Formation of Yeast Mitochondria. II. Effects of Antibiotics on Enzyme Activity during Derepression*

Carl P. Henson,† Philip Perlman,‡ Celia N. Weber, and H. R. Mahler§

ABSTRACT: Using the yeast, *Saccharomyces cerevisiae*, we have investigated long- and short-term effects of cycloheximide and chloramphenicol on the appearance of cytoplasmic and mitochondrial enzyme activities during release from glucose repression. The pattern exhibited is complex. (1) All of the enzymes studied, whether located in the cytoplasm or the mitochondria, were sensitive to cycloheximide. (2) Although enzymes located in the mitochondria, especially those of the inner membrane, were initially more sensitive to chloramphenicol, by 6 hr after the addition of the drug, both cytoplasmic and mitochondrial enzymes were affected. (3) Enzymes having similar locations, *i.e.*, the parts of the respiratory chain such as reduced nicotinamide–adenine dinucleotide–ferricyanide reductase, antimycin A sensitive reduced nicotinamide–adenine dinucleotide–cytochrome *c* reductase, and cytochrome oxidase; the soluble enzymes of the cytoplasm, malic dehydrogenase and reduced nicotinamide–adenine dinucleotide–ferricyanide reductase; or the microsomal enzymes, reduced nicotinamide–adenine dinucleotide phosphate–cytochrome *c* reductase and antimycin-insensitive reduced nicotinamide–adenine dinucleotide–cytochrome *c* reductase, showed noncoordinated patterns of sensitivity to the drugs. A model for the formation of mitochondria during derepression is proposed.

We are interested in the biogenesis of mitochondria in *Saccharomyces cerevisiae*. The paradigm we have employed in previous studies (Jayaraman *et al.*, 1966; Henson *et al.*, 1968) depends upon the fact that this organism is subject to metabolic regulation by glucose repression (Ephrussi *et al.*, 1956; Linnane, 1965; Polakis *et al.*, 1965; Polakis and Bartley, 1966; Utter *et al.*, 1967; Witt *et al.*, 1966a). In this state, when glucose is used as their sole carbon and energy source, cells contain low levels of a number of respiratory enzymes and of functional mitochondria. As glucose becomes exhausted by cell growth and division and is replaced by ethanol (plus some acetate and lactate), the levels of these enzymes rise, functional mitochondria appear in large number, and cellular metabolism is directed from aerobic glycolysis to terminal oxidation and oxidative phosphorylation. We wish to establish the causal relationship between these sets of events and especially to define the in- and interdependence of cytoplasm and mitochondria in the course of the formation of these particles. Earlier we have presented evidence for a model which postulates that as a consequence of growth during repression the average cell is characterized by a possibly heterogeneous population of immature mitochondria (Jayaraman *et al.*, 1966). In the course of derepression these precursors are reorganized and converted into fully competent mitochondria by the acquisition of additional structural and functional components in, perhaps, several definite and discrete steps. What are the origins of these additional components; where and how are they assembled into the final particle?

In the previous paper (Henson *et al.*, 1968) we have tried to probe these questions by means of studies on the kinetics of incorporation of radioactive amino acids into the proteins of a number of different subcellular and submitochondrial fractions. We concluded that, although mitochondria do contain a distinct protein-synthesizing system, its function appears to be primarily concerned with the synthesis of certain minor components, perhaps with a regulatory and integrative function, and that the bulk of even the insoluble, membrane-bound mitochondrial proteins appears to originate in the cytoplasm, and is, therefore, probably specified by nuclear genes. Many of these experiments made use of inhibitors of protein synthesis of selective, and mutually complementary, specificity.

We now wish to describe the results of the same experimental approach, but this time on the level of enzyme activity. The rationale of such studies is based on the work of Linnane and his collaborators (Clark-Walker and Linnane, 1966, 1967; Linnane *et al.*, 1967). They showed that chloramphenicol appeared

* Contribution No. 1618 from the Chemical Laboratory, Indiana University, Bloomington, Indiana 47401. *Received June 7, 1968.* This research was supported by Research Grant GM 12228 from the National Institute of General Medical Sciences, National Institutes of Health, U. S. Public Health Service.

† Receipt of a postdoctoral fellowship from the National Science Foundation during 1966–1967 and a senior traineeship from Training Grant 2 T01 GM 01046-06 from the National Institutes of Health during 1967–1968 is gratefully acknowledged.

Present address: Electro-Optical Systems, Inc., Pasadena, Calif.

‡ Receipt of a predoctoral research fellowship (2-F1-GM-37, 632-01) from the National Institutes of Health during 1967–1968 is gratefully acknowledged.

§ Recipient of Research Career Award GM 05060 from the National Institute of General Medical Sciences, U. S. Public Health Service.

