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Mitochondriogenesis Analyzed by Blocks on Mitochondrial Translation and Transcription*

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ABSTRACT: The mitochondrial contribution to the biosynthesis of the respiratory chain in yeast has been analyzed by use of the inhibitors cycloheximide, chloramphenicol, and ethidium bromide. When care is taken to isolate primary from secondary effects, a minimal lesion can be localized in the region of cytochrome oxidase (enzymatic)/cytochrome aa_3 (spectroscopic); the continued elaboration of these entities is abolished by both chloramphenicol and ethidium bromide added to either facultatively anaerobic petite-forming or to obligately aerobic, petite-negative, yeasts. We conclude that—so far as the respiratory chain is concerned—no mitochondrial transcript is wholly translated on cytoribosomes nor is any nuclear transcript translated wholly on mitoribosomes; that mitochondrial mRNA must have a relatively short half-life relative to cellular generation time; and that mitochondria in a metastable functional state, lacking certain key components of the respiratory chain are nevertheless capable of duplication.

Mitochondria are ubiquitous organelles of eucaryotic cells. They are present in yeast whether or not mitochondrial respiration and attendant energy transduction is indispensible for cellular maintenance, growth, and division (Yotsuyanagi, 1962a,b; Criddle and Schatz, 1969; Watson *et al.*, 1970; Perlman and Mahler, 1970b). They contain a system for the storage, transmission, and expression of genetic information separate from and, at least in part, independent of that localized in the nucleus (see recent reviews by Roodyn and Wilkie, 1968, Nass, 1969, Rabinowitz and Swift, 1970, and Borst and Kroon, 1971). However, in spite of intense investigations during the recent past, the extent of mitochondrial autonomy, particularly with regard to the polypeptide gene products specified and synthesized, remains uncertain (Ashwell and Work, 1970).

One possible approach to this aspect of mitochondriogenesis makes use of selective inhibitors to probe the sites and extent of transcription and translation for mitochondrial as compared to other proteins. Cycloheximide (cyclo)[1] is known to be an effective inhibitor of protein synthesis on cytoribosomes (Siegel and Sisler, 1965; deKloet, 1965, 1966; Clark-Walker and Linnane, 1967; Hartwell *et al.*, 1970) while, conversely, chloramphenicol (CAP) is an effective block of this processs on mitoribosomes (Wintersberger, 1965; Linnane *et al.*, 1967; Lamb *et al.*, 1968). Ethidium bromide (EB) is capable of producing a profound alteration in the mitochondrial genomes (the ρ^+ to ρ^- transition) of every cell in a population (Slonimski *et al.*, 1968). This agent has also been reported to inhibit mitochondrial (but not nuclear) DNA and RNA synthesis, *in vivo* (Zylber *et al.*, 1969; Zylber and Penman, 1969; Knight, 1969; Fukuhara and Kujawa, 1970; Goldring *et al.*, 1970; Perlman and Mahler, 1971) and *in vitro* (South and Mahler, 1968) as well as mitochondrial protein synthesis *in vivo* (Kroon and Jansen, 1968; DeVries and Kroon, 1970; Perlman

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‡ Portions of this study were submitted to the faculty of Indiana University in partial fulfillment of the requirements for the Ph.D. degree.

[1] Abbreviations used are: anti A, antimycin A; ATP, 5′-adenosine triphosphate; CAP, chloramphenicol; cfu, colony-forming units; cyclo, cycloheximide; cyt, cytochrome; cyt ox, cytochrome oxidase (EC 1.9.3.1); EB, ethidium bromide; GDH, L-glutamate dehydrogenase, NAD-linked (EC 1.4.1.2); GTP, 5′ guanosine triphosphate; IDH, L_S-isocitrate dehydrogenase, NADP-linked (EC 1.1.1.42); INT, *p*-iodonitrotetrazolium violet; MDH, L-malate dehydrogenase (EC 1.1.1.37); NAD(H), nicotinamide-adenine dinucleotide (reduced); NADH:*c*, NADH:cyt *c* reductase, anti A sensitive component (EC 1.6.2.1); NADP(H), nicotinamide-adenine dinucleotide phosphate (reduced); NC, nucleocytoplasmic; nuc, nuclear; PMS, phenazine methosulfate; SDH, succinate dehydrogenase measured as INT reductase, PMS assisted (EC 1.3.99.1); Succ:c, succinate:cyt *c* reductase (EC 1.3.99.1).

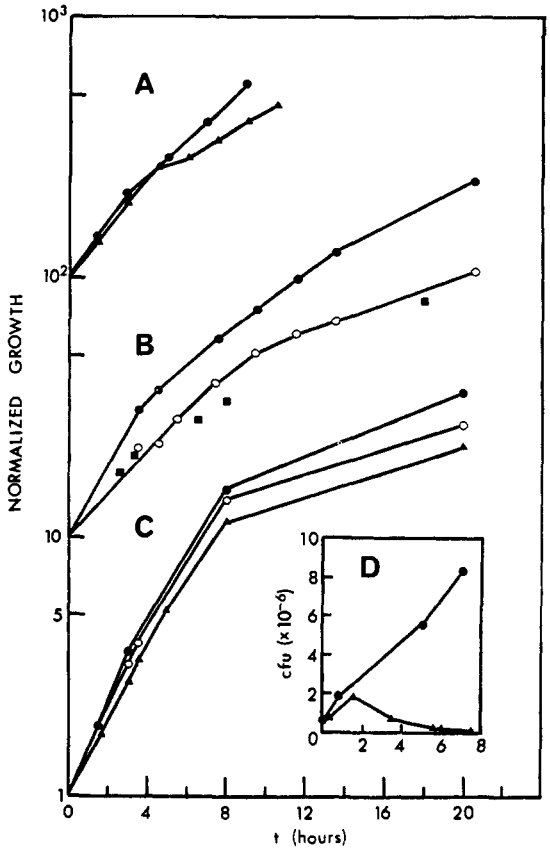

FIGURE 1: Growth data. (A) *S. cerevisiae* growing on 3% lactate in the presence of EB (20 μg/ml). Initial dry weight (milligrams per milliliter of culture) was 0.162. Filled circles, control; filled triangles, EB treated. The EB-treated culture was >99.5% ρ^- after 45 min. (B) *S. cerevisiae* growing on 3% lactate in the presence of CAP (4 mg/ml). Initial turbidity (A_{600}) was 0.09. Filled circles, control; open circles, CAP treated; filled squares, dry weight (initial value 0.075 mg/ml). No induction of petites by CAP was detected. (C) *T. utilis* growing on 5% glucose in the presence of CAP (4 mg/ml) or EB (20 μg/ml). Initial dry weight (milligrams per milliliter of medium) was 0.216. Filled circles, control; open circles, CAP treated; filled triangles, EB treated. (D) Viability of *T. utilis* growing on 5% glucose in the presence of EB. This organism grows in clumps so that cfu per milliliter was measured on plates containing 0.1% glucose and 3% lactate. The extent of clumping did not vary during growth. Filled circles, control; filled triangles, EB treated.

and Penman, 1970) and *in vitro* (Kellerman *et al.*, 1969; however, Yang and Criddle, 1970, suggest the opposite). Recently we have demonstrated that during growth of a mutagenized population in the absence (or presence) of added EB, parental mtDNA molecules are progressively destroyed and are no longer detectable after two mass doublings. The synthesis of mtDNA in the progeny is inhibited over this time span and remains inhibited so long as EB is present (Perlman and Mahler, 1971).

In this paper, we compare the effects of these three drugs on growth, viability, and enzyme biosynthesis in a petite-forming yeast (*Saccharomyces cerevisiae*) and two nonpetite-forming yeasts (*Hansenula wingei* and *Torulopsis utilis*) under identical physiological conditions, *i.e.*, in the absence of catabolite repression. This study was undertaken in order to derive answers to several questions. (1) Is the response to these drugs dependent on the viability of the petite phenotype? That is, do species of yeast which cannot grow (even) on glucose when its efficient utilization by mitochondrial respiration is no longer possible, differ in their response to these drugs from that of petite-forming, facultative anaerobes? (2) Is the response dependent on the step in mitochondrial gene expression that is blocked? That is, do the effects on enzyme synthesis produced by CAP differ from those produced by EB, and just what are the phenotypic—as distinct from the genotypic—effects of the latter? (3) Can we use experiments of this sort to (a) distinguish primary from secondary effects subsequent to a block of the expression of the mitochondrial genome (*i.e.*, can we introduce a minimal rather than the usual pleiotropic lesion resulting in respiratory deficiency?) and (b) gain insight into the cellular localization of the genes coding for various respiratory enzymes as well as their sites of translation?

