SELECTIVE EFFECTS OF CHLORAMPHENICOL, CYCLOHEXIMIDE AND NALIDIXIC ACID ON THE BIOSYNTHESIS OF RESPIRATORY ENZYMES IN YEAST

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The utility of antibiotics for studies on the development of mitochondria in baker's yeast was first demonstrated by Linnane and his collaborators (Huang et al., 1966; Clark-Walker and Linnane 1966, 1967; Linnane et al., 1967). Chloramphenicol (CAP) and other inhibitors of bacterial protein synthesis appeared to exert relatively little effect on growth of yeast cells at high glucose concentrations (5%), under conditions where the organism received its energy supply by aerobic glycolysis, and mitochondrial development was repressed, but were effective in inhibiting the elaboration of functional mitochondria, cytochromes, and other respiratory enzymes when cells were grown on glucose at lower concentrations (1%). Conversely cycloheximide (cyclo) was reported not to inhibit cytochrome formation and mitochondrial development. On the basis of these experiments, combined with the known specificity of CAP as an inhibitor for mitochondrial (Wintersberger, 1965; Mahler et al., 1967; Linnane et al., 1967) and cyclo as one for cytoplasmic protein synthesis in yeast extracts (Siegel and Sisler, 1964), Clark-Walker and Linnane (1967) postulated that cristae-bound mitochondrial enzymes are synthesized by these particles themselves, while solubilizable mitochondrial enzymes share with cytoplasmic proteins a synthesis site in the cytoplasm. A subsidiary hypothesis was advanced to account for the inhibition of fumarase and malate dehydrogenase, which may, however by generalized as follows: The most striking effects of CAP are manifest when yeast cells are known to undergo release from glucose re-

pression (Ephrussi et al., 1956; Linnane, 1965; Polakis et al., 1965; Jayaraman et al., 1966). Hence the inhibitor may affect the promulgation (or the execution) of a signal of mitochondrial origin - related to the intracellular concentration of glucose or a catabolite produced by glucose (Witt et al., 1966; Polakis and Bartley, 1966) - that triggers derepression of the biosynthesis of respiratory proteins in general. Either hypothesis would predict that CAP should inhibit the biosynthesis of mitochondrial enzymes of the same group to the same extent (i.e. the measured activity of insoluble respiratory enzymes should rise or fall co-ordinately), but the second alternative would also predict similar effects for all derepressible enzymes of the same class, including cytoplasmic ones. In addition it also suggests that effects should vary with the time of addition of CAP: effectiveness should be greatly enhanced if the inhibitor is already present when cells are fully repressed, and mitochondrial number and function is grossly impaired, i.e. when it is added with the inoculum, the paradigm employed by Linnane et al., as compared to addition at a later time, when there has already occurred a measurable rise in the respiratory activity of the cell. Furthermore this latter design should also guard against the possibility that the effect of CAP is really on repression (Ephrussi et al., 1956; Utter, 1967; Jayaraman et al., 1967) early, rather than on de-repression late during the growth cycle.

We have tested these predictions by a study of the effect of CAP (added at two concentrations and at different times) and cyclo on a number of enzymes varying in their sensitivity to repression and intracellular localization. Our results suggest that effective mitochondriogènesis requires a highly complex set of interactions between the two protein synthesizing systems and their products. In addition we have used the inhibitor nalidixic acid (NDA) which is known to affect DNA duplication of bacteria (Cook et al., 1966) and chloroplasts (Lyman, 1967), and which by analogy might be expected to inhibit the duplication of mitochondrial DNA and hence the number of mitochondria and the respiratory capacity of the cell. Such an effect has been found.

RESULTS AND DISCUSSION

Growth Curves and Derepression - Seven different conditions were investigated. In all cases 250 ml of a semi-synthetic medium using 1% glucose as a carbon source (Jayaraman et al., 1966) were inoculated with 0.25 ml of a culture of a diploid strain of Saccharomyces cerevisiae (Fleischman). Growth curves for the latter stages of growth are shown in Fig. 1, which also indicates the concentration and time of addition of the various inhibitors used, as well as the O_2 uptake [μmoles/min./mg protein at 30°C with glucose as substrate