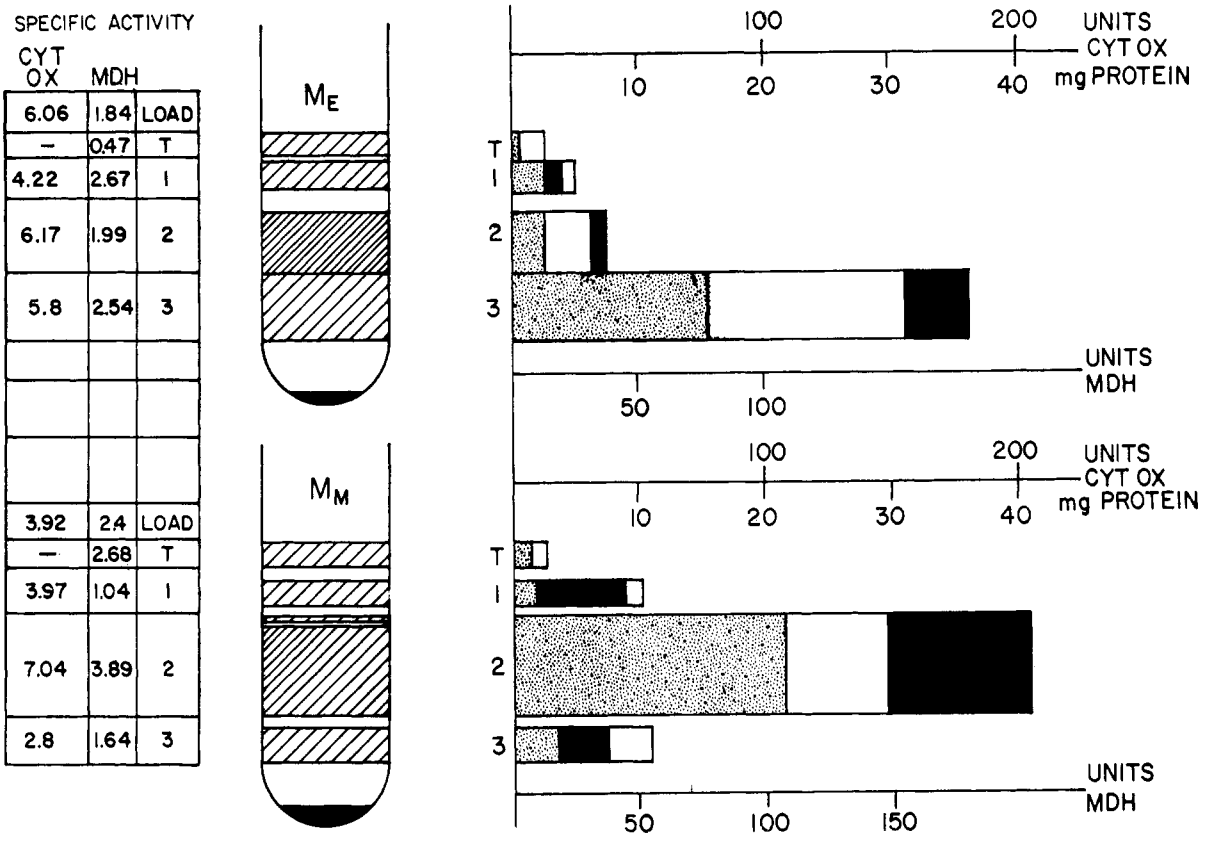


FIGURE 1: Association of malic dehydrogenase activity with mitochondrial material. Mitochondria were prepared either mechanically as described in Materials and Methods or through enzymatic lysis of the cells (Leon and Mahler, 1968) and suspended in 1 M sorbitol. Aliquots containing approximately 70 mg of protein in 3 ml of suspension were layered on a discontinuous sorbitol gradient composed of 6 ml of 70% and 3 ml each of 60, 57.5, 55, 52.5, and 50% sorbitol in 0.02 M Tris (pH 7.4). After 2.5-hr centrifugation at 63,500*g*, the tubes were sliced as indicated and the malic dehydrogenase (MDH), cytochrome oxidase (cytox), and protein content of the slices was determined. Enzyme activity is defined as micromoles of substrate oxidized per minute at 23°. Specific activities (micromoles per minute per milligram) are on the left; total activities (micromoles per minute) on the right; protein, empty bars; cytochrome oxidase, filled bars; and malic dehydrogenase, shaded bars.

to be virtually ineffective in inhibiting the growth of yeast cells under conditions of glucose repression (5% glucose), but was capable of interfering with the development of structurally and functionally competent mitochondria in cells permitted to reach stationary phase after growth on glucose at lower concentrations (1%). Conversely, the elaboration of mitochondria and their constituent cytochromes was reported to be insensitive to inhibition by cycloheximide. Based on these observations, plus the known mode of action of chloramphenicol on mitochondrial (Wintersberger, 1965; Linnane *et al.*, 1967, 1968; Mahler *et al.*, 1967), and cycloheximide on cytoplasmic protein synthesis in yeast extracts (Siegel and Sisler, 1965; de Kloet, 1965), Clark-Walker and Linnane (1967) proposed a model which postulated that cristae-bound mitochondrial enzymes were synthesized within the particles, while the more easily solubilized mitochondrial enzymes shared with other cytoplasmic proteins a site of synthesis in the cytoplasm. To account for the decrease in the elaboration of L-malate dehydrogenase and fumarase observed when chloramphenicol was added to 1% glucose, these investigators advanced a subsidiary hypothesis, namely that this lowered activity was not due to a direct effect of chloramphenicol on the synthesis of these enzymes "but a composite effect of chloramphenicol and the phenomenon of glucose repression." This model can be extended to account for all the observed effects of chloramphenicol. The inhibitor may exert its action by inhibiting the synthesis of a protein of mitochondrial origin, triggered in turn by a change in the intracellular concentration of glucose or a glucose metabolite, which must be present in order to release the biosynthesis of respiratory proteins from repression.

Our tentative interpretation of the data presented here is that neither of the two models just outlined is adequate to account for the results obtained. Effective mitochondriogenesis appears to depend upon a highly complex set of interactions between the mitochondrial and extramitochondrial protein-synthesizing systems. These conclusions confirm and extend those presented previously in a brief communication (Mahler *et al.*, 1968).

Results

Localization of Marker Enzymes. In yeast the two main segments of the respiratory chain from NADH to oxygen can be independently assayed in intact

mitochondria or submitochondrial particles since both the NADH–cytochrome *c* reductase system and the cytochrome *c* oxidase readily react with externally added cytochrome *c* (Mackler *et al.*, 1962; Mahler *et al.*, 1964a). Furthermore, the former appears completely accessible to extramitochondrial NADH and is virtually completely sensitive to small amounts (≤ 1 μg/ml) of antimycin A, and therefore its assay is free of the difficulties encountered with mammalian heart, or liver (Green *et al.*, 1954; Slater, 1958; Raw and Mahler, 1959; Lehninger, 1964; Sottocasa *et al.*, 1967). Since both the oxidase and reductase remain associated with the particulate fraction when mitochondria are converted into submitochondrial particles by a variety of means, such as exposure to high pH or various detergents, disruption by osmotic shock, freezing and thawing, sonic oscillation, etc. (Slonimski, 1956; Mackler *et al.*, 1962; Schatz and Racker, 1966; Henson *et al.*, 1968), it appears reasonable to assign these activities to the mitochondrial inner membrane in yeast in complete analogy to other cells (Blair *et al.*, 1963; Parsons *et al.*, 1966; Sottocasa *et al.*, 1967; Schnaitman *et al.*, 1967; Bachmann *et al.*, 1967). A third mitochondrial enzyme assayed was NADH dehydrogenase (measured as NADH–ferricyanide reductase), presumably the first segment of the respiratory chain, and similar to it in its localization. Finally, mitochondrial L-malate dehydrogenase has been selected as an example of an enzyme easily extracted from mitochondria and definitely not associated with the inner membrane of the particle (Allmann *et al.*, 1966; Klingenberg and Pfaff, 1966; Caplan and Greenawalt, 1968). In our current studies we have found it to be virtually completely solubilized on freezing and thawing the mitochondria in distilled water. In spite of this easy extractability L-malate dehydrogenase is an authentic mitochondrial activity. When intact mitochondria are analyzed on continuous or discontinuous sorbitol gradients, L-malate dehydrogenase can be shown to be localized in a band that coincides with one that contains the various components of the respiratory chain (Figure 1). It remains associated with the mitochondria on extensive incubation at 30° (Leon and Mahler, 1968) or washing with salt solutions such as KCl, provided the osmolarity is maintained at isotonicity or above.

