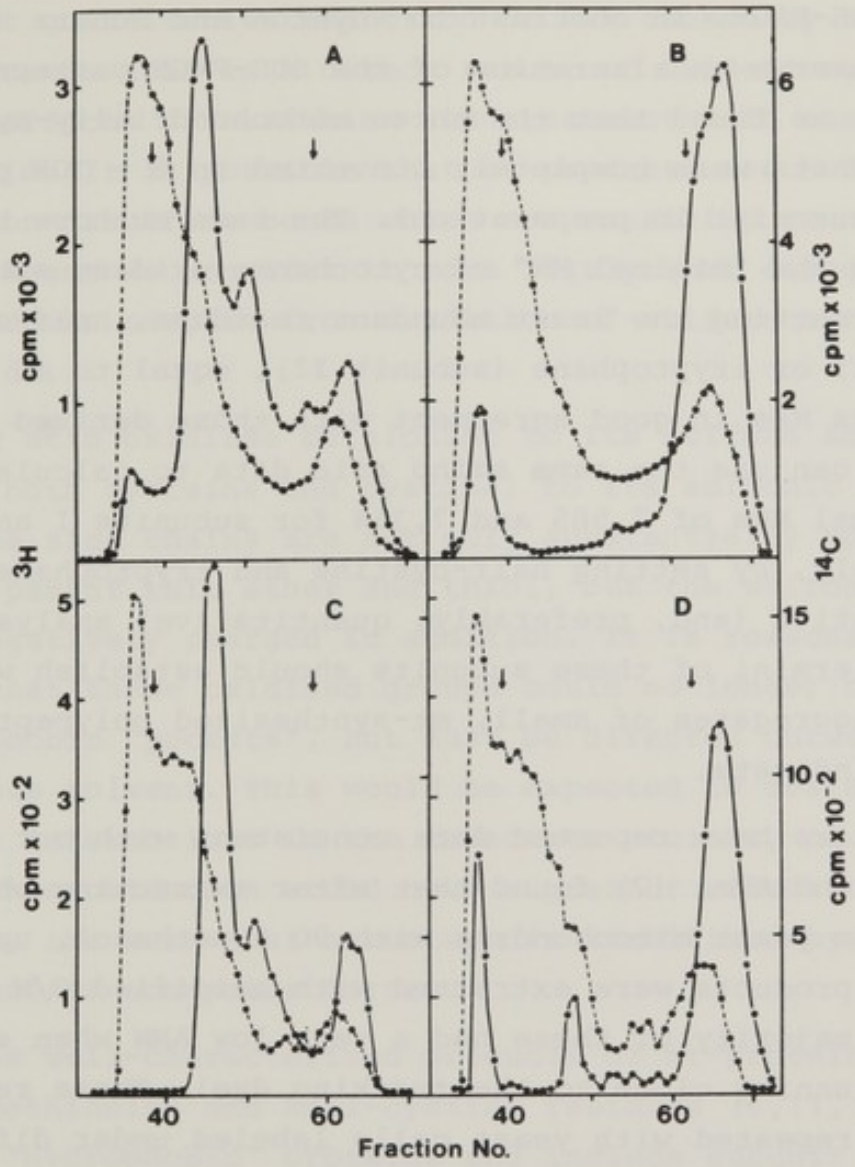


Fig. 4: SDS-Sephadex G-200 filtration of whole mitochondrial proteins from Neurospora (A,B) or yeast (C,D). Cells were double-labeled as described in Fig. 1 (o---o, [^{14}C]-radioactivity; ●——●, [^{3}H]-radioactivity). A,C: untreated proteins; B,D: oxidized proteins. The arrows indicate the elution positions of bovine serum albumin and cytochrome c.

with C/M. It is not a property of either standard proteins or of total mt-proteins.

We have also oxidized purified yeast cytochrome oxidase (heme content 10.6 µg/mg protein) and analyzed the subunit composi-

tion by SDS-PAGE. In contrast to Poynton and Schatz (6) who did not observe an alteration of the SDS-PAGE pattern upon oxidation, we found that the three mitochondrially-synthesized, large subunits were completely converted to 8 - 10K polypeptides (manuscript in preparation). The same authors have also calculated the "minimal MW" of cytochrome oxidase subunits I and II by setting the least abundant residues, half-cystine (subunit I) or tryptophane (subunit II), equal to 4. Although this yields MWs in good agreement with those derived by SDS-PAGE, one can use the same amino acid data to calculate the true minimal MWs of 7,585 and 7,364 for subunits I and II, respectively, by setting half-cystine and tryptophane equal to 1. Qualitative (and, preferably, quantitative) analyses of the N- and C-termini of these subunits should establish whether they are aggregates of small, mt-synthesized polypeptides, as our data indicate.

Other workers have reported data consistent with our results. Tzagoloff and Akai (7) found that after extracting phospholipids from yeast mitochondria with 90 % methanol, up to 60 % of the mt-products were extracted with acidified C/M and that the great majority of these had a very low AMW when submitted to PAGE (running close to the tracking dye). These results have been repeated with yeast cells labeled under different conditions (8). Hawley and Greenawalt (9) showed that early in the germination of Neurospora conidia, 80 % of mt-protein synthesis is restricted to two small proteins of about 7200 and 9000 AMW and that only later in development does the synthesis of larger AMW species predominate. Furthermore, Küntzel and Blossey (10) showed that the major, in vitro translation products of a Neurospora mt-lysate are 10 and 12K AMW proteins; identical AMW proteins were synthesized by a heterologous E. coli translation system supplemented with in vitro transcripts of Neurospora mt-DNA.

Yeast and Neurospora mt-translation products were not disaggregated by treatment with formic acid alone. Consequently,

the almost quantitative reduction of AMW caused by treatment with performic acid is a direct result of oxidation, or of the combined effects of oxidation and treatment with concentrated formic acid. It is possible that mt-products are held together through non-disulfide bonds which are sensitive to oxidation. But at present we consider it more likely that disaggregation of mt-products results from oxidation of sulfur-containing amino acids.

Performic acid oxidizes methionine to its sulfone and half-cystine (both cysteine and cystine) to its sulfonic acid. These residue side chains are not only substantially more polar than the parent thio ether and thiol, but the sulfonic acid is doubly-negatively charged in addition. It is reasonable to imagine that these oxidized groups could no longer be located in hydrophobic "pockets", but must be directed outward toward the aqueous solvent. This would be expected to destabilize non-polar interactions in the immediate vicinity of these residues. Thus oxidation would have a greater effect on protein structure, the more that structure is stabilized by disulfide bridges and/or non-polar forces.

All of the well-characterized products of mt-protein synthesis contain methionine and half-cystine residues (6,11,12). They are also "hydrophobic" proteins and include subunit 9 of yeast oligomycin-sensitive ATPase, which has the lowest known polarity of any protein (11). If the low polarity of this protein is the reason for its solubility in C/M, then the C/M-extractable mt-products might be low polarity proteins as well. These proteins may share many properties with another group of low-polarity proteins, the so-called "proteolipids" (13).

Purified proteolipid apoproteins from rat brain white matter have been isolated in two forms, one soluble in C/M but insoluble in aqueous solvents and the other soluble in aqueous solvents but insoluble in C/M (13); these forms are reversibly interconvertible (13). At least two-thirds of the half-cystine residues of these proteins are in disulfide bonds (14). All

their half-cystines are blocked by reduction and treatment with iodoacetic acid in neutral C/M (14), but the half-cystines of the proteolipid apoprotein P8 are not completely blocked by reduction and treatment with iodoacetic acid in aqueous solvents (15). The first 20 residues of P8 contain only 2 polar amino acids (polarity = 0,1) and this region contains 3 half-cystines. These half-cystines might be the ones which are not reduced and/or blocked because they are located in an unusually hydrophobic "pocket". They are however oxidized by performic acid (15).

We argue by analogy that many of the half-cystines (and possibly methionines also) in the low-polarity mt-translation products might be located in hydrophobic "pockets". Their environment might protect them from reduction and/or blocking. Although Poynton and Schatz (6) detected no change in the AMWs of any yeast cytochrome oxidase subunit by SDS-PAGE after treatment with 2-mercaptoethanol and iodoacetamide, they did not demonstrate that the treatment completely blocked all half-cystines.

We conclude that the disaggregating effect of complete performic acid oxidation on yeast and Neurospora mt-products is most probably due not only to the complete elimination of all disulfide bonds, but perhaps also to the concommitant introduction of very polar and strongly charged groups into these low polarity proteins.

ACKNOWLEDGEMENT

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The Synthesis of Yeast Mitochondrial Proteins in Cell-free Systems

H. Scragg, M.J. Eggitt, D.Y. Thomas

A number of cell-free systems have been described which are capable of transcribing and translating small DNAs (1-4). The systems have been particularly useful for the identification of the genes coded by small DNAs, and are becoming increasingly important in the study of the control mechanisms of gene expression. We are interested in the genes coded by yeast mitochondrial DNA (mtDNA).

By RNA-DNA hybridization it has been shown that per $5x10^7$ daltons molecular weight of yeast mtDNA there is a close to a single copy of each of the mitochondrial ribosomal RNAs and about 19 tRNA genes (5-7). Indirect evidence, obtained through the use of specific inhibitors of cytoplasmic or mitochondrial protein synthesis (16), indicates that the three largest polypeptides of the cytochrome c oxidase complex (E.c. 1.9.3.1., ferrocytochrome c : oxygen oxidoreductase) and the four smallest polypeptides of the rutamycin-sensitive ATPase complex (9-11) are synthesized in mitochondria. Recently it has also been shown that isolated mitochondria are capable of synthesizing the 3 largest subunits of cytochrome c oxidase (12), further indicating that the structural genes for these proteins may be located in mtDNA.

A two-step *in vitro* system capable of transcribing and translating yeast mtDNA has been developed (13). Components for this system have been isolated from *E.coli*, and identification of the *in vitro* products has been achieved by precipitation with antiserum raised against mitochondrial proteins, and subsequent dodecylsulphate-polyacrylamide gel electrophoresis of the immune precipitates. Such a system has revealed the synthesis from mtDNA of 5-6 polypeptides similar in antigenicity and size to those synthesized *in vivo*. By use of antiserum raised against cytochrome c oxidase holoenzyme we have further identified that two and probably three of the largest polypeptides of the enzyme complex are synthesized in the mtDNA directed *in vitro* system directly demonstrating their mtDNA coding (20).

In addition, yeast mitochondrial RNA has been isolated showing messenger-RNA activity when added to an *E.coli* cell-free system (14), or injected into *Xenopus laevis* oocytes (15). The *in vitro* products from these two systems were similarly analyzed and were found to have peptides in common with those synthesized *in vivo* and in the DNA directed *in vitro* system.

Materials and Methods

Strains and Growth Conditions

Saccharomyces cerevisiae strains 239 (N.C.Y.C.) and ∠DV 147 (original source, R.K. Mortimer) were cultured as described previously (16). *E.coli* MRE 600 was supplied frozen by the Microbiological Research Establishment (Porton, Wiltshire, U.K.).

Labelling of Mitochondrial Protein in vivo

(^{35}S)-methionine labelled mitochondrial proteins were prepared essentially as described previously (16). (^{35}S)-methionine labelled cytochrome c oxidase was extracted from labelled mitochondria using the method of Tzagoloff and Meagher (17). Much non-specific binding of (^{35}S)-methionine could be eliminated by dispensing the isotope into small vials storing them at -80°C and thawing them only once before use.

Extraction of total mitochondrial RNA

Total mitochondrial RNA was extracted as described (15).

Cell-free Protein-Synthesizing Systems

The DNA-directed system was prepared and run as described in detail previously by us (13) as was the RNA-directed system (14).

Injection of RNA into Xenopus laevis Oocytes

The injection, incubation and analysis of the X. laevis oocytes was done as described previously (15).

Dodecylsulphate-Polyacrylamide Gel Electrophoresis

The polyacrylamide gel electrophoresis either by the method of Laemmli (18) and treated as described (13), or with the gel system of Weber and Osborn (19) and treated as

described (15). All gels contained internal markers of small amounts of dansylated proteins (33), the molecular weights of these markers were taken to be not significantly different from those of the native proteins. The fluorescent bands were visualized under ultraviolet light (>300 nm) before slicing the gel with a multiple razor blade device. Radioactivity was solubilized by incubation at 60°C with 3% Protosol (New England Nuclear). As there are only a few polypeptides to be analyzed both from in vivo and in vitro systems this method of gel analysis is optimal.

Preparation of Antisera

Antimitochondrial antisera was either prepared as described (13) (Method I) or as described (15) (Method II). Antisera prepared against purified cytochrome c oxidase and ATPase were kindly supplied by Dr. A. Tzagoloff.

Preparation of Yeast mtDNA

Mitochondrial DNA was prepared essentially as described previously (13). In later preparations mitochondria were purified from sphaeroplasts by differential centrifugation, resuspended in 1M Nacl - 50mM Tris-Cl pH 8 - 50mM EDTA and rapidly frozen to -20°C. The suspension was then thawed at 60°C in the presence of 2% sodium-dodecylsulphate for 20 minutes. The lysate was deproteinized at 25°C with an equal volume of redistilled phenol (+1% m-cresol, 0.1% hydroxyquinoline), then with an equal volume of chloroform. The nucleic acid solution was dialysed twice against 500 volumes of 10mM Tris-Cl pH 8 - 5mM EDTA. Recrystallized sodium iodide was added to a $^{M}/_{25}$

= 1.43, ethidium bromide was added to a final concentration of 2µg/ml. Centrifugation in polyallomer tubes (previously boiled in 1% Sarkosyl) in the Beckman Ti-60 rotor was for 12 hours at 45 K.r.p.m. at 25°C, followed by 30 hours at 35 K.r.p.m. This method gives excellent separation of mitochondrial and nuclear DNA (about 1cm in a 15ml volume gradient). The mitochondrial band was collected by upward displacement, diluted with an equal volume of water, the ethidium bromide removed with Dowex 50wx-8 (Na^+ form) beads, and the DNA precipitated with 2 volumes of ethanol at 0°C for 2 hours. The DNA was dissolved in 5mM Tris-Cl pH 8 - 1mM EDTA and exhaustively dialysed against the same buffer. No contamination with nuclear DNA could be detected by analytical ultracentrifugation.

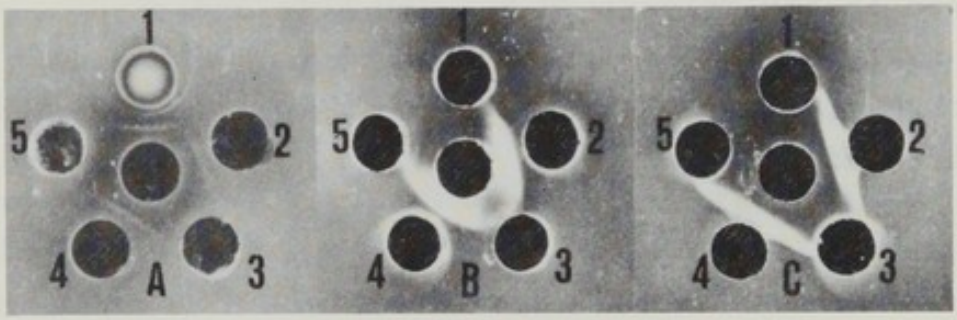
Results

Not only is there a superficial similarity between mitochondrial and bacterial transcriptional and translational apparatuses but previous studies have shown that they can be interchanged to a considerable extent in cell-free systems (27).

We developed a two-stage cell-free system (13) to transcribe and translate yeast mtDNA as we were unable to obtain consistent results using a coupled transcription/translation system for this template. It consists of the transcription of purified mtDNA using either yeast mitochondrial RNA polymerase or E.coli RNA polymerase (Ec 2.7.7.6) under optimal conditions, the RNA synthesized being used to direct the synthesis of polypeptides

in an E.coli S-30 cell-free system (13). Antisera prepared against mitochondrial proteins is used to precipitate specific products from the cell-free system which are subsequently analyzed by dodecylsulphate-polyacrylamide gel electrophoresis. The results indicate that some 5-6 polypeptides made in vitro compare in antigenicity and molecular weight with those synthesized in vivo (see ref. 13).

Fig. 1. Ouchterlony Plates of Various Antigens and Antisera



(A) The centre well contains 10 µl antisera prepared against mitochondrial proteins (Method I); well 1, 10 µl of a total mitochondrial sonnicate; wells 3 and 5, 10 µl E.coli S-30 preparation; wells 2 and 4, 10 µl of a mitochondrial sonnicate with the particulate fraction removed (10,000 x g x 10 min).

(B) The centre well contains 10 µl cytochrome c oxidase antiserum; well 1, 10 µl E.coli S-30 preparation; wells 2 and 4, 10 µl purified cytochrome c oxidase; wells 3 and 5, 10 µl purified F_1 ATPase.

(C) The centre well contains 10 µl F_1 ATPase antiserum; well 1, 10 µl E.coli S-30 preparation; wells 2 and 4, 10 µl purified F_1 ATPase; wells 3 and 5, 10 µl purified cytochrome c oxidase.

The plates are 1% agarose in 10 mM Tris-acetate buffer, pH 7.5. Incubation was at 22°C overnight, the plates were washed with phosphate-buffered saline (0.05 M KPO_4, pH 7.0 containing 0.9% NaCl), and stained with 1% Thiazolium red in 1% acetic acid, and destained in 1% acetic acid.

We have paid careful attention to the specificity of the antisera we have used in this study and the optimal conditions for precipitation of mitochondrial antigens. The availability of antisera prepared against purified cytochrome c oxidase enabled the detection of specific proteins produced in vitro (20).

Fig. 1 shows the specificity of the various antisera used in this series of experiments as determined by double diffusion against various antigens. Fig. 1a shows that the antimitochondrial protein antisera produced precipitin

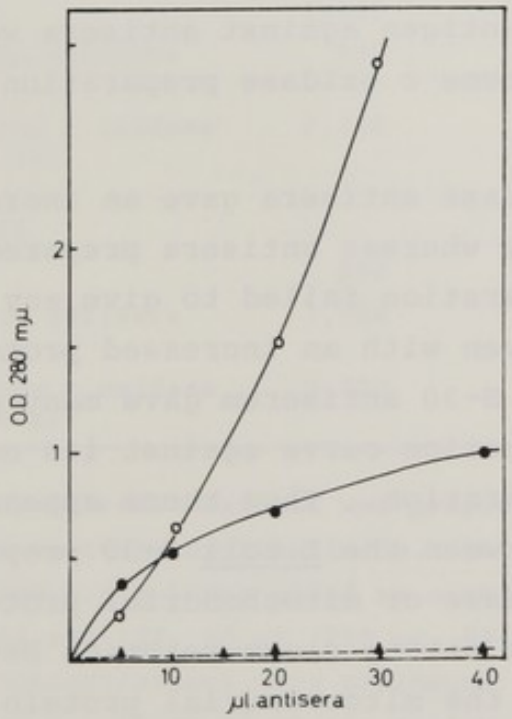


Fig. 2. Precipitation of cytochrome c oxidase by various antisera.
38 μg of purified cytochrome c oxidase was incubated with increasing amounts of cytochrome c oxidase antisera (26 mg/ml) (-o-o-), 19 μg of purified cytochrome c oxidase was incubated with increasing amounts of mitochondrial protein antisera (Method 2) (60 mg/ml) (-●-●-), 1.088 μg of cytochrome c oxidase preparation (prepared by KCl and Triton X-100 extraction) was incubated with increasing amounts of E.coli S-30 antisera (96 mg/ml) (-▲-▲-). Incubation was at 4°C overnight, 0.9 ml phosphate-buffered soline added and the precipitate removed by centrifugation at 10,000 x g x 10 min. The optical density of the resultant supernatant was read and the optical density of the protein in the pellet calculated. The values quoted have the blank values minus antisera subtracted.

lines only against mitochondrial preparations and not with the E.coli S-30 preparation. Fig. 1b demonstrates the production of a precipitin line between cytochrome c oxidase and its antisera and a failure to react with either the E.coli S-30 preparation or purified ATPase. Further in Fig. 1c the cytochrome c oxidase preparation failed to give any reaction with antisera raised against ATPase indicating no contamination of the cytochrome c oxidase with ATPase and the specificity of the antisera used.

Titration curves of antigen against antisera were prepared using a cytochrome c oxidase preparation and various antisera (Fig. 2).

The cytochrome c oxidase antisera gave an increasing amount of precipitate whereas antisera prepared against the E.coli S-30 preparation failed to give any significant precipitation even with an increased protein concentration. The E.coli S-30 antiserum gave many precipitin lines and a good titration curve against its own antigen the E.coli S-30 preparation. Thus there appeared to be no cross-reaction between the E.coli S-30 preparation and the cytochrome c oxidase or mitochondrial proteins. This is in contrast to the strong cross-reaction between cytochrome c oxidase and the mitochondrial protein antisera indicating common antigens.

The specificity of the cytochrome c oxidase antisera was proved further by precipitation with a variety of antisera from a (^{35}S)-methionine labelled cytochrome c oxidase preparation.

Table I shows the levels of precipitation achieved from an in vivo labelled preparation of cytochrome c oxidase using the cytochrome c oxidase antisera, a serum from the same rabbit obtained prior to immunization and an anti-

Table I. Precipitation of Radioactive Proteins from a Labelled Cytochrome c Oxidase Preparation

	Radioactivity in precipitates (c.p.m.)	% radioactivity precipitated
Experiment I		
Control	2,381	0.71
plus preimmune sera (100 µl)	2,880	0.86
plus cytochrome c oxidase antisera (100 µl)	12,540	3.77
Experiment II		
Control	1,120	1.73
plus ovalbumin antisera (25 µl)	990	1.53
plus cytochrome c oxidase antisera (25 µl)	2,260	3.49
Experiment III		
Control	890	1.37
plus ovalbumin antisera (100 µl)	1,020	1.57
plus cytochrome c oxidase antisera (200 µl)	3,420	5.29

Radioactive cytochrome c oxidase was prepared as described under Methods omitting cycloheximide. In experiment I 250 µl (498,000 c.p.m.) was added to each reaction and in experiments II and III, 50 µl (250 µg, 64,600 c.p.m.). The protein concentrations were preimmune sera 50.4 mg/ml; cytochrome c oxidase 26 mg/ml; ovalbumin antisera 20 mg/ml. Incubation was at 4°C overnight, the precipitate removed by centrifugation 10,000 x g x 10 min, washed 3 x 2 ml of 5 mM Tris-acetate buffer, pH 7.5, and resuspended in a final volume as described under Methods.

ovalbumin antisera. Both the preimmune and ovalbumin antisera precipitated little radioactivity more than that obtained for the control value whereas the cytochrome c oxidase antisera gave some 5 times the control value. A similar result was obtained when the antisera concentrations were increased.

The precipitates obtained in this experiment (TABLE I) were subsequently analyzed by dodecylsulphate polyacrylamide gel electrophoresis. The results are shown in Fig. 3.

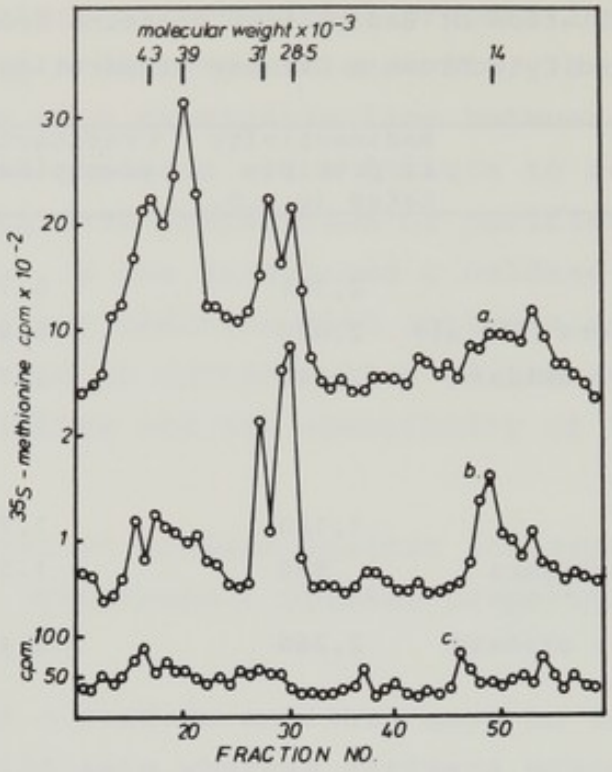


Fig. 3. SDS-gel electrophoresis of in vivo translation products

Yeast cells Saccharomyces cerevisiae, strain 239 N.C.Y.C. were labelled with ^{35}S-methionine 1μCi/ml, specific activity 164 Ci/mM for 1 hr at 30°C essentially as described previously[13]. A 1M KCl and 1% Triton X-100 extract was prepared from submitochondrial particles. This extract was either treated immediately for electrophoresis[13,18], or treated with antiserum[13]. Antiserum precipitates were prepared from 50μl of Triton X-100 extract with a predetermined optimum amount of antiserum. Incubation was at 22°C for 1 hr, the precipitate being recovered by centrifugation (9,000 x g for 10 min), and washed 3 times with 2ml 5mM Tris-acetate buffer (pH 7.5). The final precipitate was prepared for electrophoresis as described previously[13]. After electrophoresis in 15% polyacrylamide gels[18] at 5mA/gel for 16 hr at room temperature, the gels were cut into 1mm slices, and digested in 5ml toluene-based scintillant containing 5% "Protosol" (New England Nuclear). The following fluorescent labelled molecular weight-standards were incorporated in each gel run (ovalbumin, 43,000; lysozyme, 14,500; cytochrome c, 12,500). a, in vivo; b, antiserum precipitate from in vivo; c, preimmune antiserum precipitate from in vivo.

In this experiment cycloheximide was omitted enabling all 7 polypeptide components of the cytochrome c oxidase complex to be labelled in vivo as can be seen in Fig. 3A. Figs. 3B and C show that the cytochrome c oxidase precipitates only those labelled proteins corresponding in molecular weight to the cytochrome c oxidase subunits with perhaps a rather low amount of subunit III being detected. In contrast, the preimmune serum failed to give any significant precipitation of radioactive proteins (small proteins and peptides would not be detected on these gels).

Experience with the analysis of the products of cell-free systems using antisera has shown three main problems to be particularly considered when dealing with proteins. Firstly, mitochondrially synthesized protein, in particular cytochrome c oxidase subunits are relatively insoluble such that a combination of KCl and Triton X-100 is required for their solubilization (17). Consequently at the termination of the cell-free systems both KCl and Triton X-100 are added to ensure solubilization of products such as these. The effect of omission of KCl and Triton X-100 has been described (15), yielding considerable aggregation. Experiments have shown that addition of KCl and Triton X-100 has little or no effect upon production of antigen-antisera complexes.

Secondly, cell-free systems accumulate during the translational incubation (37°C) or during the immune precipitation step (4°C) a significant amount of aggregated ribosomes which trap radioactive and unfinished proteins. Although addition of KCl and Triton X-100 to the immune precipitation step reduces this, aggregated ribosomes are normally removed by low speed centrifugation.

Lastly, antigen-antisera complex formation does include non-specific adsorption and precipitation, important when involving labelled proteins. Adsorbed radioactiv-

ity can be significantly reduced by careful storage of the (^{35}S)-methionine (see Methods) and by washing of the resulting precipitates but the adsorbed larger polypeptides constitute more of a problem. We have used a pre-precipitation method to reduce this. An antigen-antisera complex is formed (i.e. using ovalbumin) and removed by centrifugation prior to the antisera precipitation of the specific products. This has the effect of removing much of the non-specific adsorbing material but does considerably reduce the final yield of the specific precipitation and also makes calculations of the efficiency of translation of these cell-free systems difficult. Under normal conditions a specific amount of cold carrier is added in the final antisera precipitate as the amounts of polypeptides synthesized in vitro are small. A second method has been used in particular with RNA-directed in vitro systems that of omitting the carrier and adding goat-antirabbit serum (14) to precipitate the first antigen-antisera complex. This gives a smaller precipitate with a consequent reduction in non-specific adsorption. The scheme used is summarized in Fig. 4.

The described method has been recently further improved by linking the antisera to cyanogen-bromide activated Sepharose 4 B which has successfully identified the synthesis in a cell-free system of isocitrate lyase (36). This method may, however, present more problems when complex mixtures of antigens are to be analyzed. Possibly antisera raised against purified cytochrome c oxidase and F_1 ATPase subunits will be easier to handle.

Similar results were also obtained by us using a double precipitation method (a predetermined titre of goat anti-rabbit antiserum was added after the initial rabbit anti-mitochondrial antiserum) (see ref. 15). Both systems require prior removal of ribosomes and have similar capabilities for non-specific trapping of radioactivity unless the precautions outlined in Fig. 4 are applied.

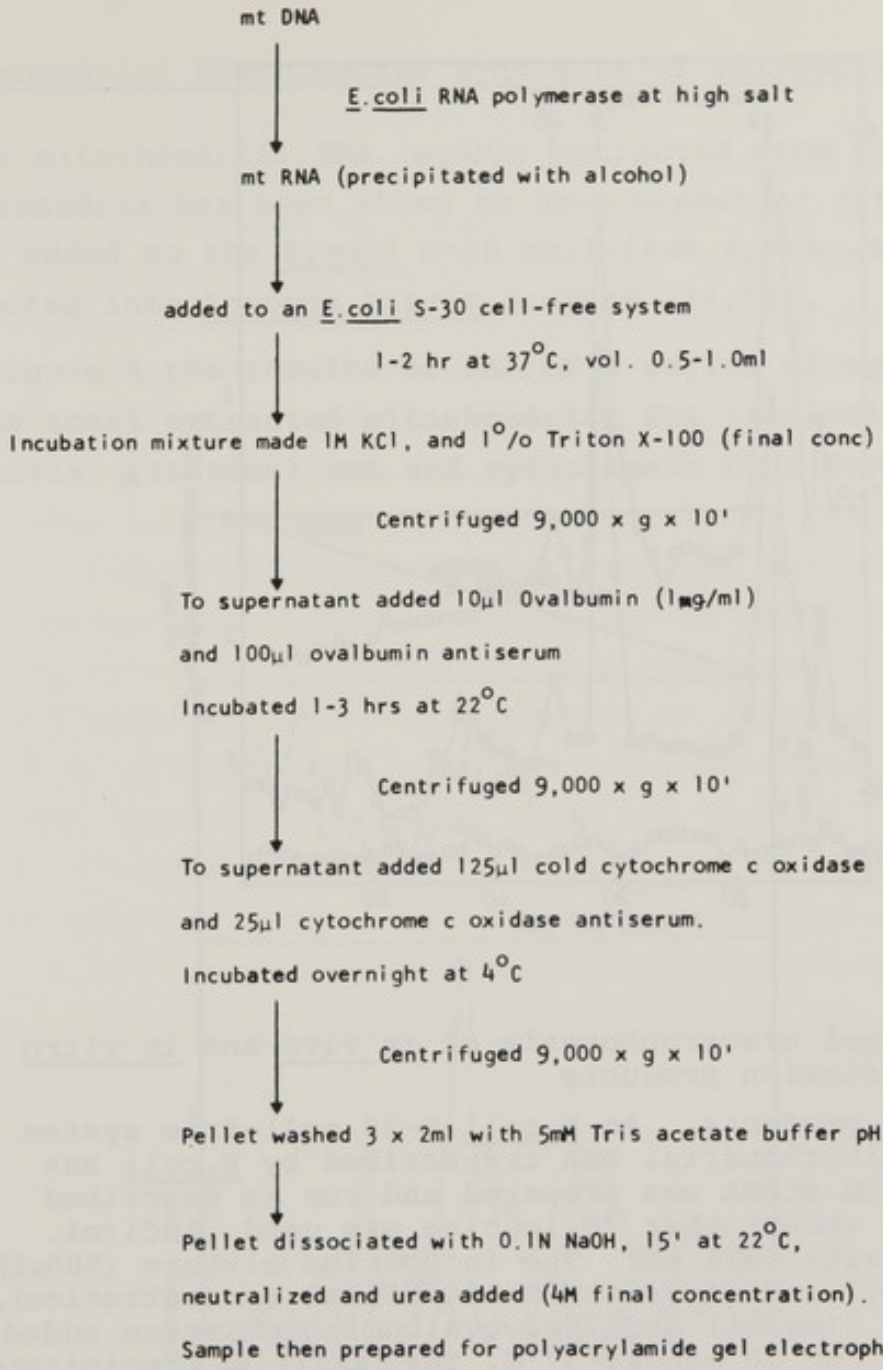


Fig. 4. Summary of the scheme used for the analysis of the mt-DNA-directed E.coli S30 cell-free system.

Using the scheme described in Fig. 4, the synthesis of three components of the cytochrome c oxidase complex has been shown in a DNA-directed cell-free system (Fig. 5).

The ribosomal pellet contained some 20% of the label incorporated, the ovalbumin precipitate 2.5% and the final pellet 1.16%. The gel radioactive profile obtained in Fig. 5A and B shows that the only polypeptide species detected *in vitro* corresponded in molecular weight to the three largest subunits of cytochrome c oxidase. Additionally, no peaks of radioactivity were observed when preimmune sera was used (Fig. 5C), or in which chloramphenicol (100 µg/ml) was added to the *in vitro* system,

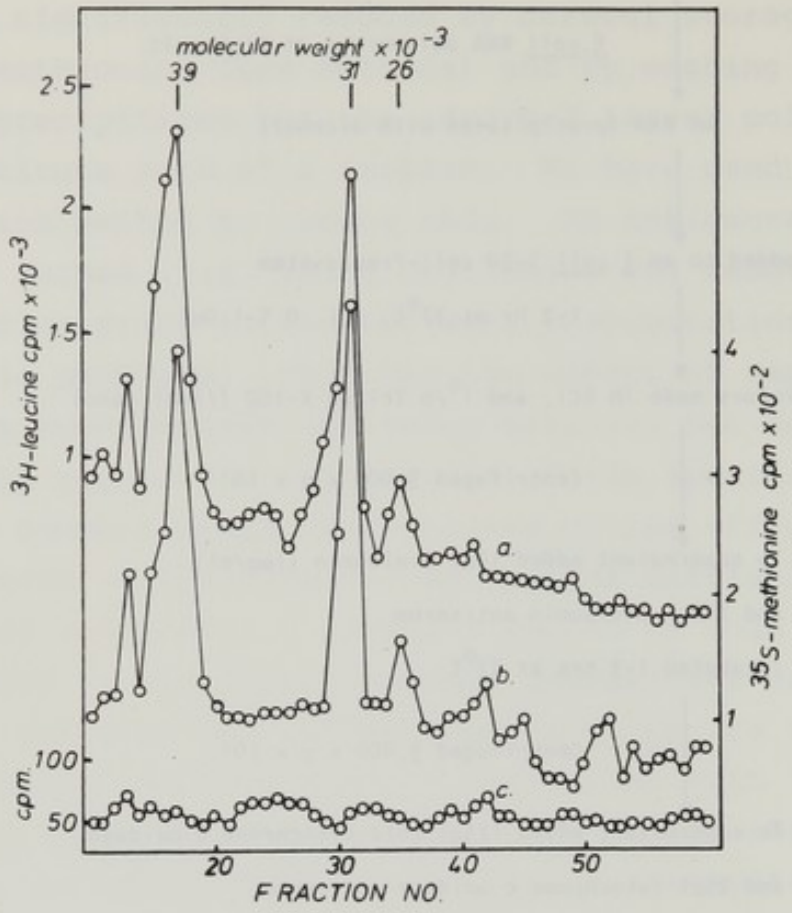


Fig. 5. SDS-gel electrophoresis of in vivo and in vitro translation products

(a) In vitro products. An E.coli S-30 cell-free system directed by mitochondrial RNA transcribed by E.coli RNA polymerase from mtDNA was prepared and run as described previously[13], except that ^{3}H-leucine was used (1µCi/ml, specific activity 46Ci/mM). The incubation mixture (500µl) was made 1M KCl and 1% Triton X-100 (final concentration), 5µl ovalbumin (1mg/ml) and 25µl ovalbumin antiserum added and incubated for 30 min at 37°C. The resulting precipitate was removed (9,000 x g x 10 min) and to the supernatant was added 125µl of non-radioactive carrier cytochrome c oxidase and 25µl of cytochrome c oxidase antiserum. Incubation was at 4°C, overnight. The precipitate was recovered, washed, and electrophoresed as described in the legend to Fig. 2.

(b) Yeast cells were labelled in vivo as described in the legend to Fig.2 with the addition of cycloheximide (100µg/ml), a KCl Triton X-100 extract was prepared and this analysed directly by gel electrophoresis as described in the legend to Fig. 2.

(c) Precipitate of the in vitro system with pre-immune antiserum of a sample prepared and run as described in Fig. 5a.

Mitochondrial RNA-Directed Synthesis of Polypeptides

Total mitochondrial RNA (mtRNA) extracted from yeast mitochondria has been shown to have messenger activity when added to the E.coli S-30 cell-free system, and when injected into Xenopus laevis oocytes (14,15).

In Figure 6 the results of one of a series of experiments using total extracted mitochondrial RNA (including mitochondrial ribosomal RNA and cytoplasmic rRNA from the

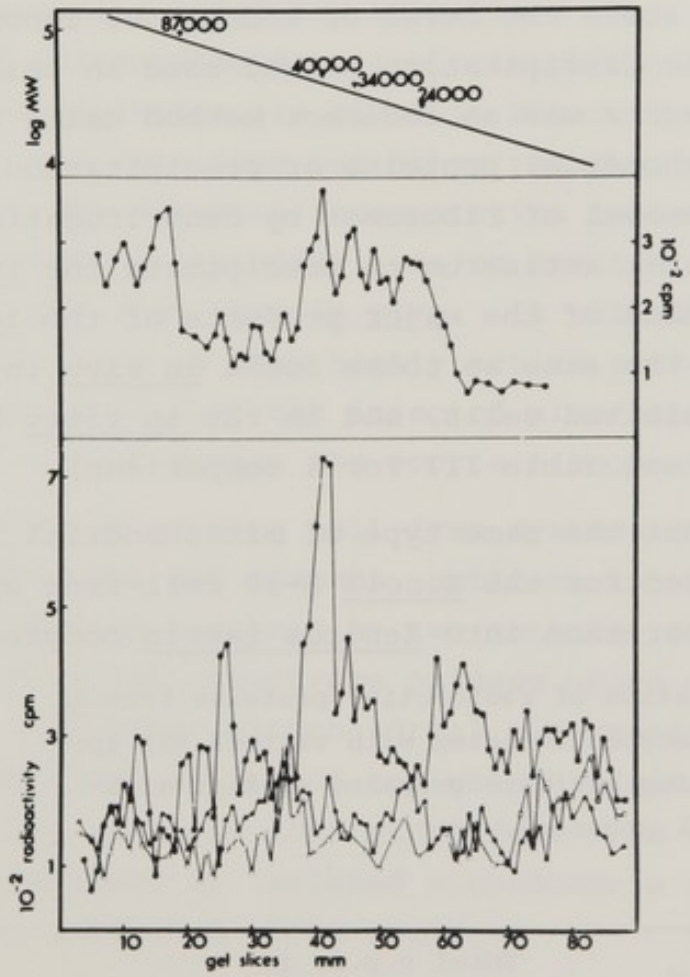


Fig. 6. Electrophoresis of the products of mitochondrial RNA directed protein synthesis in an E.coli S-30 cell-free system. The polyacrylamide gel system in this case was 7% polyacrylamide (19) + 0.46% ethylene diacrylate as the cross-linker (15). Antisera were prepared by Method II (15). Gels were sliced with a Mickle gel-slicer into 1 mm sections, digested for 2 hours with 0.2 ml of ammonia (sp.gr. 0.88), and counted in a dioxane-based scintillation fluid (15). Molecular weights of the fluorescently-labelled proteins in each gel were measured and a constructed molecular weight scale is given at the top of the figure.

-●-●- in vivo incorporation in cycloheximide-inhibited cells (14,16)
-■-■- in vitro synthesis directed by mitochondrial RNA
-Δ-Δ- in vitro system plus cytoplasmic RNA
-□-□- in vitro endogenous incorporation with no added

large ribosomal subunit, yeast cytoplasmic RNA preparations invariably include also a double stranded RNA, see ref.15 for discussion). No polyA sequences greater in length than about 20 nucleotides could be detected in these preparations (15), thus indicating the absence of cytoplasmic mRNA contamination, and the possible absence of polyA containing RNA or the presence of only short polyA sequences in yeast mitochondrial RNA. The RNA preparation stimulates the E.coli S-30 system to a high level (about 20fold above the level of endogenous incorporation). The immune precipitation method used in this series of experiments was an indirect method using no added carrier mitochondrial proteins or precipitation step (except for the removal of ribosomes by centrifugation), and goat anti-rabbit antiserum to precipitate the in vitro product. Some of the major products of the in vitro system are the same as those found in vivo in cycloheximide-inhibited cells, and in the in vitro DNA-directed system (see Table III for a comparison).

Table II shows that the same type of mitochondrial RNA preparation as used for the E.coli S-30 cell-free system stimulated incorporation into Xenopus laevis oocytes

Table II. Precipitation of radioactive proteins from X. laevis oocytes injected with various RNA species, using antisera prepared against mitochondrial proteins.

Injection	Total c.p.m. in antisera precipitate
Mitochondrial RNA	504,000
Cytoplasmic RNA	285,000
Rabbit ribosomal RNA	192,000
Incubation medium	150,000

RNA (1 mg/ml) and (^{35}S)-methionine were injected together into 40 oocytes per sample as described under Methods. After incubation for 16 hr at 20°C the oocytes were treated, antisera precipitates formed and counted as described under Methods.

3.36 times above that found in controls injected with no RNA. Mitochondrial RNA also appears to be a better source of messenger for mitochondrial proteins than cytoplasmic RNA. About 15% of the mitochondrial proteins appear to be synthesized in mitochondria the rest by cytoplasmic ribosomes. As the antigen in this case was mitochondrial membranes we are detecting in the immune precipitation of the cytoplasmic RNA-injected oocytes those mitochondrial membrane (and possibly mitochondrial ribosomal) proteins coded for by nuclear DNA. The results of SDS polyacrylamide gel electrophoresis (19) of the immune precipitates are given in Figure 7 and summarized with the data from our other results in Table III. Mitochondrial RNA directed incorporation into a few species of polypeptide including a major species at 50,000 molecular weight also seen in vivo. It will be interesting to determine in this highly active protein-synthesizing system if in fact the injected mitochondrial mRNA is being translated within the oocyte mitochondria.

The results of the various cell-free systems (Table III) using two different gel systems, antisera to whole mitochondrial membranes prepared by two methods, the use of antisera to purified cytochrome oxidase which does not cross react with other mitochondrial components and the direct and indirect method of antigen precipitation, all give compelling evidence that mtDNA does code for the proteins synthesized by isolated mitochondria and by cycloheximide-inhibited whole cells.

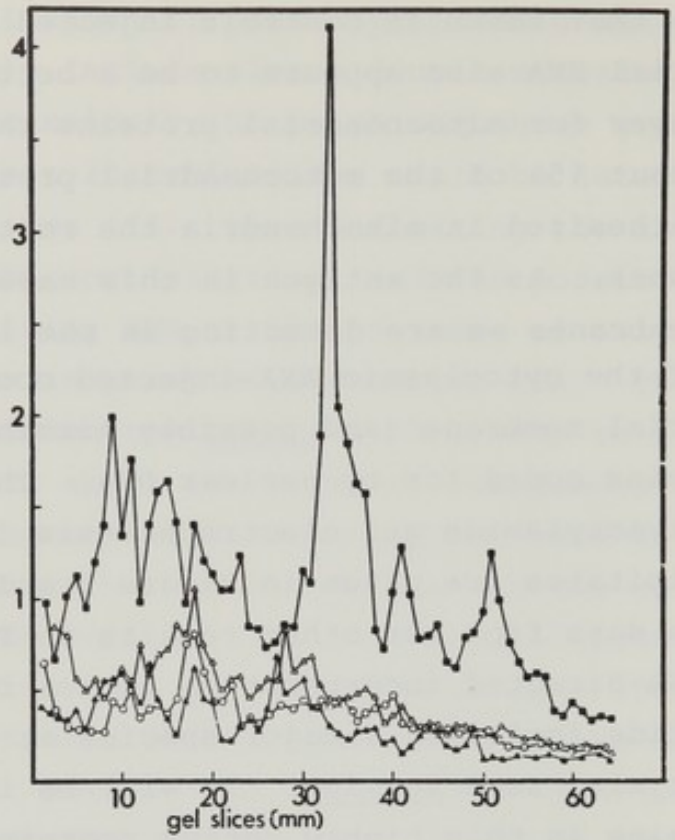


Fig. 7. Electrophoresis of the products of mitochondrial RNA directed protein synthesis in Xenopus laevis oocytes. Gel methods and antisera were essentially as described in Methods and in the legend to Fig.6 and ref. 15. In each case 40 oocytes were used and each injected 150-200 nl of a solution containing RNA (or buffer in the control) 0.7 mg/ml and a total of 30 µCi ^{35}S-methionine. Incubation was at 20°C for 16 hours and the oocytes were dispersed in 1% Nonidet P.40 (B.D.H., UK) in phosphate-buffered saline. The lysate was centrifuged at 4.5 Kxg for 10 min and the supernatant removed without disturbing the overlaying of lipid. Mitochondrial antisera prepared by our Method II (ref.15) was added (20 µl) the mixture incubated at 37°C for 30 minutes and 200 µl goat anti-rabbit serum added and the mixture incubated at 37°C for a further 45 minutes, and then at +4°C overnight. The immune precipitate was then treated as described previously (13, 15).

-■-■- mitochondrial RNA
-o-o- cytoplasmic RNA
-Δ-Δ- rabbit ribosomal RNA
-▲-▲- control no added RNA

Discussion

The results of the *in vivo* labelling experiments and both DNA and RNA directed synthesis of polypeptides analyzed by several different methods are summarized in Table III. It is clear that the molecular weights of polypeptides synthesized *in vivo* are similar to those produced in the *in vitro* system particularly in the molecular weight range of 50,000 to 20,000 daltons. The antisera precipitates from the *in vivo* preparation gave results similar to those obtained both in RNA and DNA directed *in vitro* systems, suggesting that no major polypeptide species is missing within the limitation of the antisera precipitation procedure. This is further strengthened by the use of two different antisera preparations in this work. It might be suggested from this work that polypeptides not detected here are either non-antigenic or only present in catalytic amounts.

We have investigated whether the high molecular weight polypeptide (60-70,000 daltons apparent molecular weight see Table I) regularly found in the *in vitro* systems and which does not appear to be incomplete by dissociated polypeptide represents a polypeptide which is synthesized by mitochondria *in vivo*. Such a protein is not normally found in the standard long term labelling conditions used to label mitochondrially synthesized proteins (13, 29). Several examples are known of high molecular weight precursors of structural or secretory proteins (30) which undergo a specific endopeptidic cleavage at some time during their incorporation into their final location. We have pulse-labelled (2 minutes) mitochondrial proteins with (^{35}S)-methionine and although a high molecular weight protein was detected it did not show the kinetics of labelling expected of such a precursor (see ref. 13). Similar pulse and longer

term labelling experiments in the presence of amino acid analogues (canavanine, norleucine, parafluorophenylalanine) (28) and protease inhibitors (phenyl-methylsulphonylfluoride and L-1-tosylamido-2-phenylethyl chlormethylketone) again failed to demonstrate the existence of such a precursor (D.Y. Thomas and A.H.Scragg, unpublished experiments). The question of whether the synthesis of hydrophobic mitochondrial proteins occurs via a high molecular weight precursor protein and the mechanism of their incorporation into membranes remains open. However, it is apparent that the suggestion (9,31) that the primary products of mitochondrial protein

Table III. Comparison of the Molecular Weights of Polypeptides Synthesized *in vivo* and *in vitro*

in vivo			*in vitro*			
gel system 2 [a]	gel system 1 [b]	antimitochondrial antisera ppt. gel system 1 [b]	antimitochondrial antisera ppt. from *X.laevis* oocytes. RNA-directed gel system 2 [c]	antimitochondrial antisera ppt. from *E.coli* S-30 system RNA-directed gel system 2 [a]	antimitochondrial antisera ppt. from *E.coli* S-30 system DNA-directed gel system 1 [b]	cyto.oxidase antisera ppt. from *E. coli* S-30 system DNA-directed gel system 1 [d]
90,000+			100,000	80-100,000		
			85-95,000			
70,000			69,000	70,000		
		64,000				
60,000				60,000	64,000	
50,000	48,000	48,000	50,000		48,000	
39-44,600	38,000	38,000	38-44,000	39-44,000	40,000	39,000
34,000	35,000	35,000	34,000	34,000	35,000	
28-31,000	30,000	30,000	31,000	31.5-33,000	30-32,000	31,000
22-25,000	23,000	23,000	22-26,000	18-22,000		26,000
					12,000	
Occasionally small proteins				many small proteins	many small proteins	

Gel system 1 is that described by Laemmli (18) and gel system 2 that described by Weber and Osborn (19).

a data from reference 14

b data from reference 13

c data from reference 15

d data from this paper and reference 19

synthesis are low molecular weight proteins (c. 8-11,000 daltons) seems unlikely.

In addition to coding for some 5-6 polypeptides of unknown function, the results using cytochrome c oxidase antisera demonstrate that the mtDNA does code for 2 of the highest molecular weight components of cytochrome c oxidase and probably three. These components are represented in the 5-6 polypeptides we have shown to be synthesized *in vitro* and precipitated by antisera raised against a mitochondrial membrane preparation, in view of their similar molecular weights and the cross reaction with the total mitochondrial antisera with cytochrome c oxidase (Fig. 2). The four smallest polypeptides of the ATPase complex suggested by inhibitor studies as possibly being coded for by mtDNA have molecular weights of 29,000; 22,000; 12,000; and 7,500 respectively (17). Those below 20,000 would not be adequately separated by the gel systems used here but the two higher molecular weight components may be represented in the *in vitro* products.

There are additional complications in attempting to show synthesis of specific polypeptides of such low molecular weight by the systems we have developed, in that a large proportion of incorporated label in both the DNA and RNA directed systems is into polypeptides with heterogeneous molecular weights, which show some precipitation with cytochrome c oxidase antisera. These are presumably polypeptide chains which are incomplete for a variety of reasons ("early-quitters") which are often found even in homologous cell-free systems (32).

The relatively low amount of polypeptide corresponding to cytochrome c oxidase subunit III (molecular weight 26-27,000) found *in vitro* as compared with the *in vivo* results (Fig. 5) could be due to several causes. Evidence

has been presented (12, 21) that subunit III has little or no antigenicity in rabbits when antiserum was raised against individual purified cytochrome c oxidase subunits, and is presumably only precipitated by virtue of its close association with the other subunits or exchange with complete complex. Therefore, precipitation of subunit III from either isolated mitochondria (12) or the cell-free system may rely upon its association or exchange with cytochrome c oxidase subunits present either within the mitochondrion or added as cold carrier to the system. The reduced amount of subunit III synthesized in vitro may also represent a reduced synthesis of mRNA for this component or its translation in vitro.