For the moment, by analogy to studies of bacteriophage assembly (Wood *et al.*, 1968; Laemmli, 1970; Hosoda and Cone, 1970), one might formally divide mitochondrial polypeptide gene products into two classes: (1) *stoichiometric* and (2) *integrative* polypeptides. The former are proteins which themselves appear in the membrane in a predetermined stoichiometry, including "structural" and "morphopoietic" lattice elements (Kellenberger, 1965; Cotman *et al.*, 1968; Ashwell and Work, 1970; Mahler *et al.*, 1971a). The latter catalyze the modification and activation of membrane components and may *not* be conserved in the final assembly. The experiments presented here were designed to search for the former. In fully derepressed cells any integrative factors would be expected to be present initially in high concentrations so that the effects of the inhibition of their synthesis might only be detected after a substantial lag which, however, might equally well be due to nonspecific secondary effects. Therefore experiments to probe the class of integrative polypeptides must be done separately and utilize an alternate paradigm, one in which the inability to synthesize this class of proteins could be detected as a immediate response (*e.g.*, during the release from glucose repression in the presence of drugs, see Mahler *et al.*, 1971a, and Perlman, 1971).

Methods and Materials

Strains and Growth Conditions. The following strains of yeast were used in these studies: *S. cerevisiae* (diploid, var. Fleischmann), *H. wingei* (strain 5, provided by Professor T. D. Brock, Indiana University), and *T. utilis* (from the Indiana University Microbiology Department culture collection). All were grown in a semisynthetic medium consisting of 0.2% yeast extract, 0.05% $MgCl_2 \cdot 7H_2O$, 0.1% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, and 0.05% NaCl. A carbon source (as specified in the text) was added and the initial pH adjusted to 6.2–6.5. In one experiment with *T. utilis* the phosphate concentration was increased fivefold to afford additional buffering. The increased phosphate did not alter the growth of that organism.

Cells were grown at 30° with agitation and aeration in 10-l. fermenters and divided into 500-ml aliquots which were placed in sterile 1-l. shaker flasks for drug studies. This additional manipulation was included to ensure uniformity at the time of drug addition; cultures remained sterile. Drugs were dissolved in methanol and an equivalent amount of methanol was always added to control flasks; since ethanol is metabolized by yeasts it was not used to dissolve drugs. The presence of as much as 1% methanol in the growth medium did not alter growth, viability, or enzyme synthesis.

Sample Preparation. Cell samples were rapidly chilled with crushed ice, centrifuged, and washed twice with distilled water. Cell-free homogenates were prepared by breaking the cells mechanically (by grinding cells suspended in sorbitol

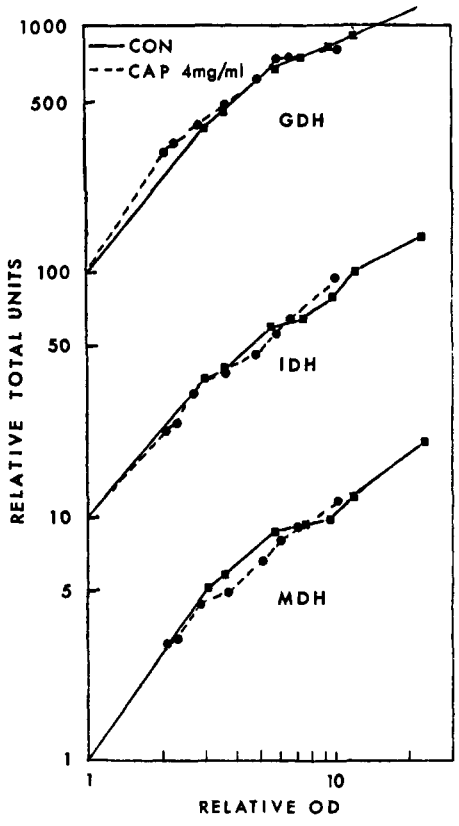

FIGURE 2: Effects of CAP (4 mg/ml) on the biosynthesis of soluble and mitochondrial matrix marker enzymes in *S. cerevisiae* growing on 3% lactate. Aliquots of the cultures whose growth characteristics are presented in Figure 1B were harvested, broken by grinding with glass beads, and assayed (cell-free supernatant) for their content of the enzymes shown. Relative total units = (nmoles min^{-1} mg homogenate $protein^{-1}$) × turbidity in the culture normalized to the initial value. The initial values of GDH, IDH, and MDH activities were 341, 107, and 2860 nmoles min^{-1} mg^{-1}, respectively. The initial turbidity was 0.09.

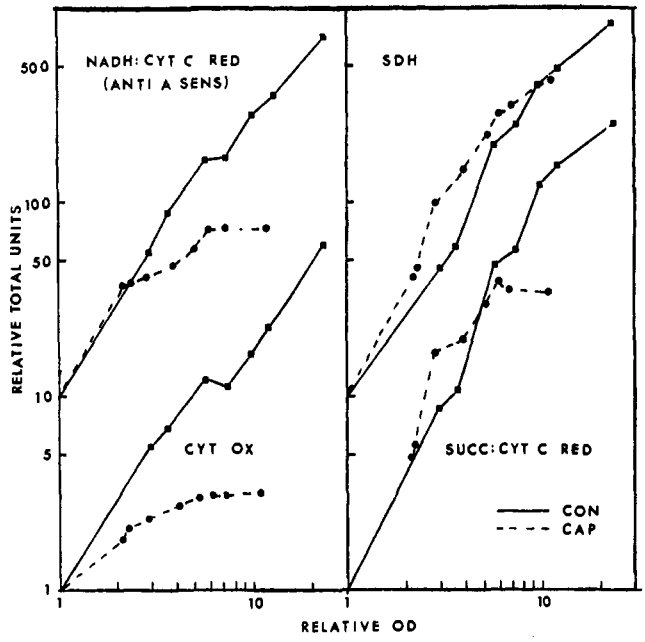

FIGURE 3: Effects of CAP (4 mg/ml) on the biosynthesis of respiratory enzymes in *S. cerevisiae* growing on 3% lactate. Aliquots of the cultures whose growth characteristics are presented in Figure 1B were assayed for their content of respiratory enzymes. Initial values of NADH:*c*, cyt ox, SDH, and Succ:*c* were 35.7, 202, 32.2, and 8.05 nmoles min^{-1} mg^{-1}, respectively. Initial turbidity was 0.09.

(0.5 M) containing Tris (0.05 M) and EDTA (1 mM) at pH 7.5, with glass beads in a chilled glass flask) as described by Jayaraman *et al.* (1966). In most experiments, the homogenization medium contained 50–100 μg of CAP/ml to retard bacterial growth. The homogenate was centrifuged once at 2000*g* for ten minutes to remove unbroken cells and debris. The 2000*g* supernatant has been shown to be a reliable fraction for estimating cellular enzyme content (Perlman and Mahler, 1970a; Perlman, 1971).

Analytical Procedures. Protein and most enzyme assays were performed as described by Perlman and Mahler (1970a). Dry weight was determined in duplicate by collecting a known volume of cell culture on a preweighed Millipore filter (0.45 μ pore size); the filter was dried at 60° for 4–12 hr and weighed. Succinate dehydrogenase (SDH) was assayed as INT reductase essentially as described by Nachlas *et al.* (1960). NADH oxidase was assayed as described by Mackler *et al.* (1962).

Liquid Nitrogen Temperature Spectroscopy. A dual-beam scanning spectrophotometer (Phoenix Precision Instruments) was used for measuring the absorbance of cytochromes in whole cells and in subcellular fractions.

Preliminary experiments demonstrated that the absorbance of a given cytochrome obeys Beer's law when whole cells of yeast are examined using a 2- or 3-mm path length with cells suspended to a protein concentration between 20 and 35 mg per ml. Partially purified mitochondrial fractions were studied at a protein concentration between 5 and 10 mg per ml. Experimental details are presented in the figure and table legends.

Materials. Adenine sulfate, α-oxoglutaric acid, antimycin A, chloramphenicol, cycloheximide, cytochrome *c* (type III), 2,6-dichlorophenolindophenol, ethidium bromide, *p*-iodonitrotetrazolium violet (grade I), isocitric acid (trisodium salt), reduced nicotinamide–adenine dinucleotide (NADH), nicotinamide–adenine dinucleotide phosphate (NADP), *cis*-oxalacetic acid, phenazine methosulfate, and L-phenylalanine were purchased from Sigma Chemical Co.

Bovine serum albumin, fraction V powder, was purchased from Pentex Inc. All other chemicals were of reagent grade.