These considerations are of importance in view of the fact that the bulk of the L-malate dehydrogenase activity is due to a second derepressible enzyme (Witt *et al.*, 1966b; Ferguson *et al.*, 1967) localized in the soluble portion of the cytoplasm (Jayaraman *et al.*, 1966). As will be seen, this entity is one of the first enzymes to be derepressed, and its increase in activity is abolished virtually completely by cycloheximide. These observations have prompted us to regard it as a marker for other proteins found in this compartment and synthesized by the cytoplasmic protein-synthesizing system. The soluble portion of the cytoplasm also contains considerable NADH–ferricyanide reductase activity, probably related to the NADH–menadione reductase described by Misaka and Nakanishi (1963).

NADPH–cytochrome *c* reductase was shown by Schatz and Klima (1964) to be a microsomal enzyme with comparable activities in anaerobically or aerobically grown wild-type cells or cytoplasmic (ρ^-) petite mutants. Similarly, Polakis *et al.* (1965) reported that this activity was not susceptible to glucose repression. We have confirmed these findings and have used this activity[1] both as a marker for microsomal proteins and as a measure of the extent of variability within any one experiment and between different experiments. Finally we have observed that the antimycin A insensitive NADH–cytochrome *c* reductase, like its NADPH-specific counterpart, is localized in the microsomes, again confirming the conclusion of Schatz and Klima (1964). In contrast to the NADPH-requiring enzyme, however, this activity appears to be weakly repressible.

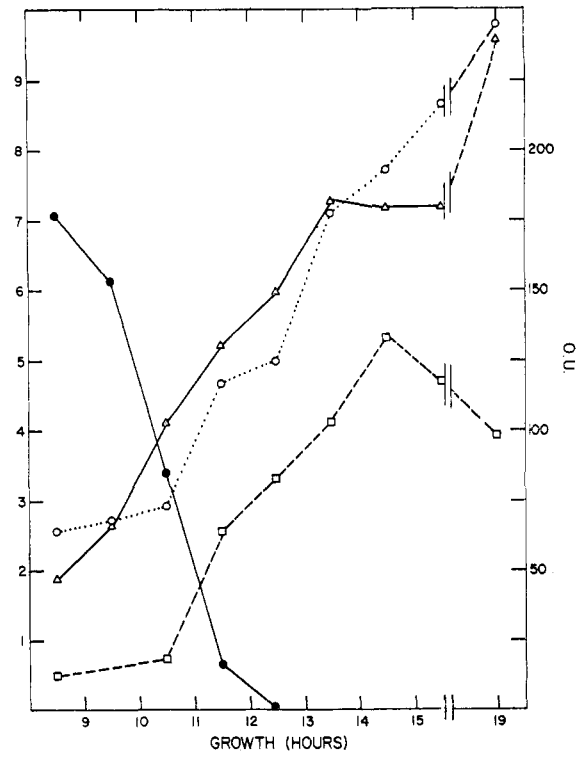


FIGURE 2: Growth and derepression of *S. cerevisiae*. The abscissa indicates length of time of aerobic growth subsequent to inoculation at $t = 0$. The left-hand ordinate is used for (—●—) glucose concentration (in micrograms per milliliter of medium), (—△—) cell protein $\times 10^{-1}$ (in milligrams per milliliter of medium), and (···○···) specific activity of antimycin-sensitive NADH–cytochrome *c* reductase $\times 10^2$ (in μmoles of substrate oxidized $\times$ min^{-1} $\times$ mg^{-1} of homogenate protein). The right-hand ordinate is used for oxygen uptake (- -□- -); values are in μmoles of O_2 consumed with glucose as substrate $\times$ min^{-1} $\times$ mg^{-1} of cell protein.

Long-Term Exposure to Antibiotics. In these experiments, we determined the effect of adding two different concentrations of chloramphenicol at the time of inoculation or after 11-hr growth on 1% glucose as carbon and energy source, *i.e.*, roughly at the time of initiation of derepression. Some parameters that define growth

[1] We have also found that mitochondria appear to contain a repressible system of low, but reproducible total activity capable of reducing cytochrome *c* at the expense of NADPH.

and derepression of control cells under our standard conditions are shown in Figure 2. We also measured the effect of cycloheximide added at 11 hr. Some of the effects of the various inhibitors on growth, on derepression of O_2 uptake, and on amino acid incorporation have already been described (Henson *et al.*, 1968; Mahler *et al.*, 1968). The two concentrations of chloramphenicol used had been shown not to affect growth, although oxygen uptake at time of harvest was 10 and 30% that of control cells with 4 and 2 mg per ml of the inhibitor added at $t = 0$, respectively. Inhibition of increase in oxygen uptake was complete when 4 mg of chloramphenicol was added at $t = 11$ hr. This level of antibiotic added at that time inhibited total protein synthesis by 20% as measured by phenylalanine incorporation into protein by whole cells. Cycloheximide, added at a concentration of 1 μg/ml at $t = 11$ hr, inhibited the increase in oxygen uptake by 84%; the extent of inhibition of amino acid incorporation at that concentration was ~40% during periods up to 30 min (Henson *et al.*, 1968).

The specific questions we wish to answer in these experiments are the following. (1) Is the derepression of all membrane-bound respiratory enzymes equally sensitive to chloramphenicol? (2) What is the effect of chloramphenicol on derepressible cytoplasmic and easily extractable mitochondrial enzymes; are they all affected to the same extent, and what is this extent relative to that of membrane-bound enzymes? (3) To what extent, if any, are the answers to 1 and 2 dependent upon the concentration and the time of addition of the inhibitor? (4) Is the derepression of membrane-bound enzymes insensitive to inhibition by cycloheximide as claimed by Clark-Walker and Linnane (1967) for cytochrome formation? (5) If the answer to question 4 is in the negative are all membrane-bound enzymes equally susceptible to such inhibition? (6) Is the derepression of all cytoplasmic and easily extractable mitochondrial enzymes equally sensitive to cycloheximide; what is the extent of this sensitivity relative to that of the membrane-bound enzymes?

Several experiments have been performed with virtually identical results. The pertinent data for one of these are tabulated in Table I in a form that uses the enzymatic activity of cells harvested at 11 hr as a standard state (set equal to 1.00) and compares it with the control and the various experimental cultures, all harvested after 17.75 hr. At this time derepression is virtually complete, as shown by various control experiments in which cells that had been allowed further growth, in the absence or presence of inhibitors, did not show any additional oxygen uptake. Per cent inhibition in these experiments is defined as [1.00 − (relative activity in experimental/relative activity in control)] × 100%, where relative activity is activity at 17.75 hr/activity at 11 hr.