Finally, although messenger RNA activity has been shown to direct the synthesis of mitochondrial proteins in vitro (14, 15), we have not detected terminal polyA sequences in this RNA, with a limit of detection of the method used of about 20 nucleotides (23). Mammalian mitochondrial mRNA has been shown to have polyA sequences (24), as previously has yeast mitochondrial mRNA (26). Recently it has been shown (25) that yeast mitochondrial mRNA contains short (20-30 nucleotides) polyA sequences, this mRNA has also been translated in an E.coli cell-free system (29). Even though these experiments like ours using mtRNA do not rigorously exclude that the RNA is imported from the nucleus, the similarity of the results provides an excellent support for the direct evidence obtained from the mtDNA-directed cell-free system (13, 20).

There seem to be several directions open to tackle the problem of which of the genetically characterized loci on mtDNA codes for each of the polypeptides demonstrated to be coded by mtDNA. The use of mRNA isolated from

petites containing known genetic markers is one such approach although as this RNA is not expressed in rho$^-$ petite cells, complete gene transcripts may not be present. Use of the mRNA isolated from the mit$^-$ cytoplasmic mutant of Tzagoloff would obviously be of value. A more promising approach would appear to be through the use of large restriction endonuclease fragments of mtDNA, directly transcribing and translating these in vitro the identity of the restriction fragments being established by hybridization to petite DNAs retaining a known marker.

Together with others we have previously (13) drawn attention to a major discrepancy between the established genetic functions of mtDNA and its molecular weight (5×10^7 daltons (34)). The evidence for some 20 tRNAs being coded by mtDNA is clear (5-7) if we further assume a single copy of each of the "23S" and "16S" ribosomal RNAs then maximally about 3.4×10^6 daltons of mtDNA can be accounted for. The proteins we have shown to be synthesized in vitro (13, 20) account for about 4×10^6 daltons of mtDNA. If we assume the proteins synthesized by mitochondria in cycloheximide-inhibited cells genuinely represent the products of the mitochondrial genome then a similar figure is arrived at (3 subunits of cytochrome c oxidase - 1.8×10^6 daltons (9), 4 subunits of ATPase 1.6×10^6 daltons (17), and 1 polypeptide of the bc_1 complex 0.6×10^6 daltons (35)). Proteins with catalytic functions would not be detected by the methods so far used. It is perhaps permissible to conjecture that S. cerevisiae has evolved a sophisticated system of control proteins and regions on mtDNA to cope with the varied carbon sources and respiratory environments it encounters.

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Translation of Mitochondrial Messenger RNAs in vitro as an Approach to the Identification of Gene Products of Yeast Mitochondrial DNA

A.F.M. Moorman, L.A. Grivell, M.B. Katan, G.J.B. van Ommen, C.M. Meiland

SUMMARY

1. Mitochondrial RNA directs the synthesis of discrete, high-molecular-weight polypeptides in a cell-free system derived from Escherichia coli.

2. With one possible exception, these polypeptides do not correspond in electrophoretic mobility with authentic products of mitochondrial protein synthesis.

3. Antibodies against yeast cytochrome oxidase and the cytochrome bc_1 complex specifically precipitate a fraction of the in vitro synthesized product.

4. Mitochondrial RNA, fractionated by a hybridization-dehybridization cycle with mitochondrial DNA, directs the synthesis of essentially the same products as given by unfractionated mitochondrial RNA. These polypeptides are thus presumably specified by genes located on mitochondrial DNA.

INTRODUCTION

Identification and mapping of the genes on mtDNA are essential prerequisites for an understanding of the function of mtDNA in the process of mitochondrial biogenesis. Analysis of mutants of mtDNA and characterization of the products of mito-

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Abbreviations: SDS, sodium dodecylsulphate; IgG, immunoglobulin G; SSC, 0.15 M NaCl, 0.015 M sodium citrate (pH 7.0).

chondrial protein synthesis are approaches which have contributed greatly to our knowledge in this area (for review see [1-4]) and it is now generally accepted - but not formally proven - that all the major products of mitochondrial protein synthesis are also specified by genes on mtDNA. Whether these are the only gene products of mtDNA and whether mRNAs originating in the nucleus are imported and translated in mitochondria is not known. Characterization of mRNAs present in mitochondria in terms of the proteins they specify by translation in vitro and of their genetic origin by DNA-RNA hybridization is, potentially, a powerful means of resolving these points. Furthermore, the localization of genes specifying proteins on the recently constructed physical map of mtDNA [5], should permit current ideas on the relationship between genes and spacers in mtDNA [6] to be tested and should give insight into the regulatory mechanisms operative during mitochondrial biogenesis.

With these aims in mind, we have developed conditions under which mRNAs present in yeast mitochondria are translated by cell-free extracts from E. coli to give discrete, high-molecular-weight products. The results of preliminary attempts to characterize these products and the RNAs which specify them are described.

METHODS

Saccharomyces carlsbergensis NCYC-74 was grown in semi-synthetic lactate medium [7]. Mitochondria were prepared as described previously [8] from protoplasts which had been incubated for 90 min at 28°C in growth medium osmotically stabilized by 1 M sorbitol (25 g cells/litre) [9]. Mitochondria were suspended in 100 mM NaCl, 1 mM EDTA, 0.02% macaloid and 10 mM Tris-HCl (pH 7.0), lysis was performed in a mixture containing 2% SDS, 1% tri-isopropylnaphthalene disulphonate, 2% sodium-p-amino salicylate, 6% sec-butanol and 0.5% diethylpyrocarbonate and RNA extracted by the hot phenol-chloroform method of Penman [10].

MS2 RNA was prepared according to Nathans [11].

The preparation of an E. coli S-30 extract was according to a slightly modified Nirenberg and Matthaei procedure [12] (Moorman, A.F.M. and Lamie, F., in preparation). Protein synthesis was assayed by incubation for 15 min at 36°C in 50 mM NH_4Cl, 10 mM Mg-acetate, 10 mM Tris-HCl (pH 7.8), 0.1 mM phenylmethylsulphonylfluoride [13] (Sigma), 0.2 mM leucovorin (folinic acid), Ca^{2+} salt (Serva, Heidelberg), 1 mM ATP, 0.03 mM GTP, 5 mM phosphoenolpyruvate, 20 µg/ml pyruvate kinase, 1 mM dithiothreitol, 6 mM 2-mercaptoethanol, E. coli S-30 extract (5 mg protein/ml) and 0.05 mM each of the 20 amino acids (minus methionine). The concentration and specific activity of [^{35}S]methionine and the quantities of exogenous RNA when added are indicated in the text. 10-µl samples of each reaction mixture were assayed for radioactivity precipitable by hot trichloroacetic acid. The remainder of each reaction mixture was stopped by adding 1/3rd volume of a mixture containing 12.5% SDS, 20% (w/v) glycerol, 25 mM dithiothreitol, 50 µg/ml bromophenol blue and boiling the mixture for 2 min. Products were then analysed on 15% SDS polyacrylamide slab gels [14]. Gels were sliced to give two slabs of equal thickness. Of each gel, one part was dried and contact autoradiograms were made to detect the labelled polypeptides (film, Structurix D10, Agfa-Gevaert). The other part was stained with Coomassie Blue to visualize the marker proteins.

For immunological analysis of products, the reaction was stopped by adding 1/10th volume 0.5 M Na-EDTA (pH 7.0) and 1/4th volume 750 mM NaCl, 50 mM NaP_i (pH 7.2), 5% Triton X-100, 2.5% Na-deoxycholate, 0.5% SDS and 150 mM methionine (modified from [15]). The reaction mixture was then centrifuged for 10 min at 250 000 x g at 5°C. The supernatant was used for immuno-assay.

Antisera were raised in rabbits by a primary injection of 2 mg of protein mixed with Freunds complete adjuvans (Difco), followed after 4, 6 and 8 weeks by booster injections of 0.2

mg in incomplete Freunds. IgG was isolated from antisera by chromatography on DEAE Sephadex A-50 in 10 mM NaP_i (pH 7.4), 60 mM NaCl. The excluded volume peak was collected and checked for purity by electrophoresis on 5% polyacrylamide gels in a discontinuous Tris-glycine buffer system and by Ouchterlony double diffusion.

RESULTS

RNA extracted from yeast mitochondria contains active mRNAs

The isolation of specific mRNAs by means of affinity chromatography or by purification of polysomes has been successful in a number of eukaryotic systems [16]. In the case of yeast mitochondria, our attempts at isolation of intact, pure polysomes as a possible source of mRNAs have met with failure [17]. Further, in contrast to the mRNAs of animal mitochondria [18,19], mRNAs from yeast mitochondria do not appear to contain poly(A) tracts of sufficient length to permit their isolation by affinity chromatography on oligo(dT) cellulose [20]. Although we have been able to reproduce the results of Hendler et al. [21] in binding of mtRNA to poly(U) Sepharose, this binding is not specific for mRNA since both bound and unbound RNA fractions are equally active on a weight basis in directing protein synthesis and yield similar products in the E. coli cell-free system (Moorman, A.F.M., Grivell, L.A. and Van Ommen, G.J.B., unpublished).

We, therefore, decided to attempt the detection of mRNA activities in total RNA extracted from mitochondria and their fractionation by physical means. Table I shows that total mtRNA, in contrast to RNA isolated from purified mitochondrial ribosomes, is highly active in directing the incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable material and, therefore, presumably contains mRNA sequences. Considering that mtRNAs consist mainly of ribosomal and transfer RNAs, it is quite surprising that the remaining RNA stimulates activity to an extent comparable with that given by a mRNA

TABLE I: Activity of the E. coli translation system in [^{35}S]methionine incorporation directed by MS2 RNA and mtRNA

Conditions of assay were as described in Methods except that Mg-acetate was 6.6 mM. [^{35}S]Methionine (54 mCi/mmol) was 2.6 µM. Hot trichloroacetic acid insoluble radioactivity was scintillation counted at 50% efficiency. Values given are the mean of duplicate determinations and have not been corrected for a zero time incorporation of 1555 cpm.

Additions or omissions	Incorporation (cpm/10 µl)
Minus RNA	3 035
mtRNA (0.7 mg/ml)	28 534
MS2 RNA (0.2 mg/ml)	55 276
15S mt rRNA (0.3 mg/ml)	804
21S mt rRNA (0.3 mg/ml)	2 731

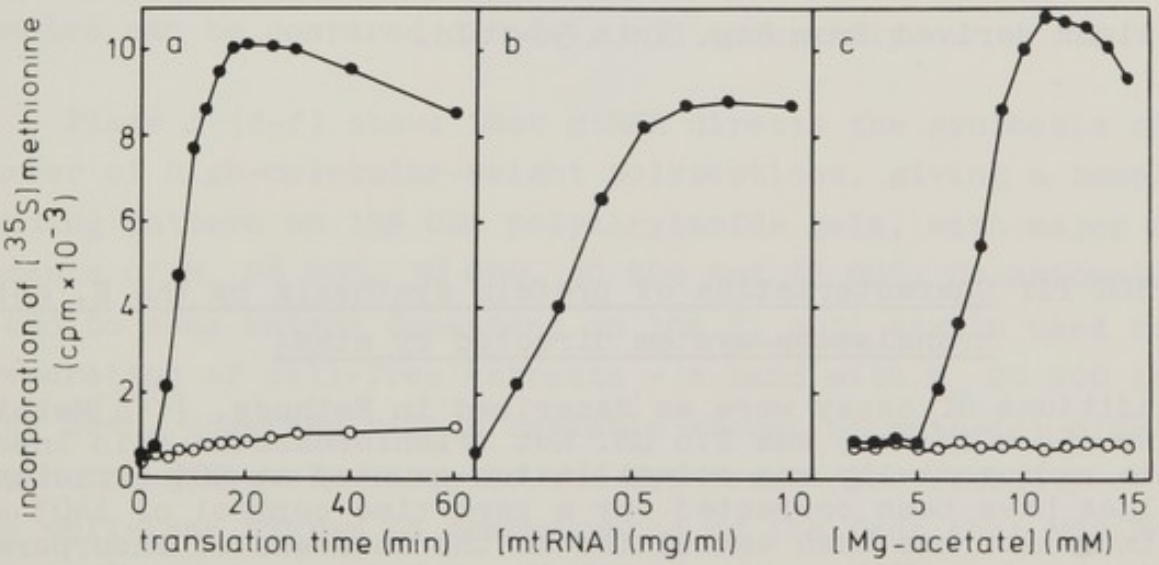


FIG. 1 - Characteristics of the mtRNA-directed translation system. Stimulation of protein synthesis as measured by [^{35}S]-methionine incorporation into hot trichloroacetic acid-precipitable material per 10 µl is given as a function of (a) time, (b) mtRNA concentration and (c) Mg-acetate concentration. Conditions of assay were as described in Methods. [^{35}S]Methionine (4.2 Ci/mmol) was 2.4 µM. Incubation time was 15 min, mtRNA 0.5 mg/ml and Mg-acetate 10 mM, unless otherwise stated. Values given are the mean of duplicate determinations. O—O, Endogenous control; ●—●, incorporation due to mtRNA.

homologous to E. coli, that of bacteriophage MS2.

Fig. 1 shows that activity is linear with time up to 15 min, is linearly dependent on the concentration of added RNA up to 0.5 mg/ml (contrast [22]) and is optimal at a magnesium concentration of 12 mM. All further experiments to be described were carried out at a magnesium concentration of 9-10 mM in order to minimize possible non-specific initiation events. The presence of the serine-protease inhibitor phenylmethylsulphonylfluoride [13] in reaction mixtures led to a higher initial rate of incorporation, which continued for a longer time (data not shown). It was, therefore, routinely included in all subsequent incubations.

Table II shows that protein synthesis directed by mtRNA is highly dependent on the presence of the formyl donor leucovorin, but is not influenced by addition of extra E. coli tRNA. Stimulation of activity by mtRNA under the optimal conditions derived from Fig. 1 is 56-fold.

TABLE II: Characteristics of protein synthesis by the E. coli translation system directed by mtRNA

Conditions of assay were as described in Methods. [^{35}S]Methionine (54 mCi/mmol) was 2.6 µM. Hot trichloroacetic acid insoluble radioactivity was scintillation counted at 50% efficiency. Values have been corrected for a zero time control of 1403 cpm. Stimulation has been calculated as the increase of incorporation due to mtRNA compared with the endogenous control.

Additions or omissions	Incorporation (cpm/10 µl)		Stimulation due to mtRNA
	Endogenous control	+ mtRNA (0.5 mg/ml)	
None	2174	124 329	56
+ tRNA (150 µg/ml)	2330	129 868	55
- Leucovorin (0.2 mM)	2073	49 271	23

Electrophoretic characterization of *in vitro* synthesized products

In order to characterize the products synthesized *in vitro*, we have compared them with authentic products of mitochondrial protein synthesis, identified by pulse-labelling of intact cells with [^{35}S]methionine in the presence of cycloheximide [23]. In initial experiments we attempted to do this by electrophoresis in cylindrical SDS polyacrylamide gels, radioactivity being determined after slicing of the gel. We have found, however, that slicing leads to a loss of the high resolution necessary for the unequivocal identification of bands in complex mixtures. Furthermore, accurate comparisons demand the use of double labelling since parallel gels are completely unsuitable for this purpose (*cf.* [22,24,25]). To circumvent these problems, we now use a combination of slab gel electrophoresis and autoradiography, in which several samples can be compared directly and accurately.

Plate I (d-f) shows that mtRNA directs the synthesis of a number of high-molecular-weight polypeptides, giving a complex banding pattern on 15% SDS polyacrylamide gels, with major components of M_r 26 000, 38 000, 40 000 and 95 000. Occasionally - and to some extent dependent on the *E. coli* strain used for preparation of cell-free extracts - a band with M_r 20 000 is also seen, together with a variable amount of material migrating heterogeneously close to the dye front. The fidelity of the cell-free system is demonstrated by the fact that MS2 RNA (plate I; a-c), translated under the same conditions used for translation of mtRNA, yields in excellent agreement with the literature [26,27] a major band with M_r 14 000 and a minor band with M_r 20 000. Bands at M_r 40 000 and 64 000 are also present, but cannot be reproduced in this figure. Incubation of extracts in the absence of added RNA yields mostly small amounts of heterogeneous material of low molecular weight.

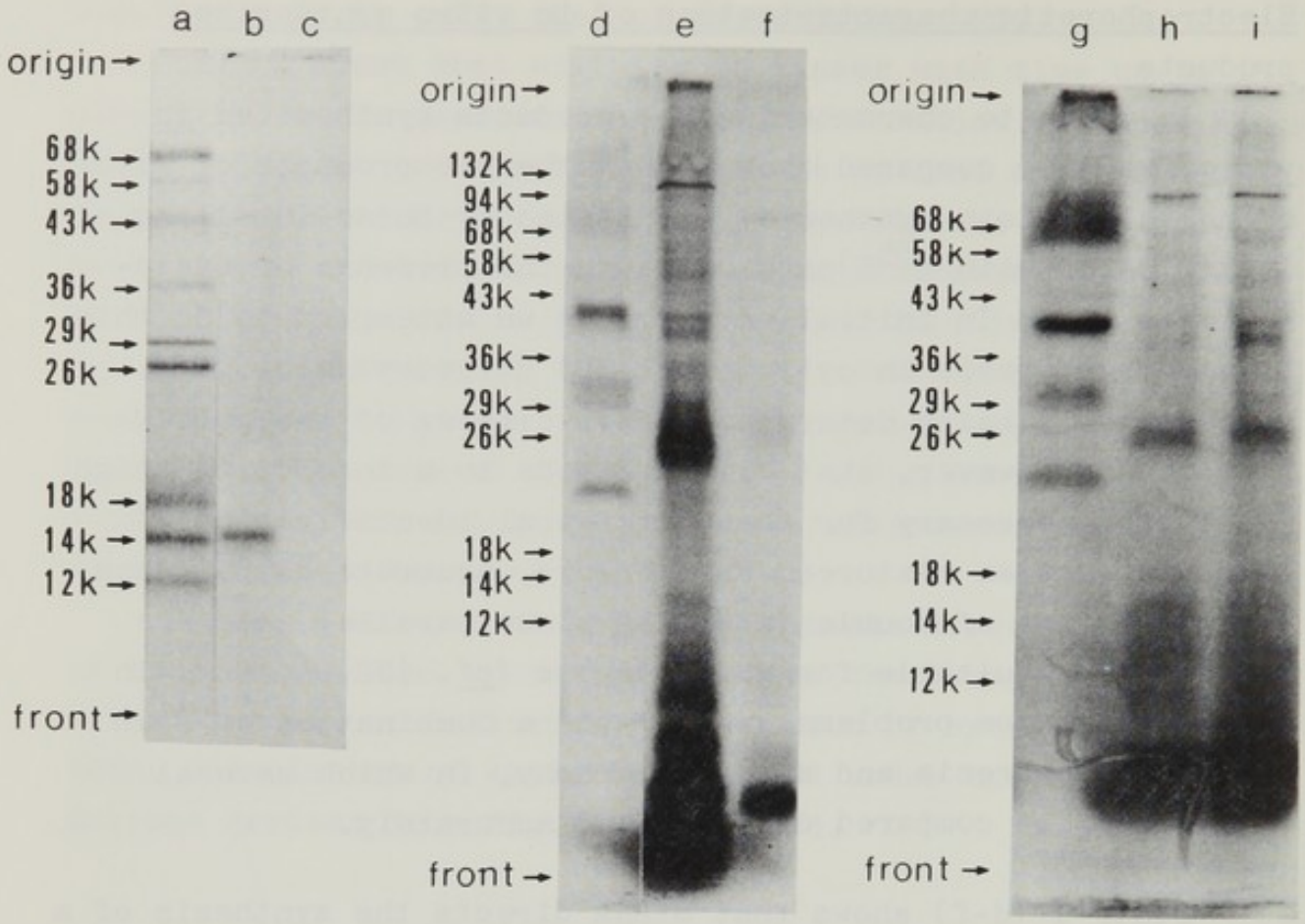


PLATE I - Autoradiograms of [^{35}S]methionine-labelled products fractionated on 15% SDS polyacrylamide slab gels as described in Methods. Strips a-c, d-f and g-i represent three different gels and protein synthesis experiments. The first two gels were exposed for 72 h, the third for two weeks. Standards of known molecular weight included β-galactosidase (132 000), phosphorylase a (94 000), bovine serum albumin (68 000), catalase (58 000), ovalbumin (43 000), lactate dehydrogenase (36 000), carbonic anhydrase (29 000), α-chymotrypsinogen A (26 000), β-lactoglobulin (18 000), ribonuclease (14 000) and cytochrome c (12 400). a) Marker proteins except β-galactosidase and phosphorylase a; b) translation products of MS2 RNA; c) endogenous control; d) *in vivo* products of mitochondrial protein synthesis; submitochondrial particles were prepared from cells pulse-labelled with [^{35}S]methionine in the presence of cycloheximide [23]; e) products of mtRNA-directed protein synthesis; f) endogenous control; g) *in vivo* products of mitochondrial protein synthesis (see d); h) translation products of mtRNA, dehybridized from mtDNA; i) translation products of mtRNA.

Comparison of mitochondrial products synthesized *in vitro* with those synthesized *in vivo* in the presence of cycloheximide (plate I; d-f), shows that with the possible exception of the band migrating with M_r 40 000, none of the major bands synthesized *in vitro* corresponds exactly in electrophoretic behaviour with authentic products. These differences led us to consider the possibility that the banding pattern found arises not from translation of mtRNA, but from protection by the added RNA of endogenous mRNA in the *E. coli* extracts. This possibility is already somewhat unlikely, since extra tRNA does not stimulate protein synthesis (Table II) and the distinctive pattern of mitochondrial products is observed even when low concentrations of mtRNA are used to programme the cell-free system (data not shown). To exclude this possibility completely, the experiment presented in Table III was carried out, however. Extracts were incubated with ribonuclease immobilized on Sepharose under conditions which led to degradation of added MS2 RNA, but left ribosomes unaffected (data not shown). The stimulation of [^{35}S]methionine incorporation by

TABLE III: *Effect of preincubation of an E. coli extract with immobilized ribonuclease on protein synthesis directed by mtRNA*

Conditions of protein synthesis were as described in Methods. [^{35}S]Methionine (6.4 Ci/mmol) was 2.4 µM. Pancreatic ribonuclease was coupled to Sepharose 4B (Pharmacia), activated with cyanogen bromide [31]. Just before protein synthesis, an *E. coli* S-30 extract was incubated for 10 min at 0°C, with Sepharose 4B (control) or ribonuclease-Sepharose 4B, washed three times with 50 mM NH_4Cl, 20 mM Mg-acetate and 10 mM Tris-HCl (pH 7.5). Ribonuclease-Sepharose added corresponds in activity to about 1 µg/ml pancreatic ribonuclease. Values given are the mean of duplicate determinations.

Treatment of S-30	Incorporation (cpm/10 µl)	
	- RNA	+ mtRNA (0.4 mg/ml)
+ Sepharose	265	5748
+ Ribonuclease-Sepharose	253	5874

mtRNA remained unchanged, suggesting that mtRNAs are indeed responsible for activity.

The lack of correspondence in electrophoretic mobility between polypeptides synthesized *in vitro*, and *in vivo* labelled mitochondrial products remains unaccounted for. It may be that use of a heterologous cell-free system results in translation errors, or that mitochondrial proteins are synthesized in the form of precursors, which are not correctly processed in this heterologous system, or that lack of specificity in mRNA recognition results in the synthesis of minor products in abnormally large amounts. Further, we cannot formally exclude the possibility of specific aggregation in the gel. Resolution of these points will require further characterization of the polypeptides synthesized *in vitro* in order to establish identity with any of the authentic mitochondrially-synthesized products, either by peptide mapping or by immunological techniques. The second approach usually employs a precipitin reaction in which equivalent amounts of antibody and antigen together form a network that can be separated from the bulk of radioactive material by centrifugation (*cf.* [22,24,25]). In our opinion this technique is less attractive for two reasons: first, the mitochondrial products formed *in vitro* may be membrane proteins, insoluble under the conditions normally used for immunochemical reactions and, second, the amount of antigen formed *in vitro* is in the sub-nanogram range, which is far too little to form a macroscopic network with specific antibodies. In the system we have used these problems are overcome by adding Triton X-100, deoxycholate and SDS in concentrations that will keep membrane proteins in solution, but do not inhibit immunochemical reactions [15] and by using an anti-globulin antibody for precipitate formation. This involves initial reaction of *in vitro* synthesized products with small amounts of rabbit antibodies raised against specific mitochondrial proteins, followed by precipitation of antigen-antibody complexes plus any remaining free antibody with a sheep antiserum against

rabbit IgG. This obviates the need for addition of carrier antigen, required in a single precipitation system to give macroscopic network formation, and thus avoids introduction of artefacts due to competition of added carrier with *in vitro* synthesized polypeptides and formation of non-precipitating complexes in excess carrier antigen. Further, aspecific precipitation due to trapping of radioactivity in networks can be quantitated, since network size and composition is unaffected by the specificity of the primary precipitating antibody. We have also found that failure to remove ribosomes from cell-free extracts before immunoassay results in irreproducible precipitation of radioactivity. Extracts were, therefore, briefly centrifuged after termination of the protein synthesis reaction (see Methods). 35-50% of incorporated trichloroacetic acid-precipitable radioactivity, presumably present in the form of nascent polypeptides still attached to ribosomes, was removed in this step. Of the remaining material 2% is bound by control IgG, while the fractions specifically bound by antibodies directed against yeast cytochrome oxidase and the cytochrome bc_1 complex are 10% and 2.9%, respectively (Table IV). Further analysis of immuno-precipitated material is in progress.

Fractionation of mRNA activities

As a first step in the identification of the mRNAs responsible for activity *in vitro*, we have attempted to fractionate them by centrifugation through isokinetic sucrose gradients (Fig. 2). In order to minimize possible aggregation artefacts, gradients contained 3% formaldehyde and RNA samples were disaggregated by heating at 65°C for 15 min in low salt (0.1 x SSC, 3% formaldehyde) and quenched immediately at 0°C before layering. Providing that formaldehyde is removed by alcohol precipitation of the RNA, this denaturation procedure does not significantly reduce total messenger activity (Moorman, A.F.M., unpublished).

TABLE IV: Immuno-precipitation of the translation product of mtRNA

Material containing 190 114 trichloroacetic acid-precipitable cpm was incubated overnight at 4°C in 150 mM NaCl, 10 mM NaP_i (pH 7.2), 1% Triton X-100, 0.5% Na-deoxycholate, 0.1% SDS and 10 mM methionine (PBSTDS) with approximately 20 µg rabbit IgG in a total volume of 50 µl. After addition of 50 µl sheep antiserum against rabbit IgG in PBSTDS incubation was continued for 2 h at 0°C. Precipitates were then centrifuged for 10 min at 15 000 x g at 0-4°C through a layer of 10% sucrose, PBSTDS. Pellets were well dispersed by mixing in PBSTDS and centrifuged for 10 min at 15 000 x g at 0-4°C. This wash was repeated once. Pellets were then dissolved in 0.5 ml 90% tissue solubilizer (Protosol, New England Nuclear), 10% water by incubation for 2 h at 37°C and scintillation counted in PPO-POPOP-toluene. Values are the mean of duplicate determinations.

Type of rabbit IgG	Radioactivity in immuno-precipitate	
	Counts/min	% of trichloroacetic acid-precipitable input
None	2 511	1.3
Control IgG	3 826	2.0
Anti-cyt. oxidase IgG	18 987	10.0
Anti-cyt. bc_1 complex IgG	5 606	2.9

Activity in protein synthesis given by each gradient fraction is displayed by the histogram in Fig. 2. There is a broad peak of activity roughly associated with the region containing the ribosomal RNAs, but stimulation can be detected as low as 12S and continues far into the gradient. The polypeptides synthesized by each of these fractions, analysed on 15% SDS polyacrylamide gels, can be seen in plate II. The banding pattern can be seen most clearly in those fractions giving the greatest stimulation. Close examination of all fractions reveals, however, that there is no significant fractionation of different mRNA activities. Possible reasons for this lack of separation are being investigated. At present, however, we cannot exclude such trivial causes as aggregation, degradation or merely lack of resolution.

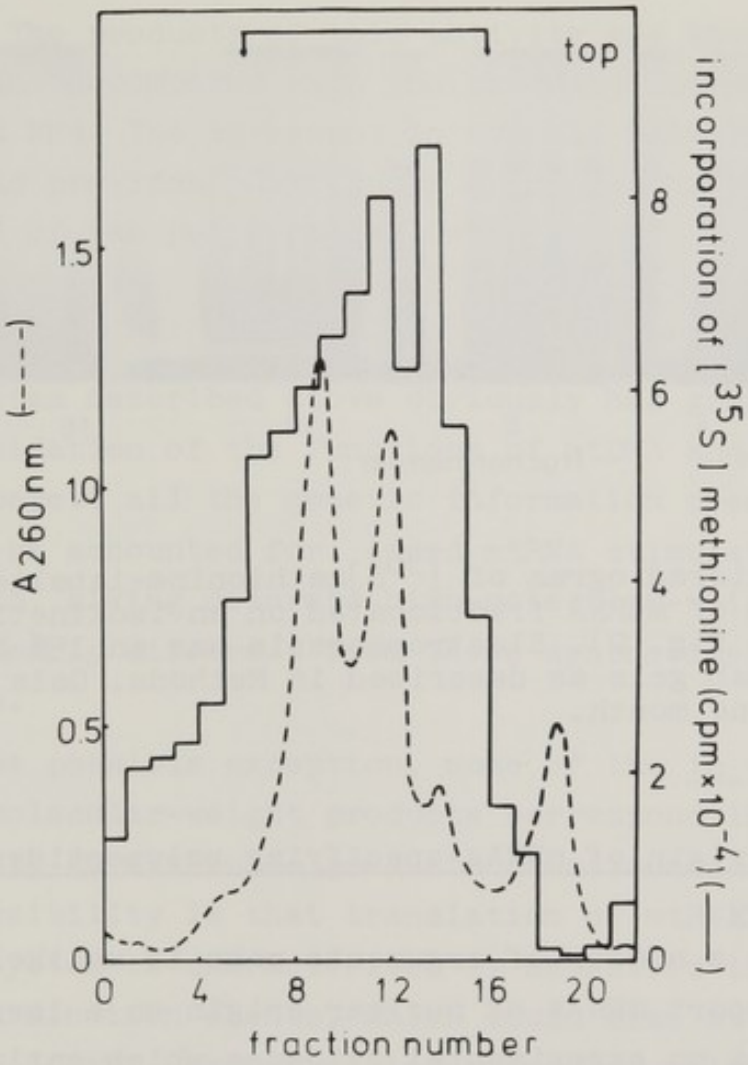


FIG. 2 - Protein synthesis directed by mtRNA separated on an isokinetic sucrose gradient containing 3% formaldehyde, 150 mM NaCl and 15 mM sodium citrate (pH 7.4). For preparing the gradients 12.0 ml 39% (w/v) sucrose was run in a mixing vessel containing 5.1% (w/v) sucrose. 100 µg mtRNA was centrifuged in the Spinco SW-41 rotor for 12 h at 40 000 rev./min at 5°C. Absorbance profiles were monitored continuously by upward displacement through a Zeiss PMQ-II spectrophotometer, modified to accept a MR-1D flow-through cuvette. Fractions were collected and after addition of E. coli tRNA to a final concentration of 50 µg/ml, the RNA was precipitated in 70% ethanol and 300 mM sodium acetate (pH 5.5) overnight at -20°C. After washing with 70% and 96% ethanol, respectively, the RNA was used for protein synthesis as described in Methods. [^{35}S]-Methionine (46 Ci/mmol) was 2 µM. 10-µl samples were assayed for hot trichloroacetic acid insoluble radioactivity and the remainder of each fraction was used for product analysis on 15% SDS polyacrylamide slab gels (plate II).

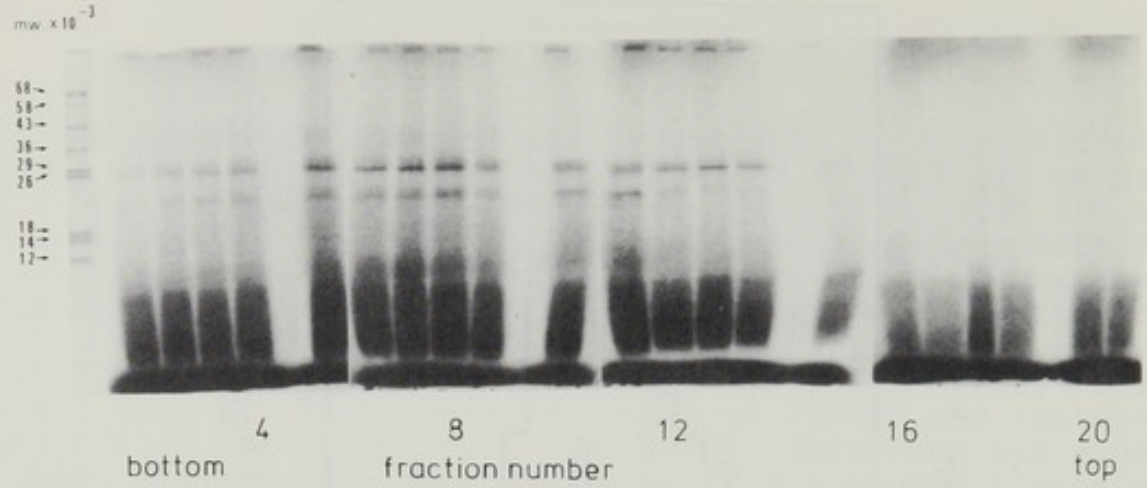


PLATE II - Autoradiogram of [^{35}S]methionine-labelled translation products of mtRNA fractionated on an isokinetic sucrose gradient (see Fig. 2). Electrophoresis was on 15% SDS polyacrylamide slab gels as described in Methods. Gels were exposed for one month.

The genetic origin of mRNAs specifying polypeptides synthesized in vitro

Although a number of arguments make it unlikely that mitochondria import mRNAs of nuclear origin on a large scale [28], there is no experimental evidence which entirely rules out this form of information transfer. In order to locate the genes specifying those polypeptides synthesized in vitro, the following experiment was carried out (plate I; g-i). mtRNA (700 µg) was hybridized to a large amount (72 µg) of mtDNA fixed on filter for 16 h at 30°C in the presence of 50% formamide and 3 x SSC. This corresponds to approximately 1000 x $R_o t$ value for plateau hybridization of ribosomal RNAs. This led to a hybridization of 7.6 µg RNA (equivalent to 21% single-strand genome size in hybrid), while a blank filter bound only 0.23 µg. After hybridization the filter was washed and hybridized RNA was recovered by a wash in 95% formamide at 45°C, followed by one in 0.1 x SSC at the same temperature. RNA which had undergone the hybridization-dehybridization cycle stimulated protein synthesis in the E. coli system 4-5 times more efficiently on a weight basis than unfractionated material (data

not shown). The products of this activity are shown in plate I (g-i) and can be compared with the products obtained with unfractionated RNA. The agreement in overall pattern is remarkably good and provides unambiguous evidence for the mitochondrial origin of the polypeptides under study.

DISCUSSION

The system described above obviously has great potential for the elucidation of the functions of mtDNA and hence should ultimately permit all the genetic information present on this molecule to be accounted for. Added mtRNA stimulates activity up to 56-fold, giving discrete high-molecular-weight products, which are clearly different from those synthesized in the absence of RNA.

With one possible exception, none of the *in vitro* synthesized high-molecular-weight products correspond in electrophoretic mobility to authentic products of mitochondrial synthesis. One possibility is that translation of mtRNA in this heterologous system is characterized by aspecific initiation and premature termination events, which could also account for at least a part of the unresolved low-molecular-weight material seen in SDS acrylamide gels. Out-of-frame translation is unlikely to give rise to high-molecular-weight products, since an approximately random occurrence of three termination triplets in 64 out-of-frame codons should ensure that peptides synthesized will be relatively short (<20 amino acids). Other possibilities are that lack of specificity in mRNA recognition results in the synthesis of minor mitochondrial products in abnormally large amounts, or that mitochondrial proteins are synthesized in precursor form, not correctly processed by *E. coli* proteases. Especially interesting in connection with the latter possibility is the presence in electrophoretic banding patterns of *in vitro* products, of a clear band with M_r 95 000 not seen in preparations labelled *in vivo*.

Other authors have recently reported on the detection of mRNA activities in mtRNA [24,29,30], but direct evidence on the genetic origin of the active species was not given. We have found that essentially the same products are synthesized either by total mtRNA or by RNA fractionated by a hybridization-dehybridization cycle with mtDNA. Since immunological tests show that polypeptides of cytochrome oxidase and of the cytochrome bc_1 complex are among the products synthesized *in vitro*, this constitutes strong evidence that genes for these polypeptides are located on mtDNA.

Although our initial attempts at the fractionation of mitochondrial mRNA activities have been unsuccessful, trivial reasons for this have not been excluded. Once achieved, localization of individual mRNAs on the recently constructed physical map of mtDNA [5] will be feasible, thus presenting us with the key to an understanding of how this molecule expresses itself within the eukaryotic cell.

ACKNOWLEDGEMENTS

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Translation Products in Isolated Mitochondria from Neurospora Crassa: Partial Assembly of Cytochrome Oxidase Components

A. v. Rücker, W. Neupert, S. Werner

Introduction

The formation of a mitochondrial membrane which is fully competent in electron transfer and oxidative phosphorylation requires the cooperation of cytoplasmic and mitochondrial protein synthesis. Particularly, this holds for the membrane constituent cytochrome oxidase. This enzyme is composed of subunits which can be divided into two groups: one group is synthesized on mitochondrial ribosomes (subunits 1, 2 and 3) and the second group on cytoplasmic ribosomes (subunits 4, 5, 6 and 7)(1, 2). This finding implies that many complex processes must be involved in the formation of the enzyme. Accordingly, we are faced with a large number of open questions, concerning e. g. the regulation of the synthesis of the two groups of subunits, the transfer of the subunit precursors to the sites of assembly, the sequence of the assembly reaction, etc.

Two types of approaches to answer these questions appear to be promising: a) *in vivo* studies, employing radioactive pulse-chase labeling techniques together with specific inhibitors of mitochondrial and cytoplasmic translation (3); b) *in vitro* studies with cell fractions and reconstituted systems.

We have described a system in which isolated mitochondria are able to form subunits 1, 2 and 3 of cytochrome oxidase

(4). Here we present experiments which show that the cytoplasmic fraction of Neurospora contains components which have a stimulatory effect on polypeptide synthesis in isolated mitochondria and on the formation of subunits of cytochrome oxidase. Furthermore, the cytoplasmic fraction inhibits proteolysis of in vitro formed polypeptides. Finally, it is shown that partial assembly of subunit precursors of cytochrome oxidase occurs in such a reconstituted system.

Results

1. Amino acid incorporation into the protein of isolated mitochondria

Fig. 1 shows the rate of incorporation of leucine into the protein of isolated mitochondria. Incorporation in an incubation medium (see ref. 4) containing 3-H-leucine, proceeds for about 30 min. When a chase of unlabeled leucine is given after 30 min it can be observed that leucine which was already incorporated is lost from TCA-precipitable protein. This indicates a breakdown of newly formed chains. Thus, in isolated mitochondria formation and breakdown of polypeptide chains take place simultaneously.

When isolated mitochondria are resuspended in an incubation medium which contains post-ribosomal cytoplasmic supernatant, incorporation of leucine is strongly stimulated. After a chase of leucine, the amount of incorporated leucine does not decrease. Thus, the cytoplasmic supernatant has two effects: it stimulates protein synthesis and inhibits breakdown. These two effects may be based on different mechanisms since it was found that a proteinase inhibitor fraction isolated from Neurospora blocks breakdown but does not stimulate the initial rate of leucine incorporation (unpublished results).

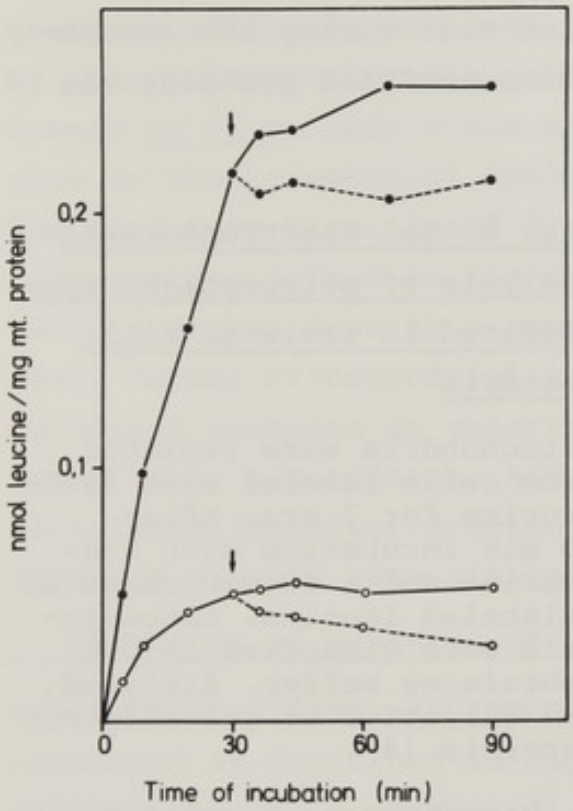


Fig. 1. Incorporation of leucine into the protein of isolated mitochondria from Neurospora crassa

Isolated mitochondria were incubated with 3-H-leucine in a medium optimized for amino acid incorporation (4) without and with cytoplasmic supernatant.

-o——o- mitochondria in incubation medium
-●——●- mitochondria in medium containing cytoplasmic supernatant

Cytoplasmic supernatant was prepared by grinding cells with sand in the presence of incubation medium (3 ml/g hyphae wet weight). The resulting suspension was centrifuged to remove cells walls, mitochondria and cytoplasmic ribosomes.

At the time indicated by arrows, a chase of unlabeled leucine (5 mM) was given to stop incorporation of 3-H-leucine

2. Products of translation in isolated mitochondria

Mitochondria were isolated from cells which were preincubated with 14-C-leucine to obtain homogeneous labeling of total mitochondrial protein. Samles of mitochondria were incubated for 30 min with 3-H-leucine in the absence and presence of cytoplasmic supernatant. Then a chase of unlabeled leucine was given for 60 min. The polypeptides synthesized under these conditions were analysed by SDS-gel electrophoresis. Fig. 2 shows the distribution of 3-H- and 14-C-label on the gels. The homogeneous 14-C-label displays the same pattern in the two samples, without (A) and with (B) added cyto-

plasmic supernatant. This pattern is very similar to the absorbance pattern after staining gels with Coomassie Brilliant Blue obtained when isolated mitochondria were subjected to gel electrophoresis. It is concluded that during the incubation no significant breakdown of mitochondrial proteins has taken place.

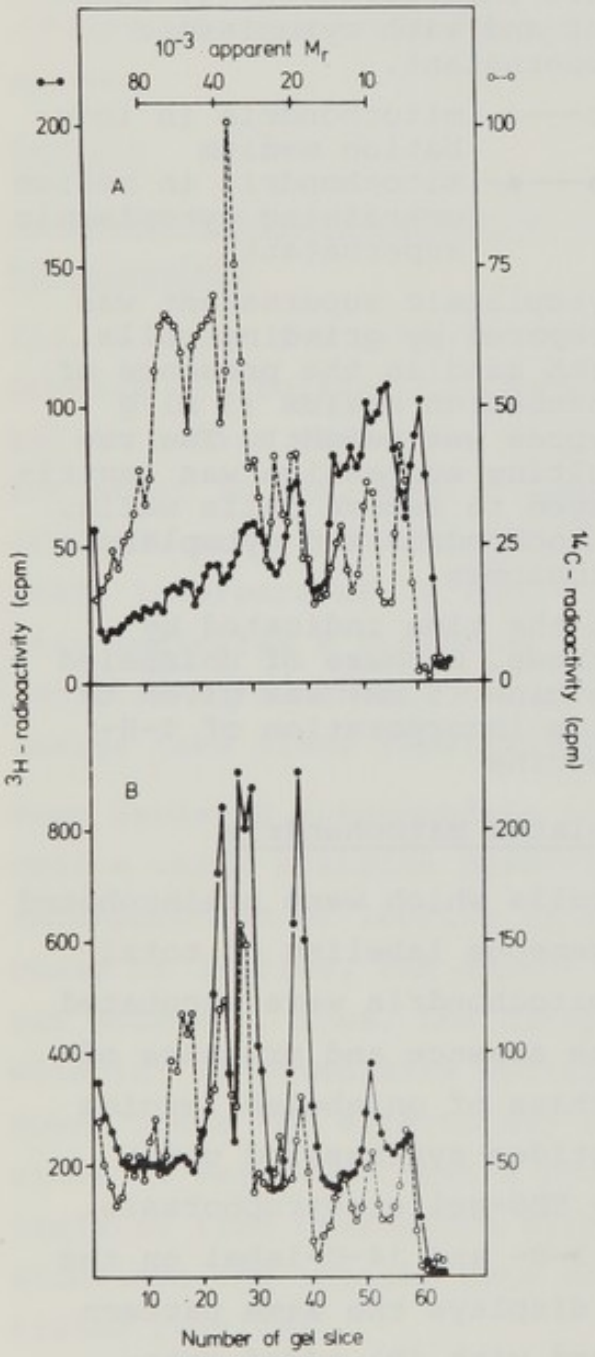


Fig. 2. Gel electrophoretic analysis of polypeptides synthesized in isolated mitochondria

Mitochondria were isolated from cells labeled with 14-C-leucine for 2 hrs. After 30 min incubation with 3-H-leucine and a 60 min chase of unlabeled leucine, mitochondria were dissolved in SDS containing buffer, dialysed, and subjected to gel electrophoresis (4).

A: Mitochondria incubated without cytoplasmic supernatant

B: Mitochondria incubated with cytoplasmic supernatant

When mitochondria are incubated without adding cytoplasmic supernatant, the bulk of mitochondrial translation products displays apparent molecular weights of less than 20 000

(Fig. 2A, 3-H-label). In contrast, the polypeptides formed in isolated mitochondria in the presence of cytoplasmic supernatant have higher apparent molecular weights and migrate as well defined bands. These polypeptides, as judged by their electrophoretic mobility, are very similar to those formed in vivo, when cells are labeled with radioactive leucine in the presence of cycloheximide (5).
The data from Figs. 1 and 2 suggest that the majority of the polypeptides formed in the absence of cytoplasmic supernatant is broken down by a specific proteinase, which attacks newly formed mitochondrial translation products but not mitochondrial proteins in general. This breakdown is blocked by cytoplasmic supernatant. After addition of the above mentioned proteinase inhibitor fraction to the incubation medium a labeling pattern very similar to that in Fig. 2A is observed.

3. Formation of subunits of cytochrome oxidase

Cytochrome oxidase was immunoprecipitated from mitochondria incubated as described in Fig. 2 with a specific antibody against the whole enzyme (3). The immunoprecipitate was analysed by SDS gel electrophoresis (Fig. 3). The 14-C-radioactivity peaks in Figs. 3A and B represent the seven subunits of cytochrome oxidase. When isolated mitochondria are incubated in the absence of cytoplasmic supernatant, only subunit 3 is found to be labeled with 3-H-leucine. The same is observed when the proteinase inhibitor fraction (see above) is added to the incubation medium. However, when the incubation medium contains cytoplasmic supernatant, 3-H-label is associated with subunits 1, 2 and 3.

These findings suggest that the cytoplasmic supernatant contains a factor which is necessary for the integration of subunits 1 and 2, and of higher amounts of subunit 3 into a complex which can be immunoprecipitated with antibody against the whole cytochrome oxidase.

In order to analyse the nature of this factor, isolated mitochondria were incubated with cytoplasmic supernatant which was isolated from cells kept for 2 hrs in the presence of cycloheximide. This cytoplasmic supernatant stimulates protein synthesis to almost the same extent as does the supernatant from control cells. However, subunits 1 and 2 were not labeled in the immunoprecipitate. These observations suggest that the factor in the cytoplasmic supernatant consists of one or more proteins, the pools of which are exhausted during cycloheximide treatment of the cells.

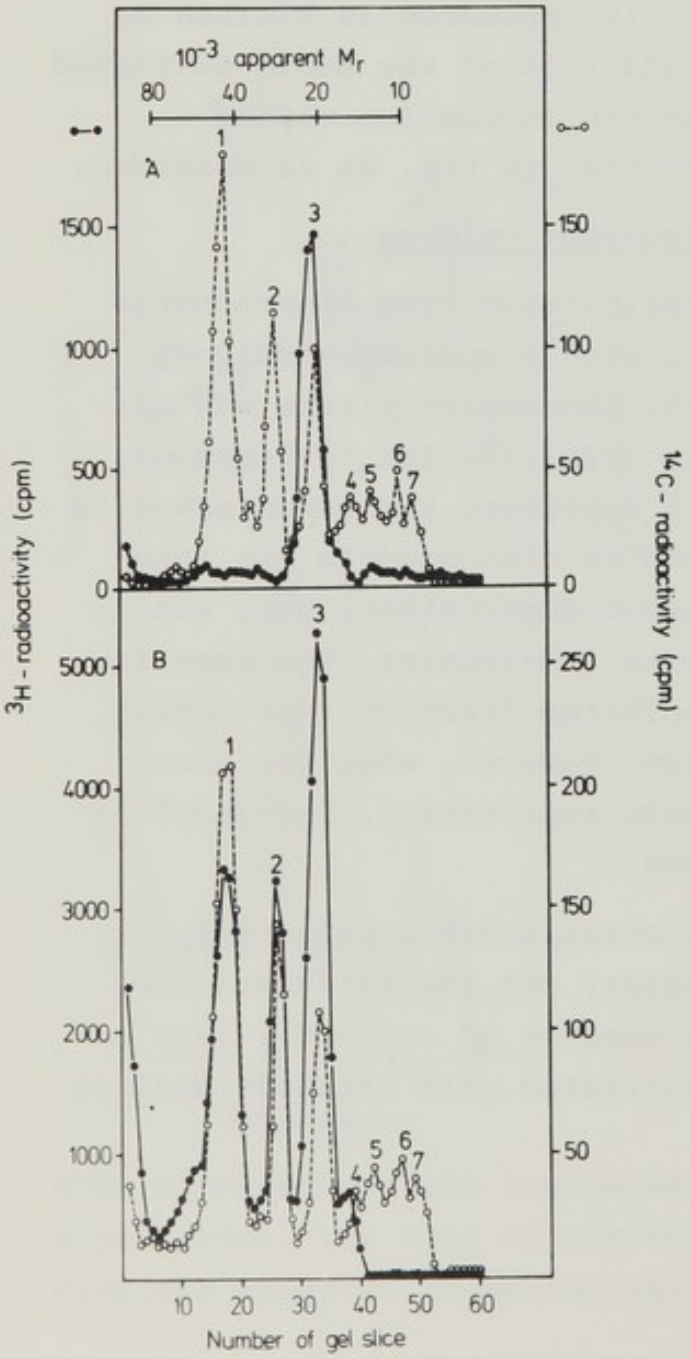


Fig. 3. Gel electrophoretic analysis of immunoprecipitated components of cytochrome oxidase from isolated mitochondria.