Results

Studies on S. cerevisiae. EFFECTS OF CAP. In contrast to its lack of effect on exponential growth of *S cerevisiae* on glucose, a fermentable carbon source (Huang *et al.*, 1966; Clark-Walker and Linnane, 1966, 1967; Mahler *et al.*, 1968; Henson *et al.*, 1968a,b), when CAP is added (in our relatively resistant strain at a concentration of 4 mg/ml) during exponential growth on lactate, a respiratory, nonfermentable carbon source, growth ceases after four generations (Figure 1B). Increases in total cell number or mass continue at a somewhat reduced rate during two generations, with the inhibition becoming progressively more pronounced in the course of two subsequent mass doublings. The percentage of respiratory-deficient (ρ^-) mutants in the population remains unchanged throughout this period; CAP induces the petite mutation in ordinary cells only after extended growth on glucose (greater than 20 generations) (Williamson, 1970; P. P. Slonimski, private communication, 1970).

One possible interpretation of the kinetics of growth in the presence of CAP is that it reflects the initial availability and

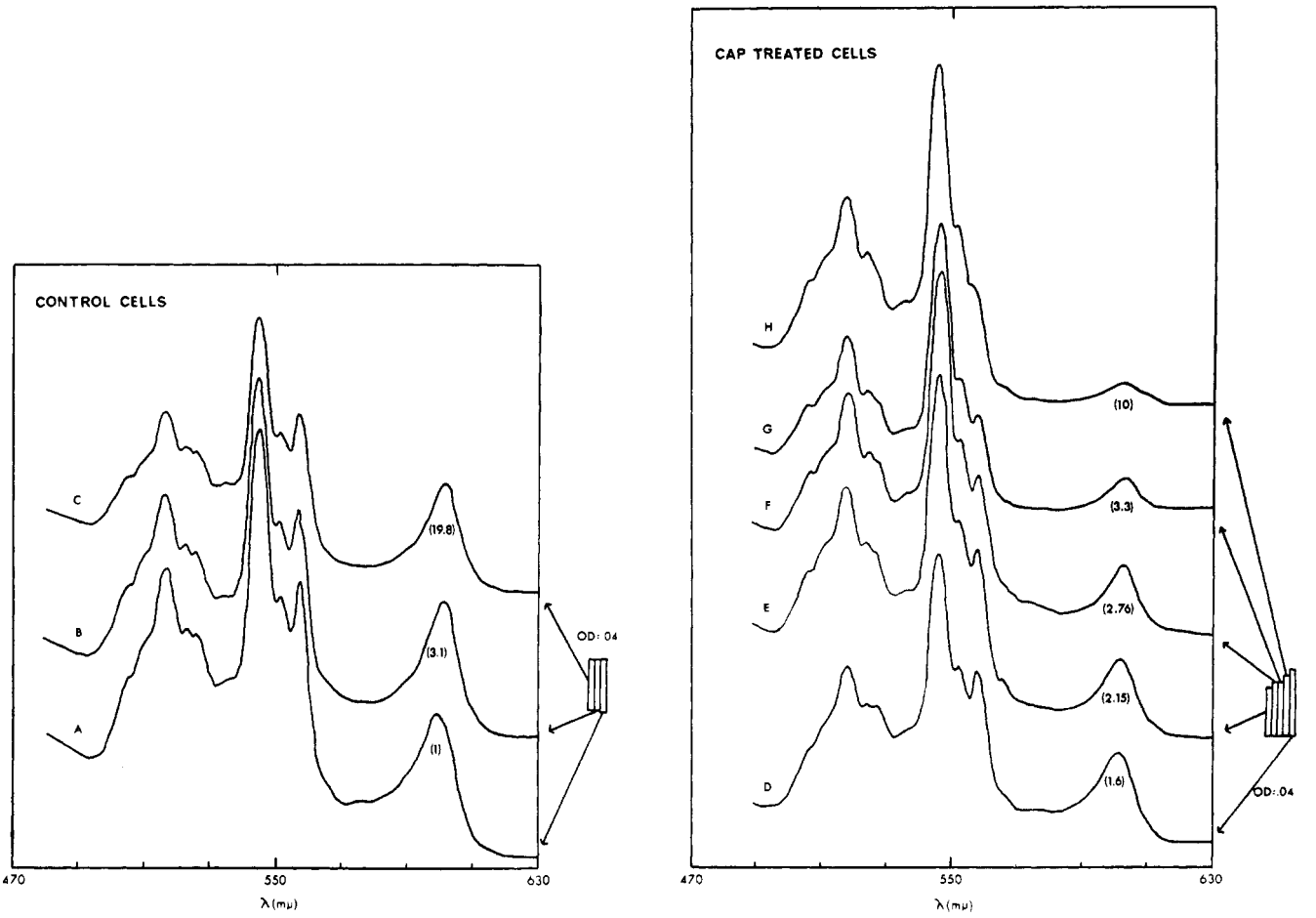

FIGURE 4: Effects of CAP on the biosynthesis of cytochromes in *S. cerevisiae* growing on 3% lactate. A 13-l. fermenter-containing medium plus 3% lactate was innoculated and incubated at 30° until the turbidity (A_{600}) reached 0.20. The cells were then transferred to sterile 1-l. flasks in 500-ml aliquots; when the transfer was completed, CAP was added to some of the flasks to 4 mg/ml and a sample of control cells was collected. Periodically samples of control and CAP-treated cells were chilled, harvested, and washed. A portion of each cell sample was suspended in 0.1 M PO_4 (pH 7.4) made 30% in glycerol for analysis of the absorption spectrum of the intact cells; the remainder of the cell sample was retained as a source of a mitochondrial fraction (see Figure 6). Spectra of dithionite-reduced cell suspensions minus 0.1 M phosphate (pH 7.4)–30% glycerol were prepared at the temperature of liquid nitrogen. All cell samples were suspended to roughly the same turbidity and a 2-mm path length was employed. The numbers in parentheses are the dry weight (milligrams per milliliter of culture) normalized to the initial value which was 0.284 mg/ml. The protein concentrations of the cell suspensions were 29.2, 23.45, 22.75, 34.7, 30.4, 30.75, 20.8, and 23.1 mg per ml for curves A–H, respectively. The height equal to 0.04 optical density unit is indicated for each curve by the bar to the right of each set of spectra.

progressive exhaustion of fermentable carbohydrate stores in cells, the respiratory apparatus of which is blocked in either its function or continued biogenesis by CAP. This interpretation is ruled out since growth of cells on lactate—in the presence or absence of CAP—ceases completely and instantly upon the addition of antimycin A (1 μg/ml), a specific respiratory inhibitor. In contrast, this inhibitor at the same concentration is without effect on fermentatively growing cells (*e.g.*, on 1% glucose). Therefore, cells stop growing on lactate (*i.e.*, they become phenotypically petite) apparently whenever their energy-producing mechanisms are no longer sufficient to support even a reduced rate of growth.

In an experiment similar to that represented in Figure 1B samples of control and drug-treated cells were collected at different times and their content of various enzymes—representatives of classes varying in their cellular localization and regulation (Perlman and Mahler, 1970a)—measured in a cell-free homogenate. The data, summarized in Figure 2, demonstrate that GDH, IDH, and MDH, enzymes representative of the cytosol and of the mitochondrial matrix, *i.e.*, the bulk of the cellular proteins, increase at the same rate as the control so long as growth continues. We conclude that when cells growing on lactate are exposed to CAP there are no immediate effects by this inhibitor either on protein synthesis by the nucleocytoplasmic (NC) system of gene expression or on generation, transfer, and utilization of ATP, GTP, or any other cofactor(s) required for this process. These conclusions are confirmed by the following observations: (1) CAP does not inhibit the synthesis of either mt- or nDNA *in vivo* (Grossman *et al.*, 1969; Perlman and Mahler, 1971). It inhibits the rate of incorporation of labeled amino acids into total cell protein by <5% (see also Henson *et al.*, 1968a, and Hawley and Greenawalt, 1970) while effectively blocking their appearance on nascent chains on mitochondrial polyribosomes and in the membrane proteins of the particles themselves (K. Dawidowicz, C. Antalis, and H. R. Mahler, in preparation).

The data presented in Figure 3 demonstrate the effect of CAP on the biosynthesis of respiratory enzymes bound to the mitochondrial inner membrane. It is clear that significant amounts of SDH and Succ:*c* are produced by these cells; NADH:*c* synthesis shows marked inhibition only coincident with the decrease in growth rate after two generations, while

cyt ox is the only enzyme whose synthesis is inhibited markedly *and* from the beginning of the drug treatment. The fact that some cyt ox synthesis did take place may indicate that the concentration of drug used was significantly but not totally effective, particularly in the beginning, in interfering with the intramitochondrial system; using an even higher concentration is not possible because the concentration of drug used in this experiment constitutes a saturated solution. An alternate explanation might be that the CAP is effective immediately and that the increase of cyt ox activity is related to activation of inactive precursors by a process no longer requiring an active mitochondrial protein-synthesizing system (this is the case for the formation of iso-2-cytochrome *c* (Sels *et al.*, 1965; Fukuhara and Sels, 1966; Fukuhara, 1966) and for cyt *c* peroxidase (Sels and Cocriamont, 1968), both during aerobic adaptation in yeast).