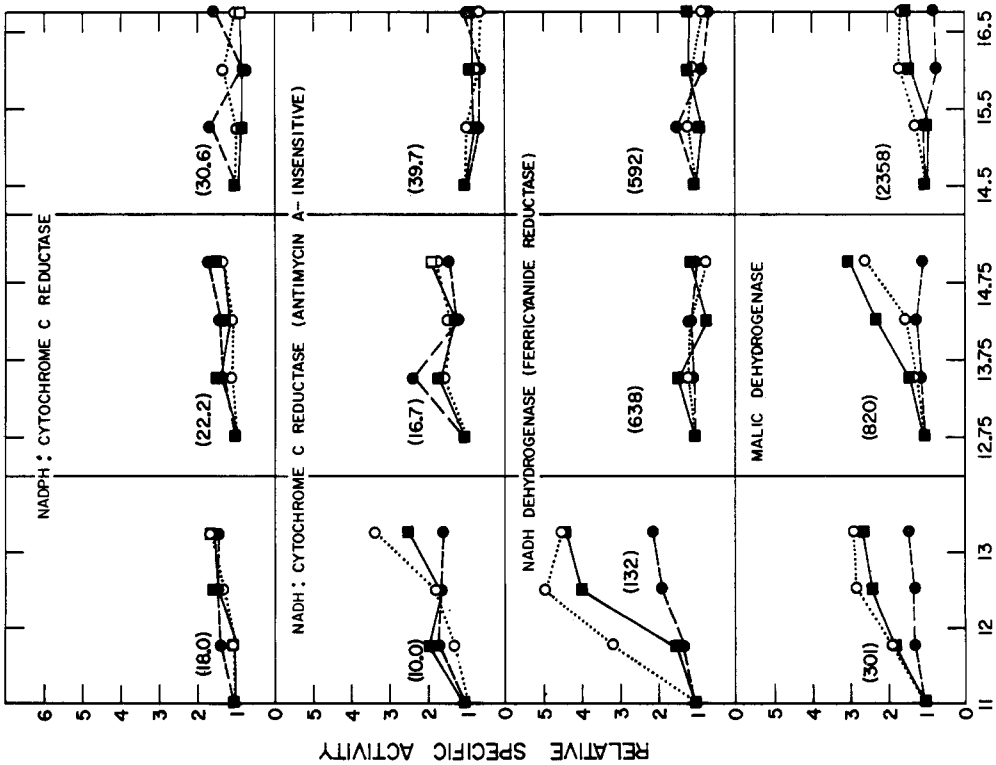
The answers to the six questions above provided by these experiments are: (1) While the elaboration of the reductase and oxidase portions of the chain appear to exhibit similar sensitivities to chloramphenicol, its first step, as measured by formation of the NADH–ferricyanide reductase, appears to be relatively insensitive. A possible interpretation of these findings is that the synthesis of functional cytochromes is more sensitive to inhibition by chloramphenicol than is that of dehydrogenases. This conclusion is weakened by the fact that we do not really know the nature of the rate-limiting reaction in the various assays used, and that the segments of the chain involved contain a variety of components other than cytochromes. (2) The derepression of all enzymes, whether mitochondrial or cytoplasmic, is subject to inhibition by chloramphenicol, with the cytoplasmic variants of the two enzyme pairs tested (malic dehydrogenase and NADH–ferricyanide reductase) only slightly less affected than are their mitochondrial counterparts. Again different enzymes exhibit significant differences in their sensitivity to the antibiotic–mitochondrial malic dehydrogenase (an enzyme *not* localized on the inner membrane) is strongly affected by the inhibitor, to almost the same extent as are the cytochrome reductase and oxidase, and significantly *more* so than is the ferricyanide reductase (yet all of these latter activities share a localization on the inner membrane). Similarly, cytoplasmic malic dehydrogenase and NADH–ferricyanide reductase are both soluble enzymes, yet the derepression of the former is greatly more sensitive to chloramphenicol than is that of the latter. (3) The effects just described can be modified somewhat by varying the concentration and the time of addition of the antibiotic, but all these modifications appear to affect all enzymes to approximately the same extent, indicating that the same *primary* mode of action, presumably the biosynthesis of some component(s) within the mitochondrion, is indeed involved. For instance, when the chloramphenicol concentration is doubled, the residual increase in activity (a more sensitive parameter in this instance than its complement, the extent of inhibition) is approximately halved for all the enzymes measured, except for the microsomal antimycin-insensitive NADH–cytochrome *c* reductase, an enzyme that appears to behave anomalously in respect to other variations of conditions as well. (4) The development of *all* mitochondrial activities, not only of those that are easily extractable, such as malic dehydrogenase, are sensitive to inhibition by cycloheximide. (5) The extent of this inhibition does vary with the respiratory chain activities, cytochrome oxidase and cytochrome reductase[2] being less sensitive to the action of the antibiotic than is malic dehydrogenase. However, NADH–ferricyanide reductase, which one might have expected to behave in a fashion similar to malic dehydrogenase, is even less sensitive. (6) Other cytoplasmic enzymes are *not* identical in the extent to which their derepression is interfered with by cycloheximide. All of them are less sensitive than is cytoplasmic malic dehydrogenase, and, even more surprisingly, than are the ac-

[2] The statement is strictly true only if total rather than just mitochondrial activities are compared. However, there is no question about the origin of this particular activity and, since mitochondria for all these experiments were prepared by mechanical disintegration of cells, it is entirely possible that in this particular instance a larger proportion of this activity could not be sedimented with the mitochondrial fraction.

TABLE 1: Inhibition of Derepression of Enzymes on Long-Term Exposure to Antibiotics.

Activity	Fractions Tested	Controls		Chloramphenicol[c]	(t = 0) %	Chloramphenicol[d]	(t = 0) %	Chloramphenicol[d]	(t = 11 hr) %	Cycloheximide[e]	(t = 11 hr) %
		11 hr[a]	RA[b]	RA[b]	Inhibn[f]	RA	Inhibn	RA	Inhibn	RA	Inhibn
Cytochrome oxidase	M[g]	4.96	10.8	1.44	86.7	1.26	88.4	2.10	80.6	3.90	63.8
	M + S[h]	(9.17)[i]	(13.3)	(2.37)	(81.9)	(1.10)	(91.5)	(3.08)	(77.0)	(4.82)	(64.0)
NADH–cytochrome *c* reductase[j]	M	1.61	25.8	3.72	85.6	1.03	96.0	4.93	81.0	16.7	38.1
	M + S	(3.60)	(21.2)	(3.97)	(80.0)	(2.14)	(90.0)	(3.97)	(80.0)	(5.67)	(73.0)
NADH dehydrogenase	M	6.65	3.59	2.18	39.1	1.40	61.0	1.71	52.4	1.88	46.2
Malic dehydrogenase	M	10.2	15.8	2.76	82.5	2.35	85.2	8.27	48.4	2.10	86.7
NADH–cytochrome *c* reductase[k]	S	7.61	5.81	2.91	49.9	3.07	46.5	3.46	40.5	2.14	63.2
NADH dehydrogenase	S[l]	234	6.27	3.86	37.3	2.49	60.3	5.88	11.0	3.47	43.1
Malic dehydrogenase	S[l]	582	9.10	3.05	66.5	1.68	81.8	4.80	47.3	1.34	85.4
NADPH–cytochrome *c* reductase	S	19.2	0.963	1.09		1.49		1.31		1.47	

[a] Actual activity, defined as mμmoles of substrate converted × min^{-1} × mg^{-1} of homogenate protein. [b] RA, relative activity = activity at 17.5 hr/activity at 11 hr. [c] 2 mg/ml. [d] 4 mg/ml. [e] 1 μg/ml. [f] % inhibn = per cent inhibition = [1.00 − (RA experimental)/(RA control)] × 100%. [g] Mitochondrial activity, determined on P_F for cytochrome oxidase, on S_F + S_K for MDH, on Mitos for all other enzymes. [h] Supernatant (S_{20}) activity. [i] Total homogenate activity. [j] Antimycin sensitive. [k] Antimycin insensitive. [l] Corrected for mitochondrial contamination.