Mitochondria labeled as described in Fig. 2 were dissolved with Triton X-100 and treated with an antibody against whole cytochrome oxidase. The precipitate was dissolved in SDS containing buffer and analysed by SDS gel electrophoresis.

A: Immunoprecipitate from mitochondria labeled as in Fig. 2A

B: Immunoprecipitate from mitochondria labeled as in Fig. 2B

There are mainly two possibilities to explain the nature of such a factor: a) it may have some unknown catalytic function in the assembly of cytochrome oxidase; b) it may represent (a) component(s) of cytochrome oxidase, i. e. cytoplasmically made subunits of the enzyme

The second possibility is in accordance with the finding of Schwab et al. (6) that cytoplasmically made subunits are present in precursor pools of different sizes in Neurospora. Furthermore, it is supported by the following experiment.

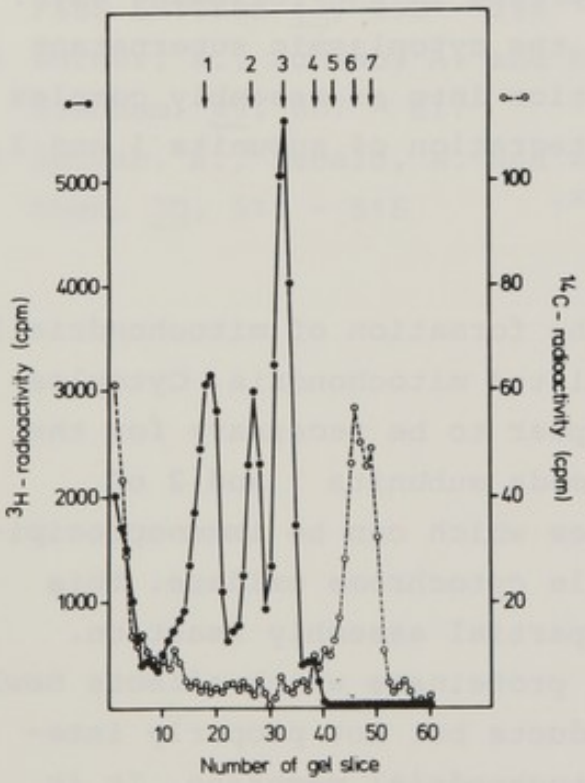


Fig. 4. Gel electrophoretic analysis of cytochrome oxidase components labeled in vitro in the presence of cytoplasmic supernatant containing 14-C-labeled proteins

Isolated mitochondria were incubated with 3-H-leucine in the presence of cytoplasmic supernatant for 30 min. The cytoplasmic supernatant was isolated from cells labeled with 14-C-leucine for 10 min. Positions of subunits 1 - 7 were determined by separating homogeneously labeled cytochrome oxidase on the same gel slab.

Whole cells were pulse labeled with 14-C-leucine and the post-ribosomal cytoplasmic supernatant was isolated. Mitochondria from unlabeled cells were incubated after addition of the 14-C-labeled supernatant in the presence of 3-H-leu-

cine. After incubation immunoprecipitation was carried out. The gel electrophoretic separation of the immunoprecipitate is shown in Fig. 4. The 3-H-label displays the integration of subunits 1, 2 and 3 into an immunoprecipitable complex, as expected from the data in Fig. 3. The 14-C-radioactivity is found in a double band. This double band coincides with the positions of subunits 6 and 7. In a control experiment it was found that no 14-C-label was precipitated when isolated mitochondria were mixed but not incubated with 14-C-labeled cytoplasmic supernatant.

These observations may be interpreted in the following way : Subunits 6 and 7 are present in the cytoplasmic supernatant as precursors and their integration into an assembly complex is necessary for the further integration of subunits 1 and 2 in an immunoprecipitable complex.

Conclusions.

Cell sap components stimulate the formation of mitochondrially synthesized polypeptides in isolated mitochondria. Cytoplasmically synthesized proteins appear to be necessary for the integration of mitochondrially made subunits 1 and 2 of cytochrome oxidase into a complex which can be immunoprecipitated with antibody against whole cytochrome oxidase. This complex may be the result of a partial assembly reaction. Mitochondria contain a specific proteinase which affects newly formed *in vitro* translation products but not properly integrated proteins of the inner mitochondrial membrane. It is supposed that this proteinase becomes active when mitochondrial translation products accumulate which cannot be properly integrated into mitochondrial membrane complexes. Thus, experiments with isolated mitochondria are considered to be a useful approach to the understanding of the mechanism and the regulation of the assembly of mitochondrial membrane complexes.

Acknowledgements. This work was supported by the Deutsche Forschungsgemeinschaft, We 107/1 and Ne 101/11 (Schwerpunktprogramm "Biochemie der Morphogenese").

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The Use of Isolated Mitochondria in the Study of the Mitochondrial Biogenesis in Yeast

G.S.P. Groot, N. van Harten-Loosbroek

INTRODUCTION

Isolated mitochondria have never been, for a variety of reasons, a favourite tool in the study of mitochondrial biogenesis. Although the observation that isolated mitochondria can incorporate radioactive amino acids into proteins [1] was one of the first indications of the presence of a separate mitochondrial genetic system, this property has subsequently been attributed to bacterial contamination [2] or aspecific adsorption [3]. The major objection, however, against the use of isolated mitochondria is the fact that the biosynthesis of the inner mitochondrial membrane is dependent on the coordinated activity of both nucleo-cell-sap and the mitochondrial genetic system [4]. An example of this is the development of cytochrome oxidase activity during respiratory adaptation of anaerobically-grown yeast. This process is inhibited by both cycloheximide, an inhibitor of cell-sap protein synthesis, and by chloramphenicol, an inhibitor of mitochondrial protein synthesis.

The coupling between the two systems of protein synthesis, however, is not completely tight as can be seen in the delayed adaptation phenomenon [5], where products synthesized in the mitochondrion in the presence of CHI can be supplemented with cytoplasmically made polypeptides in the absence of mitochondrial protein synthesis to form enzymatically active cytochrome oxidase. This observation led to the conclusion that mitochondrial protein synthesis was at least partly independent and formed the basis for the CHI label technique [6]. Moreover, it suggested that isolated mitochondria

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Abbreviations: CHI, cycloheximide; CAP, chloramphenicol; SDS, sodium dodecylsulphate; PMSF, phenylmethylsulphonyl fluoride; SSC, 0.15 M NaCl, 0.015 M sodium citrate (pH 7.0).

should in principle be capable of proper protein synthesis. Since such an "in vitro" system would be very useful for a detailed analysis of the products of mitochondrial protein synthesis, for the study of the mechanism of the regulation of mitochondrial protein synthesis and as an experimental test system for the assembly of respiratory chain complexes we have tried to find conditions optimal for mitochondrial protein synthesis [7] and with this system tried to answer some of the questions mentioned above [8].

RESULTS AND DISCUSSION

Preliminary experiments, which were intended to find conditions under which the rate of incorporation of radioactive amino acids was optimal, showed that the system described by Grivell [9] exhibited the highest rate of protein synthesis. Analysis of the products with polyacrylamide gel electrophoresis in the presence of SDS, however, indicated that relatively little material with M_r 30 000-40 000 was synthesized together with an increased amount of heterogeneous material with a low M_r (Fig. 1). Since it is known that the normal protoplast preparation affects the macromolecular synthesis in the yeast cell [10] we have, in order to reduce the deleterious effects of this procedure, changed the procedure for preparing the protoplasts and introduced a recovery period for the protoplasts [7]. When the products of in vitro protein synthesis of mitochondria prepared in this way are compared to those synthesized in vivo in the presence of CHI (Fig. 2), it appears that the size and relative amounts of products are virtually identical.

Using this experimental set-up we have shown that isolated mitochondria are capable of synthesizing subunits I, II and III of cytochrome oxidase and assembling them into holo enzyme [7], with cytoplasmic counterparts already present. Moreover, we could show that the regulatory effect of oxygen on the synthesis or integration of subunits I and II of cytochrome oxidase [11] can still be observed in isolated mitochondria [8], suggesting that one of the steps involved in transcription, translation or integration of these two subunits requires oxygen.

The simplest hypothesis to explain the effect of oxygen is to assume that it acts as an inducer capable of inactivating a repressor of cytochrome oxidase synthesis. Since we have shown that the

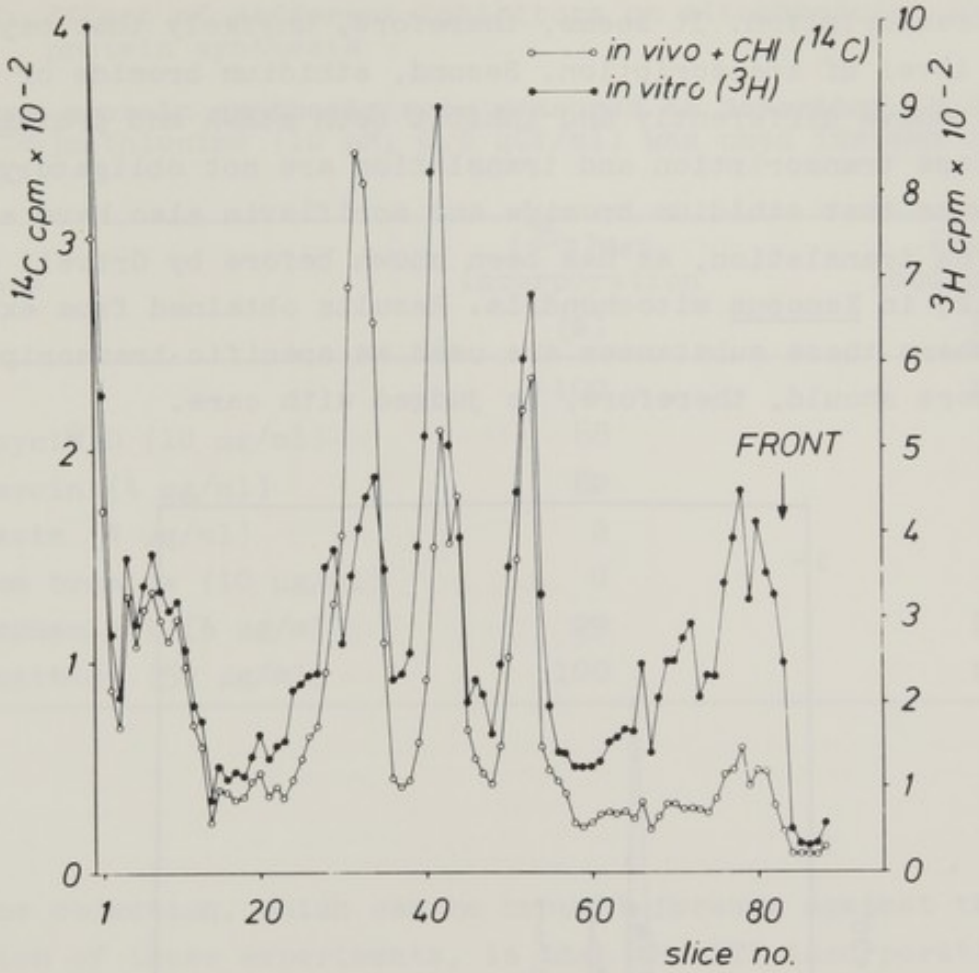


FIG. 1 - Comparison of mitochondrial translation products in vivo and in vitro. Mitochondria isolated from "non-recovered" protoplasts were labelled with [^{3}H]leucine, mixed with mitochondria, labelled in vivo with [^{14}C]leucine in the presence of 100 µg CHI per ml and sonicated. A sample was solubilized in SDS and electrophoresed on a 12% polyacrylamide gel (see [7]). O—O, In vivo + ^{14}C-labelled CHI; ●—●, in vitro, ^{3}H-labelled.

effect is reversible within minutes [8], this requires that mRNA molecules for subunits I and II should have a very short half-life. In order to see whether mitochondrial protein synthesis is dependent on continuous transcription of mtDNA we have tested the effect of several inhibitors on both mtRNA and mitochondrial protein synthesis. Table I summarizes our findings. First, inhibitors like actinomycin D and nogalamycin very strongly inhibit the incorporation of UTP into RNA while having little or no effect on the incorporation of methionine into protein. These results suggest that mitochondrial translation is not dependent on the bulk of mitochon-

drial transcription. It seems, therefore, unlikely that oxygen acts at the level of transcription. Second, ethidium bromide or acriflavin behave differently and inhibit both mtRNA and protein synthesis. Since transcription and translation are not obligatory coupled this means that ethidium bromide and acriflavin also have a direct effect on translation, as has been shown before by Grivell and Metz [12] in Xenopus mitochondria. Results obtained from experiments where these substances are used as specific transcription inhibitors should, therefore, be judged with care.

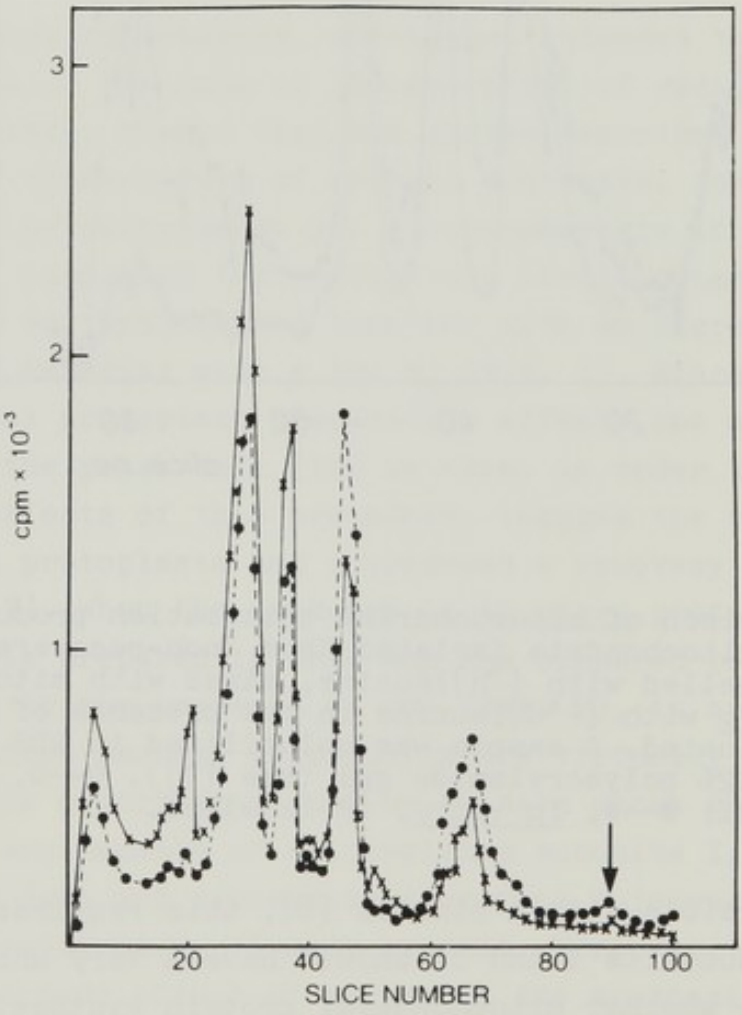


FIG. 2 - Comparison of mitochondrial translation products in vivo and in vitro. Mitochondria isolated from "recovered" protoplasts were labelled with [^{3}H]leucine and treated according to the legend to Fig. 1. ●—●, In vitro, ^{3}H-labelled; X—X, in vivo + ^{14}C-labeled CHI. (Reproduced from [7])

TABLE I: Effect of different inhibitors on mitochondrial RNA and protein synthesis

mtRNA and protein synthesis were measured as described [7,8] except that [^{35}S]methionine (10 μM, 0.6 μCi/ml) was used instead of [^{14}C]-leucine.

Additions	[^{35}S]Met incorporation (%)	[^{3}H]UTP incorporation (%)
None	100	100
Actinomycin D (10 μg/ml)	88	22
Nogalamycin (4 μg/ml)	82	0
Acriflavin (4 μg/ml)	2	10
Ethidium bromide (10 μg/ml)	0	1
Chloramphenicol (5 μg/ml)	29	102
Cycloheximide (50 μg/ml)	100	103

One objection, which can be brought forward against the interpretation of these experiments, is that the UTP incorporation we measure might be mainly in rRNA and not in mRNA. We have looked into this possibility by hybridizing *in vitro* synthesized RNA to fragments of mtDNA obtained by cleavage with the restriction enzymes HindII+III. This experiment is summarized in Table II. It can be seen that hybridization takes place with all fragments indicating that *in vitro* synthesized RNA is not transcribed from one particular region of the mtDNA. Hybridization with fragments 2 and 6 which contain the rDNA genes [13], is low because any rRNA synthesized is diluted with excess unlabelled rRNA present in the mitochondria. This experiment indicates that under these conditions no preferential transcription of rDNA takes place and invalidates the objection brought forward.

One of the main advantages of this experimental system is that it is very well suited for short pulse-label experiments, involving minimum handling and thus avoiding artefacts. It has been reported that *Neurospora crassa* mitochondria *in vivo* synthesize mitochondrial proteins as short precursors which are subsequently assembled into larger proteins. We have looked into this possibility in isolated

TABLE II: Hybridization of *in vitro* synthesized RNA to HindII+III fragments of mitochondrial DNA

HindII+III fragments of mtDNA of *S. cerevisiae* were prepared according to Sanders *et al.* [13]. Hybridization was carried out according to standard conditions with 0.6 µg ^{3}H-labelled mtRNA (specific activity 2400 cpm/µg) for 72 h at 58°C in 2 x SSC (R_{ot} excess).

DNA on filter		cpm bound
Fragment:	1	135
	2	37
	3	58
	4	133
	5	118
	6	48
	7	112
	8	122
	9	56
Phage T_7		23
None		22

mitochondria and pulse labelled for 3, 6 and 20 min. The analysis on SDS polyacrylamide gels of the products formed is shown in Fig. 3. It is clear that only a small fraction of the total proteins has a low M_r. Moreover, this fraction is constant (10%) in all three preparations and does not show the expected precursor-product kinetics. We think, therefore, that the low M_r precursors which occur in *N. crassa* do not occur in *Saccharomyces cerevisiae* and that those in *N. crassa* are probably caused by breakdown of unintegrated polypeptides.

Arguments for this conclusion come from the experiment shown in Fig. 4. Here we have added CHI during the recovery period of the protoplasts. When the isolated mitochondria are subsequently labeled *in vitro* the fraction of heterogeneous material with a low M_r is increased to 35% with a concomitant decrease of high M_r compounds, a situation reminiscent of Fig. 1. We interpret this finding by

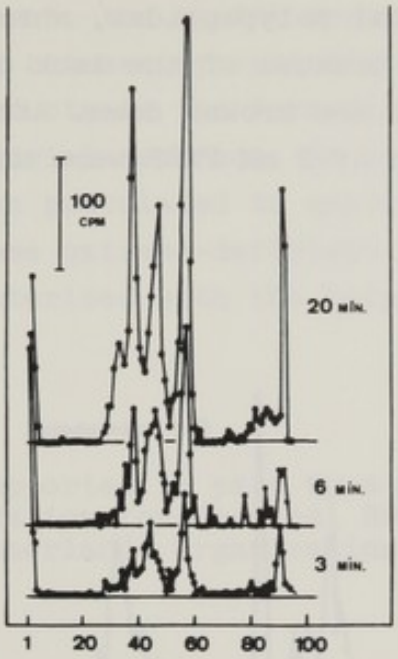


FIG. 3 - In vitro mitochondrial translation products formed during a short pulse. Mitochondria isolated from "recovered" protoplasts were pulse-labelled with [^{35}S]methionine for 3, 6 or 20 min. After the addition of 2 mM non-radioactive methionine the incubations were cooled to 0°C, washed once with 0.6 M Mannitol, 1 mM EDTA, 2 mM methionine (pH 6.8), solubilized in SDS and electrophoresed on 12% polyacrylamide gels.

TABLE III: Effect of addition of a post-ribosomal supernatant on mitochondrial protein synthesis

Mitochondrial protein synthesis was measured for 60 min as described in the legend to Table I. A post-ribosomal supernatant was prepared by centrifuging lysed protoplasts for 30 min at 100 000 x g.

Expt.	Additions	Incorporation (cpm/mg protein)
I	None	10 000
	Post-ribosomal supernatant (0.7 mg protein)	20 400
	Post-ribosomal supernatant (0.7 mg) + 0.5 mM PMSF	20 500
II	None	15 800
	Post-ribosomal supernatant (0.5 mg protein)	21 800
	Post-ribosomal supernatant (0.5 mg) + 50 µg/ml CHI	21 200
	Post-ribosomal supernatant alone	0

assuming that mitochondrial polypeptides, which cannot be integrated into holo enzymes because of the lack of cytoplasmically-synthesized counterparts, are broken down. Attempts to inhibit this breakdown by the addition of 2 mM PMSF were unsuccessful.

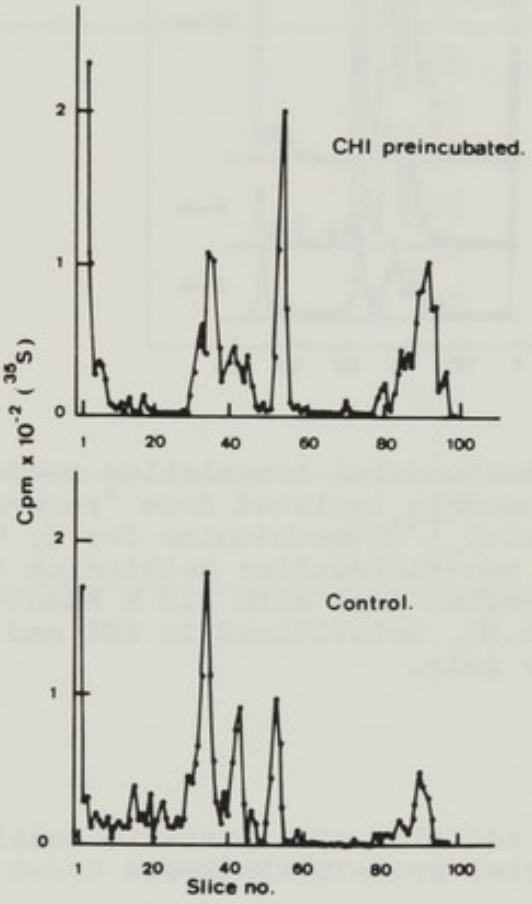


FIG. 4 - Mitochondrial translation products from mitochondria isolated from protoplasts recovered in the presence of 100 µg CHI/ml. Experimental procedure as described in the legend to Fig. 3. Label time, 20 min.

However, the addition of a post-ribosomal supernatant stimulates the rate of mitochondrial protein synthesis (Table III). In agreement with this hypothesis, Neupert [14] and Poyton and Kavanagh [15] have shown that under these conditions cytoplasmically made subunits of cytochrome oxidase are incorporated.

In summary, we feel that isolated mitochondria of Saccharomyces faithfully carry out mitochondrial protein synthesis as it

occurs in vivo. Moreover, the system seems very well suited to study the interaction between cytoplasmically made components with the mitochondrial products in the formation of the enzyme complexes in the inner mitochondrial membrane. In particular organizer proteins, which have been postulated to explain the properties of certain nuclear cytochrome oxidase-deficient mutants [16], might be identified and characterized with the help of isolated mitochondria.

ACKNOWLEDGEMENT

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Comparative Immunological and Electrophoretic Studies of the Cytochrome bc_1 Complex of Different Organisms

M.B. Katan

Organisms far apart in evolution often show a remarkable similarity at the subcellular level. More in particular, the mitochondrial respiratory chain of primitive unicellular eukaryotes has many properties in common with mammalian cells, among which are absorption spectrum and sensitivity to certain inhibitors. However, studies on *e.g.* cytochrome *c* (1) have shown that these functional similarities are often accompanied by large differences in protein primary structure. In the course of an investigation into the biogenesis of the cytochrome bc_1 complex (complex III) in *Saccharomyces cerevisiae*, I had the opportunity to compare this preparation with complex III preparations isolated from several other organisms by other workers. It was found that the cytochrome bc_1 complex shows marked variations in electrophoretic and immunological properties not just between organisms but also between different types of yeast.

Abbreviations:

EPR	Electron paramagnetic resonance
SDS	Sodium dodecylsulphate
SMP	Submitochondrial particles

METHODS AND MATERIALS

Antisera against *S. cerevisiae* cytochrome bc_1 complex were raised in rabbits by injecting 0.7 mg of antigen in Freunds complete adjuvant (Difco) into the dorsal and thigh muscles and boostering with 0.2 mg in incomplete Freunds adjuvant (Difco) after 4, 6 and 8 weeks.
Ouchterlony plates were prepared by pouring 5 ml of a solution containing 0.6% agarose (MCI Biomedical, Rockland, Maine, USA), 50 mM sodium phosphate pH 7.6 and 0.01% Merthiolate (Thimerosal, ethylmercurithiosalicylate, Sigma) onto 5 x 5 cm glass slides precoated with a thin layer of 3% agar. Wells with a diameter of 0.6 cm were cut at a centre-to-centre distance of 1.2 cm. Diffusion was for 48 h at room temperature. Precipitates were photographed either immediately with dark-field illumination (fig. 3C and 3D) or after drying followed by staining with Coomassie blue (2) (fig. 3A and 3B).
Antiserum against cytochrome *c* oxidase (3) was a gift from Dr. R.O. Poyton.
Submitochondrial particles and cytochrome bc_1 complex of *S. cerevisiae* were isolated from commercial baker's yeast ("Koningsgist", Gist-Brocades, Delft, The Netherlands) as described (4). Growth of cells and isolation of mitochondria from *Saccharomyces carlsbergensis* NCYC74, *Candida* (formerly *Torulopsis*) *utilis* CBS-621 and *Kluyveromyces* (formerly *Saccharomyces*) *lactis* NRRL Y1140 have been described (5). Submitochondrial particles of these cells were a gift from Mrs. N. van Harten-Loosbroek and were prepared by sonification of mitochondria in 0.1 M sodium phosphate pH 7.6. The particles were extracted with sodium cholate (3 mg per mg protein) plus KCl (1.2 M) according to Ebner *et al.* (6).
Beef heart complex III (7) was a gift from Dr. J. Berden and *C. utilis* complex III (8) from Dr. C. Grimmelikhuijzen. Cytochrome *b* of *Neurospora crassa* was kindly donated by

Dr. H. Weiss; its properties are described in (9). Cytochrome *c* oxidase of *S. cerevisiae* (10) was a gift from Dr. R.O. Poyton.

RESULTS AND DISCUSSION

Electrophoretic experiments

Fig. 1 shows complex III preparations from *Candida utilis* (formerly *Torulopsis utilis*), cow (*Bos taurus*) and baker's yeast (*Saccharomyces cerevisiae*) analysed by electrophoresis on 15% polyacrylamide slab gels in the presence of SDS.

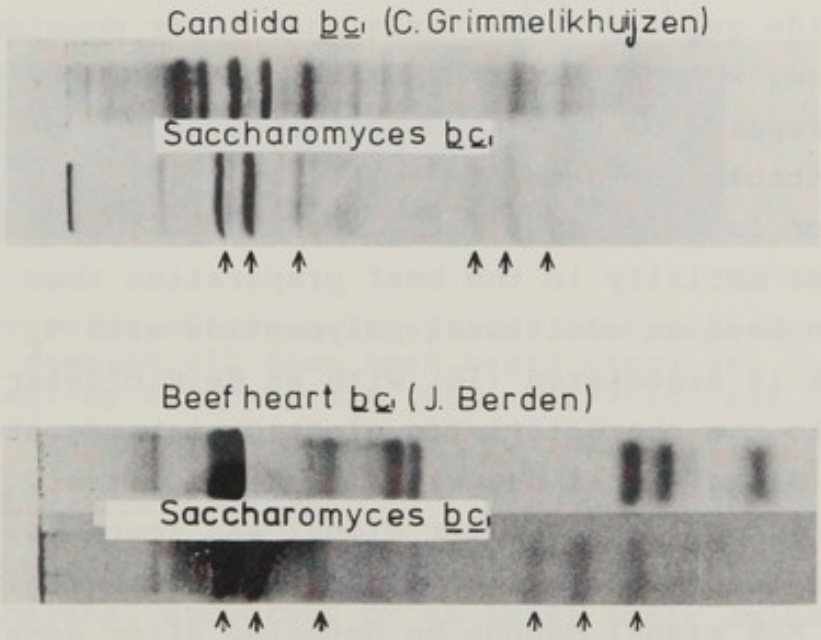


Fig.1. Electrophoresis of various complex III preparations on SDS-polyacrylamide gels. Cytochrome bc_1 complex preparations were run side by side on 15% polyacrylamide slab gels and stained with Coomassie blue and destained as described (4). Migration is from left to right. The arrows denote the 6 bands of *S. cerevisiae* complex III, with molecular weights of 44000, 40000, 32000, 17000, 14000 and 11000.

It is evident that the overall composition of the three complexes is similar with two "core protein" bands in the M_r 40000 - 50000 region, one band around 30000 and three at low

M_r, but that the molecular weights of the various subunits differ. In theory it should be possible to derive this type of information by comparing literature values; in practice, differences in electrophoresis technique and possibly in M_r values attributed to calibration proteins (2, 11, 12) make this difficult. For the "core proteins"of beef heart bc_1 complex, for instance, values of 53 and 50 (13), 52 and 50 (14), 50 and 47 (15), and 46 and 43 (16) kdaltons have been published; for the polypeptide carrying the cytochrome c_1 heme group the values range from 29 (16) to 32 (14).

Whatever the true molecular weights may turn out to be, it is clear from fig. 1 that the two largest polypeptides ("core proteins") of beef complex III have a lower mobility on SDS-polyacrylamide gel electrophoresis than their counterparts in *S. cerevisiae*, which in turn are larger than those in *C.utilis* (this preparation shows two additional bands of higher M_r that are probably contaminants (8)).

The situation is reversed for the smaller polypeptides which have a higher mobility in the beef preparation than in *S. cerevisiae*. In beef an additional polypeptide with M_r 24000 is present that is associated (16) with an Fe.S cluster which gives rise to the characteristic electron paramagnetic resonance (EPR) signal first described by Rieske *et al.* (17). In *S. cerevisiae* a very similar signal was found (4) but it has not been determined which polypeptide is the Fe.S protein because the EPR signal cannot be detected after denaturation of complex III with SDS. This problem does not hold for the polypeptide carrying the cytochrome c_1 heme group, because the porphyrin ring system is covalently linked to the polypeptide backbone and can be visualised after electrophoresis of the complex on SDS gels (18). As shown in fig. 2 the c_1 polypeptide of the beef heart complex has has the same electrophoretic mobility as that of *S. cerevisiae*.

We have shown before that the M_r 32000 band of yeast complex III contains not only cytochrome c_1 but also a polypeptide

associated with the cytochrome *b* heme. When we compare this polypeptide with the cytochrome *b* preparation isolated from *Neurospora crassa* by H. Weiss (9), we find several similarities: both are made on mitochondrial ribosomes (9, 19); they have the same mobility on SDS-polyacrylamide slab gel electrophoresis (Katan, M.B. and Weiss, H. unpublished) and the amino acid composition of the two preparations is very similar (compare (4) and (20)). The cytochrome *b* from *N. crassa* has been shown to consist of two slightly different polypeptides with the same M_r (20); for *S. cerevisiae* no information is available on this point as yet.

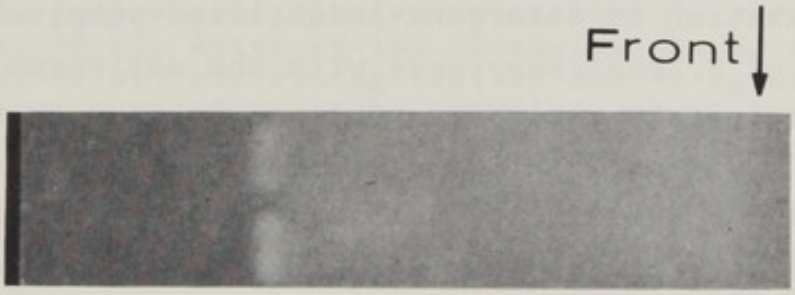


Fig. 2. Detection of the polypeptides carrying the cytochrome c_1 heme in complex III from beef heart (top) and *S. cerevisiae* (bottom) by fluorescence (18). Migration was from left to right.

Immunochemical experiments

Ideally, comparisons between proteins should involve their three-dimensional structure or at least their amino acid sequence. For mitochondrial membrane proteins such data have been slow in coming, mostly because of problems in obtaining large amounts of pure polypeptides and in the digestion of these hydrophobic polypeptides into defined smaller pieces. For the specific recognition of certain mitochondrial proteins, another approach has been used successfully (21-23), which employs specific antisera. We have used such antisera, directed against the cytochrome bc_1 complex of *S. cerevisiae*, to investigate homologies between preparations from different

organisms. The antiserum was raised against native bc_1 complex, but it was found to be reactive against SDS-denatured bc_1 complex of *S. cerevisiae* in Ouchterlony double diffusion tests (Katan, M.B. and Meiland, C.M., unpublished) and against polypeptides isolated (4) from the complex by preparative SDS-polyacrylamide gel elecrophoresis. For the latter experiments (Grivell, L.A. and Katan, M.B., unpublished) the M_r 40000 polypeptide and the M_r 32000 cytochrome *b* polypeptide were labelled with ^{125}I and then immunoprecipitated by a second antibody technique (24, 25).
These experiments suggest that the antiserum recognizes determinants in the bc_1 complex that are still present after denaturation. Incubation of detergent-solubilized submitochondrial particles of *S. carlsbergensis* with the antiserum yielded an immunoprecipitate that after SDS-polyacrylamide electrophoresis and staining with Coomassie blue showed bands with M_r 44000, 40000 and 32000, plus two additional bands corresponding in molecular weight with the heavy and light chains of immunoglobulin G (Groot, G.S.P. and Katan, M.B., unpublished). Taken together, these observations suggest that the antiserum reacts with at least some of the main components of the bc_1 complex.
Fig. 3A shows that in Ouchterlony double diffusion tests there is no cross-reactivity between beef heart complex III and the antiserum against *S. cerevisiae* complex III. In fig. 3B the antiserum is tested against submitochondrial particles of *S. cerevisiae* and *S. carlsbergensis*. A positive reaction is obtained with both and the confluence of the precipitate lines shows that the antiserum recognizes identical determinants in the bc_1 complex from *S. cerevisiae* (fig. 3B, well No 1), submitochondrial particles from *S. carlsbergensis* (well No 7) and detergent-solubilized submitochondrial particles of *S. cerevisiae* (well No 5) and *S. carlsbergensis* (well No 7'). The presence of two precipitate lines suggests that none of these antigens consists of a homogeneous

population of identical molecules or particles.

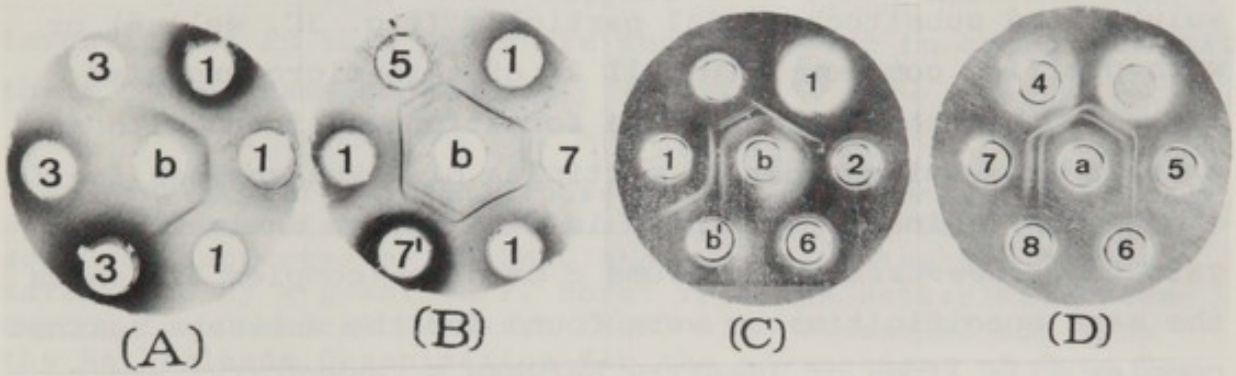


Fig. 3. Immunological analysis of mitochondrial preparations from different organisms by Ouchterlony double diffusion (*cf.* METHODS).
Fig. 3A, centre well: antiserum against *S. cerevisiae* bc_1 complex, 40 µl. Outer wells, clockwise: 1. *S. cerevisiae* bc_1 complex, 40 µl, containing 320, 80 and 40 µg of protein, respectively; 3, beef heart bc_1 complex, 40 µl, containing 1600, 200 and 50 µg of protein respectively. Dilutions were made in 150 mM NaCl, 10 mM sodium phosphate, pH 7.6 plus 0,5% Triton X-100.
Fig. 3B, centre well: antiserum against *S. cerevisiae* bc_1 complex, 20 µl. Outer wells: 1, *S. cerevisiae* bc_1 complex, 20 µl, 150 µg of protein; 5, submitochondrial particles (SMP) of *S. cerevisiae*, 20 µl, 25 µg of protein; 7, SMP of *S. carlsbergensis*, 20 µl, 65 µg of protein; 7', deoxycholate extract of same.
Fig. 3C, centre and lower left-hand well: antisera raised against *S. cerevisiae* bc_1 complex in different rabbits, 30 µl. Other wells: 1, two preparations of *S. cerevisiae* bc_1 complex, 30 µl, 300 µg of protein; 2, bc_1 complex of *C. utilis*, 30 µl, 350 µg of protein; 6, cholate extract of SMP of *C. utilis*, 30 µl, 400 µg of protein.
Fig. 3D, centre well: antiserum against *S. cerevisiae* cytochrome oxidase, 30 µl; outer wells: 4, *S. cerevisiae* cytochrome oxidase, 30 µl, 125 µg of protein; 5, SMP of *S. cerevisiae*, 30 µl, 750 µg of protein; 6, cholate extract of SMP of *C. utilis*, 30 µl, 400 µg of protein; 7, cholate extract of SMP of *S. carlsbergensis*, 30 µl, 240 µg of protein; 8, cholate extract of SMP of *K. lactis*, 30 µl, 240 µg of protein.
General key: a, antiserum against *S. cerevisiae* cytochrome oxidase; b and b', antisera against *S. cerevisiae* bc_1 complex; 1, *S. cerevisiae* bc_1 complex; 2, *C. utilis* bc_1 complex; 3, beef heart bc_1 complex; 4, *S. cerevisiae* cytochrome oxidase; 5, *S. cerevisiae* SMP; 6, extract of *C. utilis* SMP; 7, 7', (extract of) *S. carlsbergensis* SMP; 8, extract of *K. lactis* SMP.

Fig. 3C shows a similar experiment with antigens from the yeast *Candida utilis*. No cross-reactivity is seen either with solubilized submitochondrial particles (fig. 3C, well 6) or with purified complex III (well 2) of this micro-organism. The same absence of precipitate formation was found with an extract of submitochondrial particles of *Kluyveromyces lactis* (not shown). Finally, as shown in fig. 3D, an antiserum against *S. cerevisiae* cytochrome *c* oxidase (complex IV) shows the same specificities as were found for the antisera against complex III: lines of identity between *S. cerevisiae* and *S. carlsbergensis* and no immunoprecipitation with *K. lactis* or *C. utilis*.

The extracts of submitochondrial particles of different yeasts used in these experiments were prepared in parallel from simultaneously grown cultures. Also a positive reaction with *S. carlsbergensis* was obtained regardless whether the submitochondrial particles had been solubilized with cholate, with deoxycholate or with Triton X-100, or had not been in contact with detergent at all. This makes it less likely that the absence of a reaction with *C. utilis* and *K. lactis* was due to these preparations being unable to take part in immunochemical reactions at all.

Variability in polypeptide chain length and sequence is the rule rather than the exception when comparisons are made of proteins of similar functions in different organisms (1,26) and it is not surprising that this rule also holds for complex III of the mitochondrial respiratory chain. The differences found here between micro-organisms that are all classified together as yeasts, however, merit some attention. Groot, Flavell and Sanders (5) have made a careful study of the homologies between the nuclear and the mitochondrial DNA's of *S. cerevisiae*, *S. carlsbergensis*, *K. lactis* and *C. utilis* and found 100% homology between the two species of the genus *Saccharomyces*, but very little homology between the three different genera. Their conclusions and those from the se-

quence data on cytochrome *c* (26) are reinforced by the experiments reported here, which suggest once again that the yeasts investigated in this paper are further apart in evolution than their common name suggests.

I am very grateful to all those who so generously supplied me with mitochondrial preparations or antiserum. Part of this work was supported by an EMBO short-term fellowship to M.B. Katan, and by a grant to P. Borst from The Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the advancement of Pure Research (ZWO).

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Subunit Structure and Biogenesis of Cytochrome b from Neurospora Crassa Mitochondria

H. Weiss, B. Ziganke, H.J. Kolb

SUMMARY

Cytochrome b was prepared from the deoxycholate solubilized membranes of Neurospora crassa mitochondria by means of chromatography on oleyl-polymethacrylic acid resin and gel filtration on Sephadex. The specific heme content of the preparation was 38 µmol/g protein, a value which indicated a minimum molecular weight of 26 000 per mole protoheme. By measurement of the sedimentation equilibrium in a deoxycholate solution and of the deoxycholate binding of the preparation, and by analysis of the data by means of an equation appropriate for a multicomponent system, a molecular weight of 51 000 was obtained for the pure heme protein excluding the protein bound detergent. Upon dodecyl sulfate gel electrophoresis the protein moiety of the preparation migrated as a single band with an apparent molecular weight of 27 000. When previously cross-linked with dimethyl suberimidate, or glutaric dialdehyde, respectively, the preparation showed upon dodecyl sulfate gel electrophoresis two protein bands with the apparent molecular weights of 55 000 and 27 000. By means of hydroxylapatite chromatography in dodecyl sulfate the protein moiety of the preparation was separated into two fractions of about equal amounts of protein, each of which showed upon dodecyl sulfate gel electrophoresis an apparent molecular weight of 27 000. They had very similar amino acid compositions, differing essentially only in their lysine contents. Their contents of unpolar amino acids amounted to 63%. Their fragment patterns, which were obtained by means of gel filtration in formic acid after cyanogen bromide cleavage, were indistinguishable. Both fractions were radioactively labeled by in vivo incorporation of (^{14}C)-leucine in the presence of cycloheximide, an inhibitor of the extramitochondrial protein synthesis, but they were not labeled by in vivo incorporation of (^{14}C)-leucine in the presence of chloramphenicol, an inhibitor of mitochondrial protein synthesis.

From these results the conclusion is drawn that mitochondrial cytochrome b is a dimeric heme protein consisting of two slightly different heme binding polypeptides of about equal size. These two polypeptides might be derived from the same (mitochondrial) gene but (at least one) might be modified after their translation. Their translation sites are the mitochondrial ribosomes.

INTRODUCTION

The mitochondrial respiratory chain contains two different b-type cytochromes in equimolar amounts. These have been extensively studied by means of their light absorption spectra, i.e.

by means of the properties of their heme compounds (1). Information, however, about their protein compounds are extremely rare. These have now become of special interest since it has become evident that mutations of mitochondrial DNA or disturbances of mitochondrial protein synthesis may lead to a loss of these two b-type cytochromes (2). Thus, we have focussed our studies on cytochrome b on its protein compound . In previous papers we have described cytochrome b preparations from the fungus Neurospora crassa (3) and from the insect Locusta migratoria (4), respectively, which were suggested to be dimeric heme proteins consisting of two heme binding subunits of equal size. By means of in vivo incorporation of radioactive amino acids under inhibition of the extra-mitochondrial protein synthesis with cycloheximide we have shown that these subunits (or at least one of them) are translated on mitochondrial ribosomes. In the present paper we describe new results concerning the subunit structure of the cytochrome b preparation from Neurospora crassa. Some of these results have been published already in the proceedings of an international symposium (5).

METHODS

Neurospora crassa, wild type 74 a, was grown in Vogels' minimal medium (6). The hyphae were disrupted with a grinding-mill and mitochondria were isolated by fractional centrifugation (6). The mitochondrial membranes were solubilized with deoxycholate and separated by means of chromatography on oleyl-polymethacrylic acid resin (7). Cytochrome b was finally purified by gel filtration on Sephadex in a solution containing deoxycholate and potassium chloride (3,8). The cytochrome b subunits were separated by hydroxylapatite chromatography in dodecyl sulfate (9). 0.1 - 0.5 mg cytochrome b in 2-5 ml 0.2% sodium dodecyl sulfate, 0.01 M sodium phosphate pH 6.4 and 0.002 M dithioerytritol was applied on a 0.6 x 8.5 cm column of Bio Gel HT (Bio-Rad, Richmond, California) equilibrated in the above-mentioned solution. They were eluted with a linear 200 ml gradient ranging from 0.25 M to 0.55 M sodium phosphate containing additionally 0.2% dodecyl sulfate and 0.002 M dithioerytritol. The flow rate was 3.5 ml/h, the temperature 28° C.

In order to selectively label proteins according to their translation site - the mitochondrial versus the cytoplasmic ribosomes - (^{3}H)-leucine (1.5 mCi/g protein) was added first to

an exponentially growing culture and was incorporated for 2 h. Then the cells either remained untreated or were treated with cycloheximide (0.1 mg/ml) or with chloramphenicol (4 mg/ml). Two minutes later (^{14}C)-leucine (0.2 mCi/g protein) was added. The cells were harvested 2 h later.

Analytical ultracentrifugation was carried out with a Beckman-Spinco Model E instrument, equipped with absorption optics and with photoelectric scanner. The temperature was kept at 23°C. 0.5 mg cytochrome b was solubilized in 0.5% K-deoxycholate, 0.2 M KCl and 0.02 M Tris-acetate pH8. Sedimentation runs were performed at 56 000 rpm (10). The diffusion coefficient was determined at 6 000 rpm using a capillary type double-sector cell (11). High speed sedimentation velocity experiments were carried out as described (12). 30 µl silicon oil and 120 µl protein solution were filled in a standard double-sector cell. The equilibrium was reached after 18 h at a speed of 24 000 rpm.

The deoxycholate-binding of the cytochrome b preparation was measured by subjecting 0.27 ml 112 µM cytochrome b in 0.5% K-deoxycholate, 1.5 M KCl, 0.05 M Tris-acetate pH 8 to gel filtration on a 0.5 x 21 cm Sephadex G-150 column equilibrated with 0.25% (^{14}C) K-deoxycholate (0.126 µCi/ml) (Amersham-Buchler, Braunschweig) 0.2 M KCl and 0.05 M Tris-acetate pH 9 (13).

Cross-linking of the cytochrome b subunits was performed by addition of 6 mg/ml dimethyl suberimidate (Pierce Eurocompany, Rotterdam) (14) or 0.1 g/ml glutaric dialdehyde (Serva, Heidelberg) (15), respectively, to a 30 µM cytochrome b preparation in 0.5% K-deoxycholate, 0.5% K-cholate, 0.2 M KCl, 0.02 M triethanolamin HCl, pH 8.5. After incubation for 2 h at 25°C the solutions were dialysed against water for 5 h and then lyophilized. Thereafter, dodecyl sulfate gel electrophoresis was performed as described (16) using 7.5% polyacrylamide gels.

For cyanogen bromide cleavage (17) 30 µg cytochrome b was dissolved in 100 µl 90% formic acid. 3 mg cyanogen bromide (Merck, Darmstadt) was added, the tube was flushed with nitrogen and sealed. For separation of the fragments the reaction medium was applied 27 h later on a 0.6 x 250 cm Bio-Gel P 30 column in 85% formic acid. At a hydrostatic pressure of 120 cm the flow rate was 0.5 ml/h. The temperature was 25°C.

Amino acid analysis after acid hydrolysis of cytochrome b (18) was performed with a Biotronik (Biotronik GmbH, Frankfurt) amino acid analyser.

RESULTS AND DISCUSSION

Starting from mitochondrial membranes the final yield of cytochrome b preparation was 3-5%. The heme content was enriched about 40 times to 35-40 µmol/g protein. This value was obtained using an iron test for quantitative heme determination (3) and

the Lowry-method or amino acid analysis for quantitative protein determination. The preparation showed light absorption maxima with molar absorption coefficients characteristic for cytochrome b (Fig. 1.) (3). It was soluble only in the presence of potassium deoxycholate (0.5%) and potassium chloride (0.2 M).