Cyclo at 5 μg/ml, however, inhibits whole cell protein synthesis in this strain by around 98% (*cf.* also Hartwell *et al.*, 1970), and, more specifically, completely blocks the synthesis of *all* enzymes shown in Figures 2 and 3 including cyt ox. Therefore, this second alternative would require the additional postulate that this conversion of inactive to active enzyme *does* require additional *extramitochondrial* protein synthesis. The addition of cyclo to growing cultures of yeast also is known not to impair grossly energy metabolism and macromolecular synthesis for as long as 6 hr as exemplified by RNA synthesis and mitrochondrial DNA and protein synthesis (Fukuhara, 1965, 1967; deKloet, 1965; Grossman *et al.*, 1969). In any event, we conclude that of all the activities tested CAP specifically blocks the increase *only* of cyt ox.

One may propose that this effect of CAP concerns the biosynthesis of only one of the several constituent polypeptides known to be required for cyt ox *activity*[2] (Lemberg, 1969). We have therefore extended our investigations on CAP sensitivity to the formation of the spectroscopically distinguishable cytochromes aa_3, as well as of cytochromes *b*, c_1, and *c*. We are, however, cognizant that such studies do not necessarily permit any correlation with particular enzymatically active species for of inferences regarding the *net* biosynthesis of any or all components required to produce the spectroscopically identifiable entity.

Figure 4 presents absolute reduced spectra of whole yeast cells grown on 3% lactate in the presence of 4 mg of CAP/ml. Because such cells are very rich in cyt *c*, we must restrict a quantitative interpretation of these data to a comparison of the extent of synthesis of cytochromes *c* and aa_3. The specific content of the latter decreased markedly already during the first generation of growth as well as in the two subsequent generations while the synthesis of cyt *c* was slightly stimulated (Figure 5). No destruction of cyt aa_3 was detected in several experiments of this type. Qualitative data for cyt *b* and c_1 are included in Figure 5. Since these two peaks were well resolved an estimate of their absorbance was made even though a large portion of each of them is due to (a varying degree of) overlap with the adjoining larger peaks.

A more quantitative measure of the extent of synthesis of these two cytochromes under these conditions was obtained from measurements in *mitochondrial* fractions isolated from mechanical cell homogenates (Figure 6). Direct analysis of these data requires that all mitochondrial fractions studied be of equal purity; we know that this requirement is rarely met, *especially* when cells of altered mitochondrial physiology are being compared (Perlman and Mahler, 1970a,b). It was found, however, that the ratios c/aa_3, c/b, and c/c_1 (Table I) remain

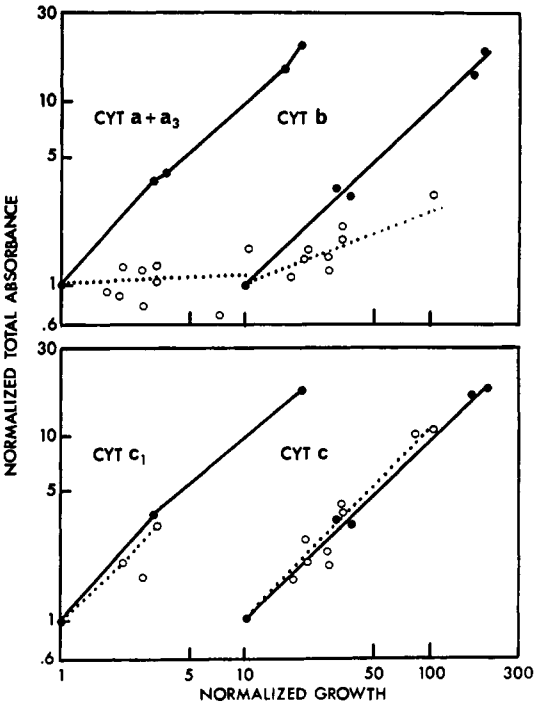

FIGURE 5: Biosynthesis of cytochromes aa_3, *b*, c_1, and *c* in *S. cerevisiae* growing on 3% lactate in the presence of CAP. Absorbance of each cytochrome was estimated by measuring $A_{600}-A_{630}$ for cyt aa_3, $A_{558}-A_{575}$ for cyt *b*, $A_{553}-A_{538}$ for cyt c_1, and $A_{547}-A_{538}$ for cyt *c*. Total absorbance was calculated by multiplying the absorbance per milligram of cell protein by the dry weight of the culture at the time that the sample was harvested. This was then normalized to the initial value which was 0.00364, 0.00563, 0.002, and 0.00626 for cyt aa_3, *b*, c_1, and *c*, respectively. Data from two separate experiments are plotted. Because reliable extinction coefficients for yeast cytochromes measured under these conditions are not available, no attempt was made to express these data in terms of micromoles of each cytochrome.

TABLE I: Changes in Relative Cytochrome Content.[a]

	Normal Dry Weight	c/a c_0/a_0	c/b c_0/b_0	c/c_1 c_0/c_{1_0}
Initial ratio		1.07	0.576	1.1
Control	1.0	1.0	1.0	1.0
	3.1	0.84	0.99	1.04
	19.8	0.82	1.0	1.35
CAP treated	1.6	1.47	1.26	1.2
	2.15	1.41	1.38	1.2
	2.76	1.37	1.34	1.25
	3.3	2.82	2.15	1.26
	10.0	9.5	4.8	1.16

[a] Ratios of absolute absorbances in mitochondrial fractions isolated from cells of *S. cerevisiae* grown in the presence of CAP (see legend to Figure 6 for details).

[2] Cytochrome *a*, cytochrome a_3, and a copper protein. Recent studies of Love *et al.* (1970) and Chan *et al.* (1970) suggest that the apoproteins of beef heart cyt *a* and a_3 may be the same; the same appears true for highly purified cytochrome oxidase preparations of *S. cerevisiae*, which furthermore contains four polypeptide chains (P. Shakespeare, in preparation).