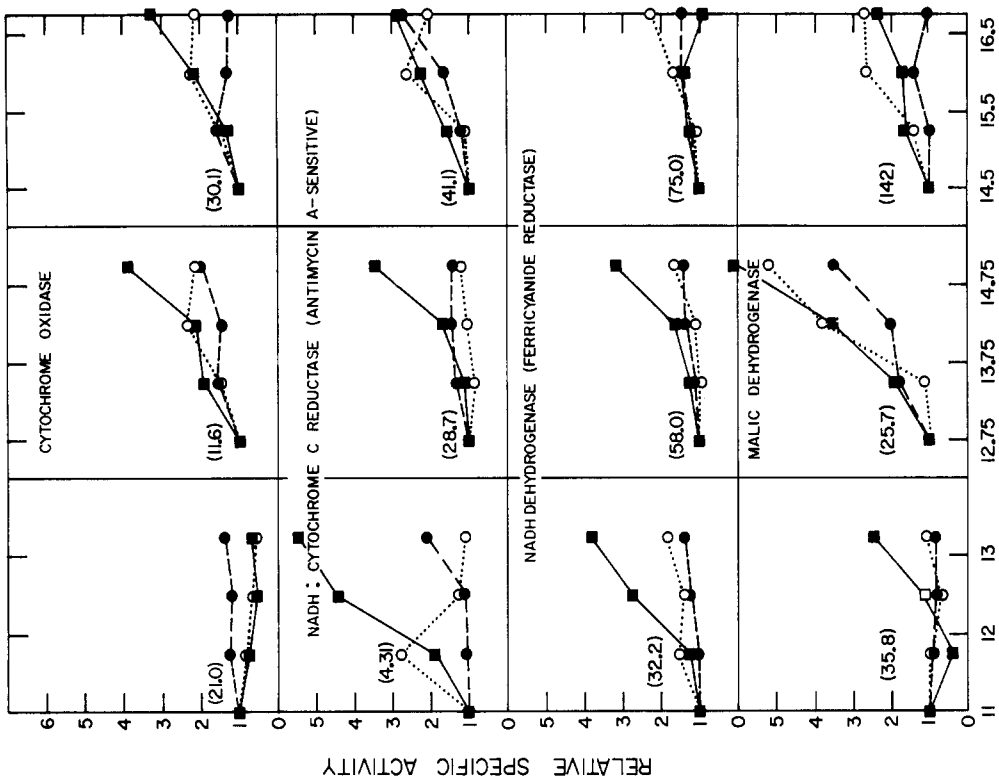


FIGURE 3: Effect of 2 mg/ml of chloramphenicol and 0.625 μg/ml of cycloheximide when added in early, middle, and late derepression. The figure in parenthesis at the lower left of each graph is the total enzyme activity (μmoles oxidized $\times$ min^{-1}) found at 11, 12.75, or 14.5 hr. Each point on the graph represents the ratio of the activity per milligrams of protein found at the indicated time to this initial activity. (—■—) Control, (···○···) chloramphenicol, and (-·-●-·-) cycloheximide. (A) left: Mitochondrial activities and (B) right: cytoplasmic activities.

TABLE II: Short-Term Effects of Antibiotics Added at Various Periods of Derepression.[a]

Activity	Tested	Chloroamphenicol			Cycloheximide		
		I	II	III	I	II	III
Cytochrome oxidase	M[b] + S[c]	−	+	+	−	++	+++
NADH–cytochrome *c* reductase[d]	M + S	++	+++	+	++	++	+
NADH dehydrogenase	M	++	++	0	+++	++	−
Malic dehydrogenase	M	+++	+	0	+++	++	++
NADH–cytochrome *c* reductase[e]	S	0	0	−	+	0	−
NADH dehydrogenase	S[f]	0	−	−	++	−	−
Malic dehydrogenase	S[f]	0	+	0	++	+++	+++
NADPH–cytochrome *c* reductase	S	0	−	−	0	−	−

[a] The three periods studied were I, 11–13.25 hr; II, 12.75–15.00 hr; and III, 14.5–16.75 hr after inoculation (see Figure 3). Degree of inhibition of increase in activity is symbolized as follows: +++ is >90%, ++ is 50–90%, + is 20–50%, and 0 indicates <20%. A dash shows that there was no significant increase in enzyme activity over that particular period. [b] Mitochondrial activity; determined on P_K for all except malic dehydrogenase. [c] Postmitochondrial supernatant. [d] Antimycin sensitive. [e] Antimycin insensitive. [f] Corrected for mitochondrial contamination by using oxidase and antimycin-sensitive reductase in this fraction to assess extent of this contribution.

tivities of the respiratory chain of the inner mitochondrial membrane.

The most parsimonious hypothesis that might account for the observations just described postulates that the formation of functional mitochondria requires components both of cytoplasmic and mitochondrial origin and should therefore be subject to inhibition by both antibiotics. Furthermore the biosynthesis of new proteins regardless of their origin and localization requires energy. Since the energy after 11-hr growth on glucose is provided by mitochondrial reactions, inhibition of the rate of formation of functional mitochondria may lead to a decrease in the available energy. This model can account on a qualitative level for both the susceptibility of respiratory chain-linked mitochondrial activities to cycloheximide and of the solubilizable mitochondrial and cytoplasmic activities to chloramphenicol. It is rendered unattractive on a quantitative level by the highly individualistic patterns of inhibition described. A more clear-cut demonstration is, however, possible. While formation of mitochondria and attendant energy production cannot be ruled out for long-term experiments, an analysis of more immediate causes should become possible if the effects of the inhibitors are studied over short time periods relative to the time required to produce a new mitochondrion. Although the doubling time of a mitochondrion during derepression is not known with certainty,[3] we do know the magnitude of this parameter for a number of mitochondrial enzymes to be of the order of 60 min or less.