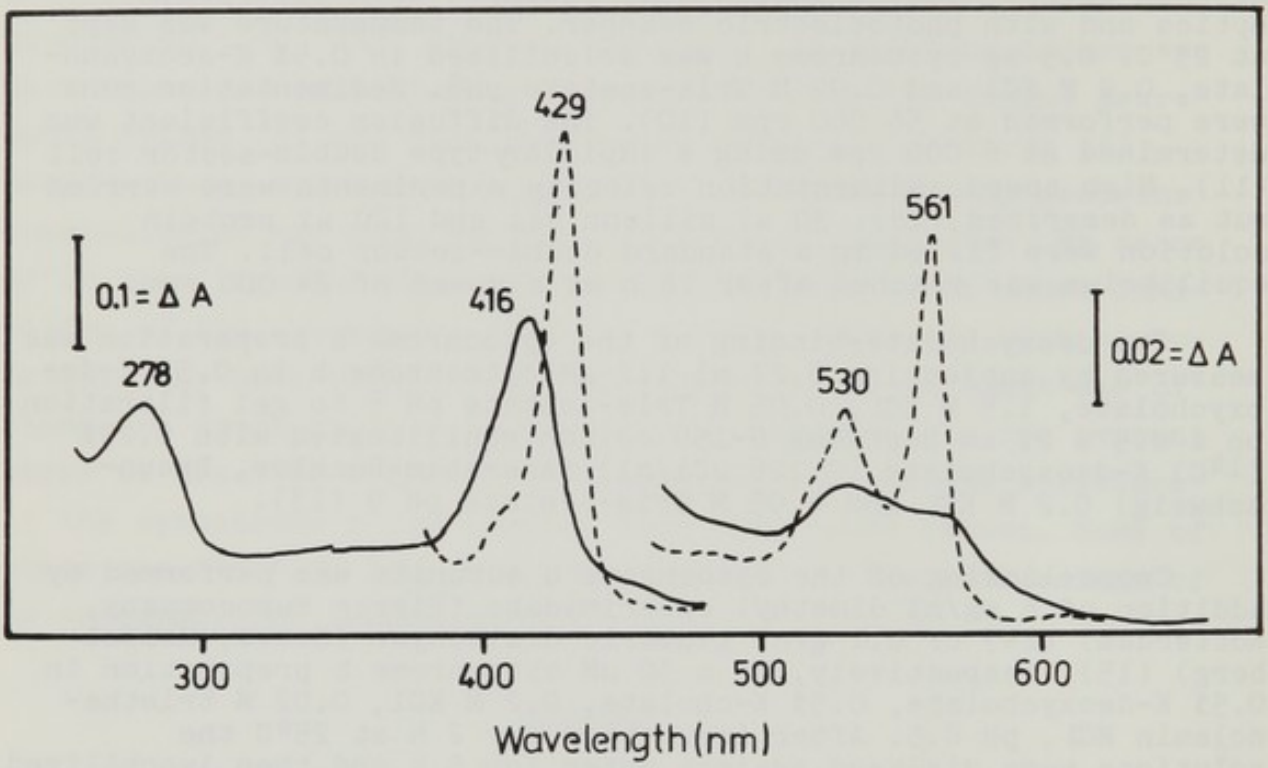


Fig. 1. Light absorption spectra of the cytochrome b preparation. (——) air-oxidized form; (----) dithionite-reduced form.

Analytical ultracentrifugation experiments in a deoxycholate solution confirmed the homogeniety of the cytochrome b preparation. Sedimentation velocity experiments gave linear relationships between the logarithm of the boundary distance from the rotation center and time. Following the absorbance of the heme compound at 416 nm or of the protein compound at 280 nm identical sedimentation coefficients were obtained (5). With high-speed sedimentation equilibrium experiments the plot of the logarithm of the absorbance at 416 nm versus the square of distance from the rotation center also yielded a straight line (Fig.2). The following hydrodynamic parameters were obtained: Sedimentation coefficient, $S_{20,w}$= 4.2; diffusion coefficient, $D_{20,w}$ = 6.3; frictional coefficient (20),f=1.25; molecular weight from sedimentation and

diffusion coefficient (21), $M_{r(S,D)}$ = 63 000; and molecular weight from sedimentation equilibrium (12), M_r = 62 000. These values were corrected to standard conditions (viscosity and density of the buffer). The used partial specific volume of cytochrome b (0.754 ml/g) was estimated from the amino acid composition (22). The values do not include correction for the protein bound deoxycholate.

In a multicomponent system, however, such as the cytochrome b preparation in the deoxycholate solution, an interaction of the detergent with the protein has to be considered. In order to determine the amount of the protein-bound deoxycholate the cytochrome b preparation was subjected to gel filtration in the presence of (^{14}C)-deoxycholate (Fig.3) (13). A quantity of 0.3 g deoxycholate per g protein resulted from the amount of radioactivity that co-eluted with cytochrome b. From the sedimentation equilibrium of the cytochrome b-deoxycholate complex and from the amount of protein-bound deoxycholate the molecular weight 51 000 was calculated for the pure heme protein excluding the bound detergent as pointed out by Tanford et al (26).

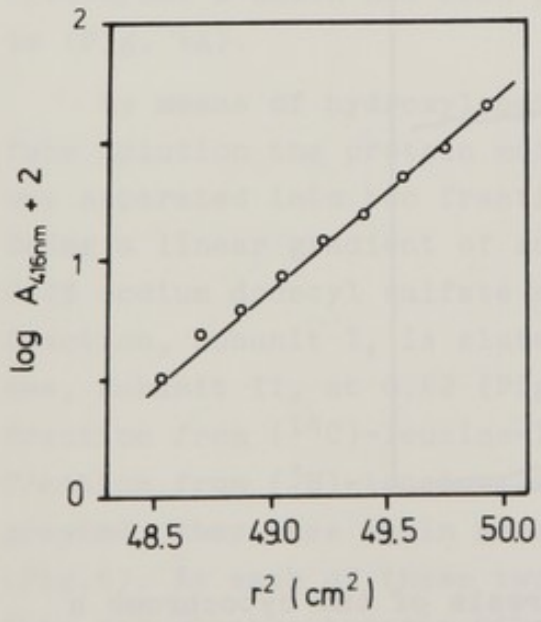


Fig. 2. Sedimentation equilibrium run of the cytochrome b preparation in the presence of K-deoxycholate.

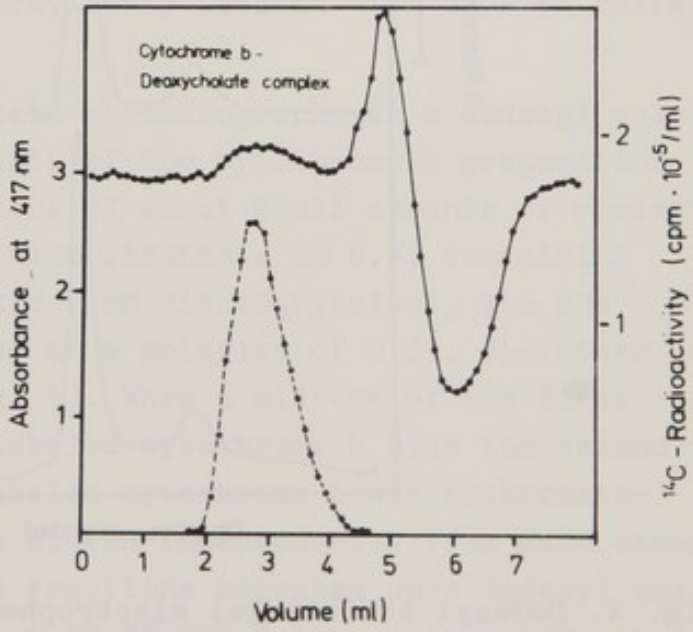


Fig. 3. Deoxycholate-binding of cytochrome b. The preparation was subjected to gel filtration in the presence of (^{14}C)-deoxycholate.

In the presence of dodecyl sulfate the cytochrome b preparation dissociated into polypeptide subunits and heme. Upon dodecyl sulfate gel electrophoresis the protein moiety migrated as one band. An apparent molecular weight of 30 000 was determined for this band, when a 12.5% polyacrylamide gel was used, and a molecu-

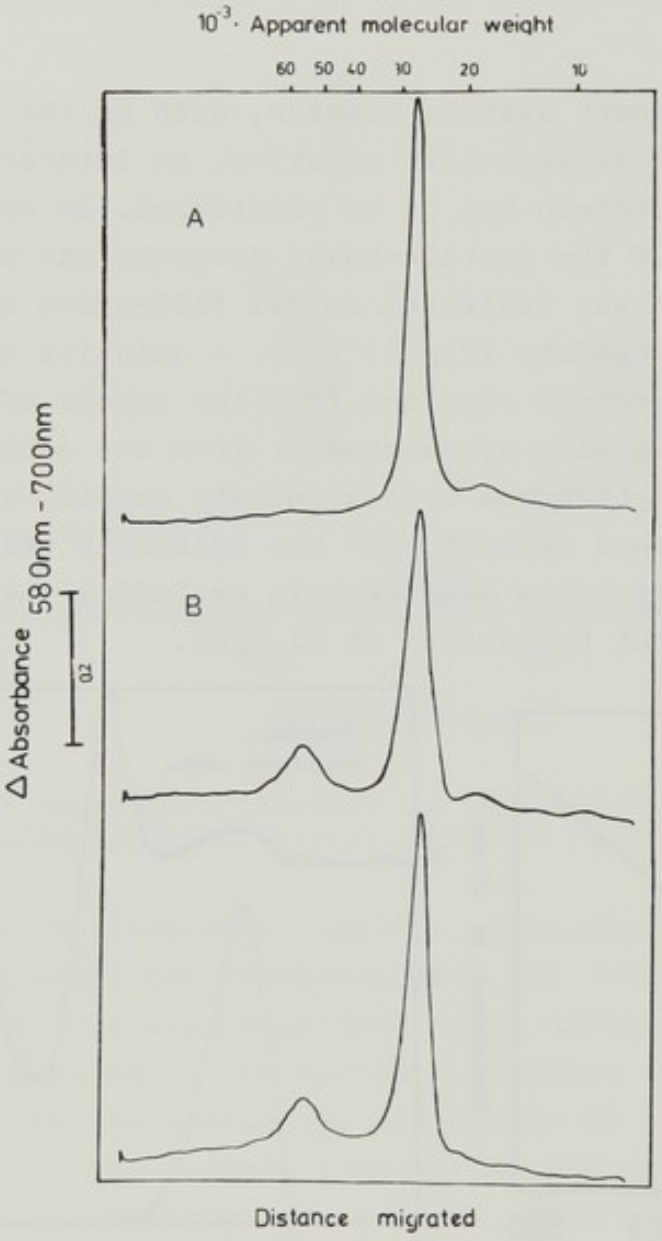


Fig. 4. Dodecyl sulfate gel electrophoresis of the cytochrome b preparation (A) previously treated with dimethyl suberimidate in the presence of dodecyl sulfate (B) previously treated with dimethyl suberimidate in the presence of deoxycholate (C) previously treated with glutaric dialdehyde in the presence of deoxycholate. Bovine serum albumin, chicken egg albumin, chymotrypsinogen, myoglobin and cytochrome c were used for calibration.

lar weight of 27 000 when a 7.5% polyacrylamide gel was used (Fig.4A).

Obviously, the molecular weight of the cytochrome b in dodecyl sulfate is equal to the minimum molecular weight per mole protoheme (which resulted from the specific heme content of the preparation), but is only one half of the molecular weight of cytochrome b in deoxycholate. This suggested that the cytochrome b preparation in the deoxycholate solution is a dimeric heme protein consisting of two heme binding subunits of equal size.

This subunit structure was further confirmed by means of dodecyl sulfate gel electrophoresis of a cytochrome b preparation whose subunits were previously cross-linked with the bifunctional reagents dimethyl suberimidate (14), or glutaric dialdehyde, respectively (15). As can be seen in figure 4B and 4C, a second protein band with an apparent molecular weight of 55 000 was revealed on the gel when these reagents were added to the cytochrome b preparation in a deoxycholate solution. This additional protein band, however, was not revealed when these reagents were added to cytochrome b which had been previously treated with dodecyl sulfate (Fig. 4A).

By means of hydroxylapatite chromatography in a dodecyl sulfate solution the protein moiety of the cytochrome b preparation was separated into two fractions of about equal amounts of protein. Using a linear gradient of sodium phosphate pH 6.4, containing 0.2% sodium dodecyl sulfate and 2 mM dithioerytritol, the one fraction, subunit I, is eluted at a molarity of 0.37, the other one, subunit II, at 0.42 (Fig. 5). When a mixture of the first fraction from (^{14}C)-leucine-labeled cytochrome b plus the second fraction from (^{3}H)-leucine-labeled cytochrome b was rechromatographed they once again were eluted independently from each other (Fig.6). As each of these two fractions migrated upon dodecyl sulfate gel electrophoresis like a 27-30 000 Dalton polypeptide they were suggested to represent the two different subunits of the dimeric heme protein cytochrome b.

The amino acid composition of these two fractions, however,

Table 1. Amino acid compositions of cytochrome b and the cytochrome b subunits from Neurospora crassa.

Amino acid	Cytochrome b	Subunit I	Subunit II
		mole %	
Aspartic acid	8.77	8.92	8.66
Threonine	4.86	4.95	4.98
Serine	8.52	8.27	8.58
Glutamic acid	6.30	7.67	5.87
Proline	5.06	4.88	4.93
Glycine	7.59	7.88	7.74
Alanine	7.77	7.56	7.61
Cysteine	n.d.	n.d.	n.d.
Valine	6.93	6.84	6.93
Methionine	2.45	2.18	2.73
Isoleucine	8.88	8.27	8.59
Leucine	12.82	12.05	13.25
Tyrosine	4.21	4.13	4.23
Phenylalanine	6.52	5.74	6.79
Lysine	3.14	3.82	2.81
Histidine	2.44	2.51	2.56
Arginine	3.74	4.32	3.72

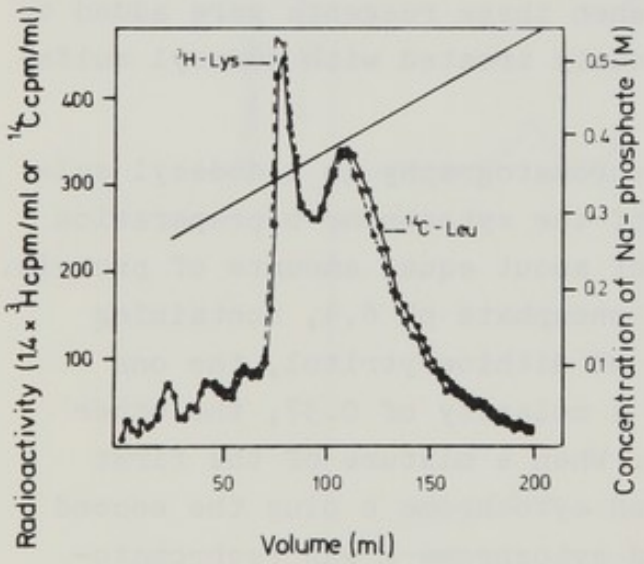


Fig. 5. Dodecyl sulfate hydroxylapatite chromatography of a (^{14}C)-leucine and (^{3}H)-lysine-labeled cytochrome b preparation.

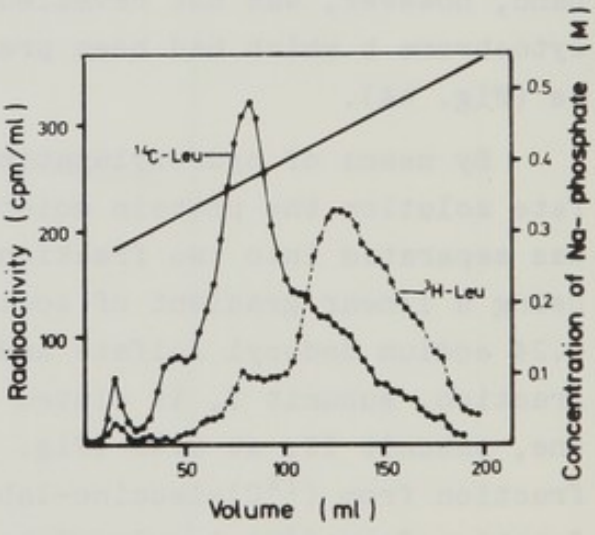


Fig. 6. Dodecyl sulfate hydroxylapatite chromatography of a mixture of subunit I from (^{14}C) leucine labeled cytochrome b plus subunit II from (^{3}H)-leucine labeled cytochrome b.

were found to be very similar (Table 1). Both displayed a high content of unpolar amino acids. The resulting low polarity of 37% is (23) characteristic for mitochondrially synthesized polypepti-

des (see below, 24, 25). Both fractions differed essentially only in their lysine content.

In order to confirm this different lysine content, cytochrome b was purified from cells which had simultaneously incorporated (^{3}H)-lysine (1.25 mCi/g protein) and (^{14}C)-leucine (0.125 mCi/g protein). Due to the fact that the catabolism of these amino acids is negligible, compared to their incorporation into proteins, and that protein break-down is negligible compared to protein synthesis, more than 85% of these radioactive amino acids were recovered five minutes after their addition to the culture medium in the proteins and remained very stable there (19). As can be seen from the data of table 2 the ^{3}H/^{14}C ratio of the cytochrome b preparation is much lower than the corresponding ratio of the cytoplasmic or mitochondrial matrix protein. This reflects the unusual low lysine to leucine ratio of cytochrome b (Table 1). When this double-labeled cytochrome b preparation was subjected to dodecyl sulfate hydroxylapatite chromatography an unequal distribution of the ^{3}H- and ^{14}C-radioactivity between the two fractions was obtained (Fig. 6). This experiment seemed to confirm the result of the amino acid analysis; namely, that the two cytochrome b subunits differ in their lysine content.

Table 2: ^{3}H/^{14}C ratio of various protein preparation from Neurospora crassa which had incorporated (^{3}H)-lysine and (^{14}C)-leucine. The molar ratio lysine to leucine of cytochrome b and of the cytochrome b subunits were taken from Table 1.

Preparation	^{3}H/^{14}C	molar ratio lysine to leucine
Cytoplasm	2.74	
Mitochondrial matrix	2.85	
Mitochondrial membrane	1.86	
Cytochrome b	0.75	0.25
Subunit I of cytochrome b	0.83	0.32
Subunit II of cytochrome b	0.59	0.21

On the other hand, when the two subunits were cleaved with cyanogen bromide and then subjected to gel filtration on Bio-Gel P 30 in 85% formic acid fragment patterns were obtained which were indistinguishable. Figure 7A shows the pattern of the total cytochrome b preparation which has been cleaved previously with cyanogen bromide, and figure 7B shows the corresponding patterns from

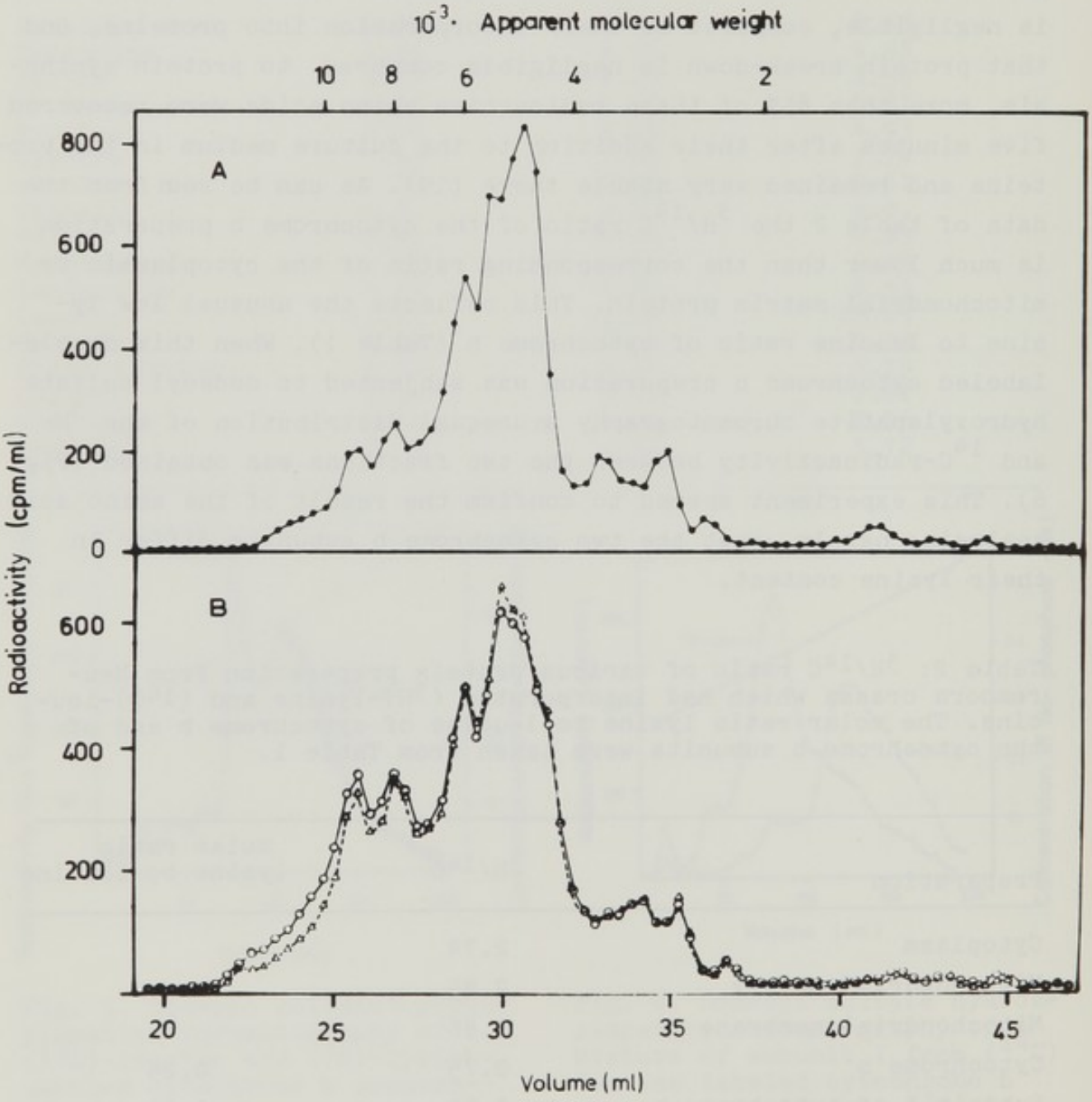


Fig. 7. Gel filtration pattern of the fragments resulting from cyanogen bromide cleavage of (^{3}H)-leucine-labeled cytochrome b (7A) and of a mixture of the (^{3}H)-leucine-labeled subunit I plus the (^{14}C)-leucine-labeled subunit II (7B).

a mixture of the (^{14}C)-leucine-labeled subunit I plus the (^{3}H)-leucine-labeled subunit II. As can be seen, cyanogen bromide cleavage of the two subunits yielded fragments with indistinguishable molecular weights. This result indicated that both subunits might have the same primary structure, i.e. both might be derived from the same gene.

From the findings that the two cytochrome b subunits could, firstly, be separated by hydroxylapatite chromatography and secondly, were differentiated in their lysine content and, thirdly, were indistinguishable on the basis of their fragment patterns obtained after cleavage with cyanogen bromide, the following conclusion might be drawn; both subunits are derived from the same type of polypeptide chain but are modified after their translation. The modification of subunit II might involve the cutting of a small lysine-containing oligopeptide. These considerations, however, must remain speculative, until more data are available.

In order to define the translation site of the cytochrome b subunits, cytochrome b was prepared from double-labeled cells. These had first incorporated (^{3}H)-leucine in the absence of inhibitors, followed by incorporation of (^{14}C)-leucine, either in the absence of inhibitors or in the presence of cycloheximide or chloramphenicol. The purified cytochrome b protein was subsequently analysed for ^{3}H - and ^{14}C -radioactivity after dodecyl sulfate gel electrophoresis and dodecyl sulfate hydroxylapatite chromatography.

Under conditions of control labeling the ^{14}C/^{3}H-ratio, which represents the relative specific ^{3}H-contents of protein, was fairly constant among all cell proteins, including the cytochrome b peaks obtained upon dodecyl sulfate gel electrophoresis (Fig. 7A).

In cells labeled in the presence of cycloheximide the proteins of cytoplasm were no longer synthesized. Their ^{14}C/^{3}H-ratios were less than one percent of the corresponding ratios in untreated cells. In contrast, a large amount of (^{14}C)-leucine was incorporated into cytocnrome b. However, the peak of ^{14}C-radioactivity obtained upon dodecyl sulfate gel electrophoresis was

asymmetrically distributed over the ^{3}H-radioactivity peak, which indicated that the ^{3}H-radioactivity peak resulted from the overlapping of two different polypeptides with different $^{14}C/^{3}H$-ratios (Fig.7B). This was confirmed after the separation of the two polypeptides by means of dodecyl sulfate hydroxylapatite chromatography (Fig.8).

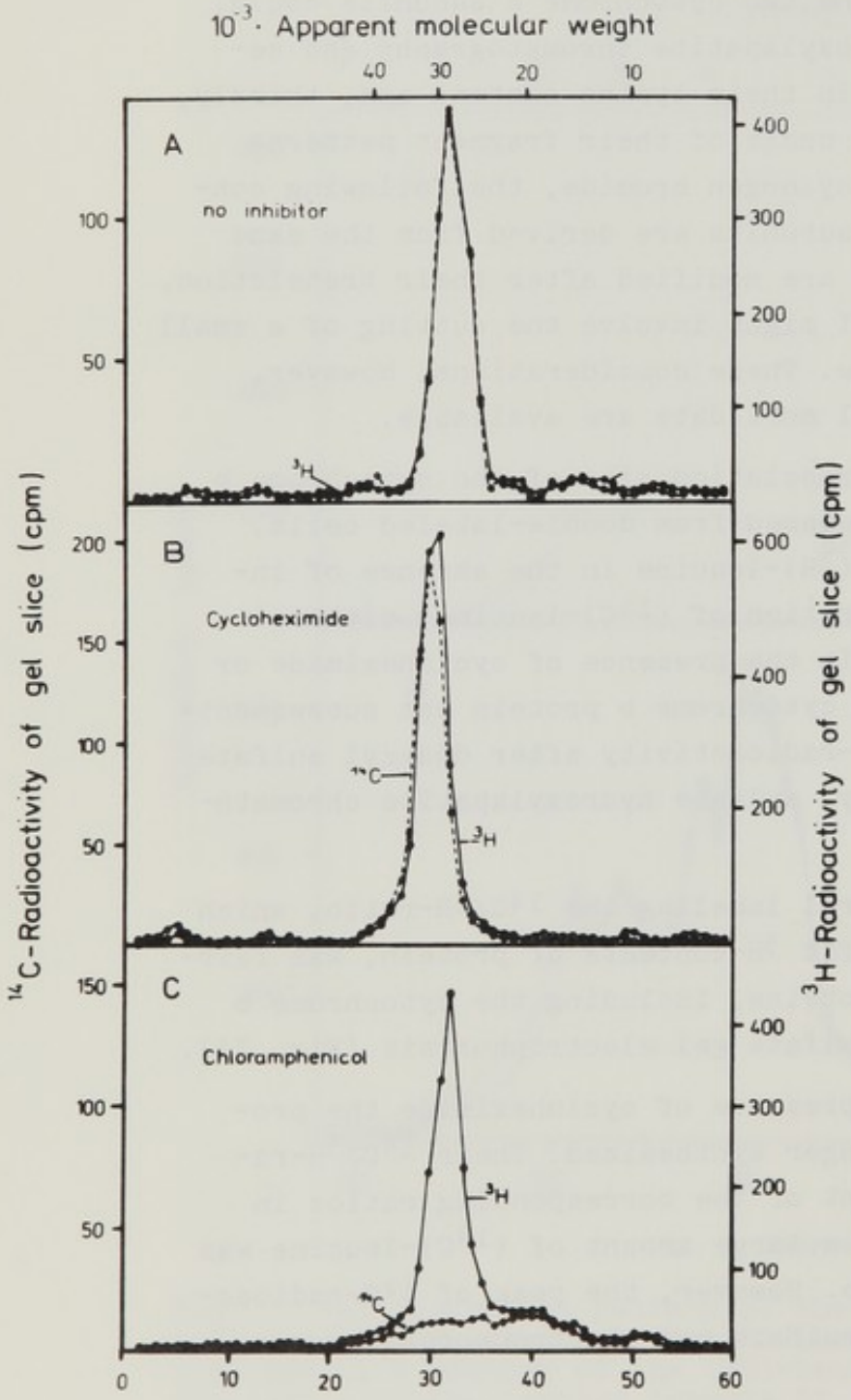
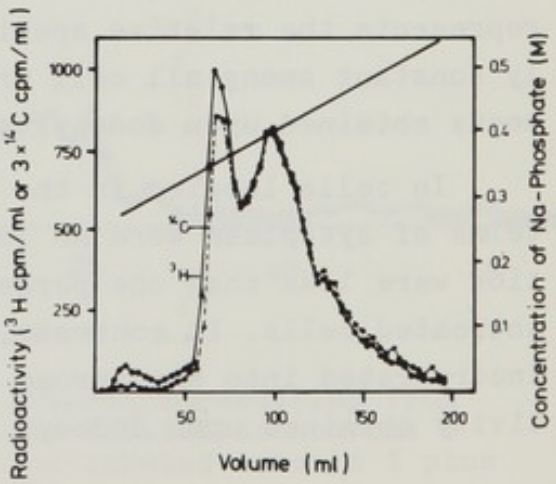


Fig. 8. Dodecyl sulfate gel electrophoresis of cytochrome b preparations from cells having incorporated (^{3}H)-leucine in the absence of inhibitors and (^{14}C)-leucine (A) either in the absence of inhibitors or (B) in the presence of cycloheximide, or (C) in the presence of chloramphenicol.

Fig. 9. Dodecyl sulfate hydroxylapatite chromatography of the cytochrome b preparation from cells having incorporated (^{3}H)-leucine in the absence of inhibitors and (^{14}C) leucine in the presence of cycloheximide (see Fig. 8B).



In the cells treated with chloramphenicol the synthesis of the cytoplasmic proteins remained unaffected but no distinct (^{14}C)-leucine incorporation into cytochrome b took place (Fig.7C).

This effect of cycloheximide and chloramphenicol, respectively, on the incorporation of (^{14}C)-leucine into the two cytochrome b subunits, infers that these two subunits are mitochondrial translation products.

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We wish to thank Brigitte Juchs and Annette Krebs for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft.

Compared Properties of the Oligomycin-Sensitive ATPase in Fungi with Short and Long Mitochondrial DNA

A. Goffeau, Y. Landry

Schizosaccharomyces pombe differs from *Saccharomyces cerevisiae* in several aspects. 1. It divides by fission instead of budding. 2. It is a "petite-negative" yeast. Thus no viable mitochondrial mutant rho$^-$ can be obtained (1, 2). 3. Its growth under anaerobic conditions is limited (3).
In these respects *S. pombe* is a much better model than *S. cerevisiae* for higher mammalian or plant cells. The similarity between *S. pombe* and mammalian cells and the differences between *S. pombe* and *S. cerevisiae* also extend to the length of mitochondrial DNA. O'Connor et al (4) have recently reported that *S. pombe* mitochondrial DNA is only 6 µm long compared to 25 µm for *S. cerevisiae* (5) and 20 µm for *Neurospora crassa* (6). It seems therefore of interest to compare the properties of the oligomycin-sensitive ATPase of *S. pombe* to those reported in the literature for the *S. cerevisiae* and *Neurospora* enzyme.

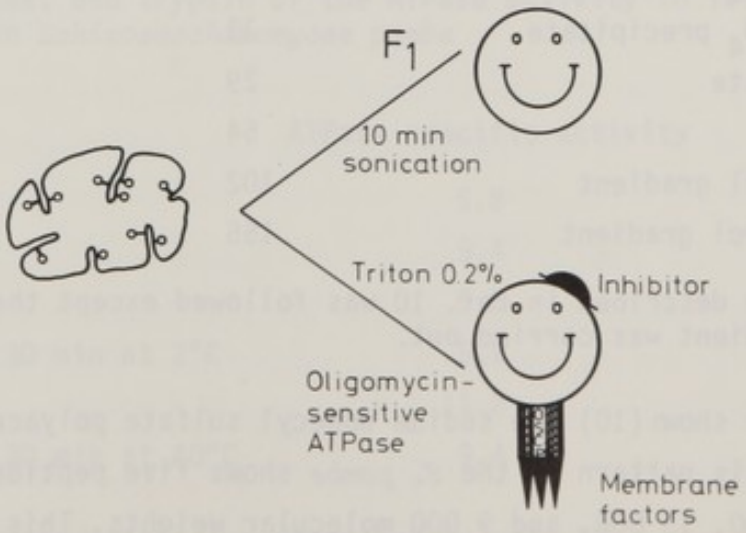


Fig. 1. Two forms of the mitochondrial ATPase.

As illustrated in Fig. 1, two forms of the mitochondrial ATPase can be obtained. Extensive sonication extracts the so-called F_1 not sensitive to oligomycin. Triton-X 100 extracts a more complex form of the enzyme which is oligomycin-sensitive and contains membrane factors and the oligomycin-sensitivity confering protein. Whether the yeast oligomycin-sensitive ATPase contains or not the ATPase inhibitor isolated in beef-heart mitochondria by Pullman and Monroy (7) was still uncertain until very recently.

None of these two forms of ATPase are extracted in *S. pombe* by using the procedures originally developed by Tzagoloff for *S. cerevisiae* (8, 9). However minor modifications such as increasing the pH to 9.0 during the sonication permits the extraction and purification of *S. pombe* F_1 of high specific activity : 150 μmoles Pi x min^{-1} x mg $protein^{-1}$ at 30°C (Table I)

Table I

Purification of the F_1 from glycerol-grown *Schizosaccharomyces pombe*

Purification steps	Specific activities (μmoles Pi x min^{-1} x mg^{-1})
Homogenate	0.8
SMP	5.8
Supernatant pH 5.6	14
40% $(NH_4)_2$ SO_4 supernatant	17
80% $(NH_4)_2$ SO_4 precipitate	33
Sephadex eluate	29
DEAE eluate	54
First glycerol gradient	102
Second glycerol gradient	155

The procedure described in ref. 10 was followed except that an additional glycerol gradient was carried out.

As previously shown (10) the sodium dodecyl sulfate polyacrylamide-gel electrophoresis pattern of the *S. pombe* shows five peptides of 58 000, 52 000, 32 000, 13 000, and 9 000 molecular weights. This pattern is slightly different from that reported for *S. cerevisiae* (11).The peptide

of 36 000 molecular weight is not observed in *S. pombe* but an additional peptide of 9 000 molecular weight is seen in the latter species. In these respects the *S. pombe* ATPase is similar to the beef heart, rat liver and *Neurospora* enzymes (see ref. 12 for review).

The Triton-extracted ATPase of *S. pombe* is much more unstable than that of *S. cerevisiae*. Oligomycin-sensitivity decreases during purification. After centrifugation through a glycerol gradient which yields an ATPase of specific activity of about 30, not more than 60% of the oligomycin-sensitivity is retained.

As described elsewhere (13) the sodium dodecyl sulfate polyacrylamide gel electrophoresis pattern of the *S. pombe* Triton-extracted ATPase purified on a glycerol-gradient contains at least 14 peptides. By superposing purified F_1 on these gels we were able to detect the five F_1 subunits and nine additional peptides of 68 000, 47 000, 37 000, 29 000, 23 000, 21 000, 16 500, 15 000 and 12 000 molecular weights. Using radioactive *S. pombe* membranes extracted by Triton-X 100 and an antibody against purified *S. pombe* F_1, we were unable to detect appreciable amounts of the so-called "subunit 9" which is a prominent peptide of low molecular weight in the *S. cerevisiae* ATPase (14). Whether the non-detection of subunit 9 is related to the low oligomycin-sensitivity of the purified *S. pombe* enzyme is not clear for the moment.

Table II

Stimulation by heat and trypsin of the ATPase activity in Triton-extracts of glycerol-grown *Schizosaccharomyces pombe*

Treatments	ATPase specific activity
None	5.8
60 µg trypsin	9.4
30 min at 2°C	5.5
60 µg trypsin + 30 min at 2°C	5.2
30 min at 40°C	11.2
60 µg trypsin + 30 min at 40°C	9.4

The experimental conditions are described in ref. 15.

On the other hand the following evidences indicate that the ATPase inhibitor is present in *S. pombe* mitochondria (15). Table II shows that the Triton-extracted ATPase of *S. pombe* is stimulated by trypsin digestion and by moderate heat treatment. The activated enzyme becomes cold-sensitive. These properties suggest the presence in *S. pombe* Triton-extract of an inhibitor similar to that associated to the beef heart ATPase. By alcaline extraction followed by acid and ammonium sulfate precipitations (15), a protein fraction was obtained which strongly inhibits purified *S. pombe* F_1 activity (Table III).

Table III

Effects of the ATPase inhibitor of *Schizosaccharomyces pombe* on the purified F_1 of *Schizosaccharomyces pombe*

Preincubation	ATPase specific activities (μmol Pi x min^{-1} x mg^{-1})
1.5 μg F_1	71
+ 3.2 μg inhibitor	12
+ 3.2 μg trypsinized inhibitor	75
+ 3.2 μg inhibitor + trypsin + trypsin inhibitor	9

F_1 purified from glycerol grown *S. pombe* as described in ref. 10 was preincubated for 10 min at pH 6.0 with an inhibitor purified from glycerol-grown *S. pombe* as described in ref. 15. The ATPase activity of preincubated F_1 was subsequently measured at pH 8.5.

The inhibitory factor is inactivated by trypsin. This fraction is enriched in a peptide of low molecular weight (Fig. 2) similar to the ATPase inhibitor recently purified from *S. cerevisiae* and *Candida utilis* (16, 17).

In conclusion, our data obtained so far suggest that the *S. cerevisiae* and *S. pombe* ATPases are similar with two possible exceptions. 1. The F_1 36 000 molecular weight peptide of *S. cerevisiae* is not seen in *S. pombe*. 2. The *S. pombe* ATPase exhibits a lower amount of the low molecular weight hydrophobic, mitochondrial product called "subunit 9" in *S. cerevisiae*.

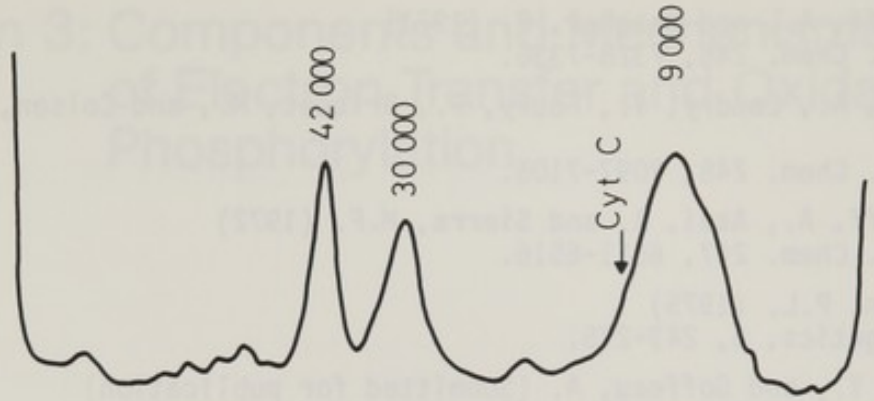


Fig. 2. Electrophoresis of a partially purified ATPase inhibitor from *Schizosaccharomyces pombe*

The ATPase inhibitor was purified from glycerol-grown *S. pombe* as described in ref. 3 and submitted to 10% acrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate (as described in ref. 10). The peaks of high molecular weight observed by staining with Coomassie-blue are probably contaminants. The estimated molecular weights are indicated on top of each peak. The arrow indicates the migration of cytochrome c.

Since a similar "subunit 9" has not been identified in the ATPase of *Neurospora crassa* (18) it seems unlikely that the presence of "peptide 9" is specifically related to the lenght of mitochondrial DNA which is three time longer in *Neurospora* (6) than in *S. pombe* (4).

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Session 3: Components and Mechanisms of Electron Transfer and Oxidative Phosphorylation

Composition and Function of Complex III from Beef Heart Mitochondria

P. Gellerfors, M. Lundén, B.D. Nelson

SUMMARY. Complex III from beef heart mitochondria binds 5-6 nmoles of C^{14}-iodoacetamide per mg of protein after 6 hours incubation at 0-4°C. The bound C^{14}-iodoacetamide is associated with a single peptide of Complex III, core protein-1. Binding is accompanied by a parallel inhibition of the enzymatic activity of the complex. These results indicate a function for core protein-1 in the cytochrome b-c_1 region of the respiratory chain.

We have recently undertaken a study on the structure and function of isolated Complex III (1-3) in order to understand the molecular mechanism involved in energy conservation in the cytochrome b-c_1 region of the respiratory chain. Isolated Complex III contains very few peptides (3-6), most of which can be identified on the basis of associated prosthetic groups (3,7). The major peptides of Complex III, the core proteins, contain, however, no prosthetic group(s) (3,7). A structural role for these peptides has been postulated by Silman et al. (7), based upon indirect evidence. In the present paper we show a direct involvement of one of the two core proteins in the structural organization of isolated Complex III.

Methods

Complex III was prepared from beef heart mitochondria by the method of Rieske et al. (8). Radioactive iodo-|1-^{14}C|acetamide was purchased from Radiochemical Centre Ltd. Binding of ^{14}C-iodoacetamide to Complex III was carried out in a medium containing 50 mM Tris-HCl pH 8.0, 0.66 M sucrose, 1 mM histidine, 1.8 mM ^{14}C-iodoacetamide (specific radioactivity 7.1 mCi/mmole), and Complex III (10-15 mg of protein/ml final concentration). Incubation was carried out at 0-4°C in the dark. The reaction was stopped by adding 20 µl of the reaction mixture to 1 ml of 0.2 M β-mercaptoethanol in 10 mM sodium phosphate, pH 7.2. The reaction mixture was then dialyzed against 10 mM sodium phosphate, pH 7.2 at 0-4°C until all free ^{14}C-iodoacetamide was removed as judged from samples in which the incubation mixture contained no Complex III. Electrophoresis of the labeled Complex III was

Abbreviations: SDS, Sodium dodecylsulfate; Q_2H_2, reduced coenzyme Q (ubiquinol-2). DQH_2, duroquinol.

done essentially as reported by Weber and Osborne (9), using 10 % acrylamide gels. The labeled, dialyzed Complex III was precipitated by 50 % ammonium sulfate (0°C) and centrifuged at 105,000 x g for 30 min. The pellet was suspended in 2 % SDS, 5 % β-mercaptoethanol in 10 mM sodium phosphate, pH 7.0, at a protein concentration of 1 mg/ml and incubated for 2 hours at 38°C. Approximately 20-50 µg of protein was applied to the gels. After 15 hours of electrophoresis gels were sliced in 2 mm slices and radioactivity measured as described by Clegg et al. (10). Enzymatic activity of Complex III was measured as Q_2H_2- or duroquinol DQH_2 cytochrome c reductase as described previously (1,2). Protein was determined by the method of Lowry et al. (11).

Results

Fig. 1 shows the inhibition of Q_2H_2-cytochrome c reductase and the parallel incorporation of ^{14}C-labeled iodoacetamide into Complex III during the first 6 hours of incubation under conditions described in Methods. Enzyme activity of the complex becomes rapidly inhibited when measured with either DQH_2 or Q_2H_2 as substrates. Inhibition is not complete and varies between 50-70 percent of the total activity depending upon the experiment. An additional 25-30 percent of the activity is lost with continued incubation for 18-20 hours. Our experiments have been concerned primarily with the first rapid phase in 5-6 hours.

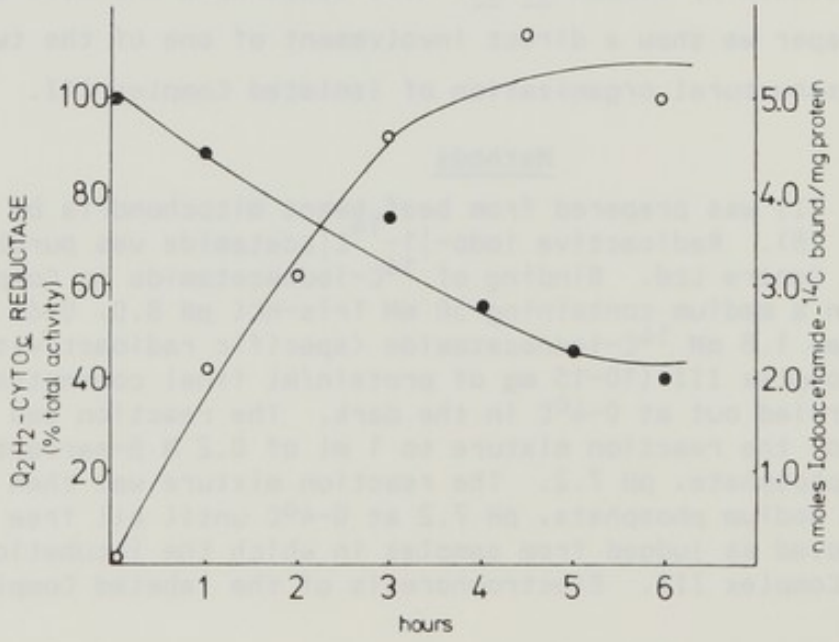


Fig. 1. The binding of ^{14}C-iodoacetamide (o-o-o) to Complex III and the parallel inhibition of the Q_2H_2-cytochrome c reductase activity (●-●-●) with time. The conditions were as described in Methods.

As seen in Fig. 1, binding of ^{14}C-iodoacetamide reaches a plateau after 5-6 hours, amounting to approximately 5 nmoles iodoacetamide bound per mg of Complex III. The average incorporation for iodoacetamide for 7 experiments carried out under identical conditions is 5.1 $\pm$ 1.1 S.E.

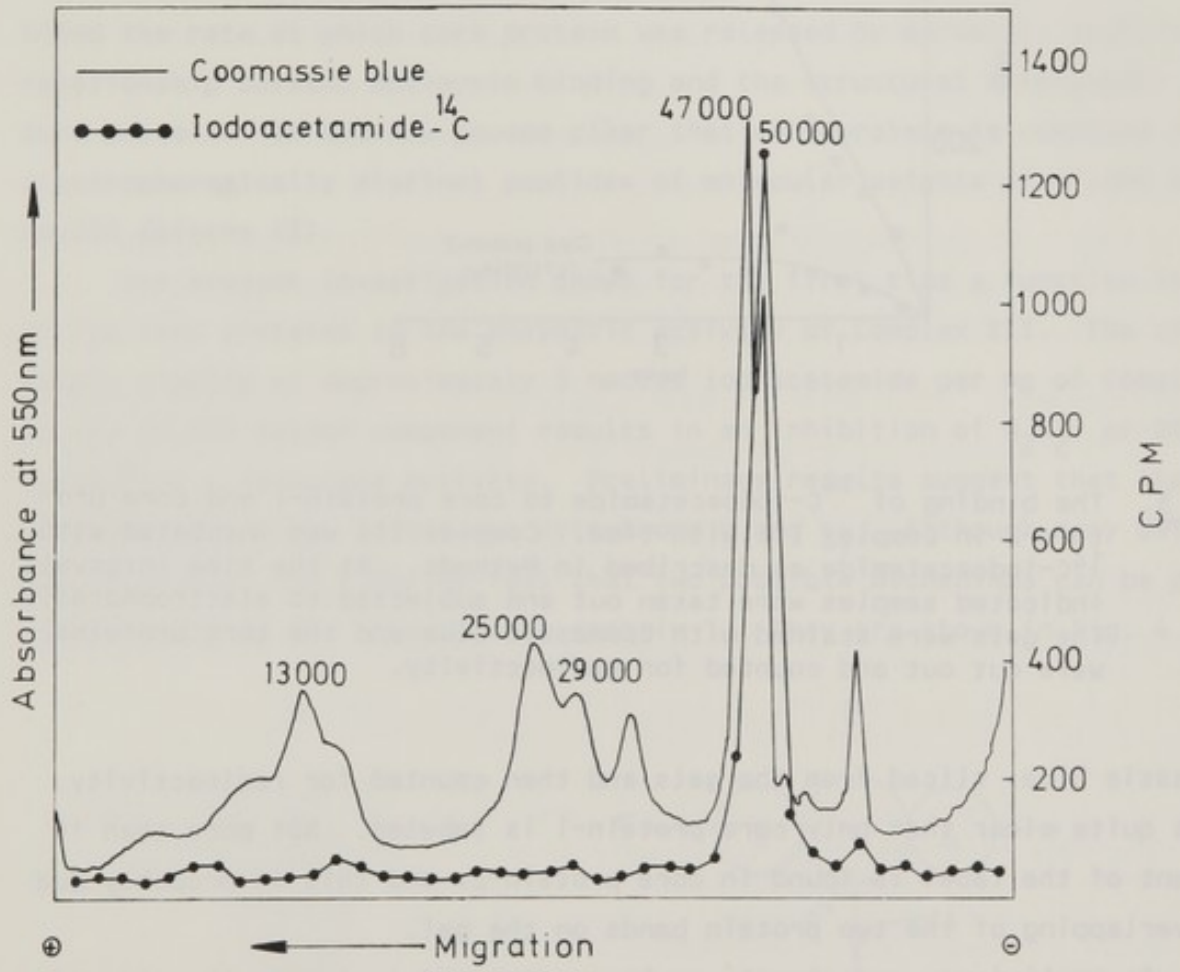


Fig. 2. SDS-gel electrophoresis of ^{14}C-iodoacetamide labeled Complex III.

Fig. 2 shows the distribution of ^{14}C-iodoacetamide among the peptides of Complex III separated on SDS-gel electrophoresis. In this experiment the incubation was for 6 hours using conditions described in Methods. It is clearly seen that all of the label is located in a single peptide of 50,000 daltons which we have previously designated as core protein-1 (3). To show that the binding of ^{14}C-iodoacetamide is only associated with core protein-1 (50,000 Mw) and not core protein-2 (47,000 Mw)(Fig. 3), the two proteins were separated by SDS-gel electrophoresis, visualized with

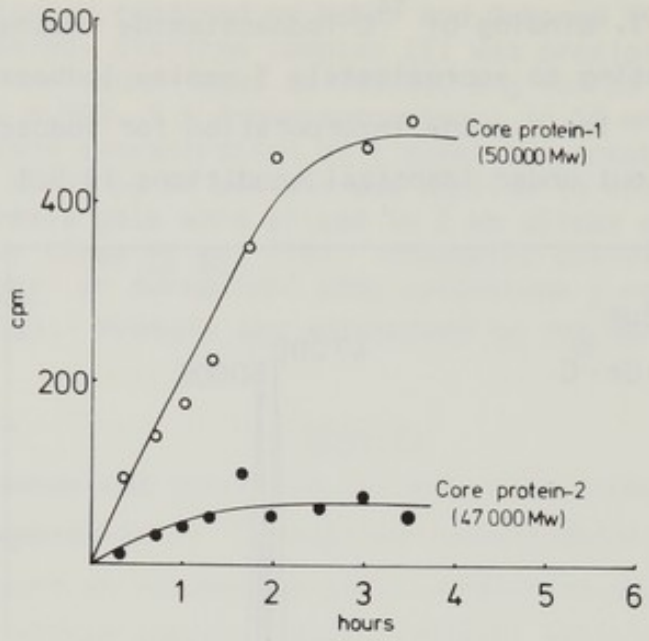


Fig. 3. The binding of ^{14}C-iodoacetamide to core protein-1 and core protein-2 in Complex III with time. Complex III was incubated with ^{14}C-iodoacetamide as described in Methods. At the time intervals indicated samples were taken out and subjected to electrophoresis. The gels were stained with Coomassie blue and the core proteins were cut out and counted for radioactivity.

Coomassie blue, sliced from the gels and then counted for radioactivity. It is quite clear that only core protein-1 is labeled. Not more than 10 percent of the label is found in core protein-2, and this is probably due to overlapping of the two protein bands on the gel.