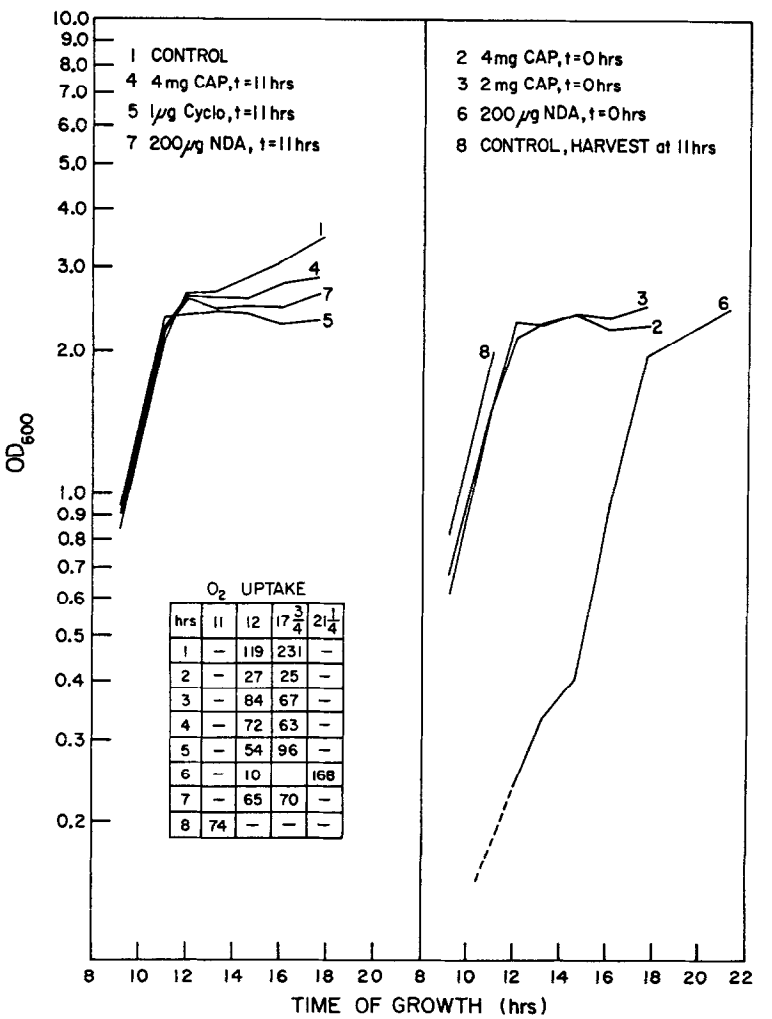

Fig. 1 - Effects of CAP, cyclo and NDA on growth and respiratory activity of yeast. Growth is measured by A_{600}; O_2 uptake is expressed as mμmoles/min./mg cell protein.

determined polarographically with a Clark electrode (Gilson Oxygraph)] at the time indicated. CAP at either concentration added at t=0 has virtually no effect on growth rate, confirming the findings of Clark-Walker and Linnane, 1967, and neither has NDA even at the highest concentration tested (200 μg/ml), although there is an extended lag at this concentration of inhibitor. No significant lag is observed at 50 μg/ml, a concentration that lowers O_2 uptake by ~ 20%. All the inhibitors tested, including cyclo and NDA, effect oxygen uptake to a varying extent without any obvious correlation with their effectiveness as inhibitors of cell number or protein content (see also Table II).

TABLE I

EFFECTS OF INHIBITORS ON DEREPRESSION OF MITOCHONDRIAL RESPIRATORY ENZYMES

CONDITIONS	ACTIVITY RELATIVE TO THAT OF 11 HR CELLS						
(Fig. 1)	Protein	A†	B†	C	D	B/A	D/A
1	1.46	19.3	17.7	2.46	10.9	0.932	0.565
2	1.58	1.06	0.656	0.89	1.48	0.566	0.535
3	1.37	2.52	2.72	1.59	2.05	1.08	0.813
4	1.44	4.00	3.38	1.19	3.46	0.845	0.865
5	1.81	5.85	11.0	1.04	1.16	1.88	0.198
6	1.42	4.52	6.98	1.50	3.95	1.55	0.875
7	1.53	2.37	4.79	1.71	3.45	2.02	1.46
8*	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	(2.60)	(2.60)	(1.61)	(6.65)	(10.2)	(0.618)	(2.56)

* Normalized to this set of data. Actual values - in μmoles converted (acceptor reduced for all except A, which is expressed as substrate oxidized) / g homogenate protein at 23° for enzymes, and in mg/g for protein, are shown in parentheses.

† Equivalent results are obtained if the portion of the activity of these (exclusively mitochondrial) enzymes not sedimented under the conditions used here (20 min. x 20,000 g) are included as well.