Short-Term Exposure to Antibiotics. With these considerations in mind we designed a set of experiments in which we added chloramphenicol or cycloheximide to cells at various stages of derepression and followed the various activities every 45 min. Several experiments of this type were performed; the results of the most comprehensive set are presented in Figure 3A,B and summarized in Table II. Several interesting features emerge, especially taken in conjunction with the results of the long-term experiments. (1) So far as inhibition of increase in activity of integrated mitochondrial enzymes by chloramphenicol is concerned, great differences now become manifest between the two parts of the chain. The derepression of the cytochrome reductase, which precedes that of the oxidase by over 1 hr, is much more sensitive to inhibition by chloramphenicol than is that of the oxidase, and even the dehydrogenase part of the reductase is now subject to this inhibition. Therefore, with longer exposure times the accumulation of non-rate-limiting oxidase components may become more sensitive to inhibition by chloramphenicol, while continued supply from a source not subject to this inhibition is apparently sufficient to overcome an initial block in the synthesis of one of the components of the reductase. (2) The block of the accumulation of mitochondrial (but not of cytoplasmic!) malic dehydrogenase exerted by chloramphenicol is apparently immediate, and as profound as on any of the membrane-bound enzymes.[4] (3) There do not appear to be any

[3] This is perhaps not really a meaningful question in this context because of the probable occurrence in the repressed cells of many membranous elements which may actually represent mitochondrial precursors. However, it is known that repressed cells contain roughly 30% of the mitochondrial DNA of the fully derepressed ones (Tewari *et al.*, 1966) and that it takes 3–4 hr for the full complement of mitochondria to become manifest subsequent to the initiation of derepression (Jayaraman *et al.*, 1966). From this we estimate a generation time of ~60 min.

[4] The differences in the kinetics and sensitivity to antibiotics of the two malic dehydrogenase and of the NADH–ferricyanide reductases indicate that one is really dealing with distinct molecular species, a conclusion for which the experiments on their localization had provided necessary but not sufficient evidence.

significant short-term effects of chloramphenicol on *any* of the cytoplasmic enzymes. Apparently, the transmission (or execution) of the chloramphenicol-sensitive signal, that affects cytoplasmic enzyme synthesis over the long run, takes time. (4) One of the more significant results obtained in these studies is the great sensitivity of the increase in *all* the mitochondrial enzymes to cycloheximide under conditions of incomplete inhibition of protein synthesis, a sensitivity similar in magnitude to that exerted by the inhibitor on increases of cytoplasmic activities.

Discussion

The patterns characterizing the inhibition of the derepression of mitochondrial enzymes are as complex and individualistic as those that define their rate and time of appearance (Jayaraman *et al.*, 1966) and the kinetics of synthesis of different classes of mitochondrial proteins (Henson *et al.*, 1968). The qualitative and quantitative relationships described are sufficient to rule out certain models, including the two enumerated earlier. If membrane-bound mitochondrial enzymes, or enzyme systems, were produced *exclusively* by an intramitochondrial protein-synthesizing system, itself sensitive to inhibition by chloramphenicol and insensitive to cycloheximide, then their appearance should all have been equally sensitive to chloramphenicol and insensitive to cycloheximide, regardless of conditions. Such is clearly not the case. If the effect of chloramphenicol were mainly on some parameter concerned with derepression as such, rather than mitochondriogenesis, then all derepressible enzymes should be affected to the same extent. This again is clearly not the case, not even for the two variants of either malic dehydrogenase or of NADH–ferricyanide reductase. If the long-term effect of chloramphenicol were a secondary one, concerned with energy to be supplied by mitochondria dead aborning, why should there be energy sufficient to support the formation of 1750 units of cytoplasmic malic dehydrogenase (or 1000 units of cytoplasmic ferricyanide reductase), but insufficient for 24 units of mitochondrial malic dehydrogenase (specific activities in Table I × protein values of Figure 1)? For that matter, if this model were correct, there should not exist any short-term effects on mitochondrial malic dehydrogenase at all. If the principal effect of chloramphenicol on cytoplasmic activities were on the elaboration of certain proteins of mitochondrial origin required for the formation of *cytoplasmic* membranes (Woodward and Munkres, 1967), then one would expect that the activity of those enzymes that are membrane bound should be more susceptible than those that exist free in the cytoplasm. Such is clearly not the case.

On the basis of the experiments presented in this series of publications (Jayaraman *et al.*, 1966; Mahler *et al.*, 1967, 1968; Henson *et al.*, 1968), we present the following model. All mitochondrial proteins not tightly integrated into the inner membrane of the particle are coded for by nuclear genes and are synthesized in the cytoplasm. Most of the proteins of the inner membrane also share this mode of specification and synthesis. As a result of derepression their synthesis is initiated and they are transported, as individual entities, from the endoplasmic reticulum to relatively undifferentiated mitochondrial membranes, characteristic of the repressed cell. For full integration into these membranes, an event coincident perhaps with formation of cristae and prerequisite for their exhibiting full enzymatic activity, these proteins require the intervention of additional entities, synthesized by the autonomous, chloramphenicol-sensitive protein-synthesizing system of the particle and probably specified by mitochondrial DNA. These entities may be certain respiratory chain enzymes themselves and/or proteins capable of affecting the configuration or conformation, and hence the activity, of the other components of the respiratory chain.[5] In turn these integrative events inside the mitochondrion themselves regulate the continued supply of proteins furnished by the cytoplasm.

Although by no means original with us in all its features[5] (see, for instance, Roodyn and Wilkie (1968) and Work *et al.* (1968) for analogous models of mitochondriogenesis) it possesses at least two sets of virtues. First, it is not inconsistent with the large body of available experimental evidence concerning yeast mitochondria. These comprise the studies on effects of antibiotics on the increases of enzyme activity and on protein synthesis both *in vivo* and *in vitro* (already referred to); estimates of the information content of mitochondrial DNA in wild-type and cytoplasmic (ρ^-) respiration-deficient cells, which suggest that it must be severely restricted in the latter if not in the former (Tewari *et al.*, 1966; Mounolou *et al.*, 1966; Slonimski, 1967; Mehrotra and Mahler, 1968); comparisons of the proteins present in these mutants with those of the wild type under equivalent physiological conditions (*i.e.*, when glucose repression is minimized), which suggest that the mutant retains information for the specification of most mitochondrial proteins (Schatz *et al.*, 1963; Sherman and Slonimski, 1964; Mahler *et al.*, 1964a,b; Mackler *et al.*, 1965; Kraml and Mahler, 1967; K. Tagawa, K. Ishidate, and B. Hagihara, private communication; Kováč *et al.*, 1967; Schatz, 1968); and comparisons of the morphology of intracellular membranes in mutant and wild-type cells under conditions that either favor or repress mitochondrial development (Yotsuyanagi, 1962; Schatz