In preliminary experiments we have attempted to locate the site of iodoacetamide inhibition in Complex III. After 6 hours of incubation with iodoacetamide both cytochrome $\underline{c}_1$ and cytochrome $\underline{b}$ show a distinct biphasic reduction by DQH_2 which is absent in non-treated controls. Binding of iodoacetamide appears to affect cytochrome $\underline{c}_1$ to a greater extent than cytochrome $\underline{b}$, since more of the former is converted to a slow reacting form. Furthermore, the rate of reduction of the slow reacting form of cytochrome $\underline{c}_1$ is slower than the rate of reduction of the slow reacting form of cytochrome $\underline{b}$. These findings suggest a possible site of action of iodoacetamide between the two cytochromes, however, further experiments are needed in order to obtain more quantitative results.

Discussion

Core protein was first isolated from Complex III by Silman et al. (7). In spite of the fact that it lacked a prosthetic group, these authors presented evidence that it was an integral part of Complex III. Their arguments were based primarily on the observation (7) that antimycin A inhibited the rate at which core protein was released by mersalyl, implying a relationship between antimycin binding and the structural arrangement of core protein. It has now become clear that core protein is composed of two electrophoretically distinct peptides of molecular weights of 47,000 and 50,000 daltons (3).

The present investigation shows for the first time a function for one of the core proteins in the enzymatic activity of Complex III. The covalent binding of approximately 5 nmoles iodoacetamide per mg of Complex III to the 50,000 dalton component results in an inhibition of Q_2H_2- or DQH_2 cytochrome c reductase activity. Preliminary results suggest that the site of inhibition might be between cytochrome b and c_1. Although many details are lacking at the time, we feel that two possible mechanisms can be proposed regarding the role of core protein-1. They are shown in Fig. 4.

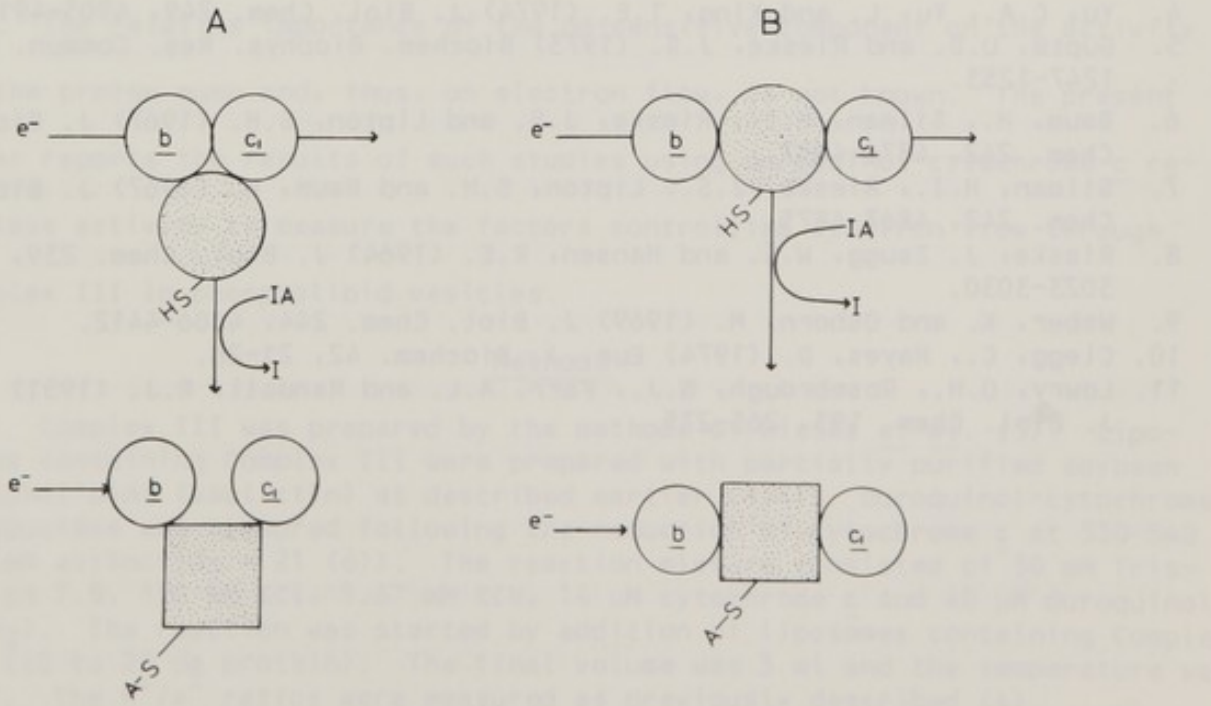


Fig. 4. Two possible roles for core protein-1 in Complex III.

In mechanism (A) the binding of iodoacetamide is depicted as inducing a conformational change in core protein-1, which in turn prevents the electron transferring proteins from interacting. It is not certain if this results in a specific separation of cytochromes $\underline{b}$ and $\underline{c}_1$ as shown in the figure, or if other electron transfer steps are also involved. In any event, in this model core protein-1 is visualized as playing a structural role in organizing the electron carriers. In the second mechanism (B) core protein-1 is shown as a participant in the electron transfer reactions, which is blocked by the binding of iodoacetamide. This possibility seems the least likely, since no redox centers are known to be associated with core protein-1 (3,7). Experiments to distinguish between these two mechanisms and the nature of the binding are in progress.

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Factors Controlling Electron Flow in Liposomes Containing Complex III from Beef Heart Mitochondria

B.D. Nelson, I. Mendel-Hartvig, F. Guerrieri, P. Gellerfors

Recent evidence from studies on submitochondrial particles (1,2) and Complex III isolated from beef heart mitochondria (3,4) demonstrate a redox-driven extrusion of protons in the cytochrome $\underline{b}$-$\underline{c}_1$ region of the respiratory chain. This redox proton pump appears to be electrogenic (1-4), creating a membrane potential which, in turn, influences the rate of electron transfer from reduced quinones to cytochrome $\underline{c}$. Recently we reported that the proton pump in Complex III-phospholipid vesicles is pH-dependent (4). The relative importance of the pH-sensitive component on the activity of the proton pump and, thus, on electron flow, is not known. The present paper reports the results of such studies using duroquinol-cytochrome $\underline{c}$ reductase activity to measure the factors controlling electron flow through Complex III in phospholipid vesicles.

Methods

Complex III was prepared by the methods of Rieske et al. (5). Liposomes containing Complex III were prepared with partially purified soybean phospholipids (asolictin) as described earlier (3,4). Duroquinol-cytochrome $\underline{c}$ reductase was measured following the reduction of cytochrome $\underline{c}$ at 550-540 nm (mM extinction = 21 (6)). The reaction mixture consisted of 50 mM Tris-Cl, pH 7.5, 150 mM KCl, 1.67 mM KCN, 14 µM cytochrome $\underline{c}$ and 40 µM duroquinol (DQH_2). The reaction was started by addition of liposomes containing Complex III (10 to 20 µg protein). The final volume was 3 ml and the temperature was 25°C. The H^+/e^- ratios were measured as previously described (4).

Results

Fig. 1 shows the release of protons which accompanies electron flow

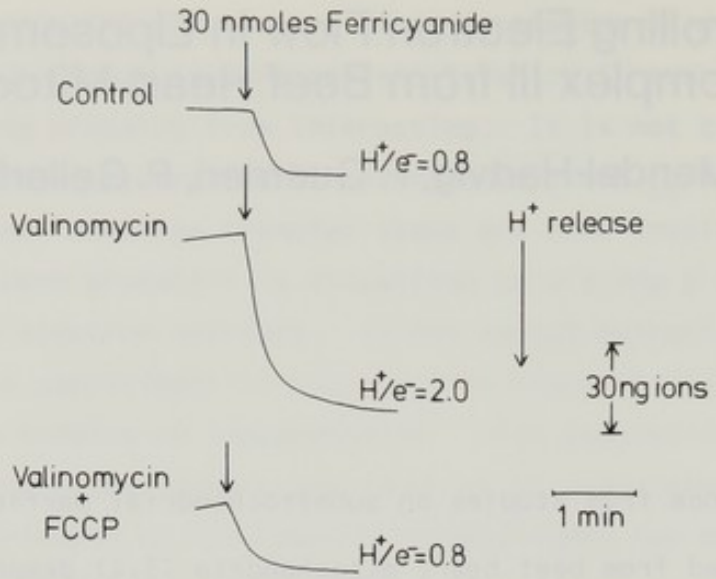


Fig. 1: Measurement of proton pump activity Complex III-phospholipid vesicles. Conditions are described in Methods.

initiated by addition of ferricyanide to Complex III-vesicles equilibrated with duroquinol (DQH_2) and cytochrome c. In the presence of valinomycin the ratio of protons released to electrons transferred (H^+/e^-) is greater than 1.0, indicating the presence of a redox-driven release of protons from the vesicles. Oxidation of DQH_2 should give a H^+/e^- ratio of l.0. As expected, H^+/e^- ratios near l.0 are observed upon oxidation of DQH_2 by Complex III which is not inlayed in a phospholipid vesicle, regardless of the incubation conditions (4). The H^+/e^- ratio of l.0 obtained in the presence of valinomycin + FCCP suggests that the pH gradient formed in the presence of valinomycin is collapsed by FCCP.

Electron flow through Complex III-vesicles is activated 3-8 fold by either valinomycin + K^+ or by FCCP (Fig. 2). Differences in the extent of activation is probably due to the efficiency of incorporation of Complex III into vesicles. This is suggested by the fact that activated rates of cytochrome c reduction obtained with FCCP or valinomycin are maximal, i.e.,

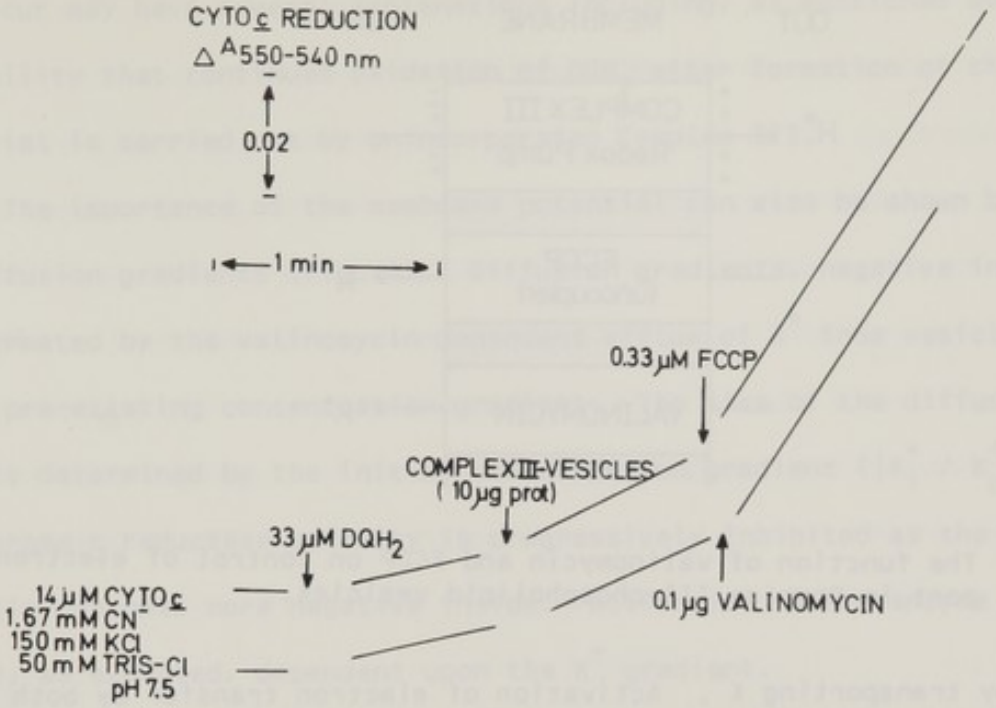


Fig. 2: Duroquinol-cytochrome c reductase activity in Complex III-phospholipid vesicles.

they are identical to those measured in un-incorporated Complex III. In contrast, the controlled, or inhibited, rates vary considerably between the different vesicle preparations, and probably represent activity of the un-incorporated complex.

The results given in Figs. 1 and 2 can be explained with the aid of Fig. 3. Complex III-vesicles contain a redox-driven proton pump which generates a pH gradient, basic inside, and a membrane potential which is negative inside. Electron flow is inhibited in the presence of the membrane potential. FCCP and valinomycin + K^+ activate electron flow by collapsing the membrane potential (Fig. 2), the former by transporting protons into the vesicles and the

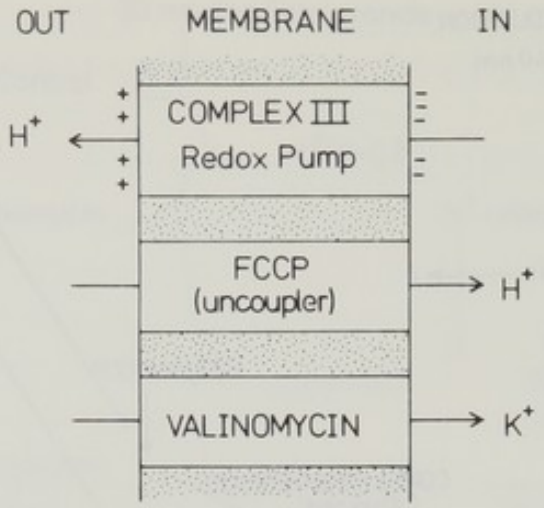


Fig. 3: The function of valinomycin and FCCP on control of electron transport in Complex III-phospholipid vesicles.

latter by transporting K^+. Activation of electron transfer by both FCCP and valinomycin indicates that inhibition is due to the electrical potential and not to the increase in internal pH. Inhibition of electron transfer in freshly prepared control vesicles (Fig. 2) is due to the rapid generation of a membrane potential (1,2) which is undoubtedly completed before initial rates can be assayed using the methods described here.

Demonstration of a H^+/e^- ratio greater than 1.0 is accomplished in the presence of valinomycin (Fig. 1) since the redox-driven proton pump functions maximally under these conditions and the pH gradient formed is stable unless a protonophore is present. FCCP, on the other hand, decreases the H^+/e^- ratio to near 1.0 since it collapses the pH gradient formed from the redox pump. A H^+/e^- ratio near 1.0 which is observed in control vesicles (Fig. 1, ref. 4) is partially explained by our inability to measure the small pH changes occurring during formation of the electrical gradient (2). However, one might expect that the continued oxidation of DQH_2 by cytochrome c should also be associated with proton pump activity and the generation of H^+/e^- ratios greater than 1.0, but at a slower rate. The reason this does

not occur may have several explanations including, as mentioned above, the possibility that continued oxidation of DQH_2 after formation of the membrane potential is carried out by unincorporated Complex III.

The importance of the membrane potential can also be shown by use of K^+ diffusion gradients (Fig. 4). Diffusion gradients, negative inside, were created by the valinomycin-dependent efflux of K^+ from vesicles containing a pre-existing concentration gradient. The size of the diffusion potential is determined by the initial concentration gradient ($|K_i^+ / K_o^+|$). DQH_2-cytochrome c reductase activity is progressively inhibited as the diffusion potential becomes more negative inside. Activation of the enzyme by FCCP is not, as expected, dependent upon the K^+ gradient.

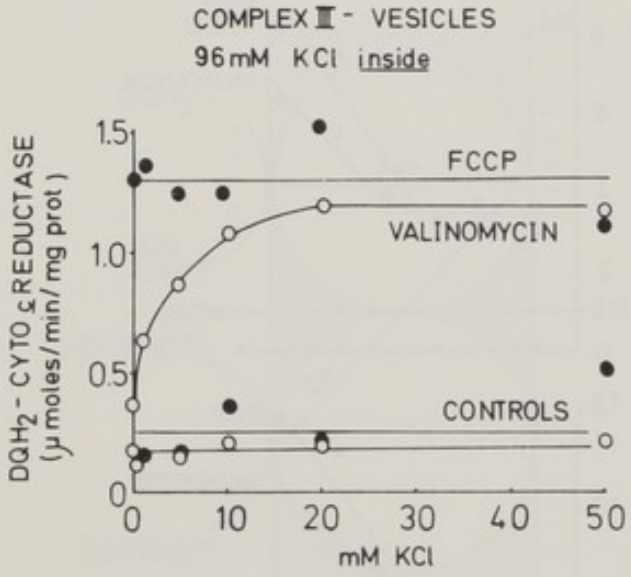


Fig. 4: Inhibition of duroquinol-cytochrome c reductase activity by a K^+ diffusion gradient. Vesicles were prepared with 96 mM KCl inside and the external K^+ altered by dialyzing against the appropriate salt buffered with 10 mM Tris-Cl, pH 7.5. Diffusion gradients were initiated by addition of 0.5 μg valinomycin.

It seems likely from the above discussion that the electrogenic proton pump controls electron transfer by generating a membrane potential. Earlier, however, we reported that the proton pump is pH-dependent (4) and is activat-

ed above pH 7.0. This raised the possibility that electron flow is ultimately controlled by the pH-dependency of the electrogenic pump. To investigate this we measured the pH-dependency of DQH_2-cytochrome c reductase in Complex III-vesicles under different conditions (Fig. 5). A pH-dependency is observed, but only in the absence of a membrane potential when the enzyme is maximally activated. These findings suggest that the pH profile shown in Fig. 5 is due to a pH-sensitive, rate limiting step in the enzymatic apparatus of Complex III. This has been confirmed by showing a similar pH dependency of DQH_2-cytochrome c reductase in unincorporated Complex III.

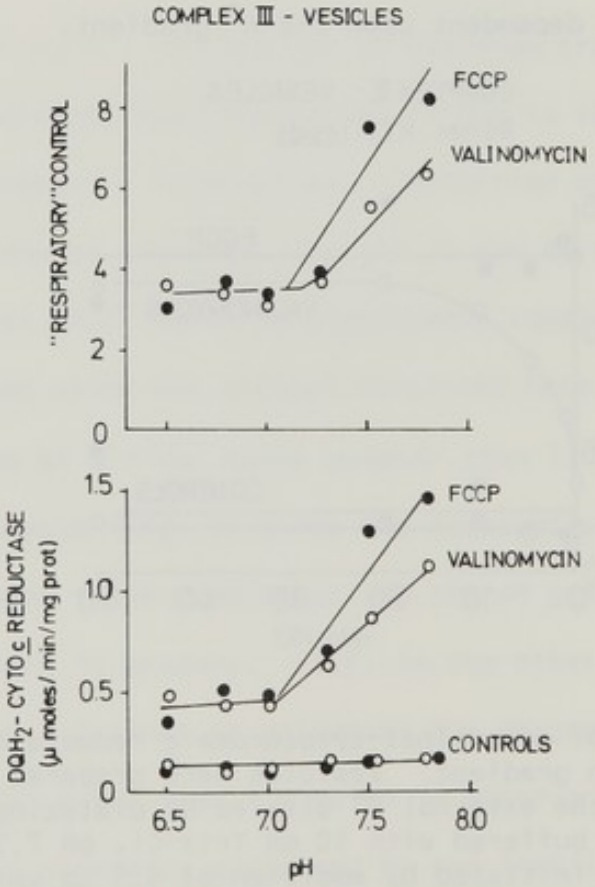


Fig. 5: Effect of pH on duroquinol-cytochrome c reductase activity in Complex III-phospholipid vesicles. Conditions are given in the Methods section.

Discussion

The present results show that two factors influence DQH_2-cytochrome c reductase activity in Complex III-phospholipid vesicles: 1) a membrane potential which can be generated either by a redox-proton pump or a K^+ diffusion gradient, and 2) a pH sensitive component located on the cytochrome c side of the membrane. The membrane potential, when negative inside, inhibits electron flow, whereas increasing alkalinity on the outside of the vesicles favors acceleration of electron transfer and proton pump activity (4). These data are in agreement with the electrogenic nature of the redox proton pump in the cytochrome b-c_1 region of the chain (1-4).

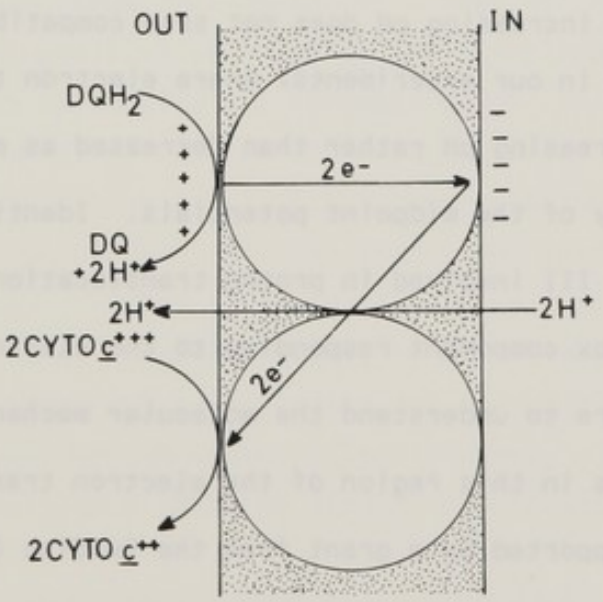


Fig. 6: The redox-proton pump in Complex III-phospholipid vesicles.

Fig. 6 shows schematically the function of the redox pump. DQH_2 reacts on the outer surface of the vesicles as indicated by the release of

protons into the external media. Similarly, cytochrome c, which is a non-penetrant molecule, reacts at the outer surface. The redox component which senses the negative internal membrane potential must, however, be located at or near the inner surface of the vesicles. Electrons thus appear to move in a loop, and in doing so are accompanied by translocation of protons.

It is not clear if protons are carried across the membrane on a proton or a hydrogen carrier (2). The latter possibility seems the least likely since Complex III contains no classical hydrogen carriers (7). Furthermore, the only electron carrier exhibiting a distinct pH-dependency in detergent-treated particles is cytochrome b-565, the midpoint potential of which drops by -60 mV/pH unit above pH 7.0 (8). A decrease in the midpoint potential of cytochrome b-565 with increasing pH does not seem compatible with its role as a hydrogen carrier in our experiments, where electron transfer activity is increased with increasing pH rather than decreased as might be expected from the pH dependency of the midpoint potentials. Identification of the components of Complex III involved in proton translocation, as well as identification of the redox component responding to the electrical potential are now necessary if we are to understand the molecular mechanism by which energy conservation occurs in this region of the electron transport chain.

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On the Role of Conformational Changes in Cytochrome c Oxidase in the Mechanism and Control of Oxidative Phosphorylation

M.K.F. Wikstrom*

1. INTRODUCTION

Cytochrome c oxidase is the only respiratory chain component that has been demonstrated to be affected structurally by the energy state of the mitochondrial membrane. Thus application of a high ATP/ADP·P_i ratio, a membrane potential with positive polarity in the cytoplasmic (C) phase, or a respiratory pulse in coupled mitochondria cause well-defined spectral shifts both in the fully reduced and fully oxidized enzyme (1-9). Moreover, as demonstrated by Wilson et al.(6,10), energization of the mitochondrial membrane alters the affinity of both ferric and ferrous cytochrome oxidase to cyanide. Finally, it has recently been shown that energization of isolated mitochondria at room temperature by ATP results in a drastic change in the rate of carbon monoxide binding and oxygen binding by the reduced enzyme measured at low temperatures, and further, a considerable change in the kinetics of electron transfer within the cytochrome aa_3 complex (11).

According to the chemiosmotic hypothesis of oxidative phosphorylation (12) the first "high energy" intermediate of the energy transduction process is the electrochemical proton gradient that develops across the mitochondrial membrane as a result of the transmembrane movement of hydrogen and electrons, resulting in a net translocation of hydrogen ions from the matrix (M) phase to the C phase. No "high energy" intermediates of respiratory carriers are involved in this process according to this proposal. Respira-

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tory control in the span between cytochrome c and oxygen is considered, according to the chemiosmotic hypothesis (13), to be simply due to a direct effect of the electric field across the membrane on the vectorial electron transfer processes perpendicular to the membrane (catalyzed by cytochrome c oxidase).

For these reasons it is of considerable interest that results indicative of "high energy" forms of cytochrome c oxidase have been obtained experimentally. However, as discussed below, this may not retract anything from the overall principle of chemiosmotic coupling although some of the detailed postulates, such as the mechanism of respiratory control, may require adjustment. It is clear, at least in my opinion, that although primary "high energy" intermediates of oxidative phosphorylation were apparently absent at the time the chemiosmotic mechanism was proposed (14), a very suggestive argument at the time in its favour, the general chemiosmotic principle does not exclude the existence of such intermediate states of energy coupling. This, of course, is true only as long as the process of energy transfer between the respiratory chain and the ATP synthetase occurs obligatorily via an intermediate electrochemical proton gradient (protonmotive force) across the membrane.

2. THE DEPENDENCE OF THE SPECTRAL SHIFT IN FERRIC CYTOCHROME $\underline{aa}_3$ ON PHOSPHATE POTENTIAL

We have recently studied the energy-linked spectral shift in ferric cytochrome $\underline{aa}_3$ of rat-liver mitochondria (Fig. 1) as a function of phosphate potential (4,5), membrane potential (4,5) and electrochemical proton gradient (5,15). Fig. 1 shows the spectral shift as induced by ATP. The specific absorptivity is about 50 mM^{-1} at 430-412 nm under the assumption that all $\underline{aa}_3$ molecules do shift at a very high phosphate potential (3 mM ATP, no added ADP or P_i).

Titrations of this shift with varying ATP/ADP·P_i ratios have previously given conflicting results. The plot of the logarithm of $\underline{aa}_3^*/\underline{aa}_3$ (where $\underline{aa}_3^*$ represents the concentration of "shifted" and $\underline{aa}_3$ the concentration of "unshifted" cytochrome oxidase molecules) against log ATP/ADP·P_i has been

reported to give a straight line with half-maximal shift at an ATP/ADP·P_i ratio of approx. 10^3 at pH 7.2 (4,5,7-9). However, Wilson and co-workers (7-9) have reported that they consistently find a slope of unity in this plot, suggesting a 1:1 stoicheiometry between the number of spectrally shifted $\underline{aa}_3$ molecules and the number of hydrolyzed ATP molecules. We have been unable to reproduce such high slopes in titrations with rat-liver mitochondria (4,5), but have obtained slopes varying between extremes of 0.4 and 0.6 (usually close to 0.5). A slope of 0.5 would mean that hydrolysis of one ATP molecule is coupled to the shift in two $\underline{aa}_3$ molecules.
From a large number of phosphate potential titrations at slightly different conditions and wavelength pairs it has now become more and more clear to us that the reason for the discrepancy is probably due to the fact that ADP and ATP cause other spectral changes than that attributable to cytochrome $\underline{aa}_3$ in the Soret region, even under the very oxidized experimental

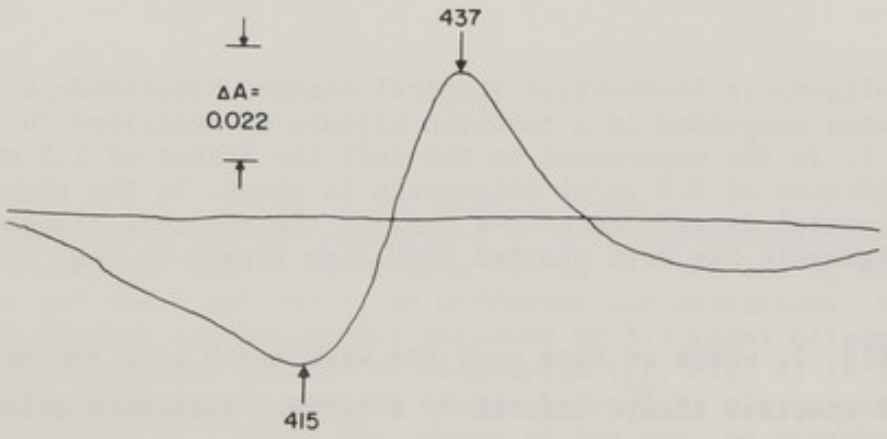


Fig. 1. ATP-linked spectral shift in ferric cytochrome $\underline{aa}_3$. Rat-liver mitochondria (3.6 mg protein/ml), suspended in 0.2 M sucrose-20 mM KCl-20 mM HEPES buffer, pH 7.2, were supplemented with 0.2 mM EDTA, 5 μM rotenone and 0.7 mM ferricyanide. A baseline was recorded after which 1.5 mM ATP was added to the sample cuvette. The spectrum represents the difference sample (increased absorption upwards) minus reference.

conditions employed. These other changes are to their extent much smaller than the spectral shift in $\underline{aa}_3$ and are therefore very difficult to identi-

fy from a simple difference spectrum such as that of Fig. 1. However, they do affect the slope of the phosphate potential titrations. The relative contribution of these unspecific changes is very much dependent on the exact choice of wavelength couple for the measurement.

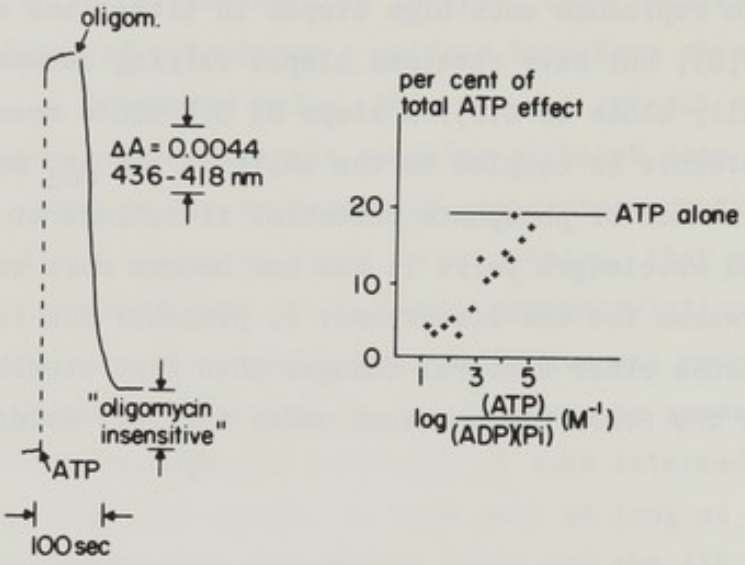


Fig. 2. The oligomycin-insensitive spectral change. Mitochondria (1.4 mg protein/ml) were suspended in a reaction mixture as described in the legend to Fig. 1. In the experiment to the left the effect of 1.5 mM ATP and subsequent addition of 3.3 μg/ml oligomycin is shown. To the right the phosphate potential dependence of the part of the spectral change not reversed by oligomycin has been plotted (see also legend to Fig. 3).

As shown in Fig. 2, where we have used the wavelength pair 436 minus 418 nm, the total spectral change induced by a certain phosphate potential is composed of two distinguishable effects: an oligomycin-sensitive and an oligomycin-insensitive increase in the absorption difference. It is clear that only the oligomycin-sensitive spectral change is relevant for the study of the energy-linked shift in ferric cytochrome aa_3. However, as also shown in Fig. 2, induction of the "oligomycin-insensitive" effect also appears to be dependent on phosphate potential. Note that oligomycin insensitivity is here defined as a lack of oligomycin-induced reversal of the spectral change. From this it is clear that it would be erroneous to correct only the maximal shift (ATP alone) for this artifact. Such a correction would give too high a slope in the plot, while no correction would result in too low a slope. This may be the reason for the earlier discre-

pancies. The portion of the spectral change that is not reversed by oligomycin is probably due to a phosphate potential-dependent reduction of ferricyanide, possibly the result of activation of the fatty acid oxidizing system. This, however, still remains to be investigated in detail. In any case, it is apparent that the extent of reversal of the total phosphate potential-dependent spectral change by oligomycin should be the best estimate for the extent of the true spectral shift in ferric cytochrome $\underline{aa}_3$.

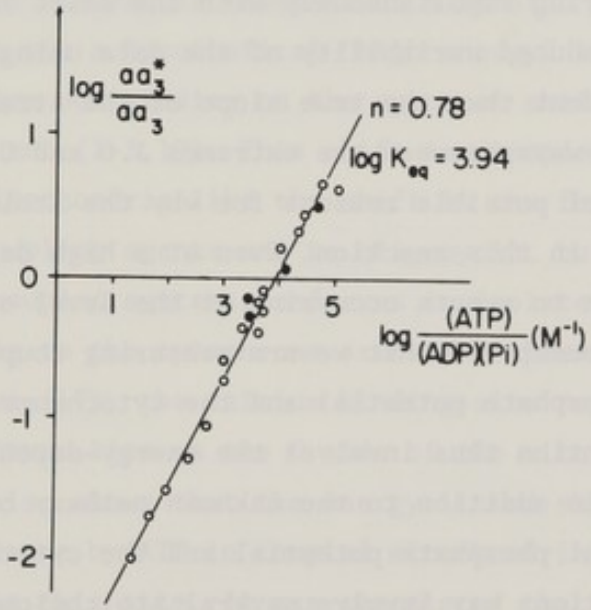


Fig. 3. Phosphate potential dependence of oligomycin-sensitive shift. Conditions as described in the legend to Fig. 1, but 1.5 mg mitochondrial protein/ml and added ADP and P_i at different concentrations. ATP was then added at different concentrations followed by 3.3 µg/ml oligomycin. The decrease in absorption induced by oligomycin (cf. Fig. 2) is termed $\underline{aa}_3^*$ while $\underline{aa}_3$ is equal to the maximum change induced by oligomycin (at 3 mM ATP without ADP or P_i) minus the change at the applied phosphate potential. The straight line is a best fit to the data points by least squares analysis. Filled points are from a separate experiment with the same mitochondria.

A plot of experimental results in this way is shown in Fig. 3. The result is a very good fit (coefficient of determination in least squares analysis was 0.98) to a straight line with a slope of 0.78 and half-maximal shift at an ATP/ADP·P_i ratio of $8.7 \times 10^3\ M^{-1}$.

Several experiments on different samples of rat-liver mitochondria have proved that the variability of both slope and equilibrium constant has been greatly reduced by this method of determining the extent of the spec-

tral shift. The results are: logarithm of ATP/ADP·P_i ratio at half-maximal shift = 3.86 ± 0.16 (S.D.),and slope = 0.76 ± 0.04 (S.D.) at pH 7.2. Least squares fits of straight lines to the data points gave coefficients of determination always higher than 0.95.

Thus it may be concluded that the earlier apparent discrepancies concerning the exact dependence of the spectral shift upon phosphate potential were most probably due to non-specific absorption changes of relatively small amplitude occurring simultaneously with the shift in ferric $\underline{aa}_3$. In view of the greatly reduced variability of the data using the oligomycin method, we feel confident that the true slope of the straight line dependence lies about half-ways between the extremes 1.0 and 0.5 reported earlier. There are several possible reasons for why the stoicheiometry may not be an even number in this reaction. Even at a high degree of coupling of the ATPase reaction to events occurring at the level of the respiratory chain, it has to be remembered that we are measuring coupling between the extramitochondrial phosphate potential and the cytochrome oxidase molecule. The pathway of interaction thus involves the energy-dependent adenylate translocating system in addition to the unknown pathway of interaction between intramitochondrial phosphate potential and the cytochrome oxidase molecule. These interactions may involve several stoicheiometric elementary steps which, when taken together, may well result in an overall stoicheiometry of, say, 0.75.

3. THE NATURE OF THE CONFORMATIONAL CHANGE UNDERLYING THE SPECTRAL SHIFTS

We have previously shown that the spectral shift in ferrous and ferric cytochrome $\underline{aa}_3$ may also be induced by an electrical diffusion potential across the mitochondrial membrane with positive polarity in the C phase (3-5). More recent data (5,15) have indicated that the spectral shift in ferric $\underline{aa}_3$ actually correlates best with the entire electrochemical proton gradient across the membrane, i.e. with the sum of the membrane potential and the hydrogen ion concentration gradient (in electrical units). We also showed that the conformational change in ferric $\underline{aa}_3$ appears to be associa-

ted with binding of a proton on the C side of the membrane (4,5), and since the shift parallels the protonmotive force rather than membrane potential (5,15), it seems reasonable to postulate that the structural change in ferric $\underline{aa}_3$ responsible for the spectral shift is also associated with release of another proton on the M side of the membrane (5). One might, of course, object to this interpretation by saying that the effect of the electrochemical proton gradient is not a direct one on the cytochrome oxidase molecule, but occurs via reversal of a "proton pump" coupled to the respiratory chain via a hypothetical "high energy" intermediate (see 16). In this respect are our studies on the spectral shift in ferrous cytochrome $\underline{aa}_3$ most relevant (1-3,17). This shift is dependent on phosphate potential in intact mitochondria like the shift in the ferric enzyme. It is sensitive to uncoupling agents and to oligomycin, but it may be mimicked in completely uncoupled systems, and even in isolated cytochrome $\underline{c}$ oxidase by calcium binding to the cytochrome oxidase complex (17). As shown by Wikstrom and Saari (17), the calcium binding-site responsible for the shift in the spectrum lies on the C side of the mitochondrial membrane. However, it was also shown that the energy-linked shift in ferrous $\underline{aa}_3$ (induced for instance by ATP in coupled mitochondria) is not the result of calcium binding to this site. It was speculated that the site may be occupied by protons in the energized state of the mitochondrial membrane. This speculation gains support from the findings described above with the ferric enzyme, but have recently been further corroborated by the observation (H.T. Saari and M.K.F. Wikstrom, unpublished) that the spectral shift in ferrous isolated cytochrome oxidase may also be induced by hydrogen ions at a relatively low pH.

It should be stressed that the spectral shift produced under uncoupled conditions by calcium or hydrogen ions obviously does not represent a complete simulation of the structural change in cytochrome oxidase upon energization of the mitochondrial membrane. Exactly how far the simulation goes remains to be investigated but calcium does not, for instance, induce the characteristic changes in half-reduction redox potential of the cytochrome oxidase hemes observed by ATP (18, see also 19). It is conceivable that the structural adjustment of the $\underline{aa}_3$ complex directly responsible for the shift in the spectrum of the ferrous enzyme may be the result of bin-

ding of a hydrogen (or calcium) ion to the site on the C side of the membrane, but that the process of "energization" induces changes in the structure of $\underline{aa_3}$ additional to those induced by the ion binding. It is obvious that the simple binding of a cation to one site of the complex would give a total effect greatly different from that induced by an electric field linked to binding and release of ions on the opposite sides of the macromolecule. Presumably, it is this anisotropic energy-linked effect that cannot be mimicked in the above simple fashion. We do, however, suggest that we may have reproduced part of the effect, namely some of the events during energization that occur on the C side of the cytochrome oxidase complex.

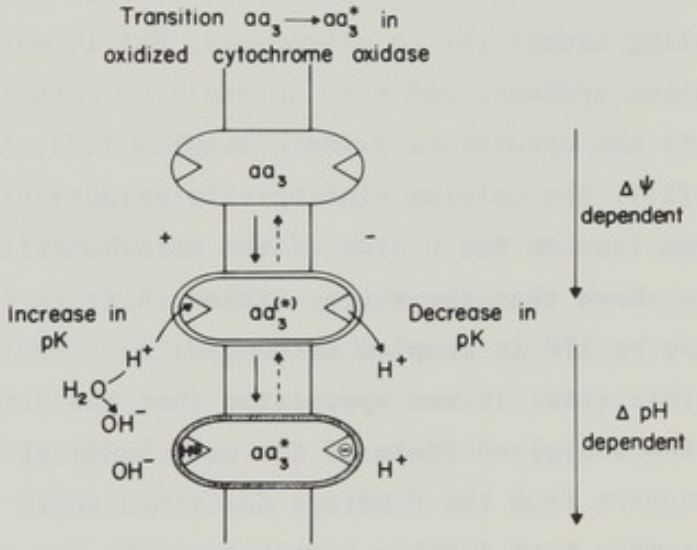


Fig. 4. Schematic picture of the energy-linked conformational change in ferric cytochrome oxidase. For details, see text.

On the basis of the above information we may now draw a tentative picture of some of the events accompanying the energy-linked conformational change in cytochrome c oxidase. It is suggested, as depicted in Fig. 4, that the primary conformational change may be brought about by the electric field accompanying the membrane potential across the membrane. The structural change is coupled to pK shifts in the opposite direction of acidic groups on the C and M sides of the membrane respectively (left and right respectively in Fig. 4), leading to binding of a proton on the C side and release

on the M side. This suggested behaviour may be compared to the recent predictions by Frohlich (20) that biological macromolecules should have metastable excited states with very high dipole moment and moreover, that these states may be further stabilized by rearrangement of counterions. Note also that our present picture (Fig. 4) differs from our original view, which was based on the belief that the conformational change in ferric $\underline{aa}_3$ was dependent on the membrane potential (electric field) alone (4). As mentioned above, we have since then shown conclusively that the spectral change, at least in ferric $\underline{aa}_3$, correlates with the total electrochemical proton gradient across the membrane (5,15).

4. THE DECAY OF THE SPECTRAL SHIFT AS INDUCED BY AN UNCOUPLING AGENT

Fig. 5 shows the effect of the uncoupling agent carbonylcyanide p-trifluomethoxy phenylhydrazone (FCCP) on the decay of the spectral shift induced by application of an electrical diffusion potential across the mitochondrial membrane by the valinomycin method (4). It may be seen that the rate of

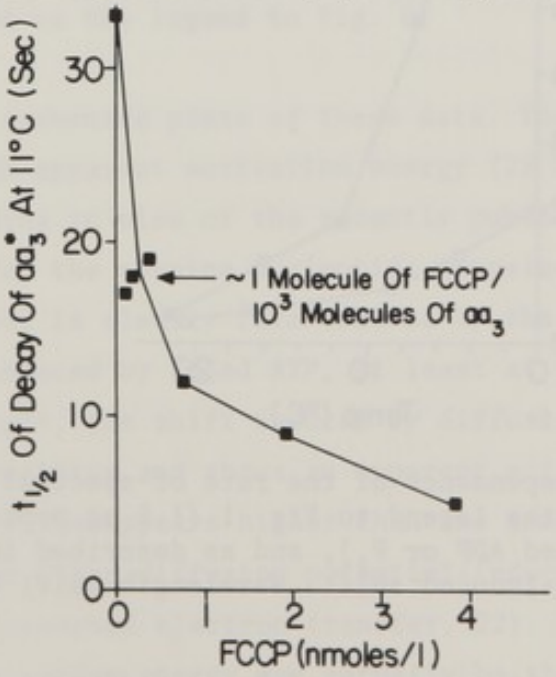


Fig. 5. Titration of the decay of spectral shift with uncoupler. Mitochondria were incubated as described in the legend to Fig. 1, but with oligomycin and without K^+. The shift was initiated by 50 ng/ml valinomycin.

decay is extremely sensitive to the uncoupler while the extent of the spectral change is much less sensitive. The experiment was performed at 11 °C as indicated in the figure. The rate of decay is doubled at a concentration of the uncoupler corresponding to about one uncoupler molecule per 10^3 cytochrome oxidase molecules, or approximately 15 uncoupler molecules for each mitochondrion. This illustrates clearly the non-stoicheiometric nature of uncoupling with respect to respiratory chains or ATPase molecules, and is consistent with the proposal that uncoupling is due to breakdown of the electrochemical proton gradient across the membrane.

5. KINETICS OF FORMATION OF THE ENERGIZED STATE OF FERRIC CYTOCHROME $\underline{aa_3}$

Fig. 6 shows the half-times of formation of the spectral shift in ferric

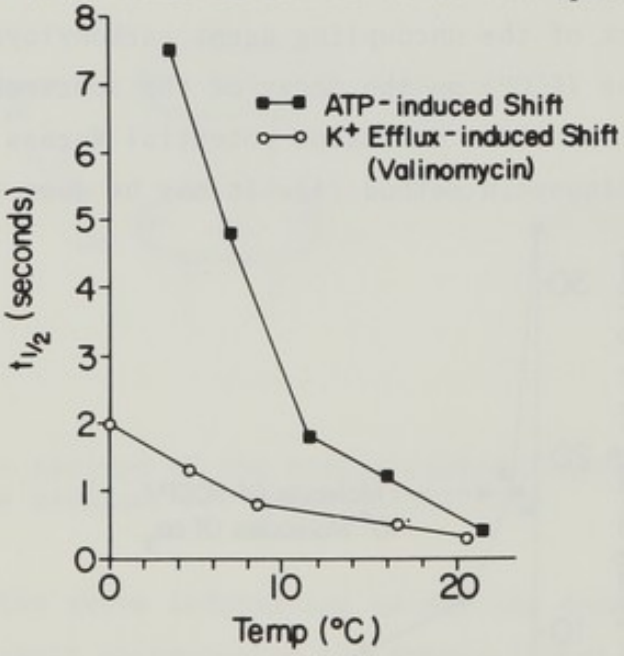


Fig. 6. Temperature dependence of the rate of spectral shift. Reaction mixture as described in the legend to Fig. 1 (1.5 mg protein/ml) for the ATP-induced shift (no added ADP or P_i), and as described in the legend to Fig. 5 for the valinomycin-induced shift. Wavelength pair: 430-412 nm.

cytochrome $\underline{aa_3}$ of rat-liver mitochondria at different temperatures. At 20°C the $t_{1/2}$ is about 0.4 seconds whether the shift is induced by ATP or by va-

linomycin-catalyzed efflux of potassium. It should be mentioned in this connection that the rate of relaxation in the presence of saturating concentrations of uncoupling agents is much faster. These rates have not yet been measured and will require the use of rapid mixing techniques. As seen in Fig. 6, the rate of the ATP-induced shift is much more sensitive to lowering the temperature than is the valinomycin-induced transition.

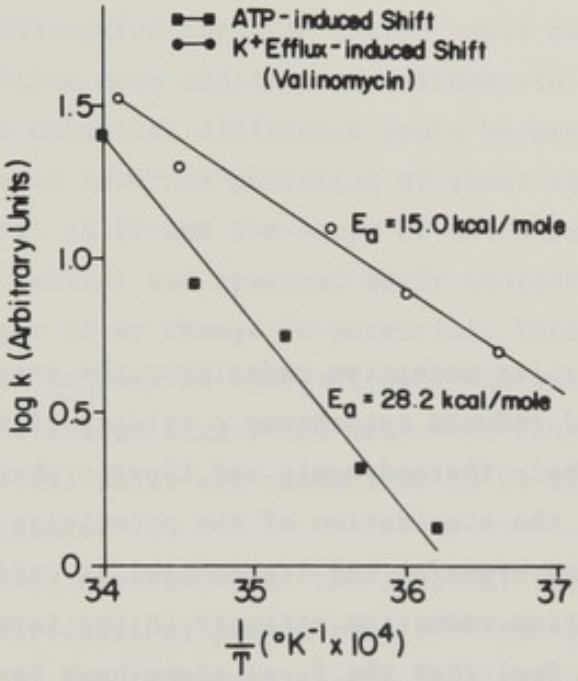


Fig. 7. Arrhenius plot of the spectral shift in ferric cytochrome aa_3. Conditions as described in the legend to Fig. 6.

Fig. 7 depicts the Arrhenius plots of these data. The ATP-induced transition shows a very high apparent activation energy (28 kcal/mole or 117 kJ), an interesting finding in view of the recently published activation energy of this magnitude for the adenine nucleotide translocator by Klingenberg (21). Thus the effect is clearly rate-limited by the adenine nucleotide translocator when induced by added ATP, at least at temperatures of 20°C and below. Accordingly, the shift induced by diffusion potential is much less temperature-sensitive and shows an apparent activation energy of about 15 kcal/mole. This value appears higher than the temperature dependences reported earlier for other diffusion potential-induced processes in the respiratory chain (reversed electron transfer, 22). Therefore it seems probable that this activation energy may actually be that of the conformational change of ferric cytochrome oxidase itself. If so, it is of interest

that the value of 15 kcal/mole is nearly identical to the activation energy of oxidation of ferrocytochrome c by mitochondria in this temperature range (23) and also to the activation energy reported by Wilson et al. (8) for the cyanide-induced spectral shift in oxidized mitochondrial cytochrome oxidase. The latter effect is spectrally similar, but not identical, to the energy-linked shift, both being consistent with a high to low spin transition in a ferric heme.

6. DISCUSSION

Knowledge of the molecular mechanism underlying the energy-linked spectral shifts in oxidized and reduced cytochrome c oxidase of the mitochondrial membrane as well as their thermodynamic and kinetic characteristics is of utmost importance for the elucidation of the principles and mechanism of energy conservation and transfer and its control in oxidative phosphorylation coupled to oxidation-reduction activity in the terminal portion of the respiratory chain. We feel that the first steps have been taken in this direction and that these data, when brought successfully together with the results from the recent studies of the kinetics of the cytochrome oxidase reaction at low temperatures (11), will help considerably to achieve these set goals. At present we must limit ourselves to the available data on the basis of which tentative working hypotheses may be built to stimulate further experimentation.

The more exact evaluation of the thermodynamic properties of the conformational change underlying the spectral shift in ferric cytochrome $\underline{aa_3}$ must apparently await more experimentation before any definite conclusions can be made. However, our recent data obtained by an improved technique (Figs. 2 and 3, section 2) suggest that the equilibrium constant of the reaction

$$(1) \qquad ADP^{ext} + P_i^{ext} + n\ \underline{aa_{3ox}^{*}} \longleftrightarrow ATP^{ext} + n\ \underline{aa_{3ox}}$$

may be close to $10^4\ M^{-1}$ at pH 7.2 and the value for n about 0.76. This would suggest that the free energy change required for half-maximal shift

is about 9.7 kcal/mole (assuming -7.5 kcal/mole for the standard free energy change of ATP hydrolysis, ref. 24), a value considerably higher than that previously estimated (4,5).

Wikstrom and Saari (4) titrated the spectral shift in oxidized cytochrome oxidase with different diffusion potentials. In those titrations the applied membrane potential was estimated from the concentration gradient of potassium under the assumption that the cation would come rapidly into electrochemical equilibrium upon addition of valinomycin to the mitochondria so that the electrical potential difference would be estimated by the Nernst equation. An estimated membrane potential of about 180 mV was required for half-maximal spectral shift and the slope of the straight line dependence between membrane potential and spectral shift indicated a tenfold change in the ratio $\underline{aa_3^*}/\underline{aa_3}$ per 60 mV change in potential. This suggests that the structural change in $\underline{aa_3}$ may be energetically equivalent with the work done in translocating one charge across the membrane. Thus the free energy required for half-maximal shift was calculated to be 180 mV x 0.023 kcal/mV·mole or 4.1 kcal/mole.