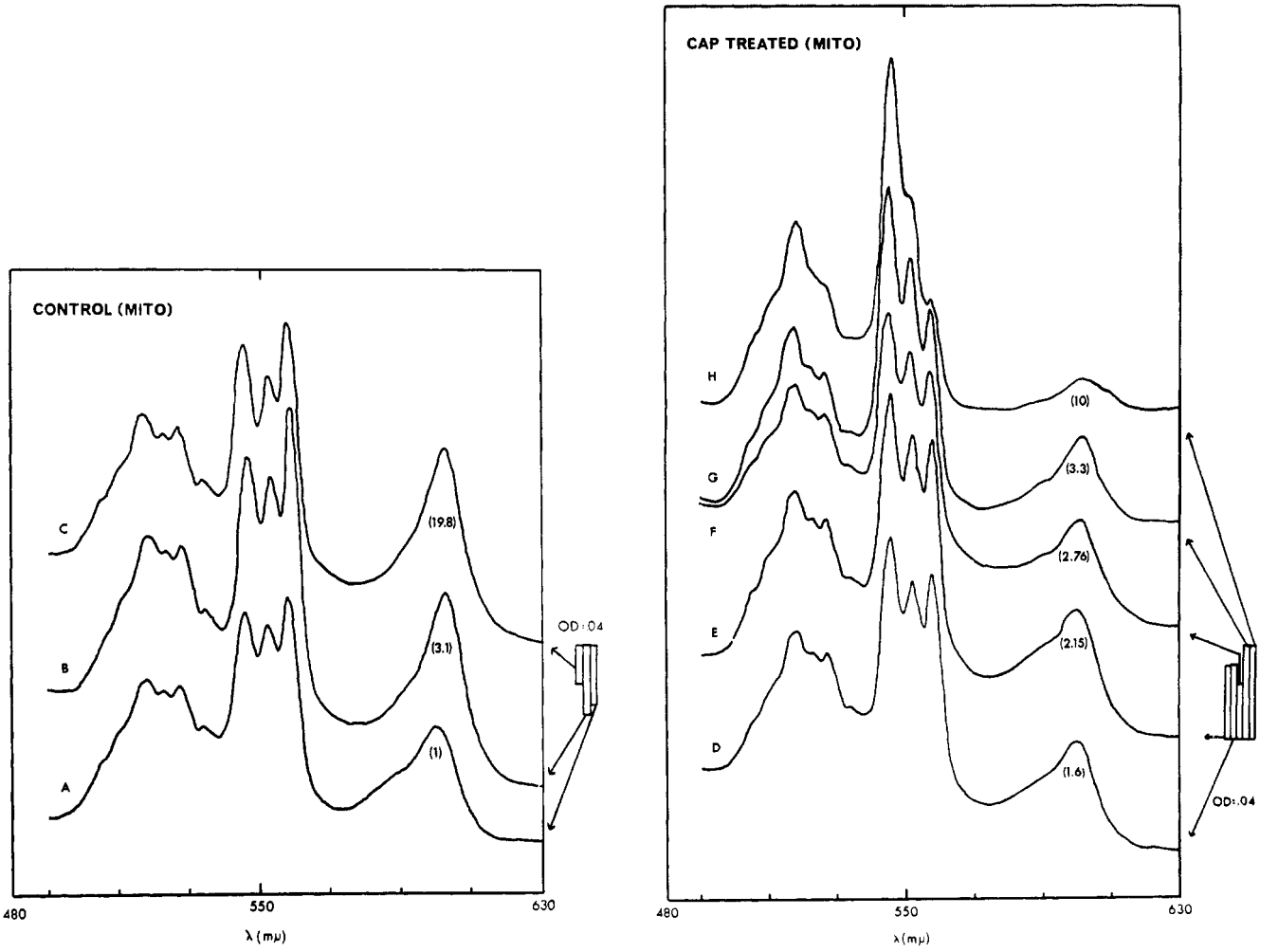

FIGURE 6: Cytochrome content of mitochondrial fractions isolated from *S. cerevisiae* grown on 3% lactate in the presence of CAP. Cell samples were obtained as described in the legend to Figure 4. Cells were broken by grinding with glass beads in STV-II (0.5 M sorbitol, 0.02 M Tris, and 1 mM EDTA, pH 7.5). Debris and unbroken cells were removed by centrifugation at 2000*g* for 10 min; mitochondria were sedimented by centrifugation at 20,000*g* for 20 min; the mitochondrial fraction was resuspended in 0.5 M sorbitol and repelleted at 20,000*g* for 20 min. This pellet was resuspended in 0.1 M phosphate buffer (pH 7.4) made 30% in glycerol for spectral analysis. Spectra of dithionite reduced samples minus buffer were obtained at the temperature of liquid nitrogen using a 3-mm path length. The numbers in parentheses are the dry weight (milligrams per milliliter of culture) normalized to the initial value which was 0.284 mg/ml. The protein concentrations of the mitochondrial suspensions were 6.62, 4.67, 9.4, 6.38, 7.99, 8.1, 3.62, and 3.7 mg per ml for curves A–H, respectively.

virtually constant in the controls; furthermore, the c/c_1 ratio also remained constant in the CAP-treated samples. The c/b ratio did not markedly deviate from the control value until the second generation, while c/aa_3 was altered from the very beginning of the experiment. We conclude that little or no cyt aa_3 was synthesized in the course of this experiment. Since data on whole cells and isolated mitochondrial fractions are in good agreement, we may rule out the possibility that there was some cyt aa_3 synthesis but that it did not become localized in the mitrochondrial fraction. This is in contrast to cyt c and c_1, substantial quantities of which were not only synthesized but continued to be localized in the mitrochondria. It is not always possible to assign the absorbance at 553–554 mμ to cyt c_1 since cyt b_1 and b_2 absorb in that region (Lindenmayer and Estabrook, 1958). Both of these species, however, are characterized by two peaks of equal intensity, at 552.5 and 557.5 for cyt b_2, and 551.5 and 557.5 for b_1. Since in our case the peak at 558 is less intense than the one at 553—and is reduced relative to the control samples—these drug-treated cells appear to have been capable of continued synthesis of cyt c_1. The question of cyt b synthesis is somewhat less straightforward; clearly, there was no significant further increase in this entity subsequent to the first generation of growth. However, it does not seem unlikely that some additional cyt b was formed coincident to the first mass doubling at a time when the synthesis of cyt aa_3 was already completely inhibited.

EFFECTS OF EB. Implicit in the work of other researchers (Slonimski *et al.*, 1968) and our earlier studies (Mahler *et al.*, 1971b) is the fact that EB does not inhibit growth of yeast on a fermentable carbon source. However, it does not follow that cells subjected to this treatment would also grow under the conditions used above. To examine this matter, aliquots of a culture growing on 3% lactate were treated with EB at several different concentrations and the fractions of mutants and wild-type survivors among the viable progeny determined periodically. Viable counts were identical with those of the control; thus there is *no* cell killing by EB under these conditions. Table II relates the rate of mutagenesis to the concentration of mutagen as an aid in the design of experiments for the study of completely mutagenized populations (*i.e.*, those known to be *fully* affected by the drug).

Figure 1A demonstrates that cells grow normally during

TABLE II: Mutagenesis of *S. cerevisiae* Growing at 30° on 3% Lactate in the Presence of EB.[a]

Time (min) of EB Treatment	EB Concn (μg/ml)					
	0	1	5	10	20	40
	% ρ^-					
0	1.0	1.0	1.0	1.0	1.0	1.0
20	1.0	2.8	1.97	8.35	29.7	92
60	1.0		45.9	98.1	100[b]	100
315	1.0	4.19	100	100	100	100
1065	1.0	99.71	100	100	100	100
1305	1.0	100	100	100	100	100

[a] A culture of *S. cerevisiae* growing exponentially on 3% lactate was divided into 6 aliquots each at 2×10^6 cells/ml ($A_{600} = 0.1$). EB (dissolved in methanol) was added at $t = 0$ to the concentrations indicated and suitably diluted aliquots of cells were plated on diagnostic agar plates (containing 0.1% glucose plus 2% lactate) using the soft-agar overlay technique. Plates were incubated at 30° for 3–4 days before being scored for % ρ^-. [b] Greater than 99.9% ρ^-.

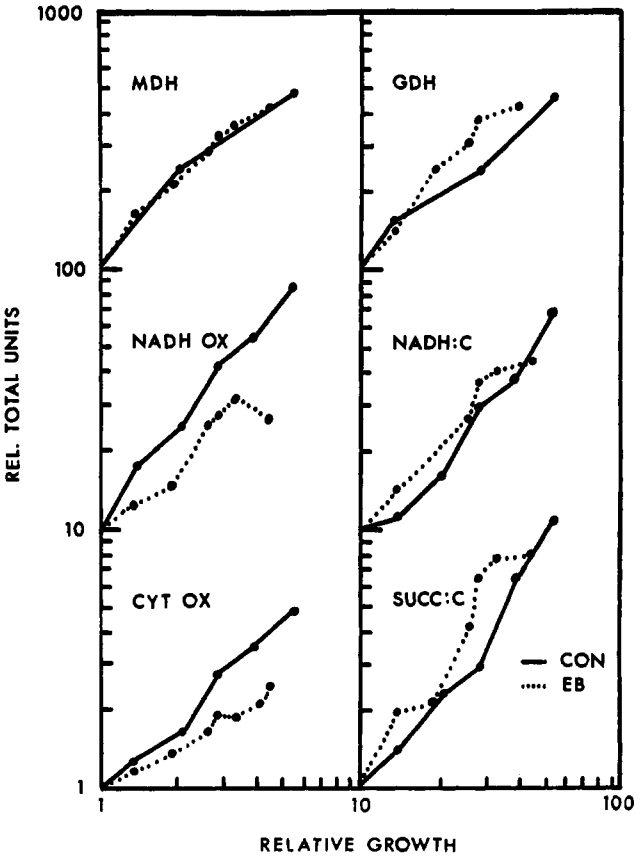

FIGURE 7: Effects of EB (20 μg/ml) on synthesis of soluble, matrix, and respiratory enzymes in cells of *S. cerevisiae* growing on 3% lactate. Aliquots of cells whose growth characteristics are presented in Figure 1A were harvested, broken by grinding with glass beads, and assayed (cell-free supernatant) for their content of the enzymes shown. Relative total units = (nmoles min^{-1} mg^{-1} of homogenate $protein^{-1}$) × (dry weight per ml of culture) normalized to the control value. The initial values of MDH, GDH, NADH ox, NADH:*c*, cyt ox, and Succ:*c* were 4090, 287, 173, 93, 215, and 47 nmoles min^{-1} mg^{-1}, respectively The initial dry weight was 0.162 mg/ml of medium.

mutagenic treatment and that once mutagenized, the population continues to grow for slightly more than two generations at a progressively reduced rate and then stops. These cultures were incubated for a total of 4 days during which time no further cell division was detected (Mahler *et al.*, 1971b). During the phase of active growth, the observed increases in turbidity, whole cell protein (per milliliter of culture), viable count, and dry weight were in excellent agreement.

Cells so mutagenized but placed on galactose, a fermentable, weakly repressing, carbon source, continue to increase in mass, total cell protein, and all the cytosol markers tested at rates indistinguishable from that of the control (Mahler *et al.*, 1971b; Perlman, 1971), for at least *six* generations. Such cells in a lactate medium are capable of supporting incorporation of appropriate precursors into nDNA (Perlman and Mahler, 1971), total cellular RNA and protein at rates no less than 90% those of the control (P. S. Perlman, C. Antalis, and H. R. Mahler, unpublished observations).