[5] The part of the model postulating a requirement for proteins responsible for mitochondrial assembly, organization, and function is closely related to Racker's (1967) concept of allotopy. Interactions with structural entities of the particle have been postulated by Sherman and Slonimski (1964), Mackler *et al.* (1965), and Woodward and Munkres (1966) to account for the pleiotropic nature of cytoplasmic mutations leading to respiratory deficiency. The application of Kellenberger's (1965) notions of orders of morphopoiesis to the problems of membrane organization have been discussed by one of us (Cotman *et al.*, 1968). One of the proteins responsible for the integration into the inner membrane of and the resultant alteration of various properties of the mitochondrial ATPase (itself probably one of the enzymes originating in the cytoplasm (Kováč *et al.*, 1967)) seems a likely candidate (Schatz, 1968; Tuppy and Swetly, 1968; MacLennan and Tzagoloff, 1968; Tzagoloff *et al.*, 1968; Schatz *et al.*, 1967; Racker, 1967; Kagawa and Racker, 1966).

et al., 1963; Huang *et al.*, 1966; Linnane, 1965; Jayaraman *et al.*, 1966; Wallace *et al.*, 1968). It is also consistent with the asynchronous and stepwise assembly and turnover of mitochondria during animal development (Lennie and Birt, 1967) and of membranes in general (Omura *et al.*, 1967). Its second virtue is that it serves us as a useful working hypothesis capable of generating certain definite predictions. These we are currently engaged in testing.

Materials and Methods

Short-Term Effects. S. cerevisiae (strain Fleischmann, diploid) was grown in a semisynthetic 1% glucose medium (Jayaraman *et al.*, 1966; Leon and Mahler, 1968) in a 10-l. New Brunswick Microferm. At 11, 12.75, and 14.5 hr, ten 250-ml aliquots were removed, mixed with 2 mg/ml of chloramphenicol or 0.625 mg/ml of cyclo, as indicated, and incubated at 30° in a rotary shaker. Growth was stopped by pouring the samples over ice. Mitochondria were isolated by diluting the approximately 2 g of cells to 15 ml with STV buffer,[6] adding 15 ml of glass beads (0.45–0.50 mm, B. Braun), and shaking the mixture 30 sec in a Braun mechanical shaker. The homogenate was decanted and, as the beads were washed with aliquots of the STV buffer, diluted to 30 ml with washes from the beads, and centrifuged 15 min at 2000*g* to remove the cell debris. The suspension was centrifuged at 20,000*g* for 20 min to give a cytoplasmic supernatant, S_{20}, and a mitochondrial pellet. This pellet was washed once with 20 ml of 1.0 M sorbitol–0.2% bovine serum albumin. The organelles were then suspended in 15 ml of water, frozen and thawed twice, and reisolated by centrifuging 20 min at 20,000*g*. The pellet was suspended in 10 ml of 0.6 N KCl, incubated at room temperature for 5 min, and centrifuged 20 min at 20,000*g* to give mitochondrial fractions, S_K and P_K. The fraction P_K was suspended in 1.0 M sorbitol for protein and enzyme assays.

The fractions S_K, P_K, and S_{20} were tested for enzymatic activity as follows. Cytochrome oxidase using the method of Smith and Conrad (1956); NADH dehydrogenase, Rao *et al.* (1963); NADPH and NADH cytochrom *c* reductase (both antimycin sensitive and insensitive), Schatz and Klima (1964); and malic dehydrogenase, Polakis and Bartley (1965). All, including cytochrome oxidase, were calculated as zero-order reactions. Malic dehydrogenase and NADH–ferricyanide reductase were corrected for mitochondrial contamination by assuming that the cytochrome oxidase and antimycin A sensitive NADH–cytochrome *c* reductase activities found in S_{20} were due to mitochondria, and that the same proportion held true also for the enzymes of interest. Proteins were determined using the method of Lowry *et al.* (1951).

Long-Term Effects. In these studies, 250-ml aliquots of yeast were grown with shaking in 1-l. erlenmeyer flasks. Mitochondria were isolated from the cells and washed in 1 M sorbitol as described above, the only change being that the cells were suspended to 10 ml with STV buffer and homogenized 30 sec with only 10 ml of glass beads. The homogenate was then diluted to 15 ml with washings from the beads. The organelles were initially suspended in 5 ml of 0.6 N KCl and incubated 5 min at room temperature. After 30 min at 30,000*g* to remove S_K, the sedimented material was suspended in 5 ml of 0.01 M Tris, 4 mM ATP, and 1 mM Cleland's reagent (pH 7.5), frozen, and thawed twice and again centrifuged 30 min at 30,000*g*. The pellet, P_F, was suspended in 1 M sorbitol. S_{20}, mitochondria, S_K, P_F, and S_F were assayed for enzyme activities as above. The tabulated data are derived from that mitochondrial fraction showing the highest activity as indicated in Table I (footnote *g*). This regime was used to take into account either inactivation or crypticity of mitochondrial activities.

[6] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: STV buffer, 1 M sorbitol–0.1 M Tris–0.001 M EDTA (pH 8)–0.2% bovine serum albumin.

Acknowledgments

The authors are indebted to Dr. R. H. Baker, Jr., of Upjohn Co. for the cycloheximide and Dr. H. E. Machamer of Parke Davis and Co. for the chloramphenicol used in these studies. We are also grateful to Mr. Richard Pflum for his technical assistance.

References

Allmann, D. W., Bachmann, E., and Green, D. E. (1966), *Arch. Biochem. Biophys. 115*, 165.

Bachmann, E., Lenaz, G., Perdue, J. F., Orme-Johnson, N., and Green, D. E. (1967), *Arch. Biochem. Biophys. 121*, 73.

Blair, P. V., Oda, T., Green, D. E., and Fernandez-Moran, H. (1963), *Biochemistry 2*, 756.

Caplan, A. I., and Greenawalt, J. W. (1968), *J. Cell Biol. 36*, 15.

Clark-Walker, G., and Linnane, A. (1966), *Biochem. Biophys. Res. Commun. 95*, 8.

Clark-Walker, G., and Linnane, A. (1967), *J. Cell Biol. 34*, 1.

Cotman, C., Mahler, H. R., and Hugli, T. (1968), *Arch. Biochem. Biophys. 126*, 821.

de Kloet, S. R. (1965), *Koninkl. Ned. Akad. Wetenschap. Proc. 68B*, 266.

Ephrussi, B., Slonimski, P. P., Yotsuyanagi, Y., and Tavlitzki, J. (1956), *Compt. Rend. Trav. Lab. Carlsberg 26*, 87.

Ferguson, J. J., Boll, M., and Holzer, H. (1967), *European J. Biochem. 1*, 21.

Green, D. E., Mackler, B., Repaske, R., and Mahler, H. R. (1954), *Biochim. Biophys. Acta 15*, 435.

Henson, C. W., Weber, C., and Mahler, H. R. (1968), *Biochemistry 7*, 4431.

Huang, M., Biggs, D. R., Clark-Walker, G. D., and Linnane, A. W. (1966), *Biochim. Biophys. Acta 114*, 434.