It was later observed, however, that the spectral shift correlated better with the total electrochemical proton gradient than with the membrane potential alone (5,15), and therefore the pH gradient,under the conditions of the diffusion potential experiments described above,would be essential in determining the energetics of the spectral shift,in addition to the electrical potential difference. Recent studies by Rottenberg (H. Rottenberg, personal communication) have indicated that there may be a very fast and rather extensive acidification of the M phase as measured by 9-amino acridine fluorescence upon addition of valinomycin to mitochondria in the absence of, or at low concentrations of external potassium. Less acidification apparently occurred the higher the external potassium concentration. This acidification appeared extensive enough to make the M phase rapidly more acid than the external medium (the C phase). Thus the protonmotive force Δp may be less than the membrane potential ($\Delta\psi$) by an amount equal to $-RT/F \cdot \Delta pH$ at low external potassium concentrations and perhaps equal or larger than $\Delta\psi$ at high external K^+. Since Δp correlates better than $\Delta\psi$ as the driving force for the shift (5,15), the free energy change of the latter estimated from the $\Delta\psi$ titrations (4.1 kcal/mole) may be an underestimate. Quantitative

measurements of both $\Delta\psi$ and Δ pH have to be performed to obtain an independent control of the free energy change of the shift estimated from the phosphate potential titrations (9.7 kcal/mole, see above).

What is then the possible function of the energy-linked conformational changes in cytochrome c oxidase? We may consider two major possibilities:

1. They represent "high energy" primary intermediates of oxidative phosphorylation implying that the free energy released by the redox reactions is conserved in these intermediates, after which follows transfer of the energy to the ATP synthetase reaction.
2. They may furnish the respiratory chain with an energy-linked allosteric device for kinetic respiratory control (4,5).

These two alternatives may also occur in combination.
It is of importance to realize that alternative 1 may imply a purely "chemical" or "conformational" energy coupling system where physical contact is established between cytochrome oxidase and the ATP synthetase complex, but that the conformational change may also be a generator of a electrochemical proton gradient via a more localized, primary charge separation across the $\underline{aa}_3$ molecule which may result in the $\underline{aa}_3 \longrightarrow \underline{aa}_3^*$ transition . The latter may well be in line with Scheme 4 (dotted arrows) and would be analogous to the mechanism by which the protonmotive force has been suggested to be generated in photosynthesis (25). It should be stressed that this model would formally involve a "primary high energy intermediate" of the respiratory chain complex although the principle of energy transfer may otherwise be purely chemiosmotic. This type of mechanism would easily account for the high sensitivity towards uncoupling agents described above (Fig. 5).

We would like to underline, however, that we observe the "high energy" state of the $\underline{aa}_3$ molecule with the proton apparently bound to it (see above, and refs. 4,5,17). Thus the transfer of the proton to the ATP synthetase complex may alternatively occur within or at the surface of the membrane (26), not necessarily involving the bulk aqueous phase in the phosphorylating steady state. Indeed, the protons may actually move both via an external (chemiosmotic) and an internal (localized) route with respect to the membrane, the proportions of which (i.e. the extent of release of H^+ into

the bulk aqueous phase) may be determined by the experimental (or *in vivo*) conditions (*cf.* 27). Such a dual-route proton transfer mechanism would easily explain why measured bulk phase electrochemical proton gradients appear to be too low to be consistent with the chemiosmotic hypothesis in its original formulation (15,28-32).

There is another aspect, that of respiratory control, which is of great interest in considering alternative 1. As shown above (Fig. 6) the $t_{1/2}$ of formation of $\underline{aa_3^*}$ from $\underline{aa_3}$ is approx. 0.4 seconds at room temperature and a high driving force. This corresponds to a turn-over rate of 1.7 $\underline{aa_3^*}$ per second which due to the experimental conditions must be considered a maximal value. This rate is at least ten times slower than the turn-over number of cytochrome oxidase (on a $2e^-$ or 1ATP basis) in State 3, but may be close to the overall turn-over in State 4. Thus we may conclude that if $\underline{aa_3^*}$ is indeed an intermediate in energy coupling (alternative 1), the $\underline{aa_3} \rightleftharpoons \underline{aa_3^*}$ transition does not come even close to equilibrium with the phosphate potential (or the Δp, or the "squiggle") in State 3, *i.e.* during active phosphorylation, but may do so in State 4. In State 3 the turn-over of cytochrome oxidase and the rate of ATP synthesis are at least ten times faster than the maximal rate by which the phosphate potential may drive $\underline{aa_3}$ "backwards" into the $\underline{aa_3^*}$ conformation.
This finding is of interest since at "coupling site 2" it has been shown that the rate of reversed electron transfer may be quite comparable with the forward rate of oxidative phosphorylation (33). Thus, if it is again assumed that the $\underline{aa_3^*}$ conformation is intermediate in energy coupling, it may be concluded that "coupling site 3" may be the only coupling site out of equilibrium under phosphorylating conditions, thus providing the irreversibility required for rapid flux of reducing equivalents in the respiratory chain in State 3. This would also imply that the cytochrome oxidase reaction would be the natural point of the respiratory chain where respiratory control is exerted, the other sites being close to equilibrium with the phosphate potential and therefore uncontrollable. This may be compared with the recent proposal by Owen and Wilson (32) that kinetic respiratory control may be exerted at cytochrome oxidase. However, their suggestion implies that "site 3" may be in equilibrium (strictly: close to thermodynamic equilibrium) with the phosphate potential in State 3, while our data

suggest that this is not the case. Whether the redox reaction of "site 3" actually comes into thermodynamic equilibrium with the phosphate potential in the controlled State 4 is not certain. Although our kinetic data are compatible with such an equilibrium it is still possible that the redox reaction is kinetically controlled due to the structural change in the cytochrome oxidase molecule (see also below).

Alternative 2 could provide a chemiosmotic energy transduction mechanism without a "high energy" intermediate (contrast alternative 1), but would anyhow (like in alternative 1) necessitate a revision of the "chemiosmotic" concept of respiratory control at cytochrome oxidase. The second alternative implies that the structural, kinetic and thermodynamic parameters of the $\underline{aa}_3$ molecule would be controlled by the protonmotive force (see e.g.Fig. 4), but without direct involvement of these changes in the mechanism of energy transduction (4,5). The latter might then occur as envisaged in the chemiosmotic hypothesis. The control would be of an "on/off" type where the $\underline{aa}_3$ molecule can exist in an active ($\underline{aa}_3$) and an inactive ($\underline{aa}_3^*$) form depending on the magnitude of the protonmotive force, the alternation between these forms being responsible for the energy-dependent kinetic control of the cytochrome oxidase reaction (4,5,11). This idea is consistent with the changes in the kinetics of the cytochrome oxidase oxygen reactions measured at very low temperatures after pre-energizing the mitochondrial membrane at room temperature with ATP (11).

It is only recently that is has become possible to study in detail the molecular events associated with conservation of the free energy released by the redox reactions of the respiratory chain. In this respect the studies of the energy-dependent changes in the structural, chemical, kinetic and thermodynamic parameters of the cytochrome c oxidase complex appear most exciting. At present we cannot state with certainty whether these observed transitions are directly involved in primary energy conservation, whether they reflect an energy-dependent allosteric control device or perhaps both. However, we feel that these and forthcoming studies along these lines may well help in elucidating the principles of energy conservation and respiratory control in mitochondria.

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The Significance of the Radicals of Ubiquinone in Electron Transport as Judged from their Chemical Properties

A. Kröger

From the investigation of the respiratory-linked reactions of ubiquinone (Q) (1,2,3) it was proposed that radicals may be formed as primary products of the reduction of Q and the oxidation of QH_2 according to reaction (a) and (b) respectively (3).

$$Q + e^- \longrightarrow \dot{Q}^{\ominus} \qquad (a)$$

$$QH_2 \longrightarrow \dot{Q}^{\ominus} + e^{\ominus} + 2H^+ \qquad (b)$$

The radical concentration in the steady state was found to be smaller than 5% of the total Q. It was concluded that the radicals are consumed only by dismutation (reaction c), and that this reaction is much faster than

$$2\dot{Q}^{\ominus} + 2H^{\oplus} \longrightarrow Q + QH_2 \qquad (c)$$

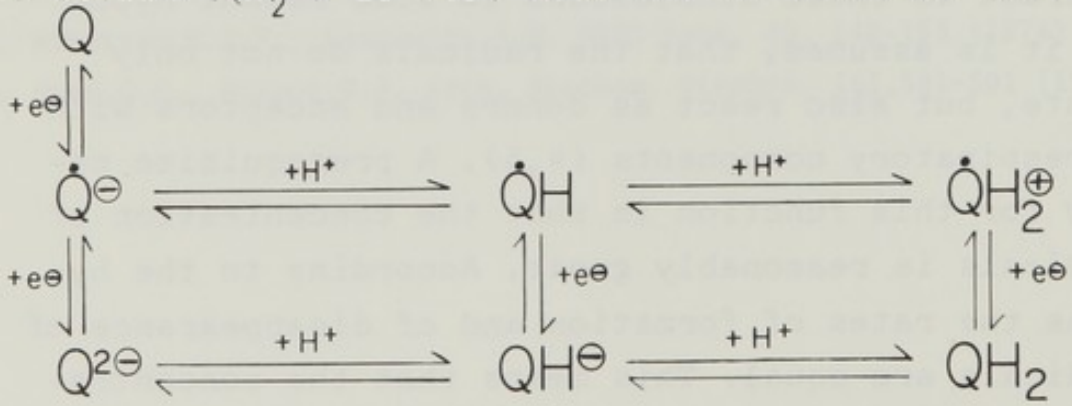
the formation of the radicals by electron transport.

In contrast to these conclusions in some recent hypothesis it is assumed, that the radicals do not only dismutate, but also react as donors and acceptors with other respiratory components (4,5). A prerequisite necessary for this function is that the concentration of the radicals is reasonably great. According to the hypotheses the rates of formation and of disappearance of the radicals are equal. This means that the concentration of the radicals is independent of electron transport and is determined only by the equilibrium constant of reaction (c).

The equilibrium radical concentration

The stability constant of the radicals of duroquinone in alkaline solution was measured with three different methods and consistent results were obtained (6,7).These data are used here for estimating the radical concentration of ubiquinone in the mitochondrial membrane, since comparably accurate data of ubiquinone are missing so far. The properties of ubiquinone in the mitochondrial membrane suggest that it is more or less solved in the lipid space of the membrane (1,2,3). Furthermore, the available kinetic data show that the influence of the structure of the quinone and of the solvent on the reaction velocities is relatively small. Thus the dismutation rate constants of the neutral radicals of p-benzoquinone, duroquinone and ubiquinone differ by less than a factor of two (8,9), and a similary small variation was measured with duroquinone in ethanol/water on one hand and in liquid paraffin on the other (10)

The quinone radical concentration is dependent on the pH of the medium. This is due to the fact that the hydroquinone as well as the radical may exist in three different states of protonization (scheme 1). The radical cation ($\dot{Q}H_2^{\oplus}$) is very acid and is detected only in



Scheme 1 Possible intermediates of the redox reactions of quinones.

the presence of mineral acids (11). Therefore, this

species can be left out of consideration. The dissociation constants of both protons of QH_2 are very small (reaction d and e) (12), whereas $\dot{Q}H$ shows the properties of a weak acid (reaction f) (10).

$$QH_2 \longrightarrow QH^- + H^+ \qquad K_1 = 6\cdot10^{-12}M \qquad (d)$$

$$QH^- \longrightarrow Q^{2-} + H^+ \qquad K_2 = 2\cdot10^{-13}M \qquad (e)$$

$$\dot{Q}H \longrightarrow \dot{Q}^- + H^+ \qquad K_3 = 10^{-6}M \qquad (f)$$

This situation provides that the radical concentration decreases with the square of the proton concentration (equn.1). Baxendale and Hardy (7) measured the stability constant (reaction f) of the radical anion of duroquinone to be 1.3 at pH > 13, where the reduced quinone is fully deprotonized.

$$Q + Q^{2-} \longrightarrow 2\dot{Q}^- \qquad K_4 = 1.3 \qquad (g)$$

With the constants of the reactions (d), (e) and (g) equn. (1) is derived which gives the radical concentration as a function of the proton concentration and is valid between pH 6 and 11.

$$\frac{[\dot{Q}^{\ominus}]^2 \cdot [H^+]^2}{[Q] \cdot [QH_2]} = K_1 \cdot K_2 \cdot K_4 = 1.6\cdot10^{-24}M^2 \qquad (1)$$

According to equn. (1) the maximum concentration of radicals at a given pH results from equal amounts of Q and QH_2. Under these conditions at pH 7 it is calculated that $6\cdot10^{-4}$% of the quinone occurs in the radical state. Thus the equilibrium radical concentration is extremely small.

At pH < 6 where $\dot{Q}H$ rather than $\dot{Q}^{\ominus}$ is stable, equn. (2) is valid which is obtained from equn. (1) and the dissociation constant of the proton from the neutral radical (reaction f).

$$\frac{[\dot{Q}H]^2}{[Q]\,[QH_2]} = \frac{K_1 \cdot K_2 \cdot K_4}{K_3^2} = 1.6\cdot10^{-12} \qquad (2)$$

According to equn. (2) only $6\cdot10^{-5}$% of the total quinone

occurs in the radical state in the presence of equal concentrations of Q and QH_2 in the equilibrium.

The stability constant of the radical anion (reaction f) was measured spectrophotometrically with duroquinone in water (table 1). The value so obtained (7) is in good agreement with that for duroquinone in water/pyridine which was measured earlier by Michaelis et al. (6) by potentiometry and confirmed by magnetometry. Also the pH-dependency of the radical concentration according to equn. (1) is consistent with the measurements of Michaelis et al.. Thus from independent measurements with three different methods a consistently very low equilibrium radical concentration for duroquinone in aqueous solutions is obtained. In contrast, the corresponding number reported by Bäckström et al. (13) for ubiquinone in ethanol (0.2%) is more than two orders of magnitude greater. This big discrepancy can neither be attributed to the different quinones nor to the different solvents used (see above). Bäckström et al. also report that the radical concentration in the mitochondrial membrane is 1.5% of the total Q which is of the same order of magnitude as their value reported for Q in ethanol. In comparison this may indicate that the radical concentration of Q in the membrane is similar as in ethanol.

In conclusion the more convincing number of the equilibrium radical concentration is that derived from the consistent measurements of Michaelis et al. and Baxendale and Hardy. This value (about 0.001% of the total Q) is four orders of magnitude smaller than the concentration of the b-cytochromes of the mitochondrial membrane. Therefore, it is very unlikely that quinone radicals interact in electron transport as donors and acceptors of reducing equivalents.

Table 1 Stability constants of quinone radicals

The fraction of Q in the radical state at pH 7 given in the first two lines is calculated from the stability constants K_4 (reaction g) using equn. (1).

author	reference	method	quinone	solvent	pH	K_4	%Q in radical state at pH 7
Michaelis et al.	6	potentiometry magnetometry	duroquinone	20% pyridine in water	13	4.6	10^{-3}
Baxendale and Hardy	7	spectrophotometry	duroquinone	water	13	1.3	$6 \cdot 10^{-4}$
Bäckström et al.	13	ESR - spectroscopy	ubiquinone	ethanol	neutral	--	0,2
				mitochondr. membrane	neutral	--	1.5

The steady state quinone radical concentration

On the basis that the radicals are formed according to reaction (a) and (b) and are consumed only by dismutation (reaction c) and not by reactions with other respiratory components, the radical concentration in the steady state is a function of the activity of electron transport. The rate constants of dismutation of quinone radicals are well known. As these data can be assumed to be valid also for Q in the membrane, the radical concentration in the steady state can be calculated from the respiratory activity and the rate constants of dismutation. The result may answer the question whether the dismutation alone is fast enough to bring about a radical concentration which is below 5% of the total Q.

The rate constants of dismutation of the radical anion (reaction h) and of the neutral radical (reaction i) are given in table 2 together with the constants of the proton exchange reactions of the radicals. The constant of the reaction of the neutral radical with the radical anion (reaction k) has not yet been measured. However, the experiments of Land and Swallow (8) indicate that this reaction is much faster than the dismutation of the radical anion. The latter reaction is two orders of magnitude slower than the dismutation of the neutral radical, probably because of the negative charge of both reactants. As only one of the reactants is charged in reaction (k), it is assumed that this reaction is as fast as the dismutation of the neutral radical.

The radical concentrations given in table 2 are calculated with the steady state condition, that the rate of formation is equal to the rate of dismutation of the radicals, and on the basis that each reaction alone ac-

Table 2 Rate constants of dismutation and of the acid-base reaction of quinone radicals.

The fraction of Q in the radical state is calculated under the assumption that the rate of dismutation is equal to the respiratory rate and that the dismutation is brought about by each reaction (h, i and k) alone. The respiratory activity is assumed to be 1200 µatoms O/min per gram of protein. This corresponds to a rate of electron transfer of $4 \cdot 10^{-2}$M/sec on the basis that 1 gram of protein is equivalent to 1ml of lipid space of the membrane which contains 5mM Q.

Nr	reaction	quinone	rate constant	reference	%Q in radical state
h	$2\dot{Q}^{\ominus} \rightarrow Q+Q^{2\ominus}$	duroquinone	$4.6 \cdot 10^{6} M^{-1} \cdot sec^{-1}$	10	2
i	$2\dot{Q}H \rightarrow Q+QH_2$	ubiquinone	$2 \cdot 10^{8} M^{-1} \cdot sec^{-1}$	9	0,3
k	$\dot{Q}^{-}+\dot{Q}H \rightarrow Q+QH^{\ominus}$	--	?	-	0,3?
l	$\dot{Q}^{-}+H^{+} \rightarrow \dot{Q}H$	duroquinone	$7.4 \cdot 10^{2} \cdot sec^{-1}$ (pH7)	10	1
m	$\dot{Q}H \rightarrow \dot{Q}^{\ominus}+H^{+}$	ubiquinone	$10^{4} \cdot sec^{-1}$	8	0,1

counts for the dismutation. The steady state rate of electron transport is assumed to be $4 \cdot 10^{-2}$M sec^{-1} which corresponds to a respiratory activity of 1200 uatoms O/ min per gram of protein, if 1 gram of protein is equivalent to 1 ml of lipid phase of the membrane as the reaction space of the quinone. With this assumption the total concentration of Q in the membrane is 5mM.

A maximum radical concentration of 2% is expected if the dismutation proceeds only via the radical anion (reaction h) as the more stable species at pH 7. A much smaller concentration (0.3%) is obtained by assuming that the radical anion reacts only with the neutral radical (reaction k). This reaction may also occur, because both species are formed in equal amounts as primary products of reaction (a) and (b). However, reaction (k) cannot alone account for the dismutation, because it is three times slower than the dissociation of the proton of the neutral radical. Therefore, the dismutation is probably achieved both by reaction (h) and (k), and an intermediate radical concentration of about 1% of the total Q is estimated to ensue under the conditions assumed. This number is consistent with the conclusion (1,2,3) that maximally 5% of the total Q of the mitochondrial membrane is in the radical state.

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The Inhibition of Electron Transfer from Cytochrome b to Ubiquinone by Antimycin

G. v. Jagow

I would like to take the opportunity, in this session entitled "mechanism of electron transfer", to discuss our latest results concerning the inhibition of electron transfer from cytochrome b to ubiquinone by antimycin (1).

The inhibitory action of antimycin on cytochrome b was reported by Potter and Reif in 1952 (2). Later Chance (3) localized the block of electron transfer between cytochrome b and cytochrome c. Data regarding the action of antimycin increased with knowledge of the function of the b cytochromes. Over a period of about two decades, interest in antimycin research was mainly focussed on kinetics and thermodynamics of the antimycin-dependent redox changes of the b cytochromes. Less attention was paid to the influence of antimycin on electron exchange between cytochrome b and ubiquinone.

In point of fact, Chance had already found in 1958 (3) that antimycin interferes not only with electron transfer from cytochrome b to cytochrome c_1, but also with that from cytochrome b to ubiquinone. He performed redox titrations of the b cytochromes with the aid of fumarate after prior addition of succinate, and vice versa. He recognized that the adjustment of the redox equilibrium needs more time when titration was performed in a positive potential direction than when performed in the opposite direction. He estimated a twentyfold slower reoxidation by fumarate when inhibitor was present and a

fiftyfold faster reduction of cytochrome b by succinate in the presence of antimycin.

The studies of Chance were resumed by his group in 1971 (4). Boveris carried out durohydroquinone pulse experiments in which the reduction kinetics of the b cytochromes and of the constituent ubiquinone were analysed. It has been reported that the kinetics of the redox equilibration between cytochrome b and ubiquinone are altered by antimycin. A direct binding of antimycin to cytochrome b with a conformational change of the cytochrome was considered as one possibility; alternatively, the hydrocarbon core of the membrane on which ubiquinone is sited might become modified, resulting in a decrease in ubiquinone reactivity (4).

However, measurements of the redox changes of constituent ubiquinone in durohydroquinone pulse experiments are rendered more difficult by a high absorbance interference of duroquinone in the ubiquinone measurements (1). Our new interest in the influence of antimycin on these redox reactions came from a result obtained in mitochondria from chloramphenicol-grown Neurospora in which the mitochondrial protein synthesis was only partially inhibited (5).

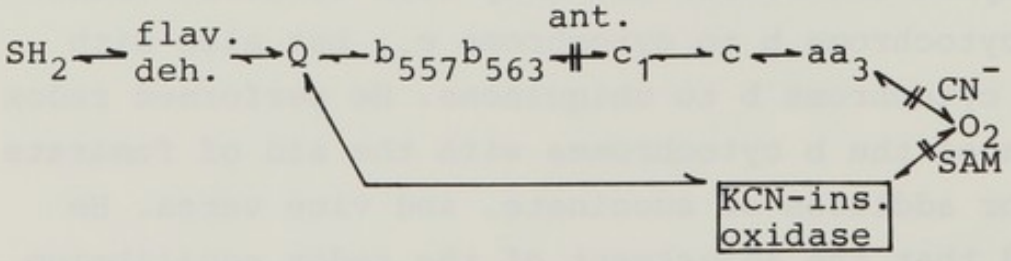


Fig. 1. Scheme of the respiratory chain of Neurospora grown in the presence of chloramphenicol. The mitochondrial protein synthesis was partially inhibited by addit-

ion of 1 g/l chloramphenicol. The hyphae were harvested in the early logarithmic growth phase. The content of cytochrome oxidase was diminished to one tenth, those of cytochrome b_{557} and b_{563} to one third of their original values under these conditions. The nature of the KCN-insensitive oxidase induced by this treatment is unknown so far with the exception of its sensitivity to salicylhydroxamate. The abbreviations used are: SH_2 for substrates as NADH or succinate, Q for the consituent ubiquinone, ant. for antimycin and SAM for salicylhydroxamate.

Fig. 1 shows the respiratory chain of such a species: Inhibition of mitochondrial protein synthesis, besides decreasing the contents of cytochromes b and aa_3, induces the formation of a KCN-insensitive oxidase which carries electrons from ubiquinone to oxygen in a side-shunt to the cytochrome chain. This branched chain is able to oxidise either NADH or succinate as well as the artificial electron donor durohydroquinone with high activity in the presence of KCN. The reaction site of durohydroquinone may be directly on the b cytochromes (6, 7), or on a component adjacent to the b cytochromes.

Thus the branched chain of Neurospora seemed to be a good model for reinvestigating the question as to whether antimycin influences solely the electron-transferring activity of the b cytochromes, or additionally that of ubiquinone, as considered in the durohydroquinone pulse experiments of Boveris (4), or as mentioned in new studies of Wilson and Erecinska (8). In the former case antimycin should separately influence the KCN-insensitive durohydroquinone respiration; in the latter the oxidation of NADH and succinate via the KCN-insensitive branch should be influenced in addition.

Fig. 2 shows an antimycin titration curve of the KCN-in-

sensitive durohydroquinone respiration. The respiratory activity, amounting to 100 units, was measured spectrophotometrically at 270-284 nm. The titration curve is non-linear; full inhibition of electron flow is reached at an antimycin concentration of one mole inhibitor per mole of cytochrome b-566 or cytochrome b-562. Experimental results obtained from the functioning branched chain suggested that electrons can be transferred from durohydroquinone to ubiquinone via cytochrome b in the KCN-inhibited state, but not in the antimycin-inhibited state.

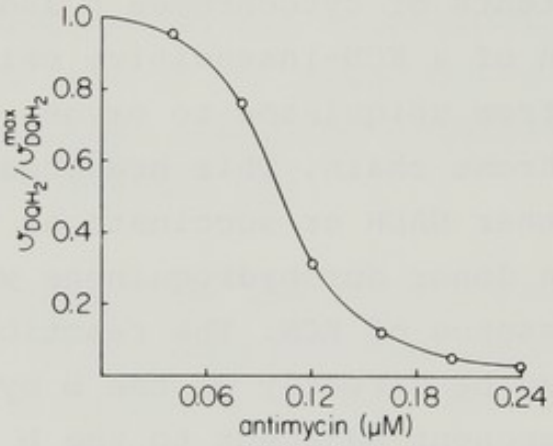


Fig. 2. Antimycin inhibition curve of the durohydroquinone respiration of Neurospora mitochondria possessing a second KCN-insensitive oxidase. The mitochondria were grown under conditions which induce a second terminal oxidase (cf. legend to Fig.1). The durohydroquinone (DQH_2) respiration was followed spectrophotometrically at 270-284 nm, its rate (DQH_2^{max}) amounted at 25° C in the presence of 1 mM KCN to 100 μmol/min per g protein.

The influence of antimycin on the KCN-insensitive succinate and NADH respiration was studied in parallel. No influence of antimycin on either respiratory rate has been found. Since especially the NADH oxidation had a very high activity (600 μmol/min per g protein), the experiments seem to indicate that antimycin does not influence the reactivity of ubiquinone.

The site of action of antimycin on durohydroquinone oxidation was studied in more detail by aid of redox patterns including ubiquinone and the b cytochromes. The patterns were obtained under near equilibrium state. The experiments are more complicated, since for special reasons discussed above (1) ubiquinone needs to be extracted from the particles with organic solvents before its redox level can be determined. We did not really expect the experiments to give reliable results, because we knew that durohydroquinone reduces ubiquinone chemically in organic solvents (unpublished). This reduction might be expected to occur in the light petroleum phase during the extraction procedure. Nevertheless, the experiments worked.

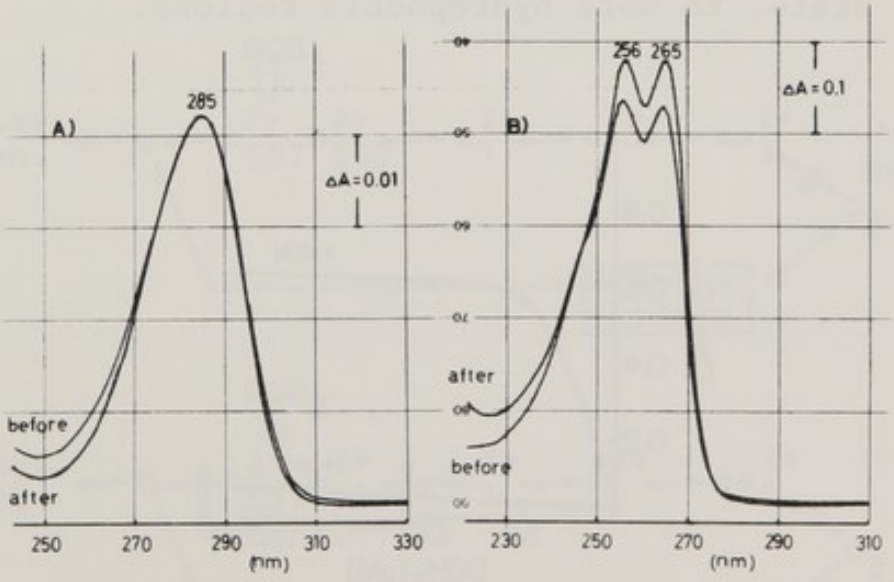


Fig. 3. Partition characteristics of durohydroquinone and duroquinone between a water/methanolic and a light petroleum phase. A) durohydroquinone was dissolved in a water/methanolic phase (v/v = 2:3) at a final concentration of 20 μM; the absolute spectrum was recorded before and after equilibration with a light petroleum phase; B) duroquinone was dissolved in a light petroleum phase at a final concentration of 20 μM; the absolute spectrum was recorded before and after equilibration with a water/methanolic phase (v/v = 2:3).

The reason is that during extraction durohydroquinone,

lacking the isoprenoid chain of ubiquinone, moves instantaneously into the water/methanolic phase (cf. Fig. 3A), whereas ubiquinone, both in the reduced and oxidized state, is recovered practically quantitatively in the light petroleum phase. Duroquinone, in contrast to durohydroquinone, is partitioned largely in the light petroleum phase (cf. Fig. 3B).

Besides the consequences for our experiments, this behaviour of duroquinone may indicate a tendency of the polar head group of ubiquinone to assume a special orientation in the mitochondrial membrane, depending on its state of reduction. Ubiquinone, situated in the lipid bilayer, may in the reduced state have its head group in close proximity to more hydrophilic regions, and, in the oxidized state, to more hydrophobic regions.

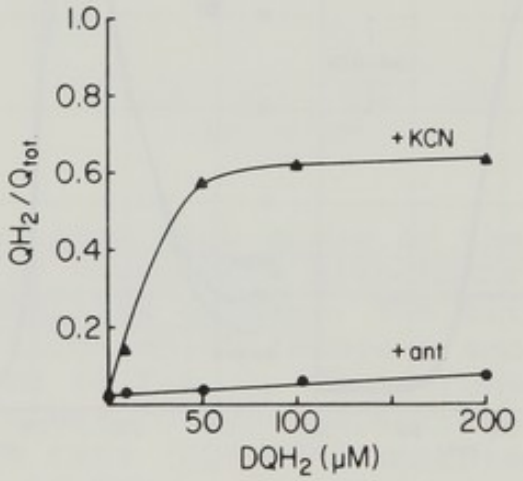


Fig. 4. Concentration dependency of the degree of reduction of the constituent ubiquinone of submitochondrial particles of beef heart on added durohydroquinone. The redox state of ubiquinone was quenched and determined in the KCN- and antimycin-inhibited state as described by Kröger and Klingenberg (12). The ubiquinone content of the preparation was 10 μmol/g protein. The designations used are: QH_2 for the content of reduced ubiquinone, Q_{tot} for the total ubiquinone content. 2 mM KCN or antimycin (2 nmol/mg protein) were added as indicated.

Let me now come back to our redox patterns which were obtained in submitochondrial particles from beef heart. The upper curve of Fig. 4 shows the extent of reduction of the mitochondrial ubiquinone in the KCN-inhibited state. It becomes apparent, in contradiction to earlier reports (9), that ubiquinone can be reduced by external durohydroquinone when cytochrome oxidase is inhibited by KCN. In the antimycin-inhibited state, as in the lower curve, a redox equilibrium between the b cytochromes and ubiquinone is not established. Although under these conditions both b cytochromes are reduced to about 70% by durohydroquinone (not shown), ubiquinone is only slightly reduced.

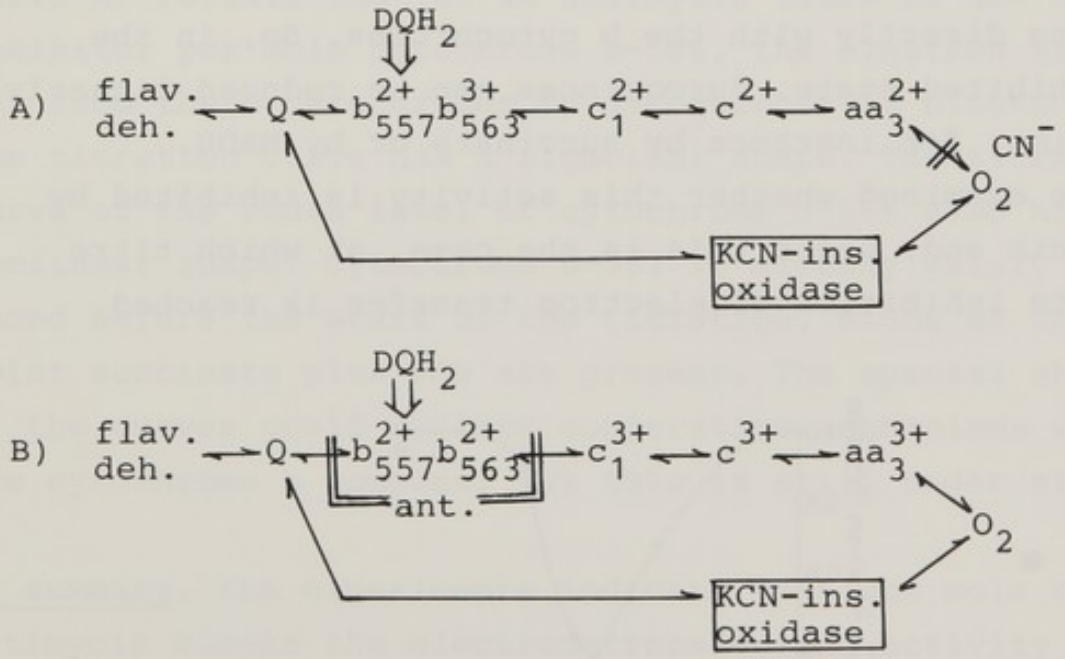


Fig. 5. Possibilities of electron flow in the KCN- and antimycin-inhibited state of the respiratory chain of Neurospora mitochondria possessing a second final oxidase. A) in the presence of KCN; B) in the presence of antimycin. The abbreviations used are: SH_2 for substrates as for instance NADH or succinate, flav.deh. for any flavin dehydrogenase as NADH or succinate dehydrogenase, Q for the constituent ubiquinone, DQH_2 for added durohydroquinone and ant. for antimycin.

Fig. 5 shows an interpetation of the results in a schematic representation of the branched chain of Neurospora. In the presence of KCN (Fig. 5A) the redox equivalents from durohydroquinone as well as those from the flavin dehydrogenases can be channelled to oxygen via the Q branch.

In the presence of antimycin (Fig. 5B), electrons can be transferred from durohydroquinone to the b cytochromes, but electron transfer from cytochrome b to ubiquinone is inhibited in the same way as from cytochrome b to cytochrome c_1. Electron flow from the flavodehydrogenases to oxygen via the second pathway is not disturbed by this mechanism.

In the normal respiratory chain the oxidized form of duroquinone can be used as electron acceptor, probably reacting directly with the b cytochromes. So, in the KCN-inhibited state, duroquinone can be reduced linearly with time, for instance by succinate or by NADH.

We have examined whether this activity is inhibited by antimycin and, where this is the case, at which titre complete inhibition of electron transfer is reached.

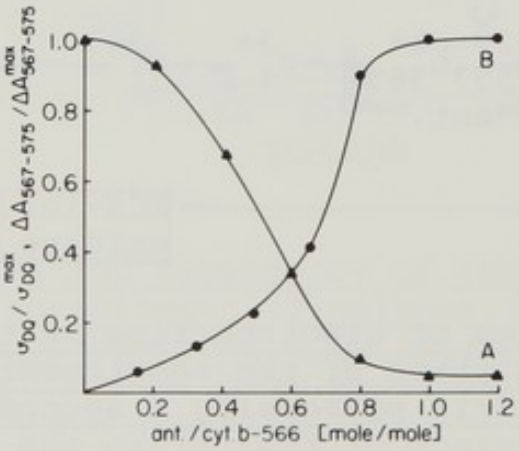


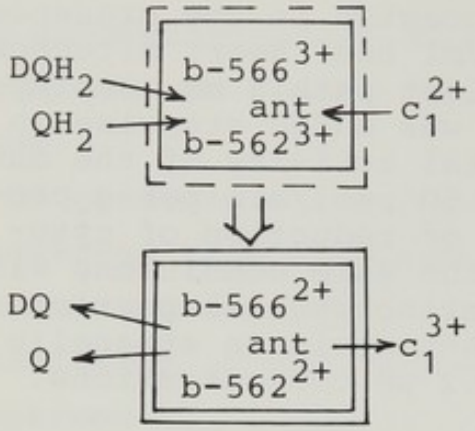
Fig. 6. Antimycin inhibition curve of the succinate dependent duroquinone reduction with its corresponding degree of reduction of cytochrome b-566 in submitochondrial

particles of beef heart. Curve A) relative activity of the duroquinone reduction; Curve B) relative redox level of cytochrome b-566. The rate of duroquinone reduction was measured with 5 mM succinate as hydrogen donor and 0.1 mM duroquinone as electron acceptor in the presence of 1 mM KCN at 25° C with 0.3 mg/ml beef heart mitochondrial particles at 275-289 nm, light path 2 mm. Antimycin (ant.) in ethanolic solution was added stepwise in 0.04 µM concentrations. The initial activity of the duroquinone reduction (v_{DQ}^{max}) was 50 µmol/min per g protein. The increase in the degree of reduction of cytochrome b-566 was measured under the same conditions with 2.5 mg protein/ml beef heart submitochondrial particles at 567-575 nm, light path 5 mm. Antimycin in ethanolic solution was added stepwise in 0.2 µM concentrations.

Fig. 6 shows in A) the antimycin titration curve of this activity and in B) the extent of reduction of cytochrome b-566 as a function of the antimycin concentration. Curve A) reveals that at an antimycin titre of one mole inhibitor per mole cytochrome b-566, the electron transfer from the b cytochromes to duroquinone is blocked. The titration curve has a sigmoidal shape. The titration curve of the redox level of cytochrome b-566 also has a nonlinear shape. Cytochrome b-562 is already mainly reduced before the start of the titration, since at this point succinate plus KCN are present. The special shape of the curves could reflect cooperative mechanisms within the cytochrome b complex, but this is still under study.

In summary, the experiments indicate that one mole of antimycin blocks the electron-transferring activity of two different molecules of cytochrome b not only to cytochrome c_1 but also to ubiquinone and duroquinone. The simplest interpretation of this finding is the assumption of a dimeric cytochrome complex, one subunit of which may bind antimycin; or the assumption of a dimeric complex which both subunits may bind simultaneously to

two different chemical groups of the antimycin molecule (10). The inhibition may involve cooperative mechanisms.



A) transitory state
antimycin is bound, the reduction of the two b cytochromes is not prevented

B) antimycin-inhibited state
the bound antimycin prevents the reoxidation of the two b cytochromes

Fig. 7. A model for the inhibitory action of antimycin on the b cytochromes. For detailed explanation see Discussion. The superpositioning of the two b cytochromes should not be taken to indicate their arrangement in the respiratory chain.

Fig. 7 gives a working scheme for the action of antimycin. As is known, antimycin is bound when the b cytochromes are in oxidized state, or when cytochrome b-562 is already reduced by prior addition of succinate or of another electron donor (11). In the scheme, the former condition can be defined as a "transitory state". On addition of redox equivalents, as indicated by the arrows in Fig. 7A), the b cytochromes are reduced instantaneously when antimycin takes effect. The redox equilibration with ubiquinone, duroquinone and cytochrome c_1 is blocked as indicated by the double line in Fig. 7B). Unfortunately, the scheme is not too different from a black (or white?) box. It shows neither where antimycin is bound, nor in which sequence the b cytochromes are arranged, nor if they really form a dimeric complex, or perhaps, for reasons of symmetry and function, a tetrameric complex.

The question cannot yet be answered because the binding site of antimycin as well as the sequence and the topographical arrangement of the b cytochromes are still unknown.

Nevertheless, I do not intend to go into the mass of speculations concerning unsolved questions; neither do I intend to enlarge the bulk of speculation by providing new ones of my own. For the moment the wiser counsel would be to hold one's horses, and to obtain new results.

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Effects of Antimycin on the b-Cytochromes in Yeast. Analysis of Antimycin Induced Effects on Cytochrome b Reduction in the Wildtype of the Fission Yeast Schizosaccharomyces pombe

R.W. Manhart, W. Bandlow

SUMMARY

The effects of antimycin on the b-cytochromes were analysed quantitatively in the presence and absence (KCN present) of electron-flow, in order to correlate them with the sequential saturation of two different antimycin binding sites in the respiratory chain of Schizosaccharomyces pombe (1). Antimycin enhances the reduction of cytochrome b-566 in the aerobic steady-state and in the presence of KCN. The extent of this effect depends in both cases non-linearily on the concentration of the antibiotic. The titration curve of this effect with duroquinol as substrate is non-linear, too, although the duroquinol-oxidase activity - in contrast to the NADH- or succinate-oxidase - is linearily affected by antimycin. Antimycin causes a spectral red-shift of cytochrome b-562 and, in the presence of mediators, an oxidation of b-562, both depending linearily on the concentration of antimycin. All effects are maximally developed after the saturation of the high-affinity binding site, i.e. after the addition of about 180 pmol antimycin per

mg protein.

INTRODUCTION

Antimycin enhances the extent of reduction of cytochrome b in beef-heart mitochondria in the presence of succinate or NADH (2). This effect of antimycin has been shown to be composite in beef-heart mitochondria, as (i) all b-cytochromes are affected by the antimycin binding, and (ii) the absorbance maximum of cytochrome b-562 is shifted by 1-2 nm to the red (3-6). The extent of the antimycin induced effects depends on the concentration of the antibiotic (4,6-8).

With NADH or succinate as substrate in the presence of mediators like phenazine methosulfate most of cytochrome b-566/558 is already reduced, and antimycin causes no further reduction of this cytochrome. Addition of antimycin leads, then, to the oxidation of a component absorbing at 562 nm (4). The extent of this oxidation depends, too, on the antimycin concentration.

Burger et al. (1) found that in yeast mitochondria two binding sites for antimycin are present in the bc_1-segment of the electron transfer chain, one with a high affinity ($K_D \cong 10^{-13}$ M) and one with a lower affinity ($K_D \cong 10^{-10}$ M). This paper will correlate the concentrations of antimycin necessary for inhibition of the electron flow and for saturation of the individual binding sites with the titres of the antimycin dependent effects on cytochrome b.

The rate of duroquinol oxidation depends linearily on the antimycin concentration in rat liver (9) and yeast

mitochondria. However, as will be shown in this paper there is a non-linear correlation between the extent of reduction of b-cytochromes by duroquinol in the presence of KCN and the antimycin concentration.

MATERIALS AND METHODS

Schizosaccharomyces pombe 972 h^- wild type was used throughout the experiments. Growth conditions were the same as described by Heslot et al. (10). Mitochondrial particles were prepared as described by Lang et al. (11) and stored in 1 ml portions at 77°K until use. All measurements shown were performed with a single preparation. Protein was determined according to the method of Lowry et al. (12). Redox changes of the b-cytochromes were measured at 25°C with an Aminco DW-2 dual wavelenght spectrophotometer (10 Å band width). The long wavelength isosbestic point for cytochrome b absorption changes was found to be at 573 nm. Further experimental details are given in the legends.

RESULTS

From Fig.1 it may be seen, that the addition of antimycin to mitochondrial particles reduced with succinate (or NADH, not shown) in the presence of KCN causes a further increase of three cytochrome b absorption bands. The spectrum succinate plus antimycin plus KCN minus succinate plus KCN shows a maximum at 566 nm with shoulders at 562 nm and 558 nm. In yeast antimycin leads not only to an enhanced reduction of cytochrome b-566/558 like in beef-heart mitochondria (5,13), but also of cytochrome b-562. Concomitant with this enhanced reduction the absorption maximum of cytochrome b-562 is shifted by

1 nm to the red (cf. Fig. 4). The spectrum illustrated by open circles is corrected for this shift and shows therefore only the enhanced reduction of the b-cytochromes.

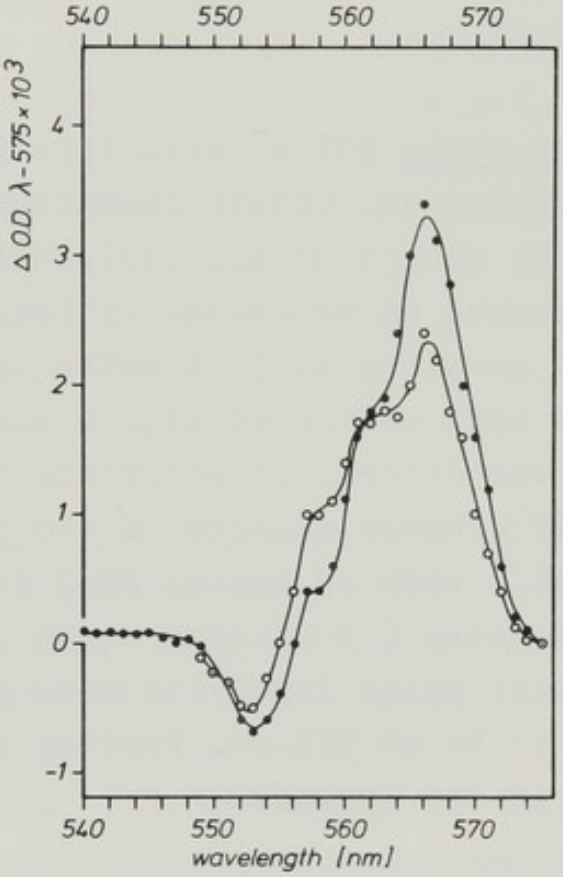


Fig.1 Enhanced reduction of cytochromes b-566/558 and b-562 in the presence of antimycin. Concentrations: 17 mM succinate, 0.5 mM KCN, mitochondrial particles 2 mg/ml, 1 nmol antimycin per mg protein. (●)succinate plus antimycin plus KCN minus succinate plus KCN. (○) same spectrum corrected for the spectral red-shift of cytochrome b-562. The correction has been performed by substracting 90% of the shift spectrum (Fig.4) from the succinate plus antimycin minus succinate reduced spectrum (●).

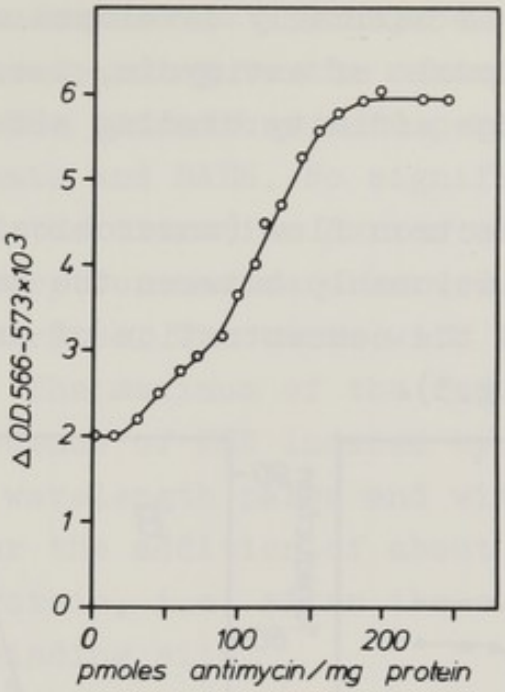


Fig.2 Antimycin induced reduction of cytochrome b-566 under aerobic steady state conditions. For each value 3 ml mitochondrial particles (2 mg/ml) was incubated with the respective concentrations of antimycin for 3 min at 25°C. After 3 min 17 mM succinate was added and the increase of the absorption difference at 566 nm minus 573 nm measured until oxygen was consumed. Anaerobiosis was indicated by a further increase of the cytochrome b reduction.

This enhanced reduction of cytochrome b is observed both in the aerobic steady state (presence of electron flow) and in the presence of KCN (absence of electron flow. Fig. 2 shows the dependence of the extent of this effect in the aerobic steady state from the amount of antimycin added. It can be seen that the redox state of cytochrome b-566 is non-linearily dependent on the antimycin concen-

tration. The effect is maximally developed after the addition of about 180 pmol of antimycin, i.e. after the saturation of the high affinity binding site (1).

In the absence of electron flow (anaerobiosis or inhibition by KCN) the relationship between the amount of cytochrome b reduced and the concentration of antimycin is non-linear, too (Fig.3).

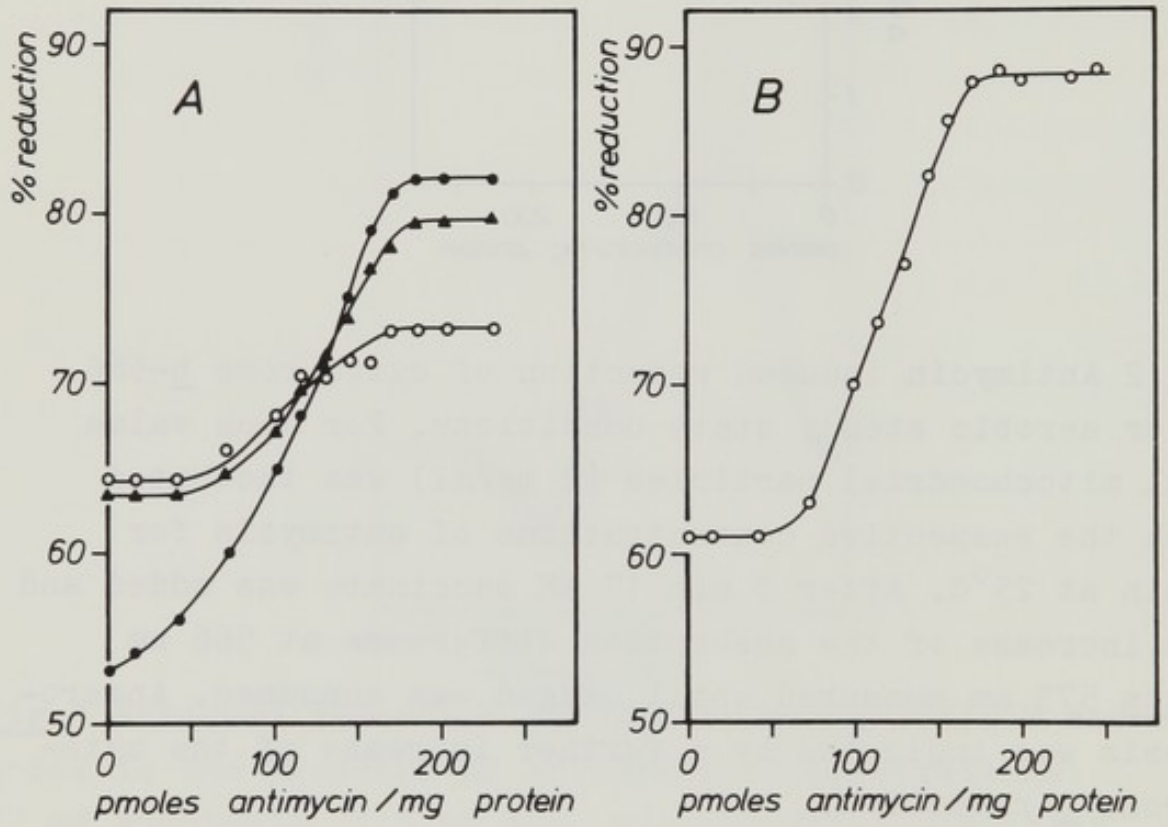


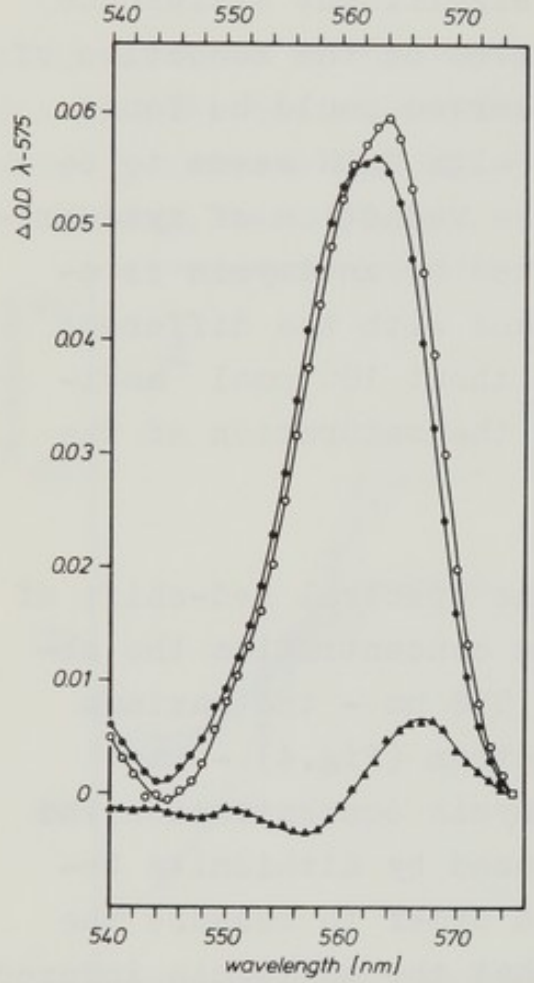
Fig. 3 Antimycin dependent reduction of b-cytochromes in the absence of electron flow after inhibition by KCN. Mitochondrial particles (2 mg/ml) were incubated for 5 min with 0.5 mM KCN before substrate was added (17 mM succinate or 3 mM NADH). Then increasing amounts of antimycin were stirred into the cuvette. 100% reduction was achieved by addition of dithionite. A: Substrate was succinate (●) Δ O.D.566 - 573 nm; (○) Δ O.D.558 - 573 nm and (▲) Δ O.D.562 - 573 nm. B: NADH Δ O.D. 566 - 573 nm.