We conclude therefore, that EB does not exert any pronounced and general effect on the expression of nuclear genes; it is also known to be ineffective as a mutagen for such genes (P. P. Slonimski, private communication, 1970).

However, in comparison to CAP, EB appeared to be a more effective inhibitor of cellular growth on lactate, particularly beyond the second mass doubling, when, however, secondary effects due to its known irreparable alterations in the mtDNA might first become manifest. To obtain a valid comparison of the two drugs, therefore, we must restrict our attention to the initial two mass doublings following mutagenesis (and the time at which the phenotypic effects of EB become evident). The data of Figure 7 describe enzyme synthesis in EB-treated cells growing on 3% lactate; comparing Figures 2, 3, and 7 demonstrates that under these conditions the differences between the effects produced by the two drugs cannot be considered significant.

An analysis of cytochrome spectra of cells grown on lactate in the presence of EB proved inconclusive because the cells were characterized by a large EB absorption band which obscured the cytochrome peaks. We have, however, examined cytochrome synthesis in EB-treated cells but grown on 3% lactate in the *absence* of extracellular EB; these increases *also* continue for two generations as the cells similarly undergo conversion from the ρ^+ to the ρ^- phenotype subsequent to the prior genotypic conversion under nongrowing conditions (Mahler *et al.*, 1971b; P. Perlman *et al.*, in preparation). The findings from these experiments (P. Perlman *et al.*, in preparation) were in excellent agreement with those presented in Figures 4–6 and Table I in showing the total inhibition of cyt aa_3 synthesis and continued synthesis of cyt *c* and c_1 measured in both cells and isolated mitochondrial fractions.

We conclude that CAP and EB give qualitatively similar results and that any remaining quantitative differences, particularly subsequent to the second mass doubling, are referable either to consequences of the petite mutation that become manifest at that time in EB-treated cells or to the somewhat leaky nature of the CAP block. However, if the latter is the case, it becomes difficult to account for the high degree of specificity *and* effectiveness of CAP in inhibiting the further elaboration of cyt ox [and cyt aa_3].

Studies on Obligate Aerobes. We next present an extension of these studies to petite-negative yeasts, species that cannot yield viable respiratory-deficient mutants (DeDeken, 1961, 1966a,b; Bulder, 1964a,b; McClary and Bowers, 1967). Ex-

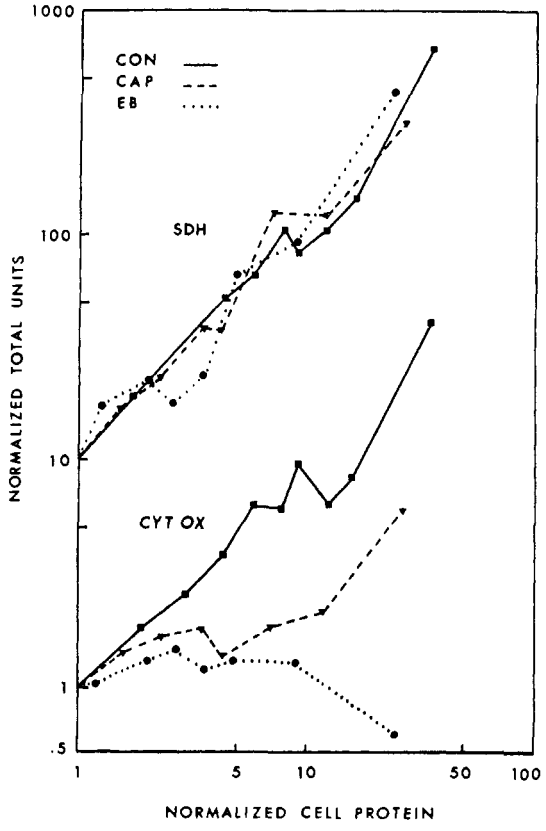

FIGURE 8: Effects of CAP (4 mg/ml) and EB (20 μg/ml) on enzyme synthesis in cells of *T. utilis* growing on 5% glucose. In an experiment similar to that shown in Figure 1C, aliquots of cells were collected and assayed for their content of several enzymes. Relative total units = (nmoles min^{-1} mg homogenate $protein^{-1}$) $\times$ (cell protein per ml of medium) normalized to the initial value. The initial values of SDH and cyt ox were 35 and 147 nmoles min^{-1} mg^{-1}, respectively. The initial cell protein concentration was 0.049 mg/ml of medium. The synthesis of MDH, IDH, and NADH:*c* was measured and was essentially the same as for SDH.

periments measuring the long-term effects of acridines on growth and respiration of such species have been reported previously (DeDeken, 1961, 1966a,b). More recently, Kellerman *et al.* (1969) compared the effects of CAP, euflavine, and low oxygen tension on the growth of the aerobic yeast, *Candida parapsilosis*, and on its cellular morphology, cytochrome content, and the levels of MDH, SDH, and fumarase, all after 16–18 hr of growth; EB was said to produce effects similar to those reported for euflavine.

Two such organisms were examined in these studies, *H. wingei* and *T. utilis*. Figure 1C presents growth data for the second organism on 5% glucose, conditions which in this instance produce no catabolite repression of respiratory biosynthesis. Neither CAP nor EB produced a profound inhibition of growth (Figure 1C); approximately 4.5 mass doublings occurred during a period of 19 hr in each case, during which time the control cells grew through five doublings. In contrast to the increase in mass in the presence of EB, the viable count (colony-forming capacity on nutrient plates after 6-days incubation at 30°) increased only for one generation after which time it fell off precipitously (Figure 1D). This lethality cannot be referrable to any interference with the nuclear genome; the expression (and presumed duplication) of the latter is unimpaired as seen from the growth curve and the absence of any effect whatever in the continued synthesis of all enzymes localized in either the cytosol or the mitrochondrial matrix (see below).

The biosynthesis of SDH (representative of most respiratory and soluble enzymes) and cyt ox under these conditions are compared in Figure 8. There was no effect of either drug on the biosynthesis of SDH while both drugs inhibited the biosynthesis of cyt ox activity after the first mass doubling. The effect of CAP was only partial during the last mass doubling, probably due to a loss of effectiveness as the pH dropped from 5.8 to 4.0 during that part of the experiment. CAP has been reported to be less effective in certain yeasts at a pH below 6.0 (P. P. Slonimski, private communication, 1970). In contrast, during the same period, the cyt ox activity in the cells exposed to EB did not increase.

In the presence of 5 μg of cyclo/ml there was no measurable increase in the amount of protein or enzymatic activity over a period of 6 hr.

Results with *H. wingei* were similar for CAP and cyclo but not for EB. The EB-treated cells managed but one mass doubling in 7.5 hr while the CAP-treated and control cells grew through 3.5 generations. A comparison and analysis of the kinetics of killing of these two aerobes is in progress.

For the moment, we may conclude that in obligately aerobic yeasts, CAP inhibits mitochondrial protein synthesis reversibly, while EB does so irreversibly. The fact that *T. utilis* continued to grow and increase in mass at a virtually undiminished rate when the viable count had dropped by 95% demonstrates that, in this organism also EB (at 20 μg/ml) does not interfere with the expression of nuclear genes.

Because *T. utilis* is rich in cytochromes *b*, c_1, and aa_3 relative to cytochrome *c*, it was possible to study the synthesis of all four cytochromes by examining spectra of whole cells. Furthermore any distortion of the cytochrome peaks by EB bound to cells was minimal so that the effects of CAP and EB on their synthesis could also be compared. Such spectra are presented in Figure 9a–c. Absorbances at the appropriate wavelengths were measured, converted into absorbance per milligram of cell protein and expressed as total absorbance (per milliliter of medium) normalized to the initial value (Figure 10).

EB quantitatively inhibited the synthesis of cyt aa_3, but allowed a substantial amount of cyt c_1 and some *b* synthesis; cyt *c* synthesis was actually stimulated over the level found in the control cells. A similar overproduction of cyt *c* appears to be the rule whenever there is a block of the mitochondrial system of gene expression (Peré, 1970).

While the effect of CAP was not so clearcut, cyt *b* and c_1 were less affected than was cyt aa_3 during generations 2 and 3. Cyt *c* synthesis was stimulated. In this organism, unlike *S. cerevisiae*, the pH of the medium drops below 6.0 in late exponential phase, and the CAP block became significantly relieved. No such effect was observed for EB.

Effects of CAP and EB on mitochondrial morphology in *T. utilis* have been presented elsewhere (Mahler *et al.*, 1971b) and are in agreement with those presented by Kellerman *et al.* (1969). Interference with the expression of mitochondrial genes produces only gradual changes in mitochondrial morphology. Only after four to five generations of growth in the presence of the inhibitors were grossly deranged mitochondrial profiles obtained. A similar analysis using *H. wingei* provided comparable results.