Cells were grown in 250 ml of a semi-synthetic medium on 1% glucose with the additions shown in Fig. 1. They were harvested after 17.75 hrs. (Expts 1-5, 7), 22.25 hrs. (Expt 6) and 11 hrs. (Expt 8) respectively and broken mechanically, and purified mitochondria isolated (Jayaraman et al., 1966). No enzymatic activity shown was increased significantly by disruption of the mitochondria (extraction with 0.6 N KCl, 2x freezing and thawing, followed by sonication). The enzymatic activities measured in Tables I, II were not inhibited when CAP was added to the assay systems.

Enzyme levels (Tables I and II) - Eight different growth conditions (Nos. 1-8 of Fig. 1) were selected for these determinations. Eight enzymatic activities (indicated by letters) were measured, chosen for their localization and susceptibility to glucose repression (abbreviated by R if present): A = cytochrome oxidase (mitochondrial inner membrane, R); B = antimycin A - sensitive DPNH: cytochrome c reductase (mitochondrial inner membrane, R); C and F = DPNH dehydrogenase (DPNH: ferricyanide reductase; two activities measured, one mitochondrial - C, one cytoplasmic - F, both R); D and G = L-malate dehydrogenase [two activities, one mitochondrial, not associated with inner membrane - D, and one cytoplasmic (soluble) - G, both R]; E = antimycin A - insensitive DPNH: cytochrome c reductase [cytoplasmic (microsomal)]; H = TPNH: cytochrome c reductase [cytoplasmic (microsomal), non-R]. Activity H can be used as a reference to assess the statistical significance of the variation found in all

TABLE II

EFFECTS OF INHIBITORS ON DEREPRESSION OF CYTOPLASMIC RESPIRATORY ENZYMES

CONDITIONS	ACTIVITY RELATIVE TO THAT OF 11 HR CELLS						
(Fig. 1)	Protein	E	F	G	H	F/C	G/D
1	0.977	5.81	6.27	9.10	0.93	2.55	0.835
2	0.796	3.07	2.49	1.68	1.49	2.80	1.13
3	0.890	2.91	3.86	3.05	1.09	2.43	1.48
4	1.08	3.46	5.58	4.80	1.31	4.70	1.38
5	0.760	2.14	3.47	1.34	1.47	3.33	1.15
6	0.985	2.90	3.81	4.15	1.08	2.54	1.05
7	0.916	3.44	3.95	2.63	1.12	2.23	0.760
8*	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	(65.4)	(7.61)	(23.4)	(58.2)	(19.2)	(3.50)	(5.70)

Cells were treated as described in the legend to Table I. The supernatants after isolation of mitochondria were tested for the enzymatic activities shown. Variation in protein content is probably due to differences in cell breakage and is not intrinsic.

other instances. The results obtained suggest that a) the specificity of CAP and cyclo is not absolute with regard to effects on mitochondrial vs. cytoplasmic

enzymes; b) de-repression of mitochondrial activities and its inhibition by the various agents is not coordinate, not even for the two main segments of the respiratory chain (variability of the ratio B/A and its relative similarity to D/A, or the patterns with the two different concentrations of CAP); c) to some extent the same holds for mitochondrial and cytoplasmic variants of the same activity (e.g. G/D); d) hence differential inhibitory effects of CAP and cyclo are observed and appear to be specific for each individual case studied; e) the pattern of inhibition by NDA is similar to that generated by CAP, especially when the effects of the two agents added at t=11 hrs. are compared; under these conditions the inhibitor is maximally effective.

CONCLUSIONS

Neither of the two sets of hypotheses mentioned in the Introduction can account for the complex results described, probably not even in combination. In their stead we would like to suggest a consideration of the following model: The synthesis (or at least the activity) of de-repressible cytoplasmic enzymes is regulated by entities of mitochondrial origin - the biosynthesis of which is sensitive to CAP. Mitochondrial proteins that are synthesized in the cytoplasm - and hence are directly susceptible to inhibition by cyclo - require integration into the particles before their enzymatic activity can become manifest. This process depends on the presence of protein(s) synthesized by a CAP sensitive process. If any of the mitochondrial respiratory enzymes are synthesized in the particles, interaction with other protein(s), of cytoplasmic origin, are required for their activity. This complex set of interactions between mitochondrial and cytoplasmic events during mitochondriogenesis is a counterpart, and perhaps represents the expression of equally complex relationships existing on the genetic level (Sherman and Slonimski, 1964). In addition we find that NDA is an effective inhibitor of the biogenesis of respiratory enzymes in yeast, qualitatively similar in its results to those produced by CAP, but presumably acting on the level of gene duplication rather than gene expression.

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