The effect was measured at 558 nm, 562 nm and 566 nm, respectively, corresponding to the maximum and the shoulders in Fig.1. The titrations were performed in the presence of succinate and NADH. No significant difference in the shape of the titration curves of the reduction of the three b absorption maxima observed could be found with these substrates. The curve with NADH seems to be a little steeper. The maximum of the reduction of cytochrome b in the presence of KCN induced by antimycin is achieved at all wavelength pairs and with the different substrates after the addition of about 180 pmol antimycin per mg protein, i.e. after the saturation of the high affinity binding site.

To determine the dependency of the spectral red-shift of cytochrome b-562 on the antimycin concentration the absorption difference 568 nm minus 558 nm - the maximum and the minimum of the shift spectrum (Fig.4) - was measured and related to the antimycin concentration.The b cytochromes had been fully reduced by dithionite before the addition of antimycin in order to measure the pure shift effect. Fig.5 shows that the antimycin induced red-shift depends linearily on the antimycin concentration and is maximally developed after the addition of 180 pmol antimycin per mg protein.

In the presence of substrate and mediators like phenazine methosulfate antimycin causes the oxidation of a component absorbing at 562 nm. This oxidation is - in contrast to the oxidant induced extrareduction - KCN sensitive (data not shown). The total absorbance at 562 nm minus 573 nm after reduction with succinate in the presence of phenazine methosulfate and in the absence of KCN decreases about 20% after the addition of

antimycin.The titration of this effect reveals a linear dependency on the antimycin concentration (Fig.6) and the maximum is reached at 180 pmol antimycin per mg protein.



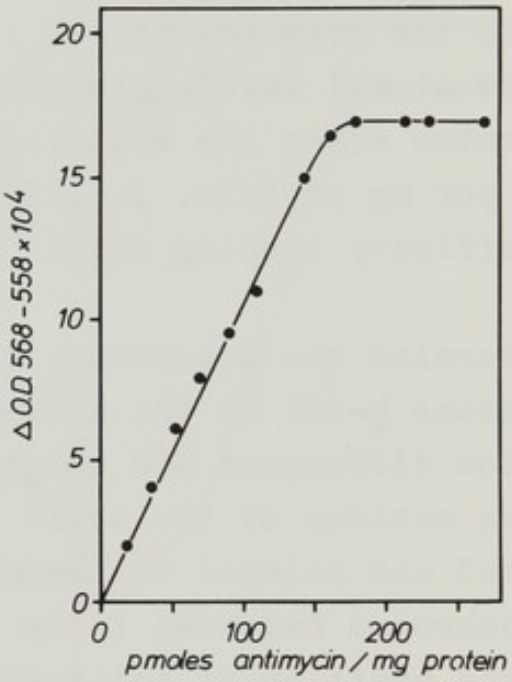


Fig.4 (left) The antimycin dependent spectral red-shift of cytochrome b-562. Concentrations: ascorbate 10 mM, antimycin 1 nmole per mg protein, mitochondrial particles 2 mg/ml. For complete reduction of the b cytochromes few grains of dithionote were added. (●) dithionite minus ascorbate; (○) dithionite plus antimycin minus ascorbate; (▲) difference between the two spectra showing the antimycin dependent shift effect.

Fig.5 (right) Titration of the antimycin dependent spectral red-shift of cytochrome b-562. 2 mg/ml mitochondrial particles were reduced by 3 mM NADH in the presence of 0.5 mM phenazine methosulfate.ΔO.D. 568 nm minus 558 nm

was measured and increasing amounts of antimycin were added.

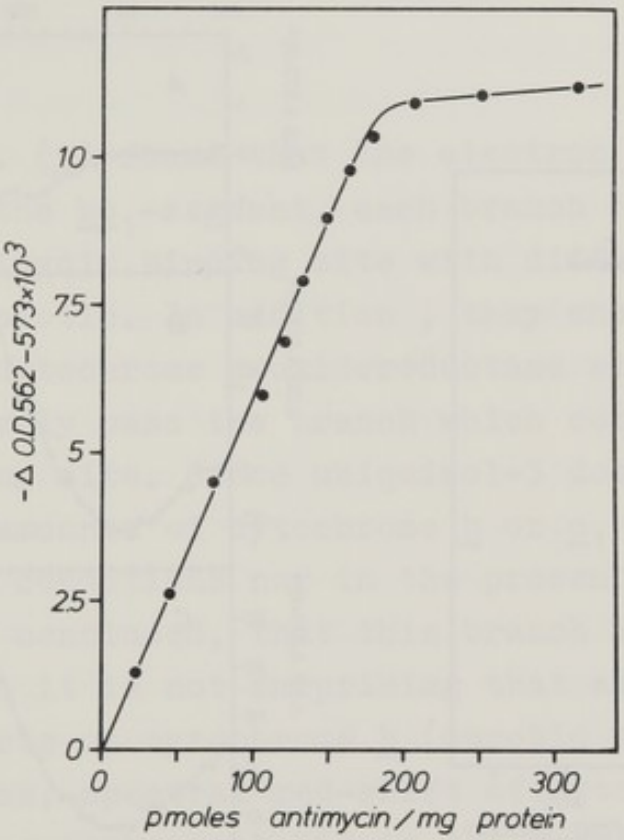


Fig.6 Titration of the oxidation of cytochrome b-562 with antimycin in the presence of substrate and electron mediators. The absorption difference at 562 nm minus 573 nm was measured in the presence of 17 mM succinate, 0.5 mM phenazine methosulfate and with increasing amounts of antimycin.

In contrast to the NADH- or succinate-oxidase duroquinol-oxidase activity is linearily affected by antimycin, as shown by Kröger and Klingenberg (9). This result holds for yeast mitochondria, too (not shown). The amount for 90% inhibition is 180 pmol antimycin per mg protein. When, however, the extrareduction of b-566 is titrated in the presence of duroquinol as substrate, the dependen-

cy on the antimycin concentration is non-linear in the KCN inhibited state (Fig.7).

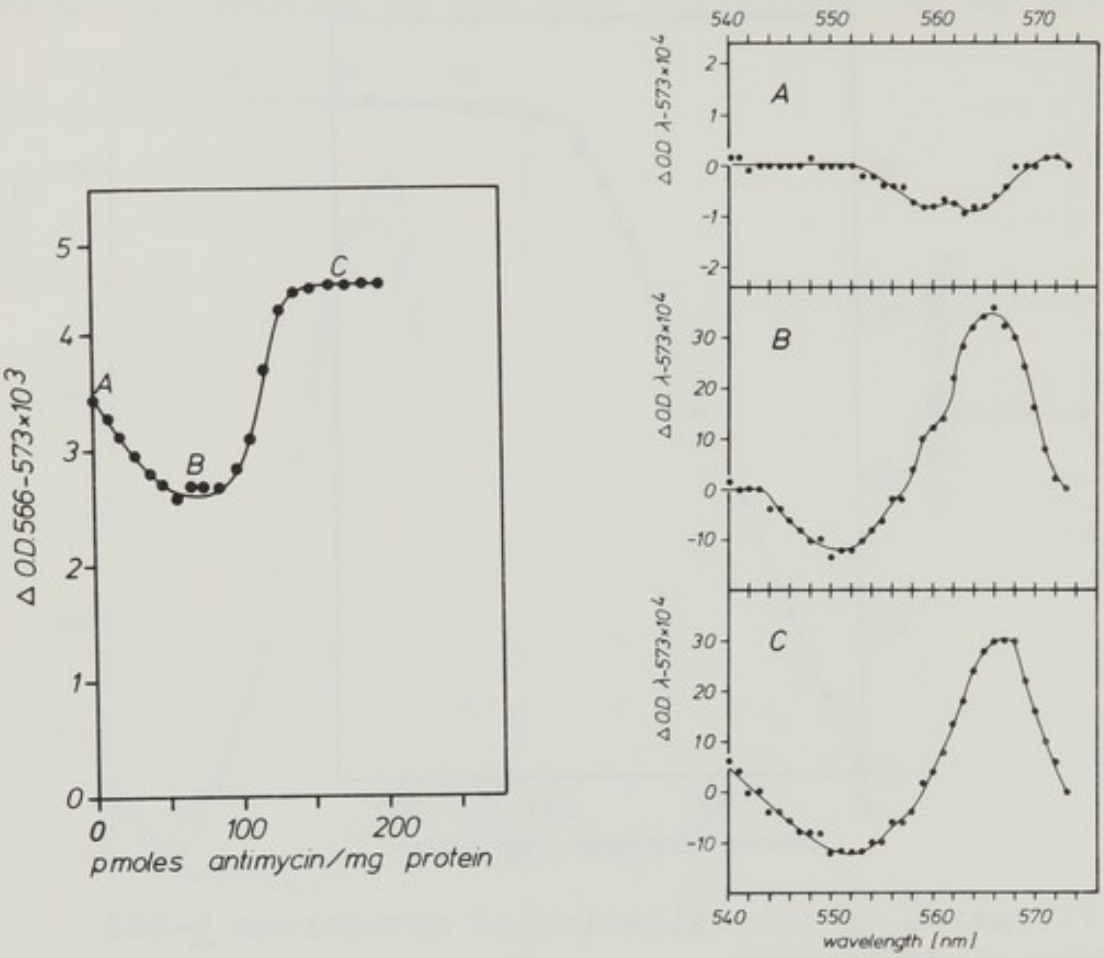


Fig.7 Antimycin dependent reduction of cytochrome b-566 with duroquinol as substrate. Duroquinone was reduced by the method of Rieske (14) exept that $NaBH_4$ was used as reductant after acidification with HCl. A, B, C points where spectra have been taken (Fig.8)

Fig.8 (right) Spectra to Fig.7. A: difference between points B and A. B: difference between points C and B. C: difference between points C and A.

From this figure it may be seen that cytochrome b is initially oxidized at low concentrations of antimycin despite the presence of KCN. At increasing concentrations of antimycin a reduction of cytochromes b-562 and b-566

is observed (Fig.8). The concentrations for maximal reduction are 180 pmol per mg protein.

DISCUSSION

Burger et al. (1) found that the electron transfer chain is split in the $\underline{bc}_1$-segment, each branch containing a separate antimycin binding site with different affinity for the antibiotic. In addition , they showed that in ubiquinol-3:cytochrome $\underline{c}$ oxidoreductase electrons almost exclusively pass the branch which contains the weaker binding site. Since ubiquinol-3 does not reduce substantial amounts of cytochrome $\underline{b}$ or $\underline{c}_1$ under aerobic staedy state conditions nor in the presence of antimycin or KCN, they concluded, that this branch is free of cytochromes. So it is not surprising that all antimycin dependent effects on cytochrome $\underline{b}$ (aerobic or anaerobic extrareduction, spectral red-shift of cytochrome $\underline{b}$-562, oxidation of cytochrome $\underline{b}$-562 in the presence of mediators) are fully developed after the saturation of only the strong binding site. This binding site must be arranged in or next to the branch containing the cytochromes. As antimycin induced effects on cytochrome $\underline{b}$ are governed by the stronger binding site only, non-linear effect curves of antimycin on cytochrome $\underline{b}$ (such as the enhanced reduction in the aerobic steady state or in the presence of KCN) are not caused by the sequential saturation of two sites. Furthermore electron flow is not a prerequisite for non-linear effect curves as they are found both in the presence (aerobic steady state) and absence (presence of KCN) of electron flow. The linear effect curves in the presence of substrate and KCN reported by Lee and Kröger (15) were obtained in the presence of ATP. Under these conditions

antimycin has almost no extra-reducing capacities, since ATP has already effected the reduction of the major portion of cytochrome b-566/558, and, therefore, the effect measured is mainly caused by the antimycin induced spectral red-shift of cytochrome b-562. Wikström and Berden (6) have proposed, that in the presence of antimycin the ubiquinol / semiquinone ratio is increased (increase in E_h), and this should cause the enhanced reduction of cytochrome b-566. After the addition of duroquinol (or ubiquinol-3, not shown) the enhanced reduction of cytochrome b-566 was observed only after the addition of antimycin, depending non-linearily on its concentration, although the quinol / semiquinone ratio might be increased under these conditions. This enhanced reduction of b-566 by duroquinol depends non-linearily on the antimycin concentration. The redox behaviour of b-566 parallels thus the inhibition of duroquinol-oxidase by antimycin via a KCN insensitive pathway in Neurospora crassa (16) in contrast to the KCN sensitive duroquinol oxidation which is linearily affected by the antibiotic (9). The incongruency in the shapes of antimycin effect curves of KCN sensitive duroquinol oxidation and duroquinol dependent cytochrome b reduction leads to the conclusion that the effects of antimycin on the electron flow and on the redox state of cytochrome b are governed by different mechanisms. The non-linear inhibition curve by antimycin of NADH oxidase activity may be quantitatively explained, as suggested by Kröger and Klingenberg (9,17) by the rate limitation of the electron transfer in the region of the dehydrogenases in combination with a mobile ubiquinone pool, whereas non-linear effects on the redox state of cytochrome b are obviously due to some mechanism intrinsic in the bc_1-segment (1,4,6,18). In the presence of substrate plus mediators and after complete

reduction with dithionite antimycin affects the b-cytochromes linearily as shown in this paper and by others (4). The linearity of the spectral red-shift is caused by the linear binding of antimycin to mitochondrial particles whereas the linearity of the oxidation effect has been interpreted by the bypassing of an " intermediate protein carrier " in the presence of phenazine methosulfate, which in the absence of mediators and in the presence of only natural substrates should be involved in the mechanism causing non-linear effect curves.(7)

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Characterization of Respiratory-Deficient and Antimycin-Resistant Mutants of Schizosaccharomyces pombe with Extrachromosomal Inheritance

B. Lang, G. Burger, K. Wolf, W. Bandlow, F. Kaudewitz

SUMMARY:

A series of respiratory-deficient mutants of S. pombe with extrachromosomal inheritance was characterized. None of them contains any cytochrome oxidase, and the content of cytochrome c and the number of b-type cytochromes is often reduced. Cytochrome b is only to a small extent reducible by substrate and is partially sensitive to CO. In mutant mit-25, which was characterized in detail, cytochrome b-566 is lost and therefore no extra-reduction by adding antimycin could be observed. The inhibition of electron transfer by antimycin, however, is about the same as in the parental strain [ANT ^{R8}]. The phenotype of the mit$^-$ mutants resembles in some respect that of respiratory-competent cells under glucose-repression. Therefore, the interpretation that a deficiency as found in mutant mit-25 is caused by a mutation in a structural gene of cytochrome a or cytochrome b , is not yet permissable.

INTRODUCTION

Mitochondrially inherited point mutations (or small deletions ?) (mit$^-$) have been studied very intensively in Saccharomyces cerevisiae in order to localize the markers for expression of mitochondrially coded components of respiratory chain and ATPase complexes (1-3). However, the biochemical analysis of respiratory deficient mutants may lead to misinterpretations, when the loss of certain mitochondrial enzymes is directly related to a genetic marker. As will be shown in this paper, regulatory processes in protein synthesis and membrane assembly make it often impossible, to decide merely by taking cytochrome-spectra what the primary lesion of the mutation is (4-6).

RESULTS AND DISCUSSION

[ANT^{R}8] is an antimycin-resistant mutant of the "petite--negative" yeast S. pombe , which shows extrachromosomal, probably mitochondrial inheritance of the resistance marker (7). From this strain it is possible to obtain spontaneously arising, extrachromosomally inherited respiratory-deficient mutants. Since revertants can be obtained from most of these mutants, they probably arise from point mutations of the mitochondrial genome (8). In 3% glucose complete-medium (9) eight different mit$^-$ strains were grown to the stationary phase. In order to characterize this type of mutant, whole cell cytochrome spectra were taken at low temperature (77°K) by comparing glucose- or dithionite-reduced cells with a reference oxidized by hydrogen peroxide (Figure 1). Each of the eight mutants has completely lost cytochrome oxidase and show altered b-type cytochrome absorption bands; some of the mutants contain almost no cytochrome c. Respiratory activity of the cells was between o.1 - o.4 % compared to [ANT^{R}8], and was completely insensitive to KCN.

In order to decide to what extent the phenotype of the mit$^-$ mutants depends solely upon growth conditions, they were grown on liquid medium with raffinose as a carbon source (which unlike glucose does not induce repression of cytochromes in respiratory-competent strains (1o)). However, a difference in the cytochrome content of mit$^-$ cells grown on glucose or raffinose was not observed, although their phenotype does resemble in some ways that of the parental strain [ANT^{R}8] cultivated under glucose repression. In Fig. 2 whole cell spectra of [ANT^{R}8] grown in the presence of various concentrations of glucose are compared : with high concentrations of glucose only part of cytochrome oxidase can be detected, the absorption maximum being shifted from 6o7 nm to 6o1 nm. Cytochrome b_{566} is absent and the content of the other cytochromes is decreased. With the same conditions or

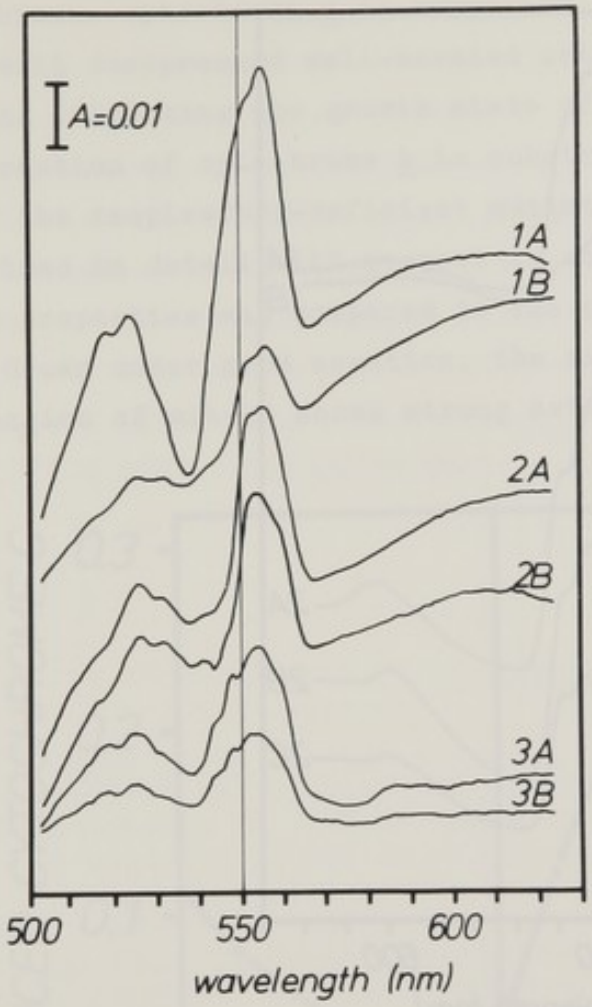


Figure 1 : Whole cell cytochrome spectra of several mit$^-$ strains

Three of the mit$^-$ strains with different phenotypes (Trace 1: mit-5 ; 2 : mit-19 and 3: mit-25) were either reduced with dithionite (A) or by 2 minutes preincubation with o.5% glucose (B). The titer of the samples was 5 x $1o^9$ cells per ml in a buffer containing o.7 M sorbitol plus 1o mM EDTA, pH 6 . The reference was oxidized by o.3 % H_2O_2 and immediately frozen in liquid nitrogen. Difference spectra were taken at 77°K from the reduced minus oxidized probes in a Cary 17. The light path was 2 mm.
The absorption maxima of cytochromes in S. pombe expected under these conditions (4, 12, 13) are:

cytochrome a, a_3	:	6oo - 6o7 nm
cytochromes b	:	553.5 - 561 nm
cytochrome c_1	:	55o nm
cytochrome c	:	547.5 nm, 544 nm and 519 nm

Only in trace 3 (mit-25) cytochrome c is clearly detectable.

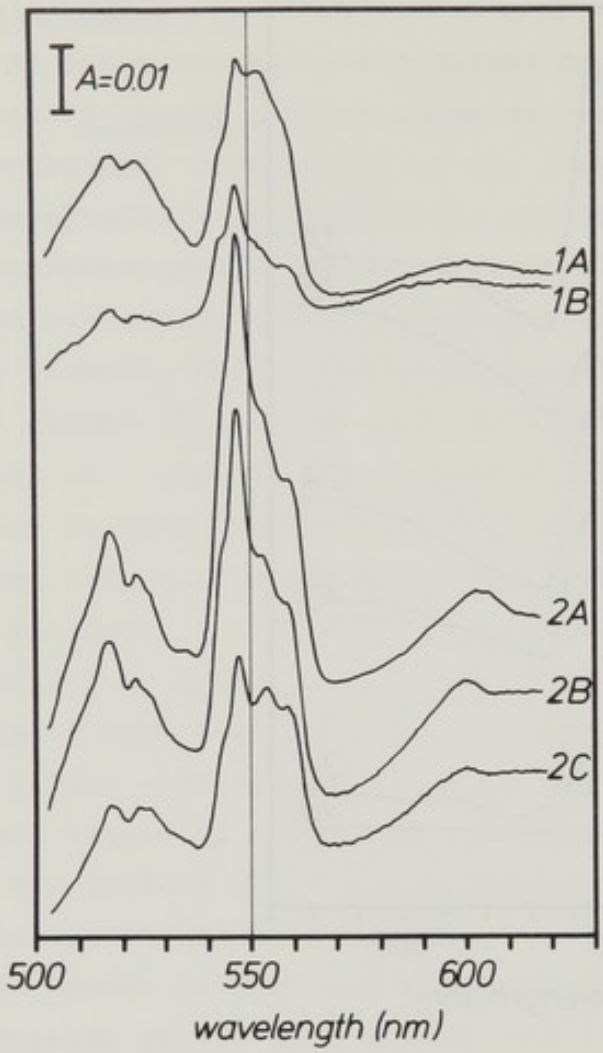


Figure 2 : Whole cell cytochrome spectra of [ANT^{R}8] under various conditions of glucose repression

In experiment (1), cells were grown in liquid medium containing 3 % glucose without aeration until the glucose was consumed. Spectra were performed as described in the legend of Fig. 1 . (1A : reduced by dithionite; 1B : reduced by glucose)
In experiment(2) a culture was grown in liquid medium containing 6 % glucose with good aeration. Spectra were taken from cells of the same culture, harvested at different times (2A : 1h after the glucose had been consumed; 2B : 2 % glucose present and 2 C : 5 % glucose present). The titer of the cells was adjusted to 4 x 10^{9} cells per ml in all cases. In the second experiment there was no significant difference between spectra of glucose- or dithionite-reduced cells (the latter being shown above).

by anaerobiosis cytochrome c is repressed to more than 5o % compared with derepressed well-aerated cells. In anaerobically grown cells (mimicking the growth state of mit$^-$ cells), only a small fraction of cytochrome b is substrate-reducible.

One of the respiratory-deficient mutants, mit-25, was characterized in detail with respect to enzymatic and spectroscopic properties and compared to the parental strain [ANT^{R}8]. Grown under good aeration, the mitochondrial membrane fraction of mit-25 shows strong heterogeneity with

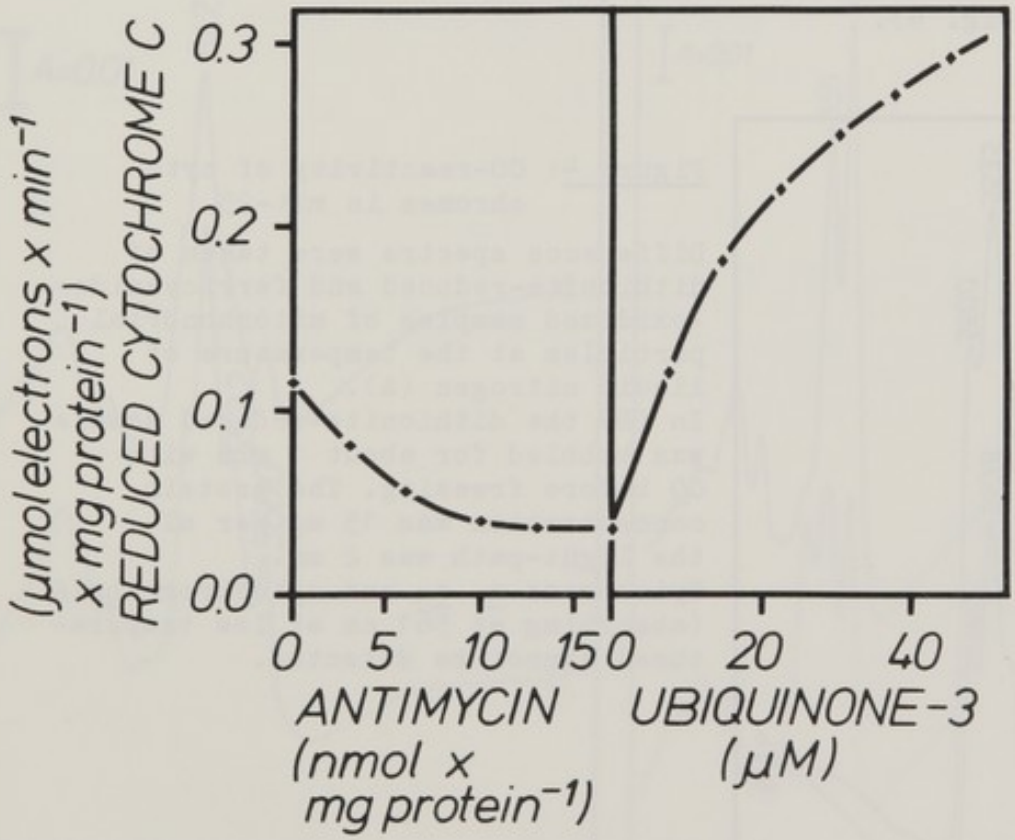


Figure 3: Inhibition of NADH : cytochrome c oxidoreductase by antimycin and restoration of the activity by competition with ubiquinone-3 in mitochondria from mit-25

The enzyme-test was performed at 25°C as described earlier (12), except that the addition of KCN proved to be negligable. Antimycin and ubiquinone-3 were added in ethanolic solution. The specific activity before the addition of antimycin was increased by 26 μM ubiquinone-3 from o.12 to o.6 (not shown in the picture).

respect to density (in a urografin gradient; compare (11)) and enzymatic activities in contrast to the wild-type and [ANT^{R}8]. The lighter fraction (between 15 and 18 % urografin) has about 7o % of succinate: and NADH: cytochrome c oxidoreductase activity compared to [ANT^{R}8]. Both, the sensitivity to antimycin and the competition of ubiquinone-3 and antimycin resembles those in [ANT^{R}8] (13). The results concerning mit-25 are shown in Fig. 3 .

In the light mitochondrial membrane fraction about 3o % of the b-type cytochromes cannot be reduced by substrate and are CO-reactive (Fig. 4).

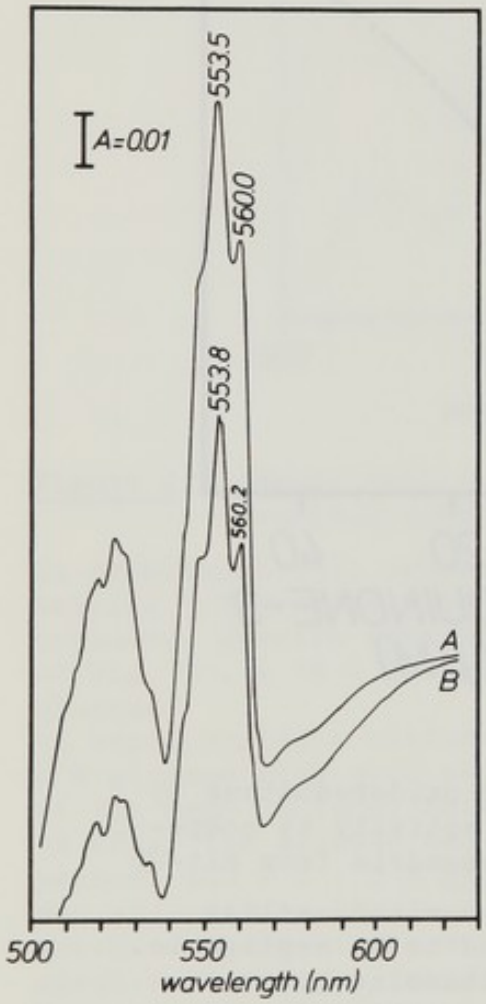


Figure 4: CO-reactivity of cytochromes in mit-25

Difference spectra were taken of dithionite-reduced and ferricyanide--oxidized samples of mitochondrial particles at the temperature of liquid nitrogen (A).
In (B) the dithionite-reduced sample was bubbled for about 1 min with CO before freezing. The protein concentration was 15 mg per ml , the light-path was 2 mm.
Cytochrome a, a_3 and cytochrome b566 (absorbing at 561 nm at low temperature) cannot be detected.

This means that the spectrally intact b-type cytochromes (7o%) are functional (with respect to their enzymatic properties). That cytochrome b_{566} is lost is even more illustrated by the fact that in mit-25 after the addition of antimycin and oxygen in the presence of substrate no cytochrome b is extrareduced. Neither adding ferricyanide nor a small concentration of wild--type mitochondria (to guarantee electron deflux from cytochrome c , which is a prerequisit for extra-reduction) was of any success (Fig. 5).

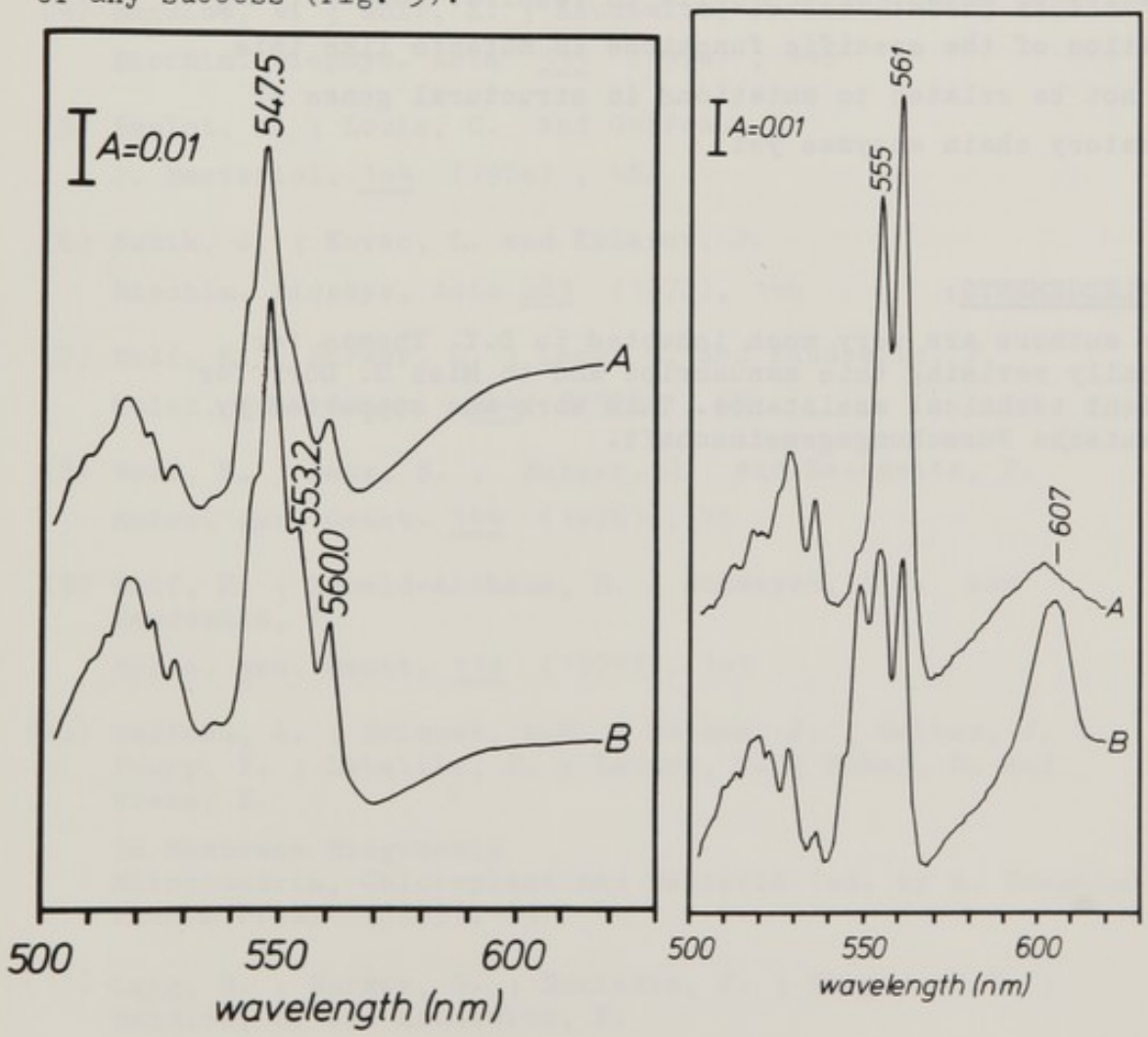


Figure 5: Extra-reduction of b-cytochromes in mit-25 and $[ANT^{R}8]$
Spectra were performed as described in the legend of Fig.4 , except that mitochondrial particles were reduced by NADH after an addition of 2 µM antimycin (A) or by NADH only (B). The left picture shows spectra of mit-25, indicating no extra-reduction but rather little oxidation of b-cytochromes. The right figure shows $[ANT^{R}8]$ for comparison (13). The protein concentration was 15 mg/ml (mit-25) and 6.9 mg/ml ($[ANTR_8]$).

The lack of cytochrome $\underline{b}_{566}$ does not considerably lower the enzymatic activity of the $\underline{bc}_1$ -segment, since the enzymatic activity of NADH : cytochrome $\underline{c}$ oxidoreductase is about the same in mit-25 as it is in [$ANT^{R}8$], each being stimulated by ubiquinone-3. The only enzymatic deficiency that could be found by enzyme tests as well as by spectroscopy in all mit$^-$ mutants is the absence of cytochrome oxidase, meaning that only the lack of this functional enzyme complex causes the incapacity of mutant mit-25 cells to respire. However, the alteration of the specific functions in mutants like this could not be related to mutations in structural genes of respiratory chain enzymes yet.

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Models for Antimycin Binding in Wild-Type Candida Utilis and Antimycin-Resistant Mutants

C.J.P. Grimmelikhuijzen*, E.C. Slater

SUMMARY

1. An attempt has been made to choose between two alternative models, A and B, for antimycin binding and action previously proposed (Grimmelikhuijzen, C.J.P., Marres, C.A.M. and Slater, E.C. (1975) *Biochim. Biophys. Acta* 376, 533-548).

2. We found that both wild-type and mutant submitochondrial particles contain such a high concentration of "aspecific" low-affinity sites, that it was difficult to use binding studies to make a choice between the two models mentioned above.

3. Submitochondrial particles of mutants 28, 29, 30 and 31 differ considerably in their resistance to the inhibitory action of antimycin. This excludes model B.

4. Mutant 31 is the most resistant of the mutants tested: up to 300 times more antimycin must be added to inhibit the respiration of submitochondrial particles by 50% when compared with the wild type.

5. A model is presented in which, in the wild type, a single antimycin molecule binds with its alkyl and acyl chains to the site affecting cytochrome *b* and with its phenolic part to the site catalytically active in electron transfer.

INTRODUCTION

Antimycin-resistant mutants of the yeast *Candida utilis* have been isolated and investigated by Butow and Zeydel [1] and Grimmelikhuijzen and coworkers [2-4]. In all mutants, the lesion resides in the mitochondrial respiratory chain. No pleiotropic effects have been detected: the cytochrome content and the activities of electron transfer and of phosphorylation are similar in wild type and mutants [2-4]. Mutants of this type could conceivably provide two types of information: (i) A changed antimycin-binding site might be identified by comparing, with the appropriate analytical method, the subunits of mutant Complex III with those of the wild type. A comparison using SDS-gel electrophoresis, however, demonstrated that the antimycin-binding site is not altered significantly in size (<300-600 dal-

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tons) by the mutation [4]. (ii) A changed antimycin-binding site might also be reflected in a change in the properties of Complex III. By studying this change, more information might be obtained on the structure and functioning of Complex III and on the action of antimycin.

Indeed, a difference in the behaviour of cytochrome *b* to treatment with antimycin was observed. Concentrations of antimycin that had no effect on electron transfer were found, in the presence of oxidant, to induce the reduction of cytochrome *b* in the mutants [3,4]. This finding is difficult to reconcile with explanations of the action of antimycin that imply an obligatory link between inhibition of electron transfer and reduction of cytochrome *b*.

The antimycin titres for other effects on cytochrome *b*, namely the reduction of cytochrome *b* in the presence of substrate, cyanide and oxygen (the so-called "extra" reduction), and the shift and lowering of E'_0 of cytochrome *b*-562 are also, in the mutant, much lower than for the inhibition of electron transfer, although this titre is somewhat higher than in the wild type [3,4]. Moreover, the titration curve of electron transfer is hyperbolic, whereas those for the reduction are sigmoidal [3,4]. It seems, then, that the effects of antimycin on cytochrome *b* and on electron transfer are two separate processes in the mutant. This implies that they are also separable in the wild type. In a previous paper [3], two alternative possibilities were given for antimycin binding and action in wild type and mutant. One possibility is that two binding sites are present in the wild type, one involved in the inhibitory action on electron transfer and the other in the action on cytochrome *b*, with equal high affinity to antimycin. The mutation causes changes in both binding sites, such that the effects on cytochrome *b* become only a little less sensitive to antimycin, but electron transfer becomes very resistant to the inhibitor (see Fig. 1A; cytochrome *b* has been drawn out of the main respiratory chain because it can be reduced without inhibiting electron transfer in Complex III [3,4]). The alternative explanation (Fig. 1B) is that there is only one high-affinity binding site in the wild type affecting both electron transfer and cytochrome

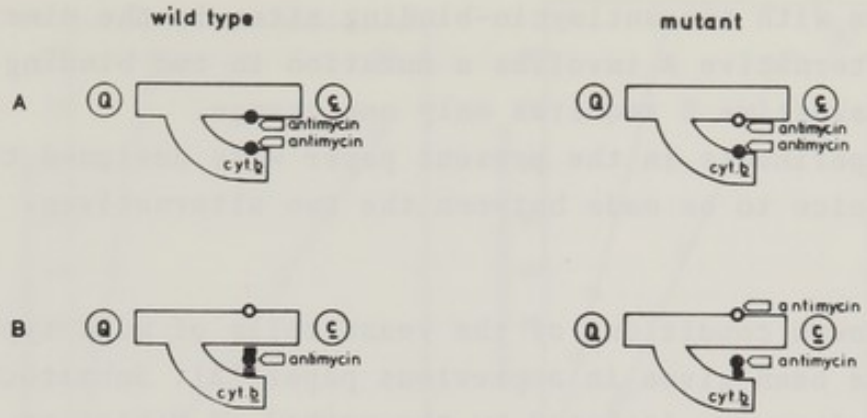


Fig. 1. Two alternative explanations for antimycin binding and action in wild type and the changes induced by the mutations. •, High-affinity site, o, low-affinity site. (A) Two binding sites in the wild type for action on electron transfer and cytochrome *b*, respectively, with equal high affinity to antimycin. Both binding sites are affected by the mutation. (B) One binding site in the wild type, with a high-affinity, where antimycin has an action on both cytochrome *b* and electron transfer. There is also a second site present, with low affinity to the antibiotic, where electron transfer can be blocked. Only one binding site is affected by the mutation. From ref. 4.

b. This simultaneous action occurs by means of a conformational change induced by the antibiotic (cf. refs 5,6). By the mutation the binding site is changed, resulting in the somewhat lowered sensitivity to antimycin and, more important, in the disappearance of the interactions between the region of the antimycin-binding site and the electron-transfer chain in Complex III, so that antimycin affects cytochrome *b* without inhibiting respiration. Inhibition by high concentrations of antimycin is explained by some other, weak, antimycin-binding site that is also present in the wild type.

The second alternative (alternative B) is the simpler and for this reason was preferred in our previous paper [3]. It is rather unlikely, as postulated in alternative A of Fig. 1, that two separate binding sites would have identical antimycin titres, antimycin-binding constants and sigmoidal antimycin-titration curves. Moreover, model A would imply a stoicheiometry of 2 strong antimycin-binding sites per molecule of Complex III, which conflicts with the experimental finding of a 1:1 ratio [7], unless we assume that this complex exists in a

dimeric form with two antimycin-binding sites in the dimer. Finally, alternative A involves a mutation in two binding sites, whereas alternative B requires only one change.

The experiments in the present paper were designed to enable a choice to be made between the two alternatives.

METHODS

The growth conditions of the yeast cells of wild type and mutants have been given in a previous paper [2]. Submitochondrial particles were isolated by the method of Mahler et al. [8]. Their fractions R_2L and R_2H were combined.

Respiratory activities were measured at 25°C in a thermostated Oxygraph vessel supplied with a Clark electrode.

Fluorescence was measured in an Eppendorf fluorimeter. The cell compartment was kept at 24°C with a thermostat. A primary filter of 313 + 316 nm and a secondary filter of 420-3000 nm were used.

Protein was determined by the biuret method of Cleland and Slater [9]. All chemicals were commercial products. Antimycin was obtained from Nutritional Biochemicals Corporation and dissolved in methanol. Its concentration was determined spectrophotometrically, using an absorption coefficient at 320 nm of 4.8 $mM^{-1} \cdot cm^{-1}$ [10].

RESULTS

The two alternative possibilities discussed in the Introduction could be tested, in principle, by antimycin-binding experiments. According to alternative A, two indistinguishable binding sites should be found in the wild type with equal high affinity to antimycin (measured as a single site) and in the mutant one high- and one low-affinity binding site, whereas according to alternative B one binding site with high affinity and one with low affinity should be measured in both wild type and mutant.

In Fig. 2A, a binding experiment is shown with submitochondrial particles of the wild type suspended in a medium with a lower concentration of serum albumin than used in previous work [2], in order better to reveal low-affinity sites. The

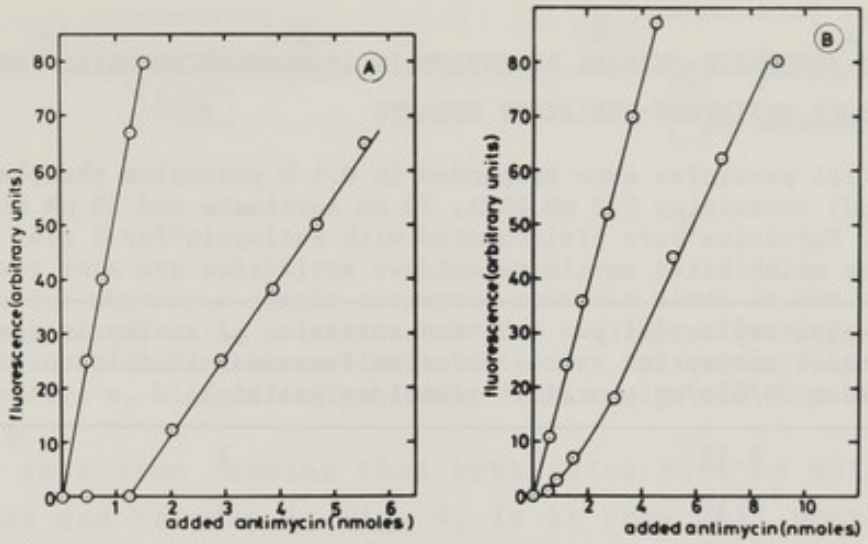


Fig. 2. Binding of antimycin to submitochondrial particles of wild-type yeast and mutant 28. The particles were suspended in 2 ml of a medium containing 250 mM sucrose, 20 mM Tris-HCl buffer, 1 mM EDTA and 1 mg/ml bovine serum albumin at pH 7.5. Antimycin was then added as a methanol solution. The binding was measured following the procedure of Berden and Slater [6], slightly modified as described in ref. 2. The fluorescence of the supernatant after spinning down the particles is shown in the lower trace of each figure. The upper trace represents supernatants to which antimycin was added after removal of the particles. A. Binding to wild-type particles, 4.1 mg/ml protein. B. Binding to particles of mutant 28, 3.1 mg/ml protein.

curve in Fig. 2A consists of two parts: at low concentrations of antimycin, all inhibitor is fully bound to the high-affinity site (cf. Fig. 4A, ref. 2 where antimycin binding is performed in the presence of high albumin concentration); when the high-affinity site has been saturated, a straight line is obtained but this is not parallel to the curve obtained in the absence of particles, as it is at higher albumin concentrations (see Fig. 4A, ref. 2). This indicates that antimycin binds to other sites with a relatively low affinity, and that the concentration of these aspecific sites is high. A similar result was found with particles of mutant 28 (Fig. 2B). The presence of a high concentration of aspecific sites makes it impossible to use the binding studies to make a choice between the two alternatives.

A second method of distinguishing between the alternatives A and B is to compare different mutants. According to model B different mutants must have the same resistance to the inhibitory action of antimycin, whereas according to model A this resistance can vary. The results given in Table I show clearly

TABLE I

INHIBITION OF SUCCINATE OXIDASE BY ANTIMYCIN IN SUBMITOCHONDRIAL PREPARATIONS OF VARIOUS ANTIMYCIN-RESISTANT MUTANTS

Submitochondrial particles were suspended in 0.1 M potassium phosphate buffer (pH 7.0) containing 0.2 mM EDTA, 20 mM succinate and 20 μM bovine cytochrome *c*. Particles were preincubated with antimycin for 5 min. For comparison the uninhibited succinate oxidase activities are also given.

Mutant	Respiratory activity without antimycin (μatom O/min/mg protein)	Concentration of antimycin required for half-maximal inhibition (nmol/mg protein)
28	0.32	2
29	0.59	5
30	0.49	6
31	0.20	30

that the mutants do vary in their resistance to antimycin.

Mutant 31 is the most insensitive: when compared with wild-type particles, 300 times more antimycin must be added to inhibit succinate oxidase by 50% and 1000 times more to inhibit by 80%. These data show that Model B is untenable and favour Model A. However, in view of the objections to this model mentioned in the Introduction, alternative explanations were sought. Indeed, in addition to Model A with *two* high-affinity sites for *two* antimycin molecules and Model B with *one* high-affinity site for *one* antimycin molecule, the logical possibility exists of *two* high-affinity sites, where *one* antimycin molecule binds (Fig. 3). Most of the objections against Model A are no longer applicable to this third model.

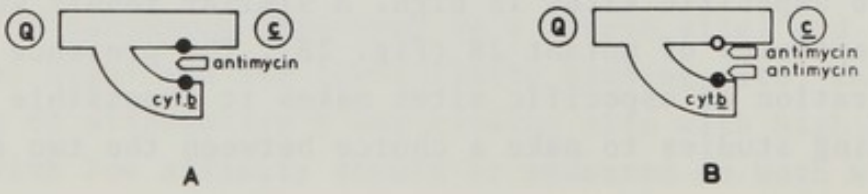


Fig. 3. A third alternative for antimycin binding in wild type (A) and mutant (B). •, High-affinity site; o, low-affinity site. For further details, see text.

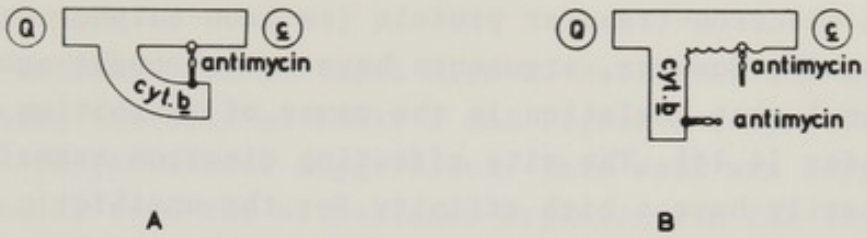


Fig. 4. A model whereby a single antimycin molecule binds in the wild type to both the site affecting electron transfer and that affecting cytochrome *b* (A), but in the mutant two antimycin molecules are required to bind to those sites (B). •, high-affinity binding site; o, low-affinity binding site.