Discussion

Rationale and Specificities. Attempts at interpreting inhibitory patterns for the biosynthesis of intracellular protein,

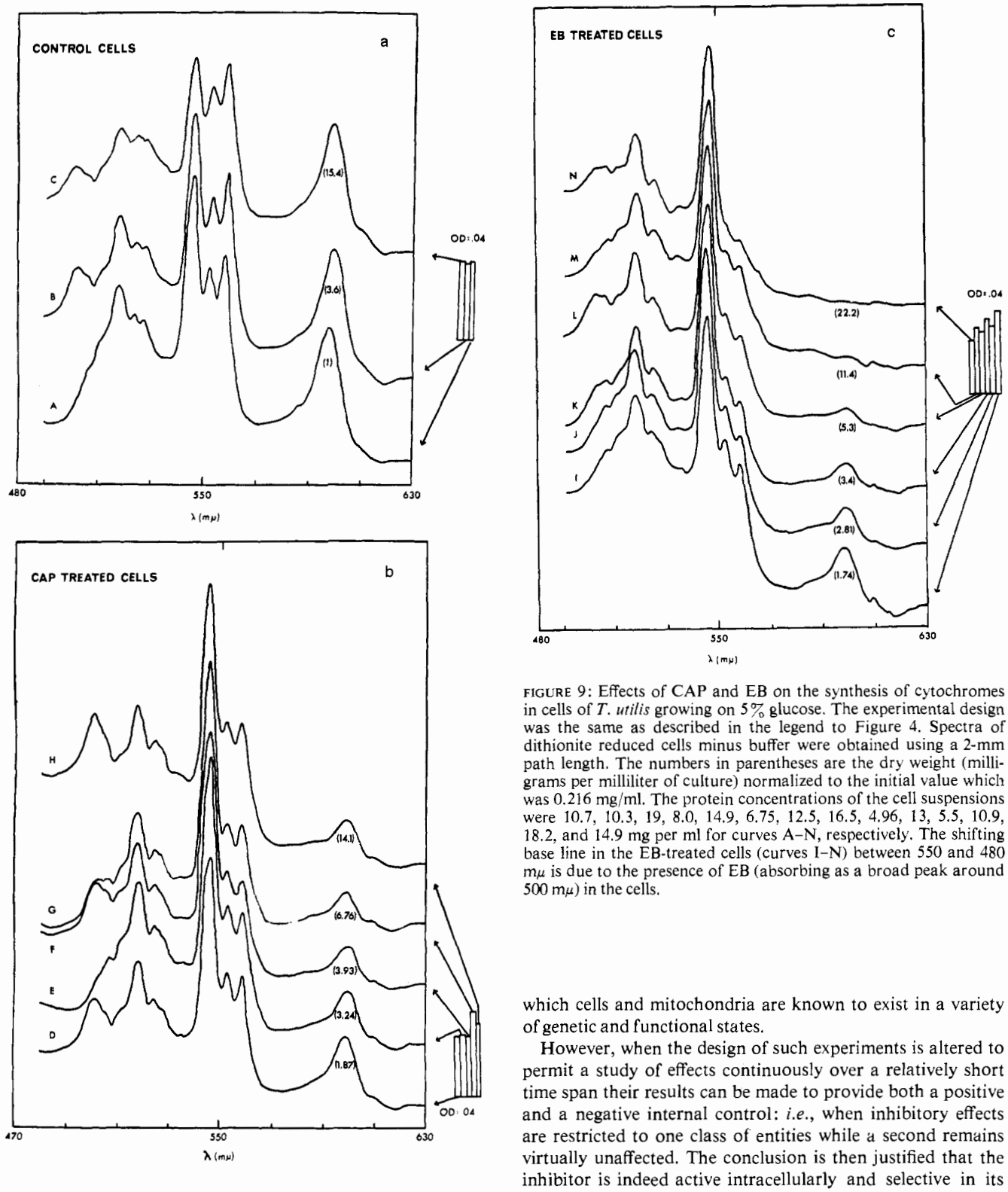

FIGURE 9: Effects of CAP and EB on the synthesis of cytochromes in cells of *T. utilis* growing on 5% glucose. The experimental design was the same as described in the legend to Figure 4. Spectra of dithionite reduced cells minus buffer were obtained using a 2-mm path length. The numbers in parentheses are the dry weight (milligrams per milliliter of culture) normalized to the initial value which was 0.216 mg/ml. The protein concentrations of the cell suspensions were 10.7, 10.3, 19, 8.0, 14.9, 6.75, 12.5, 16.5, 4.96, 13, 5.5, 10.9, 18.2, and 14.9 mg per ml for curves A–N, respectively. The shifting base line in the EB-treated cells (curves I–N) between 550 and 480 mμ is due to the presence of EB (absorbing as a broad peak around 500 mμ) in the cells.

particularly of complex catalytic or spectroscopically distinct entities, in whole cells are notoriously tenuous and ambiguous. The usual difficulties connected with specificity as to sites and mode of action become compounded when, as is frequently done, experimental observations are discontinuous and restricted to relatively long time spans (Huang *et al.*, 1966; Clark-Walker and Linnane, 1966, 1967; Mahler *et al.*, 1968; Henson *et al.*, 1968b) when sequential, cumulative, and synergistic effects may be expected to become increasingly severe. These strictures apply with particular force to yeast, in which cells and mitochondria are known to exist in a variety of genetic and functional states.

However, when the design of such experiments is altered to permit a study of effects continuously over a relatively short time span their results can be made to provide both a positive and a negative internal control: *i.e.*, when inhibitory effects are restricted to one class of entities while a second remains virtually unaffected. The conclusion is then justified that the inhibitor is indeed active intracellularly and selective in its mode of action. If the first class is composed of a sufficiently small number of subspecies and the block on their biosynthesis immediate it may even be possible to distinguish primary from secondary effects.

The known effects of CAP on macromolecular synthesis in yeast have already been discussed. Its specificity for the mitochondrial protein synthesizing system (see reviews in Nass, 1970, and Ashwell and Work, 1970) is explicitly supported by our data, since only the synthesis of certain proteins localized and *possibly* synthesized within the particles was affected at all.

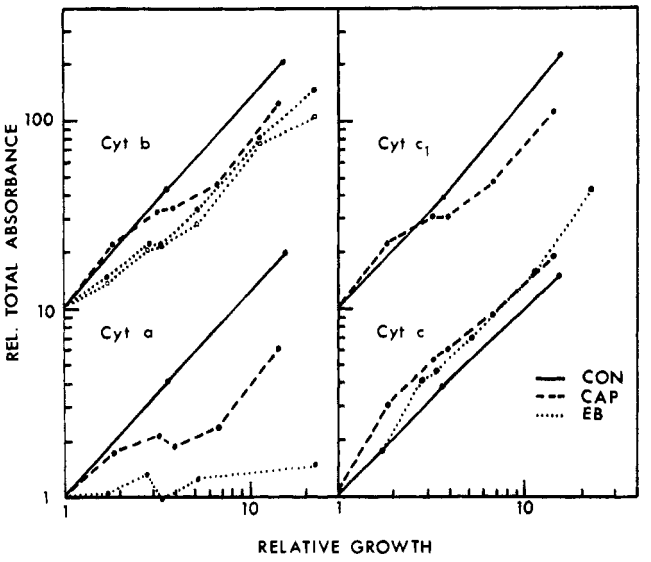

FIGURE 10: Biosynthesis of cytochromes aa_3, b, c_1, and c in cells of *T. utilis* growing on 5% glucose in the presence of CAP or EB. Absorbance of each cytochrome was estimated from Figure 9 and relative total absorbance calculated as described in the legend to Figure 5. Initial values of total absorbance were 0.0018, 0.0023, 0.0012, and 0.0025 unit per mg for cyt aa_3, b, c_1, and c, respectively. Because of the enhanced cyt c synthesis in the EB-treated samples, it was not possible to estimate cyt c_1 content in samples I–N (Figure 9). Cyt b content for EB was estimated in two ways: (1) $A_{558} - A_{575}$ (filled circles) and (2) A_{558} − base line at 558 mμ (open circles). While CAP was not as effective in inhibiting cyt aa_3 synthesis as was EB, there was a period of essentially complete inhibition (between relative growth = 1.87 and 6.96). During that time interval cyt b and c_1 synthesis were relatively insensitive to the drug.

Although similar to CAP in its effects the known mode of action of EB blocking cellular functions is not as clear-cut. To be sure, in *S. cerevisiae*, EB is a specific mutagen (Slonimski *et al.*, 1968) and effectively inhibits mtDNA and RNA synthesis (South and Mahler, 1968; Goldring *et al.*, 1970; Fukuhara and Kujawa, 1970; Perlman and Mahler, 1971) while it has no effect on the entire NC system. It may, however, inhibit mitochondrial protein synthesis directly (as noted by Kellerman *et al.*, 1969) by a close coupling to transcription; it must inhibit that synthesis indirectly and progressively as a result of the decay of mitochondrial messages, ribosomes, and even genes present at the time of drug addition.