Jayaraman, J., Cotman, C., Mahler, H. R., and Sharp, C. W. (1966), *Arch. Biochem. Biophys. 116*, 224.

Kagawa, Y., and Racker, E. (1966), *J. Biol. Chem. 241*, 2461, 2467.

Kellenberger, E. (1965), *Ciba Found. Symp. Principles Biomed. Organ.*

Klingenberg, M., and Pfaff, (1966), *in* Regulation of Metabolic Processes in Mitochondria, Tager, J. M., Papa, S., Quagliarello, E., and Slater, E. C., Ed., New York, N. Y., Elsevier, p 180.

Kováč, L., Lachowicz, T. M., and Slonimski, P. P. (1967), *Science 158*, 1564.

Kraml, J., and Mahler, H. R. (1967), *Immunochemistry 4*, 213.

Lehninger, A. L. (1964), The Mitochondrion, New York, N. Y., Benjamin.

Lennie, R. W., and Birt, L. M. (1967), *Biochem. J. 102*, 338.

Leon, S., and Mahler, H. R. (1968), *Arch. Biochem. Biophys. 126*, 305.

Linnane, A. W. (1965), *in* Oxidases and Related Redox Systems, King, T. E., Mason, H.S., and Morrison, M., Ed., New York, N. Y., Wiley, p 1102.

Linnane, A. W., Biggs, D. R., Huang, M., and Clark-Walker, G. D. (1967), *in* Aspects of Yeast Metabolism, Mills, R. K., Ed., Oxford, Blackwell, p 217.

Linnane, A. W., Lamb, A. J., Christodoulou, C., and Lukins, H. B. (1968), *Proc. Natl. Acad. Sci. U. S. 59*, 1288.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Mackler, B., Collipp, P. J., Duncan, H. M., Rao, A., and Huennekens, F. M. (1962), *J. Biol. Chem. 237*, 2968.

Mackler, B., Douglas, H. C., Will, S., Hawthorne, D. C., and Mahler, H. R. (1965), *Biochemistry 4*, 2016.

MacLennan, D. H., and Tzagoloff, A. (1968), *Biochemistry 7*, 1603.

Mahler, H. R., Mackler, B., Grandchamp, S., and Slonimski, P. P. (1964a), *Biochemistry 3*, 668.

Mahler, H. R., Mackler, B., Slonimski, P. P., and Grandchamp, S. (1964b), *Biochemistry 3*, 419.

Mahler, H. R., Perlman, P., Henson, C., and Weber, C. (1968), *Biochem. Biophys. Res. Commun. 31*, 474.

Mahler, H. R., Tewari, K. K., and Jayaraman, J. (1967), *in* Aspects of Yeast Metabolism, Mills, R. K., Ed., Oxford, Blackwell, p 247.

Misaka, E., and Nakanishi, K. (1963), *J. Biochem. (Tokyo) 53*, 465.

Mehrotra, B., and Mahler, H. R. (1968), *Arch. Biochem. Biophys.* (in press).

Mounolou, J. C., Jakob, H., and Slonimski, P. P. (1966), *Biochem. Biophys. Res. Commun. 24*, 218.

Omura, T., Siekevitz, P., and Palade, G. E. (1967), *J. Biol. Chem. 242*, 2389.

Parsons, D. F., Williams, G. R., and Chance, B. (1966), *Ann. N. Y. Acad. Sci. 137*, 643.

Polakis, E. J., and Bartley, W. (1965), *Biochem. J. 97*, 284.

Polakis, E. S., and Bartley, W. (1966), *Biochem. J. 99*, 521.

Polakis, E. S., Bartley, W., and Meek, G. A. (1965), *Biochem. J. 97*, 298.

Racker, E. (1967), *Fed. Proc. 26*, 1335.

Rao, N. A., Felton, J. P., Huennekens, F. M., and Mackler, B. (1963), *J. Biol. Chem. 238*, 449.

Raw, I., and Mahler, H. R. (1959), *J. Biol. Chem. 234*, 1867.

Roodyn, D. B., and Wilkie, D. (1968), The Biogenesis of Mitochondria, London, Methuen.

Schatz, G. (1968), *J. Biol. Chem. 243*, 2192.

Schatz, G., and Klima, J. (1964), *Biochim. Biophys. Acta 81*, 448.

Schatz, G., Penefsky, H. S., and Racker, E. (1967), *J. Biol. Chem. 242*, 2552.

Schatz, G., and Racker, E. (1966), *Biochem. Biophys. Res. Commun. 22*, 579.

Schatz, G., Tuppy, H., and Klima, J. (1963), *Z. Naturforsch. 18b*, 145.

Schnaitman, C., Erwin, V., and Greenawalt, J. W. (1967), *J. Cell Biol. 32*, 719.

Sherman, F., and Slonimski, P. P. (1964), *Biochim. Biophys. Acta 90*, 1.

Siegel, M. R., and Sisler, H. D. (1965), *Biochim. Biophys. Acta 103*, 558.

Slater, E. C. (1958), *Advan. Enzymol. 20*, 147.

Slonimski, P. P. (1956), *Proc. 3rd Intern. Congr. Biochem., Brussels*, 242.

Smith, L., and Conrad, H. (1956), *Arch. Biochem. Biophys. 63*, 403.

Sottocasa, G. L., Kuylenstierna, B., Ernster, L., and Bergstrand, A. (1967), *J. Cell Biol. 32*, 415.

Tewari, K. K., Voetsch, W., Mahler, H. R., and Mackler, B. (1966), *J. Mol. Biol. 20*, 453.

Tuppy, H., and Swetly, P. (1968), *Biochim. Biophys. Acta 153*, 293.

Tzagoloff, A., MacLennan, D. H., and Byington, K. H. (1968), *Biochemistry 7*, 1596.

Utter, M. F., Duell, E. A., and Bernofsky, C. (1967), *in* Aspects of Yeast Metabolism, Mills, R. K., Ed., Oxford, Blackwell, p 197.

Wallace, P. G., Huang, M., and Linnane, A. W. (1968), *J. Cell Biol. 37*, 207.

Wintersberger, E. (1965), *Biochem. Z. 341*, 409.

Witt, I., Kronau, R., and Holzer, H. (1966a), *Biochim. Biophys. Acta 118*, 522.

Witt, I., Kronau, R., and Holzer, H. (1966b), *Biochim. Biophys. Acta 128*, 63.

Woodward, D. O., and Munkres, K. D. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 872.

Woodward, D. O., and Munkres, K. D. (1967), *in* Organizational Biosynthesis, Vogel, H. J., Lampen, J. O., and Bryson, V., Ed., New Brunswick, N. J., Rutgers University, p 489.

Work, T. S., Coote, J. L., and Ashwell, M. (1968), *Fed. Proc. 27*, 1174.

Yotsuyanagi, Y. (1967), *J. Ultrastruct. Res. 7*, 121, 141.