The objection remains that both sites must be mutated. However, as can be seen in Fig. 4, it it very well possible that only one change has occurred. It is suggested that in the wild type (see Fig. 4A) the antibiotic binds with its apolar tails (acyl and alkyl side chains [11]) and perhaps the dilactone ring [12] (see Fig. 5) to the high-affinity site affecting cytochrome

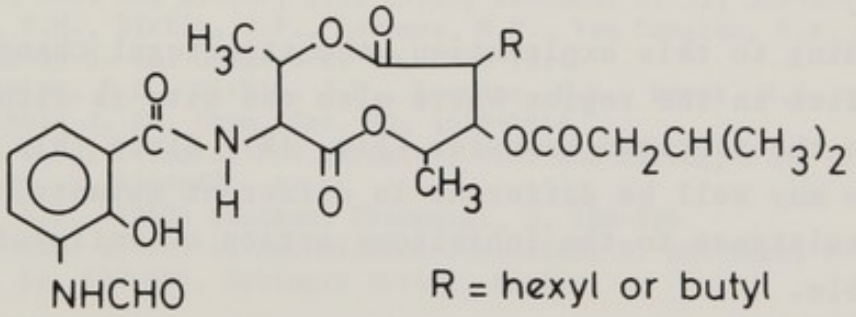


Fig. 5. The structure of antimycin [12].

b and with its phenolic part [13] to that site catalytically active in electron transfer. The choice of the phenolic head of antimycin as the inhibitor of electron transfer is quite arbitrary, although it is in line with Tappel's [13] suggestion that the phenol exerts its inhibitory action by chelating with the

iron of an electron-transfer protein (an iron-sulphur protein was suggested). However, arguments have been brought against this proposal that chelation is the cause of inhibition of electron transfer [4,14]. The site affecting electron transfer need not necessarily have a high affinity for the antibiotic, since, according to the model, antimycin, when bound with its apolar tails to the high-affinity site affecting cytochrome *b* is now in a favourable position to be attached to the other site of the complex. The mutation causes a structural change in such a way that antimycin no longer fits snugly between the two sites (see Fig. 4B). The site that affects cytochrome *b* still binds the tails (and the dilactone ring) of an antimycin molecule with high affinity. The site where electron transfer is inhibited can bind the head of a different antimycin molecule but with a low affinity. According to this model it is understandable that the site affecting cytochrome *b* in the mutant will have a somewhat lower affinity for the antibiotic than that in the wild type [3,4], since it would be energetically more favourable for antimycin to be clamped by its head and tail, than only to be held by its tail.

According to this explanation, the structural change in the mutant lies in the region where also the site is situated that affects the electron transfer (Fig. 4B). Since this structural change may well be different in different mutants, the differing resistance to the inhibitory action of antimycin is understandable.

It would be worthwhile, according to the model of Fig. 4, to test antimycin analogues. If the site affecting electron transfer binds, in the wild type, antimycin with low affinity, low concentrations of antimycin analogues with shortened acyl and alkyl chains would be expected to reduce cytochrome *b* in the wild type with no inhibition of electron transfer, i.e. have effects similar to that of antimycin in the mutants. On the other hand, analogues with lengthened acyl and alkyl chains might give effects in the mutant that are similar again to those in the wild type.

ACKNOWLEDGEMENTS

The authors wish to thank Miss P. Hoefakker and Mr W. Edam for obtaining the data of Table I and Figure 2 respectively and Mr G. van Ark for useful suggestions. This work was supported in part by grants from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under auspices of the Netherlands Foundation for Chemical Research (S.O.N.).

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The Inhibitory Effect of HQNO* Compared with Extra-reduction and Binding in the Wild-Type and in an Antimycin-Resistant Mutant, [ANT^R 8], in Schizosaccharomyces pombe

G. Burger, B. Lang, K. Wolf, W. Bandlow, F. Kaudewitz

SUMMARY

The wild-type of S. pombe and a mutant, [ANT^{R}8], which is in vitro resistant to antimycin, exhibit similar behaviour in response to HQNO (i) in growth rate, (ii) in mitochondrial electron transport, (iii) in inducibility of extrareduction of cytochrome b$_{566}$ in the presence of substrate and oxygen and (iv) in the binding to oxidized mitochondrial particles. However, with regard to antimycin, the wild type and this mutant differ considerably when compared by these tests. Therefore, the inhibitory mechanism of antimycin and HQNO cannot be identical, which is further demonstrated by spectroscopy of the b-cytochromes in the presence of the drugs.

INTRODUCTION

It has been stated (e.g. 1) that in beef heart mitochondria HQNO acts at the same site of the bc$_1$-segment of the respiratory chain as antimycin. Unlike antimycin, HQNO does not cause a shift of the b-cytochrome absorption maxima neither does it induce conformational changes in the respiratory chain in beef heart. Therefore, HQNO was said to be an inhibitor with a less complicated action than antimycin, which could be used instead of antimycin.

However, our results in yeast contradict the reported properties of HQNO. It is shown in this paper that its site of action is not identical with that of antimycin. Because of their differing effects on the bc$_1$-segment both inhibitors are valuable for studies of the respiratory chain.

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* HQNO = 2- heptyl -4-hydroxiquinoline -N-oxide (Sigma)

RESULTS AND DISCUSSION

The mutant [ANT^{R}8] and the wild type are affected in growth -rate by HQNO in the same manner. About 2 µg inhibitor per ml medium doubles the generation time (in the presence of 2o µg sodium dodecylsulfate per ml, facilitating diffusion of the drug into the cells without itself reducing the growth rate) on glycerol complete medium (2).

As is expected from inhibition of growth, in isolated mitochondria the inhibitory effect of HQNO on electron transport is much less compared to that of antimycin. For halfmaximal inhibition of NADH oxidase about 5o nmol inhibitor per mg mitochondrial protein is necessary in the wild type and in [ANT^{R}8]. This value is also found in different strains of Saccharomyces cerevisiae and Neurospora crassa. Since no significant difference between the inhibition curves of the NADH oxidase of S. pombe wild type and [ANT^{R}8] can be detected (Fig. 1 A, B), further inhibition parameters were tested.

From studies with antimycin we know that in the presence of NADH plus succinate the rate of electron transport is lowered by 5-1o % compared with NADH only, and the sensitivity to the inhibitor is enhanced. This effect is more pronounced in [ANT^{R}8]. Both substrates together also intensify the inhibition by HQNO to the same extent in mutant and wild type (3) (Fig. 1 A, B). A competition between HQNO and added ubiquinone-3 , as reported for antimycin and ubiquinone-3 in the antimycin resistant mutant (2), was also observed in both strains (Fig. 1 A, B). With increasing concentrations of ubiquinone-3 the inhibitory effect of HQNO is decreased.

HQNO, like antimycin, induces an additional reduction of cytochrome b in the presence of succinate or NADH in KCN-treated mitochondria or after anaerobiosis. Unexpectedly, 5o % of the possible extrareduction was already reached after addition of 1.5 nmol HQNO per mg protein with succinate plus NADH as substrates, which is 1/3o of the concentration needed for the same degree of inhibition of respiration (compare Fig. 2).

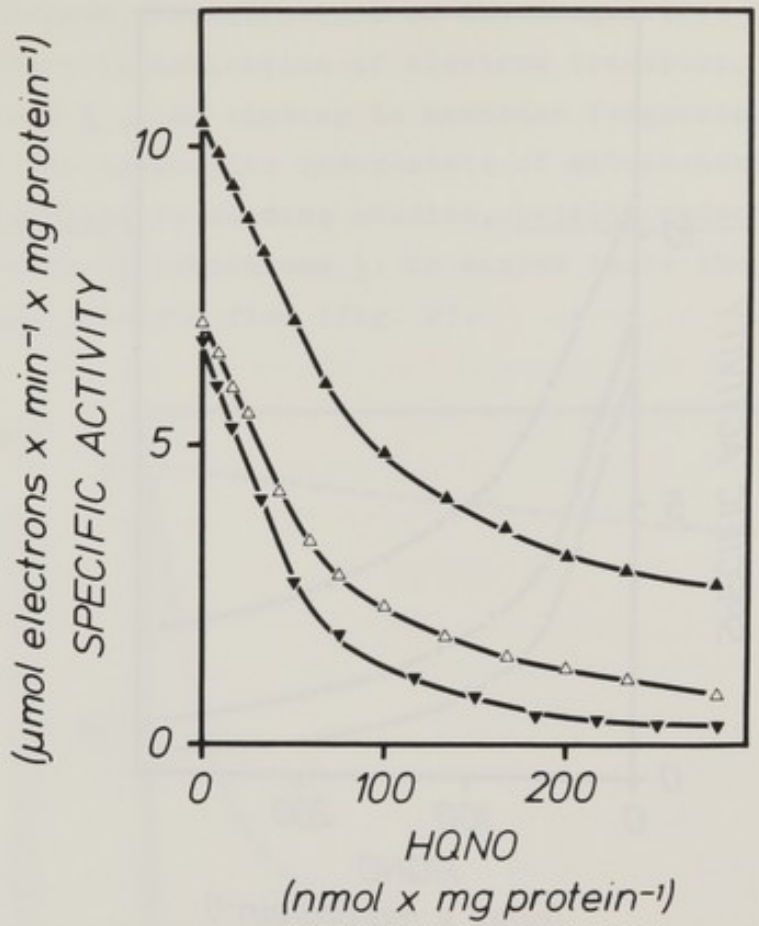


Fig. 1 A: Inhibition of respiration by HQNO in the wild type

The substrates were 1 mM NADH (△); 1 mM NADH plus 5 mM succinate(▼) and 1 mM NADH plus 26 µM ubiquinone-3 (▲).
The O_2-consumption was measured with a Clark type electrode at 25°C in a buffer containing o.1 M potassium phosphate, 1 mM EDTA and o.1 mM cytochrome c (horse heart; Boehringer). The pH was 7.4 . The protein concentration (determined by the Lowry - method) was 5o µg per 2 ml test-volume. HQNO and ubiquinone-3 were added as ethanolic solutions.

Also the extrareduction can be almost totally reversed in both strains by addition of 5o µM ubiquinone-3 (for details see reference 4).

The binding of HQNO to oxidized mitochondrial particles was much more specific than would be expected even from the extrareduction experiments. There was no significant difference between wild type and mutant in the binding parameters (TABLE I).

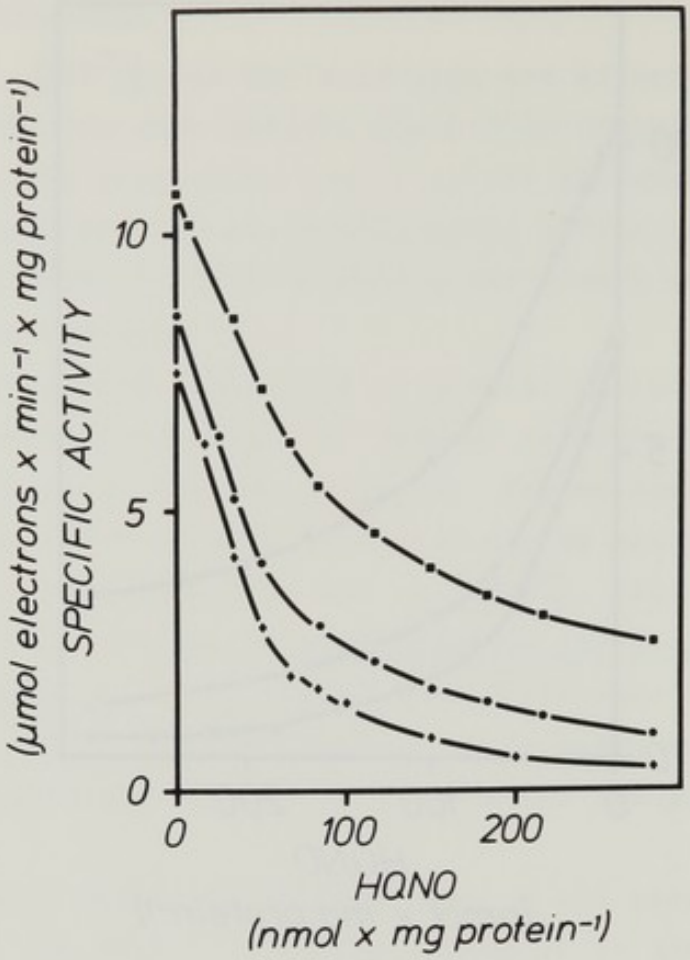


Fig. 1 B : Inhibition of respiration by HQNO in [ANT^{R}8]

The substrates were 1 mM NADH (●); 1 mM NADH plus 5 mM succinate (♦) and 1 mM NADH plus 26 µM ubiquinone-3 (■). The protein concentration was 3o µg per 2 ml buffer. For experimental details see legend to Fig. 1 A .

TABLE I : Binding parameters of HQNO to oxidized mitochondrial particles

strain	concentration of binding sites (pmol/mg protein)	dissociation constant
wild type	13o - 16o	3 - 8 x 1o^{-8}
[ANT^R 8]	1oo - 15o	2 - 8 x 1o^{-8}

A description of the method which is analogous to antimycin binding experiments in the absence of serum albumin was given earlier (6). Binding parameters were calculated by a computer program devised by others (5). The variation of the parameters is due to the individual preparations of mitochondria. Unspecific binding to the membranes occured in a concentration and with a dissociation constant similar to that found with antimycin (6) .

The different concentrations of the drug needed to give a comparable effect in inhibition of electron transport, extrareduction of cytochrome b , and binding to membrane fragments, are obviously related to the respective redox-state of mitochondrial particles: they are oxidized in binding studies, totally reduced during extrareduction of cytochrome b; in enzyme tests they are in steady-state electron flow (Fig. 2).

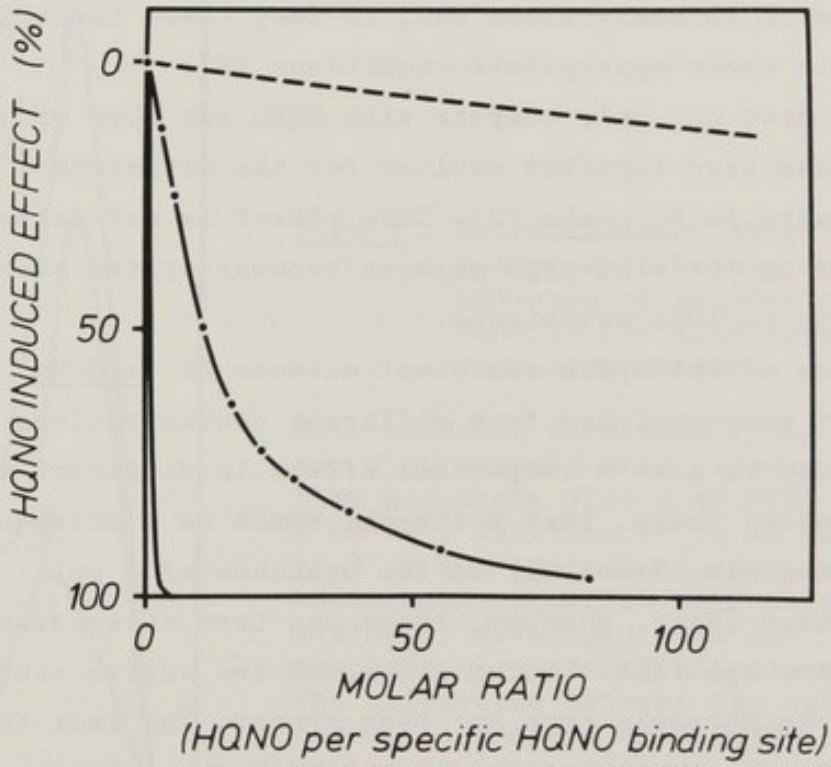


Fig. 2 : Efficiency of HQNO in binding, extrareduction and inhibition of respiratory activity

The degree of saturation of the specific binding site (———), of expression of extrareduction of cytochrome b-566 (-·-·-) and of inhibition of NADH oxidase (- - -) is plotted against the concentration of HQNO related to the concentration of the specific binding site.
Since HQNO is not only bound to a high affinity site, but also non-specifically to the membrane material, it is necessary to calculate from the binding parameters, to which degree the specific site is saturated related to the concentration of inhibitor added. This was achieved by a computer program following the graphical method of calculation of binding parameters (8,9) . 9o % saturation of the specific binding site is obtained with a molar ratio of about 2 as is indicated in the figure.

In order to resolve this discrepancy, we postulate that a redox component with a short half-life is generated during electron flow and competes with HQNO. It should be also present, however in low concentrations in reduced mitochondrial particles. Since synthetic ubiquinone reverses the effects of HQNO, the most likely candidate for a competitor with those properties is the semiquinone form of the endogenous ubiquinone-6 , a component with a similar structure to HQNO. A stabilzed form of ubisemiquinone has, in fact, been identified in mitochondria under appropriate conditions (7).

Ubiquinone does not only compete with HQNO but also with antimycin as has been reported earlier for the antimycin resistant mutants in S. pombe (2). This effect is not detected with antimycin in the wild-type strains because of the much higher affinity to this antibiotic.

From studies of antimycin resistant mutants of Candida utilis (1o) it was concluded from different concentrations of antimycin needed to give a comparable effect in different tests of the respiratory chain, that antimycin would be a divalent inhibitor acting simultaneously on two branches of a split electron transfer chain. However, there has been no evidence reported for two specific binding sites,and two active centers of the antimycin molecule have not been proved. The fact that different antimycin concentrations are needed for inhibition of electron flow to oxygen and of cytochrome b - reduction after anaerobiosis in whole cells of the mutants of Candida suggests rather a competition of endogenous ubiquinone and antimycin in this organism, too .

A difference between the action of HQNO and antimycin is also found in cytochrome b - spectra. In the presence of KCN, NADH and succinate the highest level of substrate reduction of the b-cytochromes is obtained in uncoupled mitochondria. Addition of antimycin causes, then, an additional reduction of cytochrome b - species with an absorption maximum at 565.5 nm (at room temperature) which is higher than that obtained with

HQNO under the same conditions. The difference between these two spectra exhibits a maximum at 564 nm, which cannot simply be explained by the fact that the b-cytochrome absorption maximum is shifted after addition of antimycin but not by adding HQNO (Fig. 3). The shifted spectrum in the presence

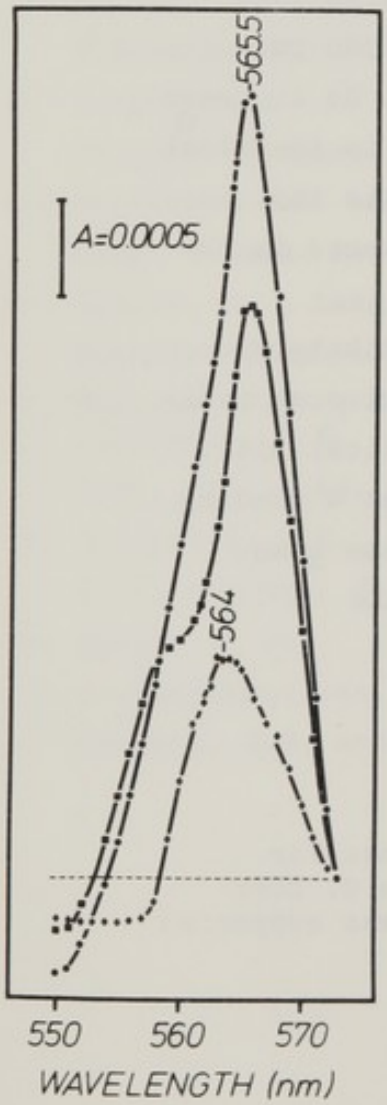


Fig. 3 : Extrareduction spectra of b-type cytochromes in the presence of antimycin and HQNO in the wild type at room temperature

Mitochondrial particles (5 mg protein per 3 ml buffer) were reduced by 5 mM succinate plus 1 mM NADH plus 2 mM KCN. Addition of 3o µM HQNO (■) or 3 µM antimycin (●) gives an additional reduction, which is maximally developed with the concentration of inhibitors, used. The difference between the extra-reduction spectra with HQNO and antimycin is a spectrum (♦) with a maximum at 564 nm.

of antimycin is much smaller and consists of a minimum and a maximum (11), while such an effect is not seen with HQNO. So under these conditions antimycin causes the reduction of more b-type cytochrome than HQNO, at least part of which is different from cytochrome b_{566}.

Now it remains to be explained, why HQNO and antimycin compete with ubiquinone, though the antimycin resistance of [ANT^{R}8] does not enable the mutant to be also resistant to HQNO. The simplest explanation is that HQNO and antimycin bind to different sites. However, it has been published earlier (6), that there exist two different binding sites for antimycin. Comparing the concentrations of HQNO binding sites (1oo - 16o pmol per mg protein) and antimycin (25o - 3oo pmol per mg protein) a ratio of about 1 : 2 is found. So it seems to be possible that one antimycin binding site is identical with the HQNO binding site in the wild type. This implies that this common binding site should not be altered in the mutant.

The competition between ubiquinone and the inhibitors or between HQNO and antimycin (1) are not an unequivocal indication of their competetive binding at an identical site. If this was so, one would have to postulate that a binding site of ubiquinone is situated between cytochrome b and cytochrome c_1 as is that of HQNO and antimycin.

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The Binding of 2-n-Heptyl-4-hydroxyquinoline-N-oxide to Submitochondrial Particles and Inhibition of Electron Transport

J.A. Berden, G. van Ark

There have been in the past and there still are problems concerning the interpretation of the data on the inhibition of respiration by antimycin. In this contribution we shall try to show that some of these problems can be resolved by using the inhibitor 2-*n*-heptyl-4-hydroxyquinoline-N-oxide (HQNO).

It seemed useful to work with this inhibitor because the indications are that it acts at the same site as antimycin [1,2]. However, no binding studies have been carried out with HQNO so far.

HQNO is fluorescent with emission maximum at 480 nm and excitation maximum at 355 nm (Fig. 1) which is in agreement with the position of the absorption maximum (348 nm, see Fig. 2). The measured absorption spectrum is identical with the spectrum published earlier by Cornforth and James [3].

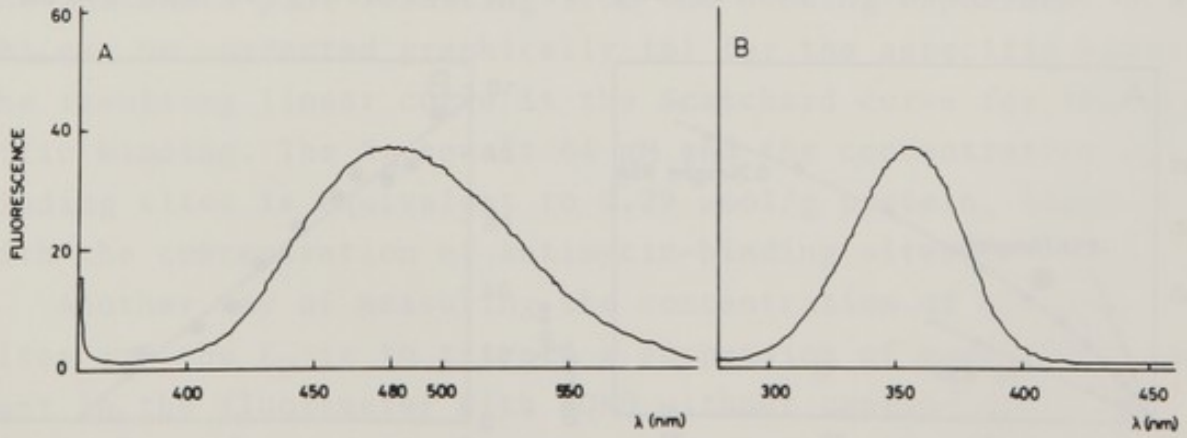


Fig. 1. Fluorescence spectra of HQNO. 58 µM HQNO was dissolved in 0.25 M sucrose, 50 mM Tris-HCl buffer, 5 mM $MgCl_2$ and 1 mM EDTA at pH 7.5. In subsequent legends this is referred to as 'standard medium'. The spectra were recorded with a Perkin-Elmer spectrofluorimeter (MPF-2A). A: emission spectrum (excitation at 355 nm). B: excitation spectrum (emission at 480 nm).

Antimycin binds specifically to bovine serum albumin with a several-fold enhancement of the fluorescence [4]. As shown

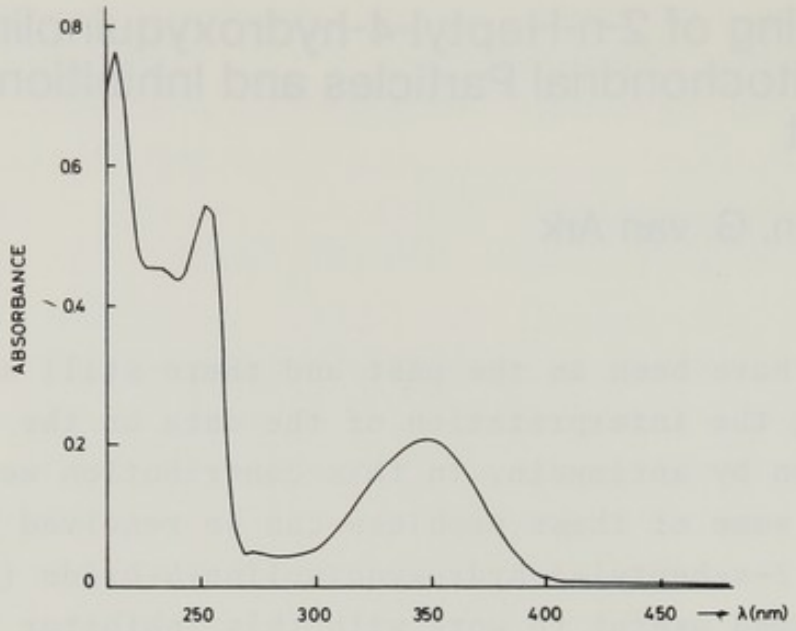


Fig. 2. Absorption spectrum of HQNO. The absorption spectrum of 23 μM HQNO dissolved in standard medium (Fig. 1) was recorded with a Cary 17 spectrophotometer. The maximum is at 348 nm ($\varepsilon = 9.45\ mM^{-1}cm^{-1}$).

in Figs 3A and B, HQNO behaves in the same way but binds less firmly, the dissociation constant being 3.5 μM, compared with 0.12 μM for antimycin.

Antimycin binds so firmly to sub-mitochondrial particles that, in order to measure the dissociation constant for binding of antimycin to particles, it is necessary to add serum

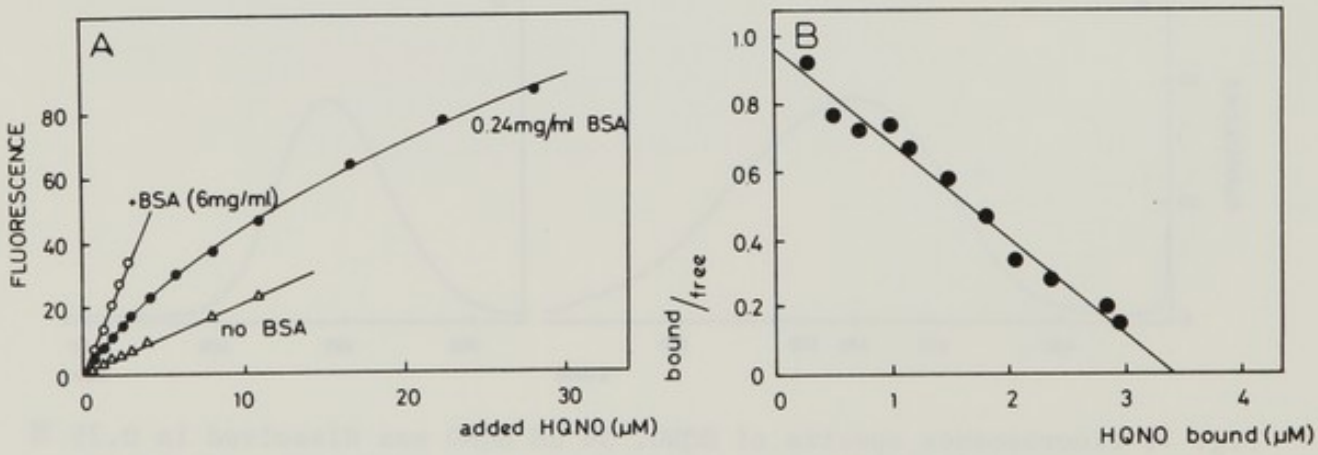


Fig. 3. Enhancement of HQNO fluorescence by binding to bovine serum albumin (BSA). A: A solution of albumin (0.24 mg/ml) in standard medium (Fig. 1) was titrated with HQNO and the fluorescence measured with an Eppendorf fluorimeter, primary filter 313 + 366 nm, secondary filter 420-3000 nm. The fluorescence of bound HQNO was determined by using a high concentration of albumin (6 mg/ml). B. The experimental points shown in the middle curve of Fig. 3A were used to construct a Scatchard plot for the binding of HQNO to serum albumin K_D = 3.5 μM and the concentration of binding sites is 3.42 μM, i.e. 1 site per molecule of albumin (0.24 mg/ml = 3.55 μM).

albumin both to compete with the particles for the antimycin and to increase the fluorescence of the antimycin not bound to the particles [4]. This is not necessary with HQNO, which binds so much less strongly that the unbound HQNO can be readily calculated by measurement of the fluorescence in the supernatant after centrifuging down the particles. One experiment is shown in Fig. 4A. The slope of the linear part of the curve obtained in the presence of particles is less than that in the absence of particles, suggesting an aspecific binding of HQNO in addition to the specific binding. Since preliminary experiments had shown that binding of antimycin to its specific site inhibits the binding of HQNO to its specific site, indicating an identity of these sites (contrast ref. 5), we measured the binding of HQNO also in the presence of a low amount of antimycin, enough to saturate the specific binding site. The resulting curve now shows the aspecific binding which results in a constant fraction of the added HQNO disappearing from the solution. The ratio bound/free of the added HQNO is 1.2, so that only about 45% of the added HQNO is free in solution, indicating a partitioning of HQNO over the membrane and water phases. Since the aspecific binding is separately measured, the Scatchard plot resulting from the binding experiment (Fig. 4B) can be corrected graphically [6] for the aspecific binding. The resulting linear curve is the Scatchard curve for the specific binding. The K_D equals 64 nM and the concentration of binding sites is equivalent to 0.29 μmol/g protein, identical with the concentration of antimycin-binding sites.

Another way of measuring the concentration of binding sites and the K_D is to titrate a suspension of particles present in the fluorimeter with HQNO without centrifuging. The experiment shown in Fig. 5A makes clear that the fluorescence of bound HQNO is quenched. Assuming full quenching upon binding, a Scatchard plot can be constructed (Fig. 5B). The resulting K_D equals 140 nM, a value much higher than found in the experiment of Fig. 4. We know from the latter experiment, however, that at the protein concentration used 1.2 times as much

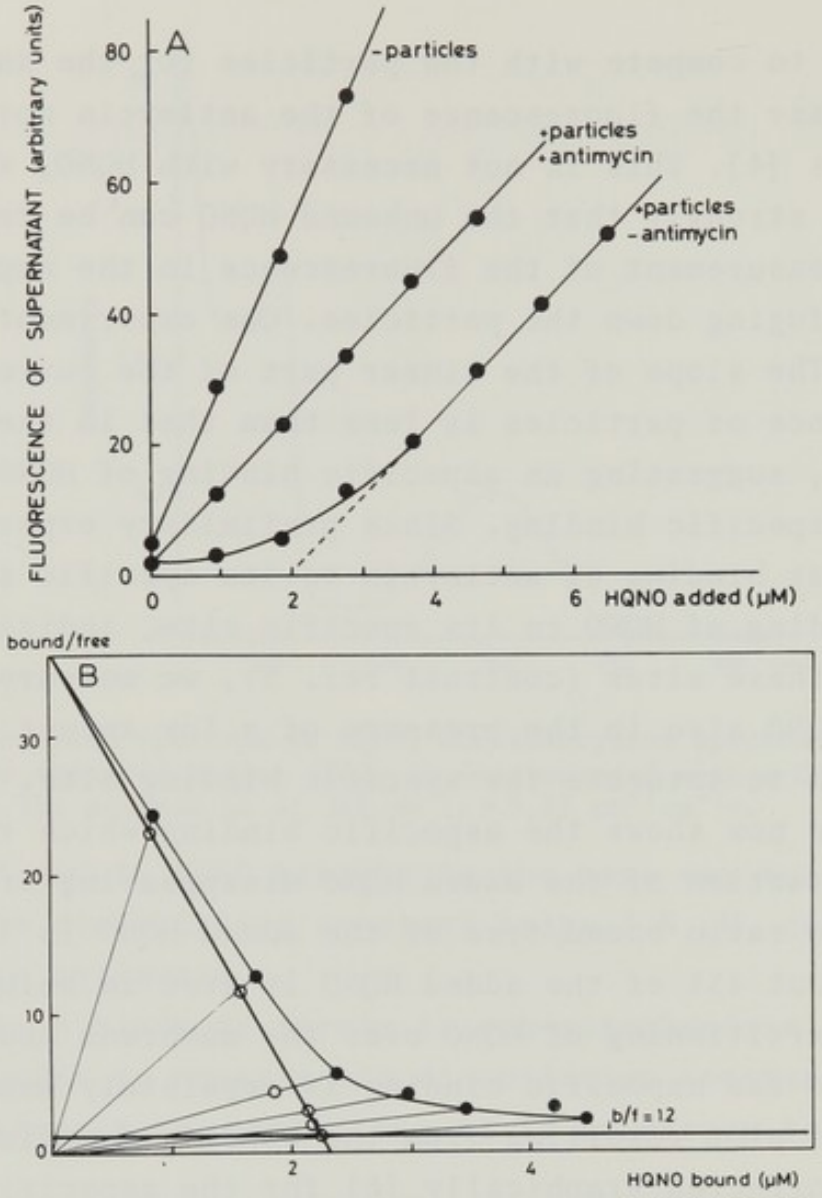


Fig. 4. Binding of HQNO to sub-mitochondrial particles. A: A suspension of particles (8.1 mg/ml) in standard medium (Fig. 1) was incubated with various amounts of HQNO for 10 min at 23°C. After spinning down the particles, the fluorescence of the supernatant was measured. For the reference curve ("- particles") the particles were incubated in the absence of HQNO and various amounts of HQNO were added to the supernatant. For the curve marked "+ particles + antimycin" all incubation mixtures contained 2.9 μM antimycin, just enough to saturate all the specific antimycin-binding sites. B. The results shown in A were used to construct the Scatchard plot (filled circles). The experiment in the presence of antimycin gave a bound/free ratio of 1.2, independent of the HQNO concentration. This is drawn as a straight line parallel to the abscissa with bound/free = 1.2. This result is due to aspecific binding and the points represented by the filled circles were corrected for this. The resulting Scatchard plot for the specific binding (open circles) gives K_D = 64 nM and n = 2.32 μM = 0.29 μmol/g protein.

HQNO is dissolved in the membrane phase as is dissolved in the water phase, so that the real free HQNO (the HQNO in the water phase) is only 1/2.2 times the amount not bound to the specific site.

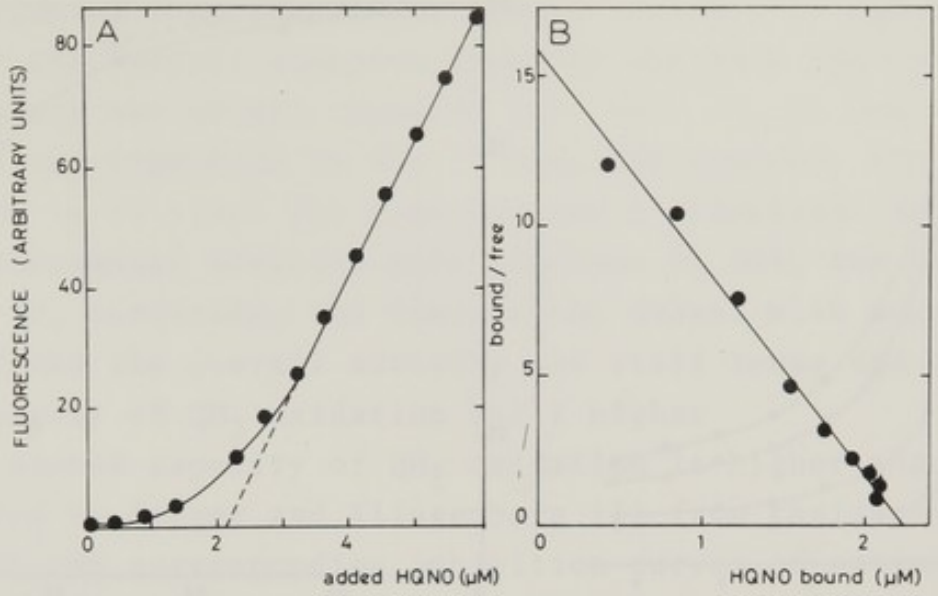


Fig. 5. Titration of sub-mitochondrial particles with HQNO. A: A suspension of particles (8.1 mg/ml) in standard medium (Fig. 1) was titrated with HQNO and the fluorescence measured after each addition. B: The data of A were used to construct a Scatchard plot on the assumption that the HQNO, bound specifically, is not fluorescent. The apparent (see text) K_D= 140 nM and n = 2.25 μM = 0.28 μmol/g protein.

If the K_D is assumed to concern the equilibrium between the bound HQNO dissolved in the water phase, the value found for the K_D has to be divided by 2.2, which results in a real K_D of 64 nM, in agreement with the result of the centrifugation experiment.

These experiments show that HQNO binds to the same site as antimycin does (at least both compounds cannot be bound at the same time) and that the binding of HQNO is much weaker than that of antimycin. In contrast to antimycin [4], no effect of adding substrate and cyanide on the binding of HQNO was found.

Since HQNO is an inhibitor of electron transfer we should examine whether binding to the specific site is really the cause of the inhibition. There could be some doubt about this since, as shown in Fig. 6, half-maximal inhibition of NADH oxidation is obtained at 2 μM HQNO, while the K_D, taking into account the concentration of protein, is below 0.1 μM. If the specific binding site is the inhibitory site one possibility is that the step inhibited by HQNO is not rate-limiting for

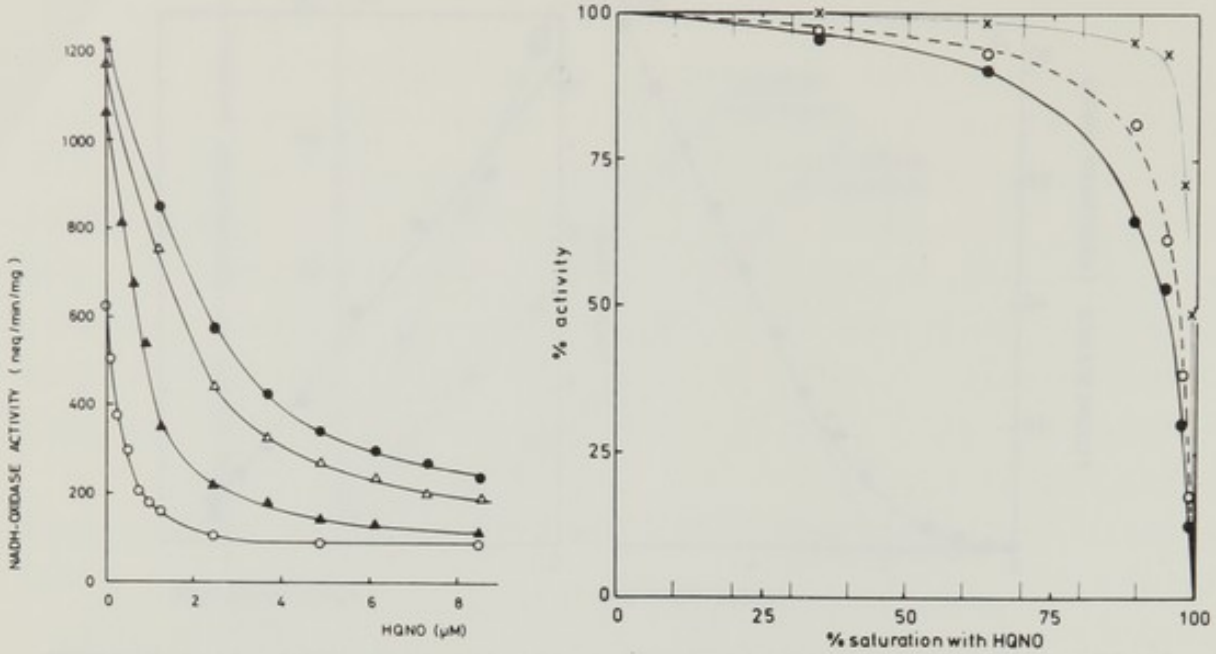


Fig. 6. Inhibition of NADH oxidation by HQNO. The oxygen uptake by a suspension of sub-mitochondrial particles (1.1 mg/ml) in standard medium (Fig. 1) was measured with a Clark oxygen electrode, in the presence of various amounts of HQNO, after addition of 0.6 mM NADH. ●——●, no further additions, $c_{\frac{1}{2}}$ = 2.0 μM; Δ——Δ, 0.136 nmol/mg antimycin present, $c_{\frac{1}{2}}$ = 1.5 μM; ▲——▲, 0.273 nmol/mg antimycin present, $c_{\frac{1}{2}}$ = 0.8 μM; O——O, 0.409 nmol/mg antimycin present, $c_{\frac{1}{2}}$ = 0.3 μM. The total concentration of antimycin-binding sites, determined in a separate experiment, was 0.5 nmol/mg.

Fig. 7. Inhibition of electron transport by HQNO. The oxidation of NADH (●) or succinate (X) was measured as described in Fig. 6, at various concentrations of HQNO. Using the K_D measured fluorimetrically for an identical suspension of particles, the saturation of the specific binding site was calculated. For the points (O) the NADH oxidase activity was inhibited by 50% by rotenone. The curve ——— , represents a theoretical inhibition curve, calculated according to the model of Kröger and Klingenberg [7], for the situation that the inhibited part of the respiratory chain (oxidation of QH_2) has a capacity 15 times that of the rate-limiting part (reduction of Q). For the curve ----, the excess capacity is 30 times.

electron transfer. To test this we saturated the site with increasing amounts of antimycin and it can be seen in Fig. 6 that the value for half-maximal inhibition decreases towards values that can be expected from the K_D and the concentration of binding sites. This is consistent with the specific binding site being the inhibitory site with this site in a part of the electron-transfer chain that is not rate-limiting. If we plot inhibition *vs* saturation of the binding site we find a hyper-

bola. Assuming that the dehydrogenase activity is rate-limiting for the overall electron transfer and that the oxidation of ubiquinol has excess capacity (cf. ref. 7), we can calculated from the hyperbola in Fig. 7 that the capacity for QH_2 oxidation is 15 times the capacity for Q reduction. Inhibiting the dehydrogenase activity with rotenone by 50%, the factor becomes 30, confirming the conclusions drawn. With succinate as substrate the overall activity was still lower and the excess capacity of QH_2 oxidation still higher

The excess capacity of QH_2 oxidation is higher than that calculated by Kröger and Klingenberg [7] from their antimycin data, but the corresponding inhibition curves of antimycin are not hyperbolic. An example is given in Fig. 8, although it should be mentioned that the deviation from a hyperbolic curve is often much less clear. It seems likely that the antimycin-inhibition curves are not hyperbolic because antimycin binding changes the conformation of the binding site (cooperative binding [4]) and apparently the conformation induced is also inhibitory, so that the inhibition is stronger than would be expected from the degree of saturation. It is hoped that comparison of antimycin-inhibition curves with HQNO-inhibition curves will make it possible to derive the allosteric parameters for the effect of antimycin.

In addition to inhibition of electron transfer, both antimycin and HQNO cause an increased reduction of cytochrome *b* in the presence of oxidant, even when cyanide is present. Since HQNO has no allosteric properties this effect cannot be caused by allosterism but is presumably due to its inhibitory effect. Since the shape of the effect curve with antimycin will be influenced by the allosteric properties of this compound, the effect can better be studied using HQNO. In Fig. 8 the increase of reduced cytochrome *b* is plotted against the saturation with HQNO. Surprisingly the reduction is already substantial at low saturation of the binding site. Since sufficient cyanide is present to block the respiration almost completely, a kinetic explanation (assuming that the effect is caused by

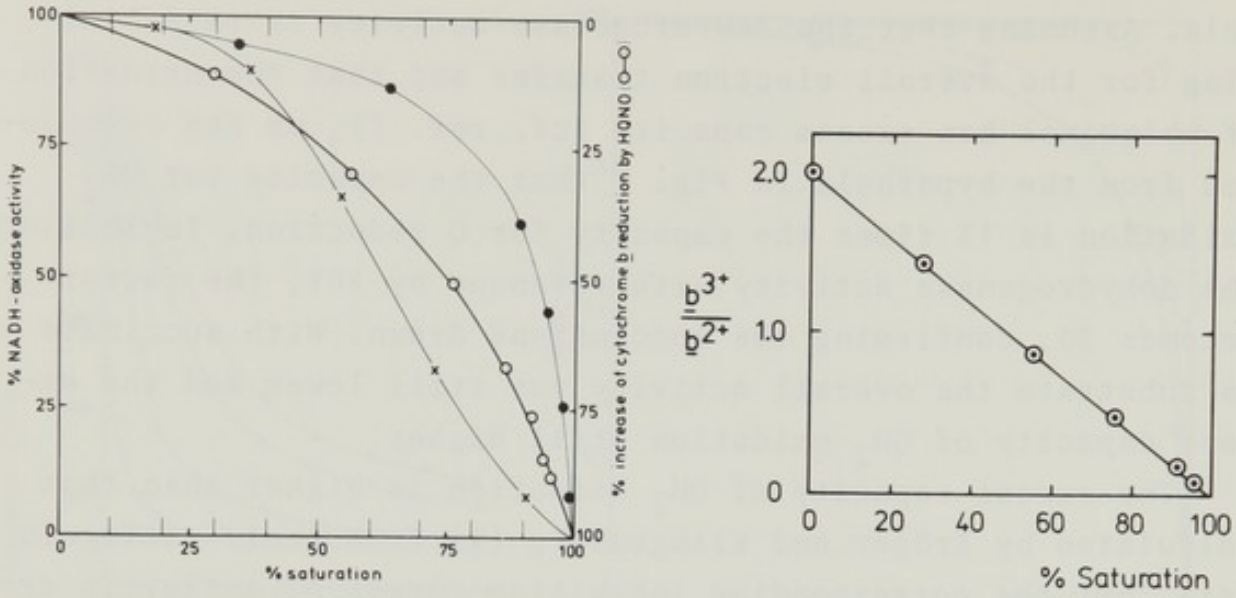


Fig. 8. Inhibition by HQNO and antimycin of NADH oxidase activity and effect of HQNO on cytochrome *b*. The oxidase activity was measured as described (Fig. 6) in the presence of various amounts of HQNO (●——●) or antimycin (X——X). The saturation level was calculated from the K_D values. For HQNO this K_D was measured, for antimycin the values in ref. 4 were used. The concentration of binding sites was measured fluorimetrically, using the quenching of antimycin fluorescence upon binding (ref. 8). The curve (⊙——⊙) represents the increase in reduction of cytochrome *b* in the presence of succinate and 2 mM cyanide upon addition of HQNO.

Fig. 9. Effect of HQNO on the redox state of cytochrome *b*. From the data given in Fig. 8 (⊙——⊙) and the redox state of cytochrome *b*-566 in the absence of HQNO the ratio b^{3+}-566/b^{2+}-566 was calculated. The maximal reduction reached in the presence of HQNO was identical with total reduction, The redox state of *b*-566 in the absence of HQNO was calculated as follows: In the absence of HQNO $\Delta A_{562-577}$ (red-ox) was 75% of the value in its presence. Since *b*-562 contributes 63% and *b*-566 37% to the total $\Delta A_{562-577}$ (red - ox) and *b*-562 was fully reduced in the absence of HQNO, the ratio $b^{3+}566/b^{2+}566$ in the absence of HQNO was 25/12 = 2.1.

inhibition of the bc_1 complex) is difficult to visualize unless a branched chain is postulated. For a quantitative description of the effect of HQNO on cytochrome *b* the redox state of cytochrome *b*, expressed as b^{3+}/b^{2+}, is a better parameter than the relative increase in reduction. Since cytochrome *b* contains two main components with different midpoint potential, the two species have to be considered separately. In the presence of substrate and cyanide *b*-562 was fully reduced, so the species involved in the increased reduction was only *b*-566. The ratio b^{3+}-566/b^{2+}-566 is plotted in Fig. 9 against the

saturation of the HQNO-binding site. The linear relationship obtained provides a good basis for further analysis. We mentioned already that this result is difficult to explain on the basis of a linear respiratory chain. In the literature branched or partially branched chains have also been proposed, eg. that of Wikström and Berden [9]. According to this scheme, depicted in Fig. 10, equilibrium between the $QH\cdot/QH_2$ and b^{3+}/b^{2+} couples would be expected in the presence of cyanide, so that, according to our result, $QH\cdot/QH_2$ would decrease linearly with the saturation of the HQNO-binding site. This fits very well with the basic idea behind the model: to explain the reduction of cytochrome *b* in the presence of antimycin (or HQNO) when an oxidant is added. The oxidized component then is the semiquinone. Wikström and Berden assume that even in the presence of

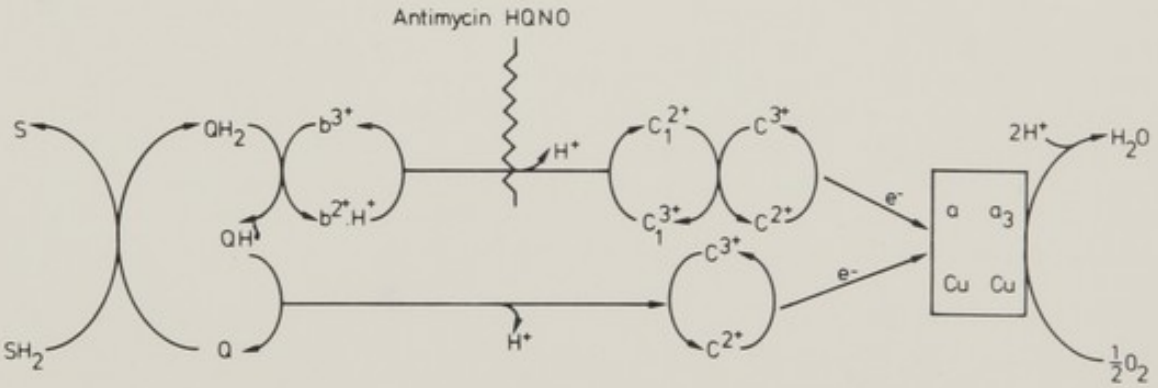


Fig. 10. Scheme for electron transfer as proposed in ref. 9.

cyanide oxygen induces an electron flow. In the steady state the rates of electron flow in the lower and upper pathway must be equal and our experimental result fits with the model if this flow is proportional to the concentration of substrate for both pathways, since then $V_{upper} = c\cdot[QH_2]\cdot f([c^{3+}]) = V_{lower} = c^1\cdot[QH\cdot]\cdot f([c^{3+}])$ and $c/c^1 = [QH\cdot]/[QH_2]$. Binding of HQNO only affects the upper pathway (c) so that $QH\cdot/QH_2$, and therefore b^{3+}/b^{2+} decreases linearly with the saturation of the HQNO-binding site. We may conclude then that the model for electron transfer, proposed by Wikström and Berden, can accommodate the results presented, provided the assumption is valid that the concentration of QH_2 and $QH\cdot$ are below the res-

pective K_m values.

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