Selective Probes for Mitochondrial Gene Expression. With the reservations just presented and remembering that any inferences may be valid only for the particular physiological state investigated (cells growing in the exponential phase in the absence of catabolite repression), we may now direct ourselves to the three questions raised earlier. (1) No qualitative difference in response to CAP or EB at the level of enzyme or cytochrome synthesis was observed despite the variety of yeast species examined. However, quantitative differences were (not surprisingly) detected. The action of EB was lethal to the aerobes under conditions that rapidly produced a viable mutant genotype in the facultative anaerobe. We therefore assume as a working hypothesis that essentially the same primary event must have occurred in both instances, namely, some irreversible alteration in the function of mitochondrial DNA. As already mentioned, binding of EB by mitochondria is known to inhibit both their DNA and RNA synthesis; it is probable that only the former would lead to rapidly irreversible consequences. As already indicated EB can also block mitochondrial protein synthesis in a more or less direct manner. However, since CAP (which exerts this last-named effect *in vivo* as well as *in vitro*) is devoid of lethality towards the obligate aerobes and does not produce mutants rapidly in *S. cerevisiae*, interference with mitochrondrial protein synthesis by itself in short-term experiments is not sufficient to produce the irreversible damage to mtDNA required in mutagenesis. (2) Again, regardless of organism, CAP and EB produced qualitatively similar effects on mitochondriogenesis. Interference, therefore, with mitochondrial gene expression, be it at the level of transcription (by EB) or of translation (by CAP) results—among the parameters tested—directly and most immediately in a selective interference with the continued biosynthesis first of cytochrome aa_3 and shortly thereafter of cytochrome oxidase activity. With time, other activities become affected progressively as does the detailed morphology of the mitochondrial inner membrane, leading, eventually to the aberrant types reported elsewhere (Mahler *et al.*, 1971b; see also Kellerman *et al.* (1969) for analogous observations on *C. parapsilosis* exposed to euflavin). (3a) The most rapid and severe and, hence, the minimal phenotypic consequences of interference with the expression of the mitochondrial genome produces an inhibition of synthesis, first of cyt aa_3 and shortly thereafter of cytochrome oxidase activity. These data do *not*, however, permit the inference that either of these entities is *itself* a product of mitochondrial transcription or translation. It will be recalled that their synthesis is also blocked completely by the addition of cyclo.

The extent of incremental increase of cyt ox and its inhibition by cyclo under these conditions suggest that at least a portion of the additional activity required the incorporation of proteins made *de novo* during the course of the experiment; it is clear that not *all* component proteins were required for additional activity. Other factors include the possible activation of enzyme precursors present at the time of drug addition and alterations in the stoichiometry of the enzyme due to changes in the membrane which result in altered kinetic characteristics.

While cyt c_1 continues to be synthesized, a secondary effect of an unusual type was observed; substantial amounts of Succ:*c* and NADH:*c activity* were synthesized under conditions where at least one of their components, cyt b, was made to a much lesser extent. It is becoming increasingly clear that the activity-to-heme ratio of respiratory enzymes need not be a fixed parameter (Chen and Charalampous, 1969).

A possible explanation for the apparent discrepancy between enzyme activity and cytochrome content may be found in the accumulating evidence for high-energy forms of cytochromes (Chance *et al.*, 1966; Wilson and Dutton, 1970; Slater *et al.*, 1970; Wegdam *et al.*, 1970) involved in oxidative phosphorylation: in this interpretation enzyme activity might continue to increase at the expense of preformed cytochrome present at the time of drug addition, but with an attendant lowering of phosphorylative efficiency. The latter effect then becomes a testable prediction of the hypothesis. (3b) If we assume for the moment that initially CAP has no effect on mtRNA synthesis and that EB inhibits mitochondrial protein synthesis only indirectly then we may draw some strong inferences from the data presented here. (1) So far as the proteins of the respiratory chain are concerned. (a) No mitochondrial transcript can have been *wholly* translated on cytoribosomes. (b) No nuclear transcript can have been *wholly* translated on mitoribosomes. (c) There is a time lag between immediate effects caused by specific blocks in the mitochondrial system and the resultant secondary inhibition of the NC system; that

is, the coupling between the two intracellular systems of gene expression may be disrupted (see also Mahler *et al.*, 1971a, b, and P. S. Perlman *et al.*, in preparation). (2) Mitochondrial mRNA must have a relatively short half-life relative to cellular generation time. (3) Since cells growing under these particular conditions contain mitochondria in a completely derepressed state, the further increases in the activity of membrane-bound enzymes (or their constant specific activity) observed subsequent to drug addition suggest that mitochondrial division remains coupled to the cell cycle. Thus new mitochrondrial mass is produced even when there is block on the expression of at least one mitochondrial gene crucial for the maintenance of respiratory sufficiency. This conclusion is confirmed by the dilution of cyt aa_3 in whole cells *and* in isolated mitochondrial fractions coupled with the lack of dilution of at least two other cytochromes (c and c_1).

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Role of Adenosine Cyclic Monophosphate in the Synthesis of Tyrosine Aminotransferase in Neonatal Rat Liver. Release of Enzyme from Membrane-Bound Polysomes *in Vitro**

Chong-Cheng Chuah and I. T. Oliver†

ABSTRACT: Previous results suggested that the synthesis of at least one form of tyrosine aminotransferase was under the control of adenosine 3′,5′-cyclic monophosphate in neonatal rat liver *in vivo*. The locus of action of the cyclic nucleotide appeared to be at a posttranscriptional step in enzyme synthesis. In postnatal Wistar albino rats of 1- to 2-days old, epinephrine and puromycin injection results in elevation of hepatic tyrosine aminotransferase activity. Microsome fractions isolated from liver homogenates prepared from such rats show a threefold elevation of tyrosine aminotransferase activity when incubated *in vitro* with cAMP, puromycin or ATP, in the absence of GTP and a nucleotide triphosphate regenerator. No additives from the supernatant fraction are necessary for the effect. Other 3′,5′-cyclic nucleotides, 5′-AMP, and 2′,3′-cAMP are without effect but cIMP and GMP inhibit the elevation of activity mediated by cAMP. Zone centrifugal analysis of the cAMP-treated microsomes shows that enzyme is released in a form having the usual molecular weight. The effect is localized to the membrane-bound polysomes which can be washed free from all apparent enzyme activity. cAMP is then required to release the bound enzyme in an active form. The ATP effect is due to adenylylcyclase activity in the microsome fraction. A microsomal protein which strongly binds cAMP is essential for enzyme release. Results with cycloheximide indicate that the system releases only finished enzyme molecules (or subunits) and that polypeptide-chain elongation does not occur. The results are discussed in relation to regulatory mechanisms of protein synthesis in higher organisms and a terminator release control mechanism is suggested which may operate in the synthesis of a range of specific proteins.

In recent papers from this and other laboratories, adenosine 3′,5′-cyclic monophosphate (cAMP)[1] has been implicated as an effector in the postnatal synthesis of tyrosine aminotransferase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5) in rat liver (for summary, see Holt and Oliver, 1971) but the evidence indicates that the effect is restricted to posttranscriptional events (Holt and Oliver, 1969a). Multiple forms of tyrosine aminotransferase in rat liver have been demonstrated by analysis in polyacrylamide gel electrophoresis (Holt and Oliver, 1969b, 1971; Sadleir *et al.*, 1970), and cAMP has its effect on the synthesis of only one of the forms *in vivo* (form C) (Holt and Oliver, 1969b).

Chuah *et al.* (1971) suggested the operation of a cAMP-dependent control at the termination or release step of tyrosine aminotransferase synthesis in neonatal rat liver and presented evidence for such a hypothesis. This evidence was largely based on an anomalous effect of puromycin *in vivo*, in that drug administration to intact animals under certain

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[1] Abbreviations used for nucleoside 3′,5′-cyclic monophosphates have the word cyclic preceding the conventional abbreviation for the 5′-nucleoside monophosphate. Adenosine 2′,3′-cyclic monophosphate (2′,3′-cAMP), N^6-2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate (dibutyryl-cAMP). Other nucleotides are abbreviated conventionally. Phosphoenolpyruvate (PEP), phosphoenolpyruvate kinase (PK), *p*-hydroxyphenylpyruvate (PHPP), reduced glutathione (GSH), sodium deoxycholate (DOC), rat hepatoma cell (HTC), tyrosine aminotransferase (TAT). TKM buffer, 50 mM Tris-HCl–275 mM KCl–5 mM $MgCl_2$ (pH 8.0). TAM buffer, 50 mM Tris-HCl–275 mM NH_4Cl–5 mM $MgCl_2$ (pH 8